AN ABSTRACT OF THE THESIS OF

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Title: EXTRACTION AND PARTIAL CHARACTERIZATION OF STRAWBERRY POLYPHENOL OXIDASE

Abstract approved: Dr. Morris W. Montgomery

The optimum conditions for the extraction of strawberry polyphenol oxidase (PPO) were examined using different levels of phenol adsorbents: Polyvinylpolypyrrolidone (PVPP), Amberlite XAD-4 (XAD-4), and Dowex AG 2-X8 Dowex 2. The effectiveness of the extraction was evaluated based on the level of PPO activity per g of strawberry tissue, the browning of the extracts, and the UV absorption scans from 240 to 230 nm.

Prevention of PPO activity loss during storage of the fresh strawberries was possible by quick-freezing the fruits in liquid nitrogen, sealing in oxygen-impermeable bags, and storing at -40°C. No loss in PPO activity was noticed after storing the strawberries for 95 days under these conditions. The first extraction step consisted in the preparation of a nitrogen powder (N₂P) by homogenizing the frozen strawberries in liquid nitrogen and storing the N₂P obtained under liquid nitrogen until used. Further steps of extraction involved mixing of the N₂P with the
desired levels of phenol adsorbents which had been previously hydrated in cold extraction buffer.

The following adsorbents, alone and in combination, and arranged in the order of decreasing efficiency, showed improved extraction of PPO when used at or above the levels specified (as g dry adsorbents per g N₂P): 1.0g PVPP plus 0.5g XAD-4; 1.0g PVPP; 0.5g PVPP plus 0.5g Dowex 2; 3.0g XAD-4 plus 0.5g Dowex 2; and 1.0g XAD-4. The samples treated with Dowex 2 only showed the presence of residual phenolics and rapid onset of browning in the extracts at any of the levels tried.

Attempts were made for the study of the isoenzyme patterns of PPO using gel electrophoresis, but interference due to the presence of pectin in the extracts made this analysis impossible.

During the concentration of the PPO extract, using dialysis and lyophilization, those extracts obtained using PVPP, alone or in combination, showed the least loss in activity. The use of PVPP combined with XAD-4 was recommended for the best adsorption of polyphenols and extraction of PPO with high activity and stability. This was the combination of adsorbents used to prepare the PPO extracts for the characterization of the strawberry PPO.

The pH optimum for activity of PPO using catechol as a substrate was 5.5 and with 4-methyl catechol, the optimum pH was 4.5. Maximum activity of PPO was extracted at pH 4.5.
using either catechol or 4-methyl catechol for the assays. The strawberry PPO showed different temperature stabilities with the different substrates used for the assays and followed first order kinetics of inactivation.

No activity against monophenols could be detected for the extracted PPO. Of the o-diphenols tried, the highest activity measured was towards catechin, 4-methyl catechol and pyrogallol in decreasing order of activity. Inhibition studies showed the effectiveness of diethyldithiocarbamate, potassium metabisulfite, potassium cyanide and dithiothreitol in the direct inhibition of PPO. It was demonstrated with the use of the polarographic assay method, that benzene sulfinic acid, cysteine and ascorbic acid did not inhibit PPO directly, but that these compounds were effective in inhibition the secondary reactions by the condensation or reduction of the quinones. The formation of color, e.g. melanin, was prevented, but there was no inhibition of oxygen uptake. Sodium chloride showed partial inhibition of PPO at high concentrations.
Extraction and Partial Characterization of Strawberry Polyphenol Oxidase.

by

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"CONCIENTIZATION refers to the process by which man, not as a recipient but as a knowing subject, reaches a deeper awareness both of the sociocultural reality on which his life is built and of his ability to transform that reality."

Paulo Freire
Cultural Action for Freedom.
TABLE OF CONTENTS

INTRODUCTION. 1

LITERATURE REVIEW 3

Polyphenol Oxidase 3
Multiple Forms of PPO 5
PPO Reactions 7
Assay Methods for PPO Activity 11
Substrate Specificity of PPO 13
pH Optima of PPO 15
The Effect of Temperature on PPO 17
Extraction of Enzymes from Plant Tissues 18
Reactions of Phenolic Compounds 22
Overcoming the Problems Caused by Phenolics 24
Removal of Polyphenols 27
  Organic Solvents 27
  Polymeric Protective Agents 27
    Inert Proteins 27
    Polyamides 27
    Polyethylene glycol 28
    Polyvinyl (poly) pyrrolidone 29
    Non-ionic Macroreticular Resins 31
    Ion-exchange Resins 33
Covalent Condensation of o-diphenols 34
Removal of Quinones 35
  Reduction of Quinones 35
  Trapping of Quinones 36
Modification of Phenolics 38
Inhibition of PPO 39
  Inhibitors Affecting the Prosthetic Group of PPO 39
  Inhibitors Affecting the Binding of Substrates by PPO 40
Purification of PPO 41
The Strawberry System 45
  Chemical Composition of Strawberries 45
  Strawberry Phenolic Compounds 45
  Phenolics, PPO and Strawberry Color Deterioration 47
The Isolation and Characterization of Strawberry PPO 51

MATERIALS AND METHODS 53

Source and Preparation of the Samples 53
Materials 53
Cleaning of the Phenol Adsorbents 54
  Amberlite XAD-4 54
  Polyélar AT 54
  Dowex AG 2-x8 55
General Extraction Procedure
Assay of PPO Activity
Measurement of Polyphenolic Content
Discontinuous Polyacrylamide Electrophoresis
Protein Concentration
Characterization of the Strawberry PPO
  Effect of pH
  Effect of Temperature
  Substrate Specificity
  Effect of Inhibitors

RESULTS AND DISCUSSION

Preparation and Preservation of the Samples
Enzyme Extraction Procedure
PPO Extracts Obtained with Polyphenol Adsorbants
  Extraction of PPO using XAD-4
  Extraction of PPO using PVPP
  Extraction of PPO using Dowex 2
  Extraction of PPO using PVPP plus XAD-4
  Extraction of PPO using PVPP plus Dowex 2
  Extraction of PPO using XAD-4 plus Dowex 2
Effect of Dialysis, Lyophilization and Electrophoresic Studies of the Extracts
  Extraction of PPO
  Dialysis of the Extracts
  Concentration of the Extracts
  Disc-electrophoresis
Characterization of the Strawberry PPO
  pH Optima of Strawberry PPO
  Effect of pH during PPO Extraction
  Heat Stability of Strawberry PPO
  Substrate Specificity of Strawberry PPO
  Effect of Inhibitors on Strawberry PPO

SUMMARY

BIBLIOGRAPHY
LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Some of the main reactions and interactions of phenolics and quinones.</td>
<td>19</td>
</tr>
<tr>
<td>2</td>
<td>Phenol adsorbents; general structures and proposed mode of action.</td>
<td>32</td>
</tr>
<tr>
<td>3</td>
<td>Structure of some phenolic compounds present in strawberries.</td>
<td>46</td>
</tr>
<tr>
<td>4</td>
<td>Basic extraction procedure of strawberry PPO.</td>
<td>68</td>
</tr>
<tr>
<td>5</td>
<td>UV-absorption spectra of PPO extracts obtained using different levels of XAD-4.</td>
<td>72</td>
</tr>
<tr>
<td>6</td>
<td>UV-absorption spectra of PPO extracts obtained using different levels of PVPP.</td>
<td>75</td>
</tr>
<tr>
<td>7</td>
<td>UV-absorption spectra of PPO extracts obtained using different levels of Dowex 2.</td>
<td>79</td>
</tr>
<tr>
<td>8</td>
<td>UV-absorption spectra of PPO extracts obtained using different levels of PVPP and XAD-4 in combination.</td>
<td>82</td>
</tr>
<tr>
<td>9</td>
<td>UV-absorption spectra of PPO extracts obtained using different levels of PVPP and Dowex 2 in combination.</td>
<td>85</td>
</tr>
<tr>
<td>10</td>
<td>UV-absorption spectra of PPO extracts obtained using different levels of XAD-4 and Dowex 2 in combination.</td>
<td>88</td>
</tr>
<tr>
<td>11</td>
<td>pH optima for activity of strawberry PPO using catechol and 4-methyl catechol as substrates.</td>
<td>99</td>
</tr>
<tr>
<td>12</td>
<td>Effect of pH during the extraction of strawberry PPO.</td>
<td>103</td>
</tr>
<tr>
<td>13</td>
<td>Heat inactivation of strawberry PPO; catechol used as substrate.</td>
<td>107</td>
</tr>
<tr>
<td>14</td>
<td>Heat inactivation of strawberry PPO; 4-methyl catechol used as substrate.</td>
<td>108</td>
</tr>
<tr>
<td>Table</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>-------</td>
<td>-----------------------------------------------------------------------------</td>
<td>-------</td>
</tr>
<tr>
<td>1</td>
<td>Techniques used to prevent the formation and reactions of quinones during</td>
<td></td>
</tr>
<tr>
<td></td>
<td>the extraction of enzymes from plant tissues.</td>
<td>25</td>
</tr>
<tr>
<td>2</td>
<td>Chemical composition of strawberries with emphasis on the Tioga variety.</td>
<td>44</td>
</tr>
<tr>
<td>3</td>
<td>Strawberry PPO activity changes after storage at -40°C.</td>
<td>67</td>
</tr>
<tr>
<td>4</td>
<td>Characteristics of PPO extracts obtained using different levels of XAD-4.</td>
<td>71</td>
</tr>
<tr>
<td>5</td>
<td>Characteristics of PPO extracts obtained using different levels of PVPP.</td>
<td>74</td>
</tr>
<tr>
<td>6</td>
<td>Characteristics of PPO extracts obtained using different levels of Dowex 2.</td>
<td>78</td>
</tr>
<tr>
<td>7</td>
<td>Characteristics of PPO extracts obtained using different levels of PVPP and</td>
<td>81</td>
</tr>
<tr>
<td></td>
<td>XAD-4.</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Characteristics of PPO extracts obtained using different levels of PVPP and</td>
<td>84</td>
</tr>
<tr>
<td></td>
<td>Dowex 2.</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Characteristics of PPO extracts obtained using different levels of XAD-4 and</td>
<td>87</td>
</tr>
<tr>
<td></td>
<td>Dowex 2.</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Effect of the different treatments on strawberry PPO.</td>
<td>90</td>
</tr>
<tr>
<td>11</td>
<td>Effect of dialysis and lyophilization on strawberry PPO.</td>
<td>93</td>
</tr>
<tr>
<td>12</td>
<td>Effect of pH on the activity of strawberry PPO.</td>
<td>98</td>
</tr>
<tr>
<td>13</td>
<td>Effect of pH during the extraction of strawberry PPO.</td>
<td>102</td>
</tr>
<tr>
<td>14</td>
<td>Heat inactivation of strawberry PPO.</td>
<td>106</td>
</tr>
<tr>
<td>15</td>
<td>Substrate specificity of strawberry PPO.</td>
<td>110</td>
</tr>
<tr>
<td>16</td>
<td>Effect of inhibitors on strawberry PPO.</td>
<td>112</td>
</tr>
</tbody>
</table>
EXTRACTION AND PARTIAL PURIFICATION
OF STRAWBERRY POLYPHENOL OXIDASE

INTRODUCTION

Production and processing of strawberries are important industries in the Pacific Northwest. Information obtained from the Northwest Food Processors Association (Fruit and Berry Concentrate Processing in the Pacific Northwest Seventh Quarterly Report, 1979) report that 9,824,245 pounds of strawberries were processed into concentrate during 1978. The price per gallon of strawberry concentrate was $35.00 and with a yield of 1 gallon per 115 lbs fruit, the value of the strawberry concentrate produced amounted to $2,989,980. This value was only surpassed by the concentrates produced from grapes, apples and blackberries. The most serious problem faced by the strawberry food processors is the color degradation of the strawberries during harvesting, storage and processing. Strawberries are delicate fruits and bruising can occur very easily.

The degradation of the strawberry color is viewed as a consequence of two possible kinds of reactions occurring in the fruit, either separately or at the same time. The compounds responsible for the red color of strawberries are the anthocyanin pigments. These pigments are very unstable and their stability or degradation rate is affected by the conditions of storage and processing. High temperatures
and high pH affect their stability adversely. The presence of active PPO or peroxidase is reported to decrease the anthocyanin stability.

The degradation of the strawberry color can also be due to the interaction of the active PPO and the polyphenols present naturally in the fruit. The action of PPO on the polyphenols causes the formation of highly reactive quinones and the reaction of the quinones with proteins, amino acids, or with other phenolics present in the fruit may lead to the formation of brown polymers which mask the red color of strawberry products if formed in high enough levels. Some workers suggest the possibility of PPO reacting directly with anthocyanins; or that the quinones formed from the PPO reaction react with the anthocyanins, degrading them, and leading also to the formation of brown pigments. Thus, the two principal processes that can affect the color stability of strawberries are anthocyanin degradation and enzymic browning by PPO.

The purpose of this study was to obtain an extract containing active and stable PPO, and the characterization of the PPO as to the effects of pH, temperature and inhibitors. The specificity of PPO towards several substrates was also studied.
LITERATURE REVIEW

POLYPHENOL OXIDASE

The enzyme \( \text{diphenol:oxygen oxidoreductase} \) (EC 1.10.3.1) is also known as tyrosinase, polyphenol oxidase, catecholase or phenolase. It has been classified by the Commission on Enzymes of the International Union of Biochemistry together with laccase, \( \text{diphenol:oxygen oxidoreductase} \), into a single enzyme function, monophenol monooxygenase (EC 1.14.18.1) (Mayer and Harel, 1979). For the purpose of this work, the name polyphenol oxidase (PPO) will be used, and the cresolase-catecholase activities will be mainly considered.

PPO is widely distributed in the plant kingdom. It is found in all orders of the angiosperms studied so far (Mayer and Harel, 1979). The levels of PPO in plants varies markedly with development and variety, and also with the parts of the plant or type of tissue studied. This variation has been observed in olives (Ben-Shalom et al., 1977b), avocados (Golan et al., 1977), tomatoes (Hobson, 1967) and many other fruits.

The levels and types of phenolic compounds that could serve as substrates for PPO activity have also been observed to vary with the development and variety of the fruit, and with the region where the plant was grown (Walker, 1962; Amberger and Schaller, 1975; Mayer and Harel, 1979). The variation in the levels of phenolics and of PPO was directly
related to the potential enzymatic browning capacity of the tissue. This is of great importance in the fruit and vegetable processing industry, as well as in the isolation of plant cell components.

Some of the natural functions of PPO in vivo that have been suggested are the synthesis of o-diphenols; regulation of plant growth; activity in the photosynthetic pathway; wound sealing; disease resistance; changing the permeability of some seed coats; causing the tanning of plant proteins, making them unavailable or toxic to some herbivores (Mayer and Harel, 1979; Synge, 1975).

The subcellular location of PPO has been reported to vary considerably. Many reports suggest the presence of PPO associated or bound to "chlorophyl" of "mitochondria" containing extracts (Harel et al., 1964; Ben-Shalom et al., 1977b). Some workers reported PPO to be associated with RNA (Balasingham and Ferdinand, 1970). Most of these works lack proper protection against the action of endogenous phenolics during the enzyme extraction procedure and the interaction of the phenolics with the enzymes, e.g. tanning reactions, could be the cause of the "associated" or "bound" PPO reported. Characterization of the particulate PPO by electron microscopy was not reported in these works.

It has been suggested that the presence of bound PPO could sometimes be due to the precipitation of proteins by bound phenolics (Loomis et al., 1979) or perhaps by the interaction between the enzymes' hydrophobic areas with cell
membrane lipids.

Many workers have pointed out the importance of the prevention of protein-phenolic interactions during the extraction of plant enzymes (Sanderson, 1964; Coggon et al., 1973; Anderson, 1968; Loomis, 1969, 1974; Kelley and Adams, 1977; and Rhodes, 1977). The use of phenol absorbents, such as polycaprolactam (Sanderson, 1964) and Polyclar AT (Coggon et al., 1973) prevented the precipitation of the PPO from tea leaves which was reported earlier to be bound to the particulate fraction of the extract. The protein-phenolic complexes formed when no protection against the tanning reactions have been taken, have often been reported as "particulate" or "bound" PPO by some workers. From these observations it can be concluded that careful evaluation of the extraction procedure used during the extraction of plant enzymes is needed, and that the results reported by many workers should not be taken at face value.

**MULTIPLE FORMS OF PPO**

The multiplicity of PPO from some sources could be caused by the association-dissociation of the enzyme subunits, and/or by the conformational changes of a single protein (Jolley and Mason, 1965). Differences in the substrate specificity of the subunits as well as differences in their response towards inhibitors were found (Stafford and Dresler, 1972). According to Clayton (1959), the dichotomy among the PPO subunits would be missed if only one
substrate was tried with the different fractions.

The presence of multiple forms of PPO has been reported in pears (Halim and Montgomery, 1978; Smith, 1980), mushrooms (Jolley and Mason, 1965; Kertesz and Zito, 1965) and apples (Harel and Mayer, 1968).

Differences in the isozyme patterns of PPO from different sources have also been reported. Isozymes are enzymatically active proteins that catalyze the same reaction and occur in the same species, but differ in certain of their physicochemical properties (Wilkinson, 1966). The variability in the conformation and properties of an enzyme gives differences in the isozyme patterns during electrophoresis and ion-exchange chromatography.

Artifacts caused during the isolation are often identified as isozymes. The production of artifacts has been shown to occur when enzymes were made to interact with phenolic compounds (Gregory and Bendall, 1966; Smith, 1980) or with organic isothiocyanates (Loomis et al., 1979). The formation of various complexes that could be mistakenly identified as isozymes occurred. Kaplan (1978) demonstrated that electrophoretically separable "isozymes" can be merely artifacts formed during the extraction procedure. Smith (1980) developed an optimized extraction procedure for pear PPO with which the number of isozyme bands could be reduced to only three compared to the eight PPO isozyme bands reported previously by Halim and Montgomery (1978) for the same
fruit, and the eleven obtained by Smith (1980) without phenol adsorbents.

**PPO reactions**

PPO is a copper-containing enzyme. The state of the copper and the mechanism of action of PPO are not exactly known. According to Coleman (1974) PPO is a colorless enzyme which contains Cu$^+$ as a prosthetic group. No evidence for a reversible oxidation-reduction of Cu$^+$ during the reaction exists. The presence of an antiferromagnetically coupled Cu$^{++}$ pair has also been suggested (Makino et al., 1974). But even though the apoenzyme obtained from mushroom PPO could be reactivated by the addition of Cu$^{++}$ to the medium (Kertesz and Zito, 1965), the inhibition of PPO with carbon monoxide and the absence of an ESR absorption suggested the presence of Cu$^+$ (Malmstrom, 1965). According to Kertesz and Zito (1962), no thiol groups are involved in the fixation of copper to the apoenzyme.

PPO can catalyze two kinds of reactions. The two reactions are trivially called the cresolase reaction, or hydroxylation; and the catecholase reaction, or dehydrogenation of a diol (Mayer and Harel, 1979). During the cresolase reaction, a monophenol is oxidized to the corresponding diphenol. The required co-substrate is a catechol which is oxidized to an o-quinone. The diphenol can also be replaced by other reducing agents.
Cresolase reaction:

\[
\begin{align*}
\text{Cresolase reaction:} \\
\text{OH} + O_2 + BH_2 & \rightarrow \text{OH} + H_2O + B
\end{align*}
\]

The catecholase reaction can occur without the need of hydroxylation of monophenols, and consists in the oxidation of an \( \alpha \)-diphenol to the corresponding \( \alpha \)-quinone with the participation of molecular oxygen.

Catecholase reaction:

\[
\begin{align*}
\text{Catecholase reaction:} \\
\text{OH} + \frac{1}{2} O_2 & \rightarrow \text{O} + H_2O
\end{align*}
\]

According to Ingraham (1957) the Michaelis constant for oxygen is not an equilibrium constant. It approaches a value of ca. 1.5% \( O_2 \) at low concentrations of hydrogen donor, regardless of substrate structure. The enzyme combines with the oxygen before combining with the substrate.

In a mechanism of reaction proposed by Hamilton (1969), the \( Cu^+ \) ion in the active site of the enzyme brings the reactants catechol and oxygen together to form a pseudo-aromatic complex where transfer can occur readily if assisted by general acid or base catalysis from the protein molecule.

Some authors question if during the cresolase reaction the diphenol formed is released from the enzyme, or if the reaction goes directly to completion to give an \( \alpha \)-quinone (Mayer and Harel, 1979). The requirement of an \( \alpha \)-diphenol or a reducing agent for the cresolase activity was indicated by the presence of an induction period which continued until
sufficient \textit{o-diphenols} had been produced (Karkhanis and Frieden, 1961). 

A non-enzymatic mechanism by which an \textit{o-quinone} produced from the catecholase reaction causes the hydroxylation of the monophenols was postulated by Kertesz and Zito (1962). Arguments against this theory were given by Mason (1956). Mammalian tyrosinase has been found to be specific for the oxidation of \textit{L-tyrosine} and \textit{L-dopa}; catalytically produced \textit{o-quinones} were not found effective in bringing about the hydroxylation of monophenols; cresolase activity has been found to be increased by reducing agents; and, Mason \textit{et al.}, (1955) demonstrated that hydroxylation of 3,4-dimethylphenol involved the incorporation of \textit{\textsuperscript{18}O} from molecular oxygen and not of oxygen from water.

The presence of cresolase activity in PPO extracts is difficult to determine because this activity sometimes disappears upon purification. Cresolase and catecholase activities have been found for PPO in mushrooms (Long \textit{et al.}, 1971), grape (Harel and Mayer, 1971) and potato (Patil and Zucker, 1965).

The PPO catalyzed reaction is a non-radical process. In the oxidation of catechol by PPO, careful investigation of the radical kinetics have demonstrated that the rate of the radical formation is very much less than the rate of the enzyme reaction (Mason, 1979). The formation of free radicals is not due to the PPO activity, but due to rapid reverse dismutation reaction (Mason \textit{et al.}, 1961).
The catecholase activity is the most evident of the two activities of PPO. The quinones formed as products of this reaction are highly reactive compounds and lead finally to the formation of brown polymeric pigments, e.g. melanin. This reaction is normally referred to as enzymic browning and is responsible for the color deterioration of many fresh fruits and vegetables. Although browning is often considered detrimental in fruit and vegetable processing, it is considered essential in the manufacture of black teas, coffee, cocoa and cider.

The oxidation of phenolics may be catalyzed by either PPO or peroxidase, or may occur non-enzymatically (Loomis, 1969). The concentration of \( \alpha \)-quinones will vary depending on the level of either PPO or peroxidase present in the tissues, or on the enzyme extraction procedure used. Laccase is responsible for the formation of \( \beta \)-quinones (Broman, et al., 1963). In the case of peroxidase, the catechol derivatives can be oxidized in the presence of \( \text{H}_2\text{O}_2 \) to the corresponding \( \alpha \)-benzosemiquinone free radicals (Mason, 1979). These free radicals are highly reactive and can function as reductants or oxidants of other compounds present in the system (Ohnishi et al., 1969). In tissues containing high levels of peroxidase, measures against this possible reaction should be taken.

In general, PPO is the main agent causing browning in plant tissues. Some sources for which only PPO catecholase activity has been detected are carrot callus cells (Habaguchi,
1979), cherry (Benjamin and Montgomery, 1973), Concord grapes (Cash, et al., 1976) and peaches (Jen and Kahler, 1974).

**Assay methods for PPO activity**

Various methods have been developed for the measurement of PPO activity. These methods can be divided into two groups, one involving the measurement of oxygen consumption by the PPO reaction, and the second involving the measurement of the formation of the products of the reaction.

The direct measurement of oxygen consumption follows the oxidation of the phenolic substrates with the decrease of oxygen in solution. One disadvantage of this method is that it also measures the oxygen consumption due to secondary oxidation of the intermediates of the reaction during the formation of melanin. The activity measured will be therefore slightly higher than the actual PPO activity. This could be overcome by the addition of sufficient quinone scavenging agents to the reaction mixture.

There are two procedures that can be used to measure the oxygen consumption. One is the use of a Warburg apparatus or manometric method, and the second is the use of a Clark-type oxygen electrode, or polarographic method. In general, the polarographic method has given better results. The advantages of this method are that better initial rate curves are obtained and a lower concentration of enzyme is
required (Rivas and Whitaker, 1975). Only the initial rates of oxygen consumption are determined since the enzyme may undergo rapid inactivation during catalytic performance (Mayer and Harel, 1979).

The rates of the reaction can also be measured spectrophotometrically. These methods are very convenient and have been widely used. The initial rates of the reaction, that is, the formation of the \(\alpha\)-quinones, cannot be determined by these methods. In the presence of reducing agents or quinone scavengers the formation of color will be prevented, even though oxygen uptake still occurs, that is, the PPO remains still active. The rates of the reaction obtained are those of the melanin formation or polymerization reactions, and even these will vary since each substrate will form polymers with different absorption coefficients. The results obtained are difficult to compare (Walker, 1977).

An indirect spectrophotometric method has been developed by Pifferi and Baldassari (1973). In this method, the \(\alpha\)-quinones formed condensed with Besthorn's hydrazone to form a product that is read at 500nm after extraction with chloroform. The disadvantage of this method is that many measurements are required to follow the rate of the reaction.

Purr (1975) developed two "Testpapiere" (test-papers) which were used to detect either catecholase or cresolase activities from tissues, juices or extracts obtained from plants. The test-paper used for cresolase activity detection
was impregnated with α-naphthol and Merck color developer #3. Cresolase activity caused the formation of blue spots on the test-paper after application of the sample. The catecholase activity was detected using filter paper impregnated with catechol and Beethorn's hydrazone. In this case, the spots on the test-paper were red. A similar technique was used by Thomas et al. (1978) for the detection of PPO on thin-layer electrofocusing gels on Sephadex. The filter they used was impregnated with 1% substrate in methanol. Bruynicks et al. (1978) developed a specific assay for PPO detection. With this method, it was possible to locate the presence of PPO on gels by detecting the presence of copper. Levels as low as 0.1 nmoles of copper, either bound or free could be detected. Interference due to other copper-containing enzymes have to be taken into account.

**Substrate Specificity of PPO**

As explained above, the basic substrates for PPO are substituted mono and o-diphenols. The Km values of PPO for substrates are generally high, ca. 1.0mM. Lanzarini et al. (1972) suggested the formation of an enzyme-substrate complex with the direct participation of copper, in which the aromatic substrates with available pi orbitals can form a complex with the transition metal.

Catechol is structurally the simplest substrate for PPO, but the nature of and the position of any substituent groups have profound effects on the rate of substrate oxidation.
Substituents in the 3 position decrease the $K_m$ value of the substrate significantly. Substituents in the 4 position with a high electron-donating power increase the rate of oxidation of the substrates (Mayer and Harel, 1979). Examples of substrates in this group are chlorogenic acid, 4-methyl catechol and 3,4-dihydroxycinnamic acid. Substrates with an electron-attracting group at the p-position could lower the activity or inactivate PPO. In this group of compounds are 4-nitro catechol, protocatechuic acid and 2,3-dihydroxynaphthalene. Halim and Montgomery (1978) noticed a decrease in the activity of PPO with increasing size of the substituent group of the substrate except for chlorogenic acid, which showed a higher activity than caffeic acid.

According to Corse (1964), chlorogenic acid is the principal substrate present in natural form in many fruits, and many plant sources show highest PPO activity towards this compound. This was the case for potato (Abukharma and Woolhouse, 1966), sweet cherry (Lanzarini et al., 1972), carrot callus cells (Habaguchi, 1979), and eggplant (Knapp, 1965). Cling peaches (Luh and Phitakpol, 1972) and pear PPO (Rivas and Whitaker, 1973) showed highest activity towards catechin, which is also found naturally in fruits. Another substrate found naturally in fruits is dopa or dopamine, which serve as the best substrates for PPO from avocado (Kahn, 1976), parsnips (Kaldy and Markakis, 1972), banana (Palmer, 1964) and mango (Joshi and Shralkar, 1977). Broad
bean leaf (Robb et al., 1966), d'Anjou pears (Halim and Montgomery, 1978) and Concord grape (Cash et al., 1976) showed their highest activity towards catechol, while apple PPO (Stelzig et al., 1972; Walker and Wilson, 1975) was most active towards 4-methyl catechol.

Sakamura et al. (1966) claims that two PPO fractions could be extracted from eggplant, one fraction showing higher activity towards chlorogenic acid, while the second fraction could use eggplant anthocyanin as well as catechol for substrates.

Various phenolics that do not serve as substrates for PPO can act as synergists or inhibitors of the enzyme. Phloroglucinol, orcinol and resorcinol are examples of avocado PPO synergists, while p-coumaric acid, protocatechuic acid and p-cresol inhibit its activity (Golan et al., 1977). Indole-3-acetic acid was found to increase the activity of tobacco PPO (Vernon and Straus, 1972).

Coggon et al. (1966) observed that highly purified tea leaf PPO, prepared by adsorbing phenolics with PVPP, was capable of epimerizing tea flavonols at their C-2 position and that the highest activity of the purified PPO was towards tea flavonols. The substrate specificity was much greater than in crude preparations.

pH Optima of PPO

A wide range of pH optima has been reported for the PPO
of various plant materials. Values between pH 5.0 and 7.0 are most commonly found. The pH optimum for a particular PPO often varies with the substrate used (Mayer and Harel, 1979). Gregory and Bendall (1966) found a pH optimum for tea PPO of pH 5.7 using pyrogallol as a substrate and a pH optimum of pH 5.0 when 4-methyl catechol was used. They also reported that high levels of auto-oxidation of the substrates occurred at pH values higher than pH 6.0 and that auto-oxidation levels were prohibitely high above pH 6.6.

A possible reason for the differences in pH optima for the same PPO using different substrates could be that the pKₐ values of the substrates and the pH used for the assay affected the activity of PPO towards the different substrates. Another reason could be that the conformation of the active site at a certain pH is such that faster reaction rates are possible towards one substrate than others. It is also possible that more than one isozyme is present in the extract and that each isozyme is more active towards a specific substrate.

The presence of two pH optima was suggested for apple PPO by Harel et al. (1965), Pratt (1967) and Stelzig et al. (1972). Two pH optima were also reported for potato PPO (Abukharma and Woolhouse, 1966) and for grape PPO (Harel and Mayer, 1971). Other workers have reported a single pH optimum for potato PPO. Using chlorogenic acid as a substrate, Balasingham and Ferdinand (1970) found a pH optimum
for potato PPO of pH 5.0, while Amberger and Schaller (1975) reported that at a pH lower than 5.0 the activity of PPO is strongly inhibited.

The variability in the pH optima reported for the same PPO by different workers confirms the necessity of a careful evaluation of the data, and consideration of possible modification of the native PPO due to the lack of proper protection against phenolic-protein interactions during the extraction of the enzyme.

Optimum pH reported for sweet cherry PPO was 4.2 using catechol (Lanzarini et al., 1972); pH 4.5 with 4-methyl catechol for green olives PPO (Ben-Shalom et al., 1977a); between pH 5.5 and 6.5 with 4-methyl catechol for carrot callus cells PPO (Habaguchi, 1979); pH 6.2 and 7.0 with catechol, respectively, for Bartlett and d'Anjou pears (Tate et al., 1964; Halim and Montgomery, 1978); pH 7.0 with dopamine for banana PPO (Palmer, 1963); and pH 7.0 for cherry using catechol (Benjamin and Montgomery, 1973).

The Effect of Temperature on PPO

The rate of inactivation of PPO during reaction catalysis at a constant temperature has been shown to be different for every fruit and to follow first order kinetics. The action of temperature on the activity of PPO was affected also by the type of substrate used during inactivation (Mihalyi et al., 1978). These workers also observed a
increase in the activity of PPO from 15°C to 35°C for all the fruits they tested. They also concluded that there was variation in the results depending on the variety of the fruit used, the degree of development and also with different harvests of the same fruit. They concluded that the results were difficult to compare unless the same conditions of extraction and assay were always used.

Two other factors that have a great influence during heat inactivation are time and pH. Some half-lives reported for PPO were 8 min at 70°C for avocado PPO (Kahn, 1977); 15 min at 70°C for green olives PPO (Ben-Shalom et al., 1977a); and 11.7 min at 70°C, 6.25 min at 75°C, 2.25 min at 80°C and 1.1 min at 85°C for d'Anjou pears (Halim and Montgomery, 1978).

**Extraction of Enzymes from Plant Tissues**

Polyphenols, tannins, resins, terpenes and alkaloids do not exhibit an inhibitory effect in the living cell because they are separated from the surrounding cytoplasm (Swain, 1965). When this compartmentalization is broken during homogenization of the tissue, these compounds may come in contact with the enzymes and react or cause inhibition. This problem together with the low protein content found in plant cells as compared with animal cells poses a special problem if an active native enzyme is to be isolated from a plant source (Anderson, 1968; Locmis, 1969, 1974).
Sulfinic sulfinic acids; sulfones and sulfonamides derivatives.

Figure 1. Some of the main reactions and interactions of o-diphenols and o-quinones.
Reactions of o-diphenols.
Figure 1. Continued. Reactions of α-quinones.
Figure 1. Continued.

B Hydrogen bond formation with polyamides, peptide bonds of proteins, PVPP, etc. (Loomis, 1974).
C Hydrophobic interactions with lipids; hydrophobic sites of proteins; hydrophobic compounds like Amberlite XAD-4 as exemplified here; etc. (Loomis, 1974).
D Ionic interactions. (Loomis, 1974).
F Enzymic ring cleavage. (Kelly and Finkle, 1969).
G Reduction of o-quinones. (Harel et al., 1965).
K Reaction with thiols; amino acid residues, i.e. cysteine. (Pierpoint, 1966).
N Polymerization reactions of o-quinones and phenolic compounds. (Pierpoint, 1966).
R Base-catalyzed auto-oxidation of o-diphenols. (Loomis, 1974).
Reactions of Phenolic Compounds

Among the compounds that have the highest potential of inhibiting enzymes during extraction are the phenolic compounds. A general scheme of the main reaction of the phenolic derivatives is shown in Fig.1. There are two principal classes of plant phenolics: phenyl propanoid derivatives, including hydrolyzable tannins, and flavonoids, including condensed tannins (Loomis, 1974).

The most reactive form of the phenolics are the quinones and free radicals (Synge, 1975). They originate mostly by the action of PPO, but some can also originate by the action of laccase, peroxidase or by the auto-oxidation of phenolics at high pH. Quinones can undergo nucleophilic attack very easily with substrates possessing \(-\text{NH}_2, >\text{NH}, -\text{SH}\) or activated methylene groups, e.g. 1.4-addition (Pierpoint, 1970; Loomis and Battaile, 1966).

When the quinones react with amino or sulfhydryl groups of amino acid residues of enzymes, stable bonds are formed which can lead to inactivation or modification of the enzyme (Anderson, 1968). Even PPO can be inactivated by these reactions (Mayer and Harel, 1979) (K,L, and M; Fig.1). Firenzuoli et al. (1969) and Goldstein and Swain (1965) proved that the protein-tannic acid interaction lead to the formation of complexes and to total inhibition of enzymes. Loomis and Battaile (1966) found that extracts of peppermint leaves underwent rapid browning if prepared conven-
tionally, and only PPO activity could be detected.

In the case of fruits and vegetables containing large amounts of phenolics and high PPO activity, the reactions of quinones with proteins may lower their nutritional value (Pierpoint, 1970). This could be the case in the browning of whole wheat meal flour of Mexican wheat, where browning by PPO occurs readily (Singh and Shedran, 1972). Synge (1975, 1976) studied and reviewed in depth the interactions of polyphenols with plant proteins and the damage to the nutritional value of plant proteins caused by these reactions both during processing and storage.

In addition to covalent addition of quinones to proteins there also exists the possibility of interactions of unoxidized phenolics with the proteins. Three kinds of interactions can occur (Loomis, 1974). The peptide bonds can form very stable hydrogen bonds with the hydroxyl groups of the polyphenols (B; Fig.1). At high pH levels, i.e. above 8.45, the phenolic hydroxyl groups and carboxyl groups (i.e. cinnamic acids) may become ionized and form salt linkages with the basic amino acid residues in the so-called ionic interactions (D; Fig.1). The phenolics possess in their structure hydrophobic aromatic rings which can interact with the hydrophobic regions of proteins, with membrane lipids or with other hydrophobic compounds, e.g. polar solvents (G; Fig.1).

Quinones can also undergo condensation and polymeriza-
tion reactions (L, M, and N; Fig.1). The complexes formed between phenolics and the proteins are often characterized by a brown color, and sometimes a dark precipitate is formed. Crosslinking of proteins by quinones with a second reactive group can also occur during these reactions (N; Fig.1) (Loomis, 1969).

Krueger (1955) noticed that the reaction of quinones with \( \beta \)-ketoacids resulted in an increase in the rate of oxidation of the substrates, as well as, an increase in the pigmentation and the final decarboxylation of the \( \beta \)-ketoacids (P; Fig.1).

Another reaction that can result is the loss of the color of some fruits by the degradation of their anthocyanin pigments. Markakis (1974) and Peng and Markakis (1963) suggested that the degradation was due to the oxidation of the anthocyanin pigments by the action of quinones (O; Fig.1). Sakamura et al., (1966) found that a PPO from eggplants was able to decolorize the anthocyanins directly.

Overcoming the Problems Caused by Phenolics

Since the production of phenolics is a function of the growing conditions, as well as the species and variety of plant, any one procedure for enzyme extraction cannot be expected to be equally effective with all plant materials (Loomis, 1969). The best results have been obtained from the 'cleanest' extracts prepared with the fewest inhibitory chemicals and simplest extraction procedures (Kelley and
Table 1. Techniques used to prevent the formation of quinones during the extraction of enzymes from plant tissues.

<table>
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<tr>
<th>ANAEROBIC CONDITIONS</th>
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<td>Inert atmospheres</td>
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<td>Liquid nitrogen homogenization</td>
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<th>PREVENTION OF AUTO-OXIDATION OF PHENOLIC COMPOUNDS</th>
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<td>Use of low pH</td>
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<tr>
<th>REMOVAL OF POLYPHENOLS</th>
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<tr>
<td>Solvent extraction of polyphenols</td>
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</tr>
<tr>
<td>Methyl alcohol</td>
<td>Diethyl ether</td>
</tr>
<tr>
<td>Isopropyl alcohol</td>
<td>n-butanol</td>
</tr>
<tr>
<td>Tertbutyl alcohol</td>
<td>Acetone</td>
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| Enzymic modification of o-diphenols | |
| Protocatechuate-3,4-dioxygenase | |
| O-methyl transferase | |

| Polymeric protective agents | |
| Inert proteins: BSA, hide powder, gelatin, collagen | |
| Polyamides: Nylon 6, Nylon 66 | |
| Polyethylene glycol | |
| Polyvinyl(poly)pyrrolidone | |
| Non-ionic adsorbents: Amberlite XAD-2, XAD-4, XAD-7; Polyacrylamide | |
| Ion-exchange adsorbents: Dowex AG 1-X8, AG 2-X8; Amberlite IRA 938 | |

| COVALENT CONDENSATION OF o-DIPHENOLS | |
| Borate | Germanate | |

| REMOVAL OF QUINONES | |
| Reduction of quinones | |
| Ascorbic acid | Dithionite | Mercaptobenzothiazole |
| Thioglycollate | Glutathione | |
| Metabisulfite | Cysteine | |
| Trapping of quinones | |
| Cysteine | Benzenesulfonic acid | |
| Metabisulfite | Diethylidithiocarbamate | |

| INHIBITION OF POLYPHENOL OXIDASE | |
| Inhibitors affecting the prosthetic group of PPO | |
| Diethyldithiocarbamate | Thiosulfate | |
| Ethylxanthate | Dithiothreitol | |
| Mercaptobenzothiazole | Potassium cyanide | |
| Phenylthiourea | Carbon monoxide | |
| Mercaptoethanol | EDTA | |
| Inhibitors affecting the binding site for the substrates | |
| 4-chlorophenol | p-coumaric acid | |
| 4-chlororesorcinol | Protocatechuic acid | |
| 4-nitrocatechol | Ferulic acid | |
| 2,3-naphtalenediol | Fisetin | |
| Benzoic acid | t-cinnamic acids | |
| p-nitrophenol | |
Adams, 1977), requiring the fewest steps performed in the fastest way possible.

The first step in the isolation of enzymes from plant tissues involves the homogenization of these tissues. This is the most critical step and measures must be taken to avoid the modification and/or inhibition of the enzymes at this stage. The objective of all techniques is to remove secondary products as quickly as possible while preventing the formation of covalent complexes between phenolics and enzymes (See Table 1).

The formation of quinones requires oxygen. It is useful, therefore, to maintain an inert atmosphere by homogenizing the sample in liquid nitrogen. Liquid nitrogen not only provides an oxygen-free atmosphere, but it also cools the extracts and allows the fracture of tough tissues (Kelley and Adams, 1977). An inert atmosphere can be obtained by use of nitrogen gas in chambers or glove boxes, or argon in an open box, but liquid nitrogen provides additional advantages.

The control of the pH of the extracts is also of great importance. High pH levels are in general undesirable because they increase the ionization of phenolic hydroxyl groups and thus promote auto-oxidation of the phenolics to quinones (R; Fig.1) (Loomis, 1974). Base-catalyzed oxidation of o-diphenols was high above pH 6.0 and prohibitely high above pH 6.6 (Gregory and Bendall, 1966). A high pH also increases
the proportion of protein amino groups in the reactive \(-\text{NH}_2\) form, making them more susceptible to addition reactions (L,M; Fig.1).

**Removal of Polyphenols**

**Organic Solvents**

Many workers have used organic solvents for the removal of phenolics. The increasing degree of phenolic-stripping ability for some solvents used is: methyl-, isopropyl-, tertbuthyl-alcohols; methyl cellosolve; methyl cellosolve acetate; acetone and dioxane (Loomis and Battaile, 1966). Kelley and Adams (1977) used n-butanol and diethyl ether to remove phenolics.

**Polymeric Protective Agents**

The use of polymeric agents takes advantage of the ability of polyphenols to form complexes with other compounds by binding with them through hydrogen bonds, hydrophobic or ionic interactions or by a combination of any or all of these reactions.

**Inert Proteins**

The interaction between proteins and phenolics has been described above and the formation of complexes explained. Many workers have, therefore, added inert proteins to the extraction media to bind the polyphenols. Bovine
serum albumin (BSA), collagen and hide powder have been most widely used (van Sumere et al., 1975). BSA has been found to bind phenolics through hydrophobic interactions or by hydrogen bonding with the peptide bonds. Quinones are also bound through reactions with the amino or sulfhydryl groups of the amino acid residues (Loomis, 1974; Pierpoint, 1969). Using collagen in the preparation of extracts from peppermint leaves resulted in active enzymes (Loomis and Battaile, 1966).

Polyamides

Polyamides are polymers rich in -CO-NH- groups and are, therefore, able to form complexes with the phenolics through hydrogen bond formation (B; Fig. 1). Sanderson (1964) was able to obtain soluble PPO from tea leaves using polycaprolaktam, (Nylon 6) to absorb phenolics.

Polyethylene Glycol

Polyethylene glycol is a polymer with a \((-\text{CH}_2\text{-O-CH}_2\text{-O-})_n\) structure that binds phenolics through hydrogen bond formation. The bonds formed with PEG are presumably weaker than those formed with the N-substituted amides, i.e. polyamides or peptide bonds. Benjamin and Montgomery (1973) obtained extracts with high PPO activity using PEG during the extraction, but they observed that much of the PEG was retained in the extract. The excess
PEG was removed by the preparation of an acetone powder with a retention of a high specific activity. Badran and Jones (1965) used PEG to bind high molecular weight phenolics during the extraction of PPO from bananas. Montgomery and Sgarbieri (1975) obtained better extracts using PVPP than using PEG.

Polyvinyl(poly)pyrrolidone

This polymer contains -CO-N\textless groups that are analogous to the peptide bonds of proteins. This group is a strong proton acceptor and forms stable H-bonded complexes with phenolic -OH groups (Loomis and Battaile, 1966; Loomis, 1969, 1974) (Fig. 2). Two forms are available, water-soluble polyvinylpyrrolidone, PVP; and insoluble cross-linked polyvinyl(poly)pyrrolidone, PVPP or Polyclar AT. PVP(P)'s are functionally related to the polyamides, but have found much wider application.

The amounts of phenolics bound to PVP(P)'s decrease as the pH increases (Loomis, 1974; Andersen and Sowers, 1968). An optimum pH of 3.5 for PVPP-phenolic binding was reported by Andersen and Sowers (1968) but Loomis (1974) pointed out that the actual difference between the binding at pH 6.0 and pH 3.5 was very small.

Several workers reported low binding levels of some phenolics to PVP(P)'s. Phenolics such as catechol derivatives and tyrosine, could not be removed effectively using PVPP (Loomis et al., 1979). Gray (1978) noticed that PVPP
showed low affinity for chlorogenic acid but a high affinity for quercetin, catechin and proanthocyanin. Lam and Shaw (1970) suggested that PVPP bound only those phenolics of molecular weight higher than chlorogenic acid.

Siegel and Enns (1979) noticed that soluble PVP adsorbed the excess polyphenols from the medium of a soybean-cell culture, thus preventing discoloration and cell aggregation. Jones et al. (1965) obtained near maximum activity for all the enzymes other than PPO using PVP, MW ca. 28,000 during their extraction from apple peel mitochondrial fraction.

Inhibition of PPO when soluble PVP was used was also noticed during the extraction of PPO from potatoes (Abukharma and Woolhouse, 1966), apples (Walker and Hulme, 1965; Harel et al., 1964) and green olives (Ben-Shalom et al., 1977a).

Insoluble PVPP is being used more widely than water-soluble PVP. It has the advantage that it can be easily removed from the extracts. Good removal of phenolics and extracts with high enzyme activity were obtained by Kelley and Adams (1977), Schneider and Hallier (1970) and Ulbricht and Zenk (1979) using PVPP. Benjamin and Montgomery (1973), Chan and Yang (1971), Halim and Montgomery (1978) and Kaldy and Markakis (1972) obtained extracts with high activity during the extraction of PPO. No inhibition of PPO by PVPP was reported by these authors; however, inhibition of PPO
when high concentrations of PVP or PVPP were used has been reported (Ben-Shalom et al., 1977a; Walker and Hulme, 1965).

Non-ionic Macrocreticular Resins

Among the non-ionic polymers, Amberlite XAD-2, XAD-4 and XAD-7 have been used as polyphenol binders. Of these polymers, Amberlite XAD-4 has found widest application.

XAD-4 is a styrene-divinylbenzene polymer with a surface area of 725 m² g⁻¹ dry wt., and average pore size of 40 Å (Rohm and Haas, 1978). Binding of phenolics occurs by interactions between the polymer surface and hydrophobic portions of the polyphenols (Loomis 1969, 1974; Rhodes, 1977; Loomis et al., 1979) (Fig. 2). Neither BSA nor horseradish peroxidase treated with XAD-4 showed adsorption to this resin (Loomis et al., 1979).

Rhodes (1977) showed that the Amberlite resins were able to remove phenolics such as chlorogenic acid and tannins (tannic acid) from solutions. XAD-4 binds terpenoids as well as other hydrophobic and surface active materials (Loomis et al., 1979). Thus, XAD-4 and PVPP appear to complement each other in the removal of high and low molecular weight phenolics, but the relative contribution of the two adsorbents vary from one tissue to another (Loomis et al., 1979).

Consistent isoelectric focusing patterns for horseradish peroxidase were obtained using dried XAD-4 to adsorb isothiocyanate vapors by Loomis et al. (1979). Amberlite
Proposed mode of action:

-CH₂CH⁻\text{hydrogen bond formation (\ldots)}

a) POLYCLAR AT (PVPP); water insoluble polyvinylpyrrolidone.

Proposed mode of action:

-CH₂CH⁻\text{hydrophobic interactions}

b) AMBERLITE XAD-4 (XAD-4); polystyrene resin.

c) DOWEX AG 2-X8 (DOWEX 2); polystyrene-quaternary ammonium cation exchange resin.

Figure 2. Phenol adsorbents. General structures and proposed mode of action during the adsorption of polyphenols.
XAD-2 was effective in the removal of polyphenols from spinach, bean and tobacco leaves (Gray, 1978).

Ion-exchange Resins

The use of ion-exchange resins for the removal of phenolics is not as widespread as the use of the other adsorbent polymers. Of the ion-exchange resins, Dowex AG 1-X8 and Dowex AG 2-X8 have been used most. Both Dowex resins contain quaternary ammonium groups on a styrene-divinylbenzene matrix. These resins bind phenolics by a combination of ionic and hydrophobic interactions, with probably some H-bonding in the case of Dowex 2 (Loomis et al., 1979) (Fig. 2). High pH levels as those used by Lam and Shaw (1970) and Gray (1978), pH 8.3 and 7.5, respectively, are not necessary to bind plant phenolics by Dowex resins (Loomis et al., 1979). Fasold et al. (1961) observed that at pH levels of 5.0 to 5.8 or lower, there seemed to be non-specific binding of many proteins to ion-exchange resins. It is suggested that protonation of protein carboxyl groups permits partial unfolding of the protein, and hydrophobic adsorption of the unfolded protein on the polystyrene surface may occur (Loomis, 1980).

Dowex 1 and 2 are more efficient in binding chlorogenic acid and quercetin than PVPP or XAD-4 at pH 6.0 with Dowex 2 being more efficient than Dowex 1 (Loomis et al., 1979). Smith (1980) obtained the most effective extraction and the
lowest number of isozyme bands of PPO from pears using Dowex 2, followed by Dowex 1, XAD-1, and PVPP in decreasing order of efficiency.

Rhodes (1977) tested a series of macroreticular ion-exchange resins for their ability to bind polyphenols. These resins are similar to XAD-1 and XAD-2 but have in addition charged groups attached to them. These characteristics combine the adsorptive capacity of the ion-ionic adsorbents with the ion-exchange properties of agents such as Dowex 1 or 2. From all the resins tested in this group, Amberlite IRA-938, a strongly basic cation exchanger, was the most effective, and prevented browning in extracts of potato tubers. There was no significant inhibition of phenolase, and thus the effect must be in removing endogenous phenolics from solutions.

Covalent Condensation of o-Diphenols

Some compounds are able to react with o-diphenols and form stable covalently-bound complexes. Among these agents, borate and germanate have been shown to yield good results. The basic mode of action is similar for both compounds, which is to inhibit the action of the phenolase complex by binding to o-diphenols, forming a covalently bound phenol-borate or phenol-germanate ester complex. This complex is not a substrate for PPO (Kelley and Adams, 1977) (H; Fig. 1). Borate and germanate show the most effective binding at pH
7.5. The complexes formed with germanate have twice the stability of those formed with borate (Loomis, 1974). Loomis and Ewing (1979) reported effective and specific binding of polyphenols from plant extracts and standards using pure proteins and phenolics through the use of a boronated resin (Affi-Gel 601, Bio-Rad Chemical Co.) in an affinity column.

**Removal of Quinones**

The formation of quinones in an extract containing polyphenols and having high PPO activity can occur even if the protective measures described above have been taken. It is, therefore, desirable to add an agent to the extract that will react with the quinones and avoid the inhibition or modification of enzymes (Pierpoint, 1970; Loomis and Battaille, 1966). Two main procedures can be used for this purpose:

**Reduction of Quinones**

Reducing compounds can be oxidized by the quinones and the o-diphenols regenerated (G; Fig.1). Ascorbic acid is a reducing agent that is very widely used and has been included during enzyme extraction to create a reducing medium and avoid the accumulation of quinones (Harel et al., 1965). Thus, ascorbic acid, as many other reducing agents, acts mainly as a quinone scavenger by reversing the reaction of PPO, but does not generally inhibit PPO. This characteristic
makes ascorbic acid very useful in the prevention of enzymic browning of fruits and vegetables. Loomis and Battaile (1966) suggested that the use of reducing agents in extracts could be harmful since in the presence of oxygen, they could activate the \( \alpha \)-hydroxylation of monophenols by PPO.

Other reducing agents that have been used are thio-glycollate (Anderson, 1968); metabisulfite (Anderson, 1968; Kelley and Adams, 1977; Haisman, 1974; Kahm, 1977; and Muneta and Wang, 1977); dithionite (Rhodes, 1977); mercaptobenzothiazole (Anderson, 1968; Loomis, 1974; Mayer and Harel, 1979; Palmer and Roberts, 1967; and Stelzig et al., 1972) and mercaptoethanol (Loomis and Battaile, 1966).

Reduced glutathione (Mayer and Harel, 1979) and cysteine (Cys) (Pierpoint, 1970) can also reduce quinones. Most of the reducing compounds mentioned can also inhibit the PPO reaction by trapping the quinones through covalent bond formation.

**Trapping of Quinones**

Some agents react with quinones forming stable complexes that are no longer reactive and thus protect enzymes from inactivation (Loomis and Battaile, 1966; Pierpoint, 1966). The most representative compounds having this characteristic are cysteine (Cys) and benzenesulfinic acid (Bsu).
When Cys or Bsu is used during the spectrophotometric assay of PPO activity there is a lag period before the formation of brown pigments can be observed (Kahn, 1977; Benjamin and Montgomery, 1973; and Pierpoint, 1970). The quinones formed through PPO activity react with Cys or Bsu forming stable covalent complexes, in the case of Cys thioethers (Loomis and Battaile, 1966) and with Bsu, phenylsulfonyl derivatives (Davies and Pierpoint, 1975) (J,K; Fig.1). When Cys or Bsu is present in excess all quinones will be complexed immediately after formation, thus no browning will occur and the solution will remain clear. The reaction is restricted to the oxidation of o-diphenols to o-quinones, and no secondary reactions will occur. After all the Cys and Bsu present have reacted with the quinones, secondary reactions leading to browning take place.

Lower levels of Bsu or Cys will shorten the lag period and browning will occur sooner. When Cys is used in low concentrations in the extracts, oxygen uptake is increased, and the reaction in these conditions may be a reduction of the quinones by the -SH groups, but it may also be a reaction of the type between quinones and amino groups and sulfhydryl groups of amino acids or proteins. This would be expressed as an increase in the oxygen uptake (Pierpoint, 1966) and with the formation of brown pigments (Mathew and Parpia, 1971).

Other thiols act similarly (Bouchilloux, 1962).
According to Walker (1977), the chemical combination of thiols with oquinones occurs only when the molar ratio exceeds a critical value of 1.5 thiol to 1.0 quinones.


The real mechanism of action of metabisulfite is not known. Sulfite has been shown to react with the substrates, quinones or intermediary products of the reaction (Embs and Markakis, 1965) (I, I'; Fig.1). The action of sulfite is pH dependent (Muneta and Wang, 1977). The bleaching effect of sulfite appears to be counteracted when ascorbic acid is used at the same time and the browning reactions are accelerated in this case (Haisman, 1974).

Modification of Phenolics

The modification of the phenolic substrates of PPO with the use of enzymes has also been suggested. The enzymatic cleavage of the aromatic ring is possible through the use of a specific oxygenase, i.e. protocatechuate-3,4-dioxygenase (Kelley and Finkle, 1969) (F; Fig.1). The methylation of the hydroxyl groups is also possible using O-methyl transferase (Walker, 1977) (E; Fig.1). These modified phenolics
are not substrates for PPO.

Inhibition of PPO

PPO is the main source of quinones, and the direct inhibition of this enzyme should lower the production of quinones during the extraction of enzymes from plant tissues. Of course, if PPO is the enzyme that is to be extracted, the production of quinones should be prevented using mechanisms other than inhibition, unless this inhibition could be reversed.

The PPO's obtained from different plant tissues may react in a different manner to the same inhibitor (Mayer and Harel, 1979). The conditions of assay, as well as the type of substrate used, affect the degree and type of inhibition of PPO.

Inhibitors Affecting the Prosthetic Group of PPO

The prosthetic group of PPO is copper, and agents that are good copper chelators inhibit PPO action. Many thiols function as copper chelators and a high degree of inhibition was obtained when Dieca was used (Mayer and Harel, 1979; Kelley and Adams, 1977; Habaguchi, 1979; and Loomis, 1969). Anderson (1968) reported a loss in the efficiency of the action of Dieca when the pH exceeded pH 7.5. Halim and Montgomery (1978) and Gregory and Bendall (1966) noticed a lag period when Dieca was used at low concentrations.
Other sulfur compounds that inhibit PPO by chelating the copper ion are ethylxanthate (Pierpoint, 1966; Anderson, 1968; Rhodes, 1977); dithiothreitol (Habaguchi, 1979; Rhodes, 1977); mercaptobenzothiazole (Rhodes, 1977; Loomis, 1974); phenylthiourea (Mayer and Harel, 1979; Mayer, 1962); and tiosulfate (Harel and Mayer, 1979) among others.

EDTA showed different degrees of inhibition of PPO. Palmer (1964) and Cash et al. (1976) found that EDTA strongly inhibited the PPO from bananas and Concord grapes, respectively. Luh and Phithakpol (1978), and Wong et al. (1971) reported low inhibition of peach PPO by EDTA.

Potassium cyanide and carbon monoxide were reported to bind to the copper ion of PPO and to compete with oxygen (Mayer and Harel, 1979). Anderson (1968) reported that potassium cyanide was not totally ionized at pH 5.2 or lower and that only little inhibitory effect towards PPO existed. But levels of potassium cyanide equal to or higher than 1mM showed complete inhibition of tea PPO (Gregory and Bendall, 1966).

**Inhibitors Affecting the Binding of Substrates by PPO**

Another group of PPO inhibitors are compounds that compete with the phenolic substrates for the active site on PPO. Duckworth and Coleman (1970) found that benzoic acid was competitive to phenolics. Balasingam and Ferdinand (1970) used benzoic acid to inhibit reversibly the PPO of potatoes during extraction and noticed that some darkening still
occurred. Kuttner and Wagreich (1953) listed a series of 40 inhibitors of PPO related to benzoic acid derivatives, benzyl alcohol derivatives and aliphatic alcohols. These workers noticed that all good inhibitors had the benzene ring moiety in common. 4-chloro resorcinol, 4-chlorophenol, 4-nitro catechol, 2,3-naphthalenediol and phenylhydrazine showed competitive inhibition of PPO (Mayer and Harel, 1979; Cash et al., 1976; Lerner et al., 1971; Mayer et al., 1964 and Pifferi et al., 1974).

Some phenolics naturally present in plants also inhibit the action of PPO. Examples of these are fisetin, p-coumaric acid, protocatechuic acid, ferulic acid and t-cinnamic acids (Kahn, 1976; Macrae and Duggleby, 1968; Mapson, 1970; Shannon and Pratt, 1967; Pifferi et al., 1974; Walker and Wilson, 1975; Walker, 1976, 1977). Pifferi et al. (1974) found that aromatic carboxylic acids inhibited PPO better than their corresponding aldehydes and that esterification lowered their inhibitory strength.

Purification of PPO

PPO is claimed to be present in plants in soluble or particulate forms. In the case of soluble PPO, purification of the enzyme can proceed as in the case of enzymes extracted from animal tissues, after measures against the action of phenolics have been taken. Acetone or ammonium sulfate precipitation, gel filtration and ion-exchange chromatography
are methods that have been used for the purification of soluble PPO (Rhodes, 1977; Ben-Shalom et al., 1977a; Benjamin and Montgomery, 1973; Flurkey and Jen, 1979a; Wisseman, 1980).

Another technique that has been recently used for the purification of PPO is affinity chromatography. Gutteridge and Robb (1975) used 4-aminobenzoate attached to Sepharose-4B resin with a C7 spacer arm. The benzoate moiety functioned as a specific competitive inhibitor of PPO and a 70% recovery of this enzyme was possible. O'Neal et al. (1973) used dopamine bound to the sepharose 4B beads and obtained a 10- to 14-fold purification of PPO, but the cycles of use of the column were limited due to enzymatic oxidation of the dopamine. Flurkey (1978) and Flurkey and Jen (1979a,b) used columns of Phenyl-Sepharose Cl-4B in the isolation of peach PPO. Isolation in this case was through hydrophobic interactions between the column and the peach PPO. Highly purified PPO from grapes was obtained by Wisseman (1980) using this technique.

Coggon et al. (1973) obtained 200-fold purification of PPO from tea leaves using isoelectric focusing.

It is important first, to evaluate if the presence of particulate PPO is not due to modification of the soluble PPO by the action of the polyphenols during the extraction. In many reports, this had been the case. The extraction of tea leaf PPO is an example. Li and Bonner (1947) used an
extraction procedure that did not involve protection of proteins against the action of endogenous phenolics. They claimed that PPO was associated with the tea leaf "chloroplasts". Later work by Sanderson (1964) and Coggon et al. (1973) demonstrated clearly that with the use of phenol adsorbents during the extraction of PPO, it was possible to extract soluble PPO from the tea leaves. The lack of protection against the action of phenolics was the cause of the precipitation of PPO due to the formation of phenolic-protein complexes which were in many occasions reported as particulate PPO but were indeed artifactual organelles formed during the extraction.

If PPO is found in the particulate form, solubilization is necessary before proceeding to purification steps. Kato et al. (1976) found that PVPP protected the organelle integrity during homogenization and that high levels of PVPP increased the concentration of PPO in the light fraction of the sucrose gradient. Mayer (1966) used proteolytic enzymes to liberate PPO from sugar beet "chloroplasts" and obtained a 4-fold activation of PPO. The most effective enzyme combination was trypsin plus carboxypeptidase A.

Palmer (1963) used detergents to solubilize bound PPO of bananas, and Stelzig et al. (1972) and Walker and Hulme (1966) obtained good solubilization of apple PPO using Triton X-100. Walker and Hulme (1965) reported that anionic detergents could reverse the inhibition of PPO caused by
Table 2. Chemical composition of strawberries with emphasis on the Tioga variety.

<table>
<thead>
<tr>
<th>Component</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>3.54</td>
</tr>
<tr>
<td>Water content</td>
<td>3.54</td>
</tr>
<tr>
<td>Total solids</td>
<td>10.20 %w/w</td>
</tr>
<tr>
<td>Soluble solids</td>
<td>7.80 %w/w</td>
</tr>
<tr>
<td>Insoluble solids</td>
<td>2.40 %w/w</td>
</tr>
<tr>
<td>Proteins</td>
<td>0.68 g%</td>
</tr>
<tr>
<td>Lipids</td>
<td>0.47 g%</td>
</tr>
<tr>
<td>Total carbohydrates</td>
<td>8.06 g%</td>
</tr>
<tr>
<td>Pectin (as Ca-pectate)</td>
<td>0.54 %w/w</td>
</tr>
<tr>
<td>Reducing sugars</td>
<td>4.13 %w/w</td>
</tr>
<tr>
<td>Sucrose</td>
<td>0.87 %w/w</td>
</tr>
<tr>
<td>Free amino acids</td>
<td>442.00 μM%</td>
</tr>
<tr>
<td>Calcium</td>
<td>16.00 mg%</td>
</tr>
<tr>
<td>Magnesium</td>
<td>11.00 mg%</td>
</tr>
<tr>
<td>Manganesium</td>
<td>7.10 mg%</td>
</tr>
<tr>
<td>Sulphur</td>
<td>13.40 mg%</td>
</tr>
<tr>
<td>Sodium</td>
<td>0.88 mg%</td>
</tr>
<tr>
<td>Potassium</td>
<td>186.00 mg%</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>31.00 mg%</td>
</tr>
<tr>
<td>Copper</td>
<td>0.80 mg%</td>
</tr>
<tr>
<td>Iron</td>
<td>1.30 mg%</td>
</tr>
<tr>
<td>Zink</td>
<td>1.70 mg%</td>
</tr>
<tr>
<td>Boron</td>
<td>1.20 mg%</td>
</tr>
<tr>
<td>Organic acids and Phenolics</td>
<td></td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>18.80 mg%</td>
</tr>
<tr>
<td>Citric acid</td>
<td>0.92 %w/w</td>
</tr>
<tr>
<td>Malic acid</td>
<td>0.09 %w/w</td>
</tr>
<tr>
<td>Total phenolics (as Tannic acid)</td>
<td>312.00 mg%</td>
</tr>
<tr>
<td>Anthocyanins</td>
<td>27.60 mg%</td>
</tr>
<tr>
<td>Leucoanthocyanins (as Abs.Units)</td>
<td>565.00 AU%</td>
</tr>
<tr>
<td>Flavanols (as Abs.Units)</td>
<td>87.60 AU%</td>
</tr>
<tr>
<td>Flavonols (+)-O-catechin</td>
<td>1.70 mg%</td>
</tr>
<tr>
<td>(-)-O-epicatechin</td>
<td>1.00 mg%</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>1.00 mg%</td>
</tr>
<tr>
<td>p-coumaric acid</td>
<td>1.50 mg%</td>
</tr>
<tr>
<td>4-hydroxybenzoic acid</td>
<td>1.30 mg%</td>
</tr>
<tr>
<td>Protocatechuic acid</td>
<td>0.60 mg%</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>1.40 mg%</td>
</tr>
<tr>
<td>Methyl gallate &amp; Ellagic acid</td>
<td>1.00 mg%</td>
</tr>
<tr>
<td>Chlorogenic &amp; Neochlorogenic acids</td>
<td>Traces</td>
</tr>
<tr>
<td>Salicylic acid</td>
<td>Traces</td>
</tr>
<tr>
<td>Gentisic acid</td>
<td>Traces</td>
</tr>
<tr>
<td>Vanillic acid</td>
<td>Traces</td>
</tr>
</tbody>
</table>

1 - Values specific for strawberries of the Tioga variety.
2 - %w/w = for fresh fruit weight.
3 - g%, mg%, μM%, AU%= g, mg, μM, AU / 100 g fresh fruit.
4 - Green, 1971.
5 - Adams, 1975.
7 - Stöhr and Herrmann, 1975.
soluble PVP. Goldstein and Swain (1965) reported a similar reversion of inhibition caused by tannic acid with the use of cationic or non-ionic detergents. Similar results were obtained by Firenzuoli et al. (1969) using Tween 80 or PVPP to reverse the inhibition caused by tannic acid. In some of these reports, the particulate PPO reported could actually be artifactual "bound" PPO formed during the extraction.

The Strawberry System

The degradation of the red color of strawberries has been a problem for the food industry for a long time. Color degradation can occur during harvesting, storage and processing of the fresh strawberries, and various mechanisms for the color loss have been postulated. Knowing and evaluating the importance of each of these mechanisms becomes very important if the color degradation is to be prevented.

Chemical Composition of Strawberries

The Tioga variety of strawberries was used for the present work. The partial chemical composition of strawberries is listed on Table No.2. Not all the data listed are for the Tioga variety, but the figures given are in the range of values naturally found for all strawberry varieties.

Strawberry Phenolic Compounds

Phenolics are present in relatively large concentrat-
Figure 3. Structures of some phenolic compounds present naturally in strawberries.
ions in strawberries. These phenolics are divided into two main groups, the flavonoid derivatives with a C₆-C₃-C₆ skeleton, and the hydroxycinnamic acid derivatives with a C₆-C₃ skeleton (Herrmann, K. 1976; Co and Markakis, 1968).

Among the flavonoids, leucoanthocyanins, anthocyanins (Acn) and flavanols are present in the highest concentrations (Abers and Wrolstad, 1978; Stohr and Herrmann, 1975). The hydroxycinnamic acid derivatives most commonly found are p-coumaric acid and caffeic acid in measurable amounts, and chlorogenic and neochlorogenic acids in trace amounts.

The flavonoids and hydroxycinnamic acid compounds are present in the form of glycosides or esters in the intact cell (Ryan, 1971; Co and Markakis, 1968).

Some phenolic compounds derived from the C₆-C₃ derivatives are also present naturally in strawberries. Stöhr and Herrmann (1975) reported the presence of 4-hydroxybenzoic acid, protocatechuic acid, methyl gallate, gallic acid and ellagic acid in measurable amounts; and salicylic acid, gentisic acid and vanillic acid present only in traces. The structures of the main phenolics present in strawberries are shown in Fig.3.

**Phenolics, PPO and Strawberry Color Deterioration**

The red color of strawberries deteriorates very rapidly after harvesting. This is mainly due to the high instability of the Anthocyanin (Acn) pigments (Cash and Sistrunk, 1971;
Markakis et al., 1957). The main Acn pigment present is pelargonidin glycoside with cyanidin glycoside present in much lower concentrations (Markakis et al., 1957; Co and Markakis, 1968) (Fig. 3).

Many factors cause the degradation of Acn. Hydrolysis to the aglycones lowers the stability of the pigments (Chichester and McFeeters, 1971). Enzymes such as the fungal glycosidases or anthocyanases, have been reported to cause such hydrolysis (Pifferi and Cultrera, 1974). High acidity and heat are also causes of hydrolysis of Acn.

Acn can be degraded more easily with high temperatures (Adams and Ongley, 1973; Kertesz and Sondheimer, 1948). Stability is dependent upon the pH of the medium and low pH's, at which the ionic form is predominant, increase the stability of the Acn (Chichester and McFeeters, 1971). In the presence of heavy metals, Mg, Fe, Sn or Al, Acn form complexes and undergo a bathochromic shift or decoloration (Markakis, 1974).

Degradation of Acn occurs also through oxidation. The degradation occurs mainly through the rupture of the flavilium structure with the formation of ketones. Ascorbic acid, in the presence of hydrogen peroxide, will cause degradation (Markakis et al., 1957). Poei (1980) reported that added ascorbic acid could accelerate the destruction of the Acn through oxidation or condensation reactions both in aerobic and in nitrogen atmospheres. Oxidized sugars, in particular fructose, i.e. hydroxymethyl furfural and
related compounds, also degraded Acn pigments (Sistrunk and Cash, 1970; Markakis, 1974).

Bleaching of Acn with SO$_2$ may be reversible or irreversible with the probable formation of a colorless chromen-2 (or -4) -sulfonic acid with similar structure and properties as an Acn pseudobase (Markakis, 1974).

Condensation of Acn with other phenolics can also cause a color loss. This copolymerization can occur between the Acn and other flavonoids, mainly leucoanthocyanins (Markakis, 1974). Co and Markakis (1968) found that the class of leucoanthocyanins present in the highest concentration in strawberries of the Midway variety, were those with the highest solubility in water and not extractable by ethyl acetate.

Abers and Wrolstad (1978) compared the degree of color degradation of strawberry preserves made from Tioga and Hood varieties with respect to their phenolic content and PPO activity. They found that in the Tioga preserve, color deterioration occurred at much faster rates due to a faster rate of browning. The Tioga variety contained higher levels of leucoanthocyanins, flavonols and total phenolics, while the Hood variety contained higher levels of anthocyanins, ascorbic acid and free amino acids. Abers and Wrolstad (1978) concluded that the loss of color was mainly due to the formation of brown pigments which masked the red color. The degradation of Acn, although possible, was less important.

Browning of strawberries and their products can be
either enzymatic or non-enzymatic. Studies on color loss in other fruit systems have also shown enzymic browning to be the main factor (Sistrunk and Cash, 1972; Pifferi and Cultrera, 1974). Cash and Sistrunk (1971) reported that PPO appeared to increase the rate of color loss in juice systems.

A controversy exists about the mechanism through which PPO degrades the color of many fruit systems. One mechanism would be the formation of melanin pigments which would mask the natural color of the fruits. Some phenolics present naturally in strawberries, such as quercetin, catechin, epicatechin, caffeic acid and chlorogenic acid (Fig.1) contain o-dihydroxy groupings and could, therefore, serve as PPO substrates. The reaction of the quinones formed with other phenolics and with proteins would lead to the browning of strawberries.

A second mechanism would be the direct oxidation of the Acn by PPO or other enzymes. Sakamura et al. (1966) found that one of the PPO isozymes from eggplants was specific for eggplant Acn and for catechol. Grommeck and Markakis (1964) reported the decolorization of purified Acn by horseradish peroxidase.

A third mechanism was proposed initially by Peng and Markakis (1963). These authors studied the effect of mushroom PPO on cherry Acn in the presence of catechol and noticed that Acn were poor substrates for PPO and that an
increase in the concentration of catechol increased the rate of decoloration to a maximum. Excessive catechol caused no higher decoloration, probably due to PPO inactivation. Peng and Markakis (1963) proposed a mechanism by which the o-phenolic substrates are enzymatically oxidized to quinones and the quinones formed cause the non-enzymic degradation of Acn:

\[ \text{O}_2 \rightarrow \text{PPO} \rightarrow \text{o-diphenols} \rightarrow \text{o-quinones} \rightarrow \text{non-enzymic} \rightarrow \text{degraded Acn} \]

\[ \text{H}_2\text{O} \rightarrow \text{Anthocyanins} \]

Goodman and Markakis (1965), using a similar system, inhibited the degradation of Acn using 8ppm of SO₂. Acn were protected from the enzymic oxidation products of chlorogenic acid only for a short period using ascorbic acid. After all the ascorbic acid had reacted with the quinones, the Acn level decreased (Pifferi and Cultrera, 1974). o-Quinones by themselves have not been shown to oxidize anthocyanins (Simpson et al., 1976).

**The Isolation and Characterization of PPO**

The rapid degradation of the color in strawberries and its possible causes have been described. No published work dealing with the extraction and study of the strawberry PPO was found. A method using acetone powder extraction followed by resolubilization in acetate buffer pH 6.2 and precipi-
The purpose of this work was to obtain a PPO extract from strawberries, Tioga variety, showing a high activity and good stability. This extract was used to partially characterize the strawberry PPO.
MATERIALS AND METHODS

Source and Preparation of the Samples

The strawberries, Tioga variety, were obtained from Jim Fuji Farms, Troutdale, Or., on June 14, 1979. They were picked up the same morning as harvested and all showed the same degree of ripeness, being fully red and firm. Upon arrival in Corvallis, the strawberries were washed under running water and sorted for homogeneity.

After washing, aliquots of 100g of strawberries were counted and placed in a Dewar flask (Lab-Line Instr. Inc., Melrose Park, Ill.) containing liquid nitrogen (LN). After the LN stopped boiling, the strawberries were transferred into plastic bags (John Vlasick Co., Seattle, Wa.) which are impermeable to oxygen. The filled bags were sealed under vacuum and the samples stored in a cold room at -40°C until used.

Materials

All chemicals used for the experiments were obtained from Sigma Chemical Co., except for the following. Benzene sulfinic acid was obtained from Eastman Kodak Co., and 4-methyl catechol was from K&K Lab. Inc. Gallic acid was prepared by B. Watson of the O.S.U. winery. All chemicals were used without further purification. Polyclar AT (PVPP) was from GAF Corporation; Dowex AG 2-X8 was obtained from
Bio-Rad Laboratories, and Amberlite XAD-4 came from Rohm and Haas Co.

**Cleaning of the Phenol Adsorbents**

The phenol adsorbents were cleaned by the procedures suggested by Loomis (1974) and Loomis et al. (1979).

**Amberlite XAD-4**

XAD-4 was suspended in distilled water several times and the fines decanted. Next, the XAD-4 was placed in a 2 liter Soxhlet extractor and extracted with acetone for ca. 25 cycles. The acetone was changed when it became colored. After the resin had been washed in the Soxhlet apparatus with three volumes of 10% HCl, it was transferred to a large Buchner filter covered with a double layer of nylon cloth and washed with glass distilled water until the effluent was free of chloride ions as determined by the silver nitrate precipitation. The XAD-4 was kept moist until used.

Before use, sufficient XAD-4 was placed in a Buchner filter fitted with a Whatman #1 filter and washed twice with glass distilled water and filtered under vacuum until no more water could be removed. This provided constant weights of XAD-4 for the assays. Moist XAD-4 was approximately 50% dry resin.

**Polycar AT (PVPP)**

The PVPP was mixed with 10% HCl to form a thick slurry
and boiled for 10min with continuous stirring before being placed in a large Erlenmeyer flask, and washed with glass distilled water. After each washing, the PVPP was allowed to settle for ca. 30min and the supernatant was decanted. This was repeated for ten times or until the supernatant was clear. The PVPP was neutralized with 1 N KOH and washed again with glass distilled water in the same manner until the supernatant was free of chloride ions as determined by the silver nitrate precipitation. After the clean PVPP was collected on a Buchner filter covered with a double layer of nylon cloth to remove the excess water, it was oven-dried at 80°C overnight. The PVPP was stored dry and used for the assays without further treatment.

**Dowex AG 2-X8**

The anion exchange resin, Dowex 2-X8 100-400 mesh, was washed successively with glass distilled water to remove the fines, one volume of 1 N HCl followed by two volumes of methanol containing 1% concentrated HCl. After washing with glass distilled water until the effluent had no UV-adsorbing material (260nm), the Dowex 2-X8 resin was stored moist at 4°C until used. Before use, the resin was treated like the XAD-4 resin for constant weights. Moist Dowex 2-X8 contained 37% dry resin.

The phenol adsorbents were placed in the extraction buffer for at least overnight to allow thorough wetting as
well as equilibration with the buffer. Different columns of buffer were required with the different proportions of the resins used. The dilution caused by the moisture content of XAD-4 and Dowex 2-X8 was considered throughout the experiments.

General Extraction Procedure

The samples were prepared fresh each day. The basic procedure followed was the same as Smith (1980). The frozen strawberry sample was placed in a Dewar flask containing liquid nitrogen (LN). The strawberries were ground under LN using a Waring Blender with a stainless steel container for 45 seconds. The fine nitrogen powder (N₂P) was stored under LN in a Dewar flask until used.

The extraction solution consisted basically of 0.1M Na citrate-0.2M K-phosphate buffer pH 5.8 containing different levels of the phenol adsorbents. The extraction solutions were prepared the day before and stored at 4°C.

To the extraction solution, 3g of N₂P were added and mixed first manually with a stirring rod and then gently stirred with a magnetic stirrer for 4 min. The suspension was transferred into stoppered centrifuge tubes and centrifuged in a Sorvall RC 5 superspeed centrifuge at 14,500 G at 0°C for 10 min. The supernatants were filtered through a Whatman #1 filter paper to remove floating debris, and stored in stoppered glass tubes in crushed ice until used.
Assay of PPO Activity

PPO activity was measured using the polarographic method. A Clark-type oxygen electrode, YSI Model 53 (Yellow Springs Instrument Co.) was covered with teflon film (YSI No. 5252). The reaction chamber was held at 30°C with a Lauda K2/2 (Brinkmann Instruments) constant temperature circulator. The electrode was standardized with 3ml of air-saturated water at 30°C by adjusting the YSI Model 53 to 100%. The YSI Model 53 was switched to O₂ and the recorder was adjusted to 100%. The scale on the recorder read from 0 to 100% oxygen, or from 0 to 711 nmoles of oxygen for the 3ml assay mixture (Cooper, 1977, p.32).

For the assays, 1.5 to 2.5 ml of the enzyme extracts were placed in the reaction chamber and 0.1M Na-citrate-0.2M-K-phosphate buffer, pH 5.2, was added to a final volume of 2.5ml. The enzyme solution was then allowed to reach temperature equilibrium for at least 3 min. To initiate the reaction, 0.5ml of freshly prepared 0.1M catechol in 0.02M Na-citrate buffer, pH 3.5 @ 30°C, was injected through the access groove. The final concentration of the catechol in the assay mixture was 16.7mM.

The oxygen consumption was recorded for at least 4 min, and the rate of the reaction was calculated from the initial portion of the curve. The oxygen consumption, or activity, was expressed as nmoles of oxygen consumed per minute per gram of strawberry nitrogen powder (nmoles O₂/min/g N₂P).
Measurement of Polyphenolic Content

The phenolic content of the different extracts was measured in two forms. The total phenolics content was measured using the method described by Singleton and Rossi (1965). The phenolic compounds reacted with the Folin-Ciocalteau's phenol reagent giving a colored solution that had a maximum adsorption at 675 nm. The standard curve was made using twice recrystallized gallic acid and the phenolic content was expressed as gallic acid equivalents, 1 GE = 1 μg gallic acid per ml of sample.

The effectiveness of the removal of phenolics from the samples was also observed from the UV absorption scans of the treated samples. The scans were made from 360 to 200 nm using a Beckman Acta CIII recording spectrophotometer at a scan speed of 4 nm/sec and a scale of 20 nm/in. The reference cuvette contained 0.25 ml of the extraction buffer and 2.75 ml of distilled water. The sample cuvette consisted of 0.25 ml of the enzyme extract and 2.75 ml of distilled water.

Discontinuous Polyacrylamide Gel Electrophoresis

The procedure for the anionic Page was performed by following the general principles described by Davis (1964), Gabriel (1971) and Smith (1980).

The following stock solutions were prepared in advance and stored at 4°C until used:
1. Electrode buffer: 0.025M tris (tris-hydroxymethylamino-methane), 0.162M glycine, pH 8.3; 9.0 g tris was mixed with 43.2 g of glycine, in 2.5 l of distilled water. The pH was adjusted to pH 8.3 with 1.0N HCl and the volume brought to 3.0 l with distilled water.

2. Spacer buffer: 0.062M tris-HCl, pH 6.7; 22.5 g of tris, 1.5 ml of TEMED (N,N,N',N'-tetramethylenediamine) and 12.0 ml HCl were added to 2.0 l of distilled water. The pH was adjusted with 1.0N HCl and made up to a final volume of 3.0 l with distilled water.

3. Running gel buffer, 7% (w/v): 0.38M tris-HCl, pH 9.0; 138 g tris, 3.0ml TEMED and 12.0 ml of concentrated HCl were combined in 2.0 l of distilled water. The pH was adjusted with 1.0N HCl and brought to a final volume of 3.0 l with distilled water.

4. Running gel buffer, 5% (w/v): 3.0M tris-HCl, pH 9.0; 181.5 g tris, 48.0 ml of 1N HCl and 1.15 ml of TEMED were combined in 750 ml of distilled water. The pH was adjusted with 1.0N HCl and brought to a final volume of 1.0 l. Acrylamide/bis solution; 200 g of acrylamide were combined with 10 g bisacrylamide in 750 ml of distilled water, dissolved thoroughly and brought to 1.0 l with more distilled water. Riboflavin solution; 20 mg of riboflavin were dissolved in 500 ml of glass distilled water.
The following working solutions were prepared immediately before used:

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

2. Running gel solution, 5% (w/v): one volume of the 5% running gel buffer was combined with two volumes of the acrylamide/bis solution, one volume of the riboflavin solution and four volumes of distilled water. The riboflavin-catalyzed photopolymerization was accomplished by placing a fluorescent lamp within 10cm of the tubes containing the solution.

3. Spacer gel solution, 4% (w/v): Cyanogum 41, 0.8g, was dissolved in 20.0ml of spacer buffer and filtered through Whatman No. 1 filter paper. Polymerization was initiated by adding 0.01g of ammonium persulfate to the solution.

The polyacrylamide gels were prepared in glass tubes of 0.5cm (i.d.) and 12cm in length. The tubes were cleaned in nitric acid, washed thoroughly with distilled water and then soaked in a 0.01% photoflo (Eastman Kodak Co.) solution. After drying, the tubes were marked at 9.0cm and 10.0cm with a Parafilm. The tubes were placed vertically in a tube rack.
The running gel working solution, either 5% or 7%, was placed into the tubes using a syringe (No. 20 needle) up to the 9.0cm mark immediately after preparation. About 3mm of water was carefully layered on top of the gels using a syringe (No. 18 needle) avoiding mixing water with the gel solution. Gelation occurred in ca. 20min as indicated by a faint opalescence.

After gelation had occurred, the water was extracted from the surface of the gels by putting the tubes upside down in a beaker. The tops of the gels were washed once with distilled water and dried using paper tissue. After the gels were placed in the tube rack, the spacer gel solution was prepared and poured on top of the running gels to the 10cm mark using a syringe (No. 18 needle). Again, 3mm of water was placed carefully on top of the gels. Gelation occurred in ca. 20min.

The enzyme sample for electrophoresis consisted of 0.25ml of 50% sucrose containing a very small amount of bromophenol blue. To this, 1.0ml of enzyme extract was added and mixed, and 300μl of this preparation were applied on top of each gel. The electrophoresis apparatus used was the Model 155 Electrophoresis Cell (Bio-Rad Laboratories). The central cooling core of the apparatus was connected to a Laude K2/2 constant temperature circulator and the temperature inside the cell kept at 4°C. In this form it was possible to work outside a cold room. After assembling of the apparatus, the
constant voltage power supply (EC-Model 454, EC Apparatus Corp.) was connected to the system. The cathode lead was attached to the upper chamber and the anode lead to the lower chamber. The initial voltage was 125 V (ca. 2ma/tube) and applied until all the sample had entered the stacking gel. The voltage was then increased to 225V for the remainder of the run (ca. 2hrs).

The tubes were taken out of the apparatus when the tracking dye had almost reached the bottom of the gels. The gels were carefully removed from the tubes by gently forcing water between the gels and the glass tubes with a syringe (No. 20 needle) filled with distilled water.

The PPO activity was detected by immersing the gels in 10mM catechol in 0.1M Na-citrate-0.2M K-phosphate buffer, pH 5.0 containing 0.05% D-phenylenediamine for ca. 30min. Relative migration (Rm) and relative intensities of the bands were determined visually.

**Protein Concentration**

The protein content of the extracts was measured using a turbidimetric method as described by Layne (1957). The turbidity due to the protein precipitation by potassium ferrocyanide was measured at 600 nm and related to a standard curve obtained using bovine serum albumin as a standard protein. The UV absorption data (280 and 260nm) were also used to compare the relative protein content of the extracts (Layne, 1957).
Characterization of the Strawberry PPO

Effect of pH

PPO activity as a function of pH was determined at pH values ranging from pH 2.5 to pH 9.5. The enzyme extracts were adjusted to the desired pH by the addition of either 1.0N HCl or 1.0N NaOH. The assay mixture contained 2.5ml of the adjusted enzyme extract and 0.5ml of the substrate. Dilutions were taken into account.

The effect of the pH during the extraction was determined by the use of 0.2M Na-citrate buffers at pH values from 2.5 to 6.5. The activity was measured after adjusting the extracts to the optimum pH for PPO activity. The assay mixture contained 2.5 ml of the adjusted enzyme extract and 0.5ml of the substrate and dilutions were taken into account.

PPO extracts in which the PPO was inactivated by boiling for 10min were used as controls throughout the experiments.

Effect of Temperature

For the heat treatment of PPO, enzyme preparations extracted at optimum pH for extraction were used. The glass tubes used for the heat treatments were preheated in a water bath (Lauda K2/2) at the desired temperature, and 8.0ml of the enzyme extract was placed into each tube. After each heating interval, the tubes were taken out and immediately chilled in an ice bath. Residual PPO activity was determined with 2.5ml of the treated sample and 0.5ml of substrate.
Substrate Specificity

For the substrate specificity assays, 2.5ml of the enzyme extracts obtained using optimum pH for extraction was mixed with 0.5ml of the substrate. The substrates were prepared by dissolving each substrate in 0.02M Na-citrate buffer pH 3.5.

Effect of Inhibitors

For the determination of the effect of inhibitors on PPO, 2.5ml of the enzyme extract was mixed with 0.25ml of the inhibitor and 0.25ml of the substrate was added. Both the substrates and the inhibitors were prepared by dissolving in 0.02M Na-citrate buffer, pH 3.5.
RESULTS AND DISCUSSION

Preparation and Preservation of Samples

From the discussion presented in the Review of Literature, the importance of preventing the interactions of secondary plant products with the plant proteins throughout the extraction of an enzyme from plant tissues has been confirmed. Also appropriate storage conditions must be used to minimize these interactions. A normal practice is to store the samples in frozen state until they are used. Slow freezing of the samples should be avoided due to the formation of large ice crystals that cause extensive breakage of the cells and subsequent contact between the secondary products and the proteins. Using quick-freezing, small ice crystals are formed with the retention of cell integrity (Potter, 1973). Successful quick-freezing of samples has been obtained using liquid nitrogen. Ulbricht and Zenk (1979) quick-froze cell suspensions of higher plants with storage at $-20^\circ$C and no change in enzyme activity after storage was evident. Similar results were obtained by Smith (1980) for d'Anjou pears stored at $-40^\circ$C after quick-freezing. Palavicini (1969) noticed some changes in the activities of some enzymes of strawberries after deep-freezing and storage at $-20^\circ$C for a long period of time. After 4.5 months of storage, no peroxide activity was detected, however no change in catalase activity and a loss of 16% of PPO activity was found.
In the present work, quick-freezing of the fresh strawberries with liquid nitrogen was, therefore, favored, followed by storage at -40°C under vacuum in sealed plastic bags. No change in activity of PPO was noticed from buffer extracted samples after 95 days of storage (Table 3).

**Enzyme Extraction Procedure**

The extraction procedure used for the preparation of a crude enzyme extract from strawberries is shown in Fig. 4. To obtain a homogeneous extract, the frozen strawberries were blended in liquid nitrogen. This allowed for the fracture of the tough cell walls and kept the homogenate at a very low temperature in a very fine powder. This reduces the rate of reaction, which may cause changes in the enzyme (Loomis, 1974; Kelley and Adams, 1977).

The nitrogen powder (N$_2$P) was added directly to the extraction buffer containing the desired levels of previously washed and prehydrated phenolic adsorbents. The extraction buffer used was 0.1M Na-citrate-0.2M K-phosphate, pH 5.8. There were various reasons for the choice of this buffer. The pH of the homogenized fresh strawberries was 3.5. This low pH might have caused inhibition of the enzyme, and therefore, a higher pH was desirable. At the same time, Loomis (1974) and Gregory and Bendall (1966) noticed high degrees of auto-oxidation of phenolics at pH values higher than pH 6.0. Smith (1980) noticed an increase in activity of pear
Table 3. Strawberry polyphenol oxidase activity changes after storage at -40°C. Quick-frozen strawberries stored under vacuum and extraction of the enzyme using 0.1 M citrate - 0.2 M K-phosphate buffer pH 5.8.

<table>
<thead>
<tr>
<th>DAYS</th>
<th>21</th>
<th>31</th>
<th>46</th>
<th>61</th>
<th>84</th>
<th>95</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACTIVITY&lt;sup&gt;a&lt;/sup&gt;</td>
<td>41</td>
<td>37</td>
<td>45</td>
<td>52</td>
<td>54</td>
<td>45</td>
</tr>
</tbody>
</table>

n=21  \( \bar{x} = 44.2 \)  s=10.7

<sup>a</sup> Polyphenol oxidase activity as moles O<sub>2</sub>/ min/g N<sub>2</sub>P.
Figure 4. Basic extraction procedure of strawberry polyphenol oxidase.
PPO when the extraction medium was buffered above pH 5.0. Using the buffer at pH 5.8 during the extraction of strawberry PPO the final pH of the extracts was generally 5.7.

In order to allow thorough contact of the solubilized NₙP with the adsorbents the mixture was mechanically agitated for 4 min while the temperature was maintained below 5°C. Debris and resins were separated by centrifugation followed by filtration. The filtrate was the crude enzyme extract and PPO activity was determined using catechol at a final concentration of 16.7 mM as substrate.

**PPO Extracts Obtained with Polyphenol Adsorbents**

Polymeric adsorbents with high affinity for plant secondary products were used during the extraction of PPO from strawberries. Three different polymers were used, alone or in combinations, at different levels to obtain an extract with high PPO activity and low residual polyphenolic content. The three polymers were Amberlite XAD-4 (XAD-4), water-insoluble polyvinylpolypyrrolidone (PVPP) and Dowex AG 2-X8 (Dowex 2) anion exchange resin. The different preparations were evaluated by comparing the levels of PPO activity in the extracts. Activity was expressed as the nmoles of oxygen consumed by the extract per minute per g of strawberry nitrogen powder used (nmoles O₂/min/g N₂P). The 280/260 and 280/240 adsorption ratios were also compared. The UV scans were used for the evaluation of the different extracts. The use of the turbidimetric method suggested by Loomis (1974)
failed to give measurable protein concentrations, perhaps due to the very low protein content in the different extracts, or as discovered in later studies, due to the presence of pectin. The limit of the turbidity method is from 1.5 to 0.5 mg protein/ml.

The activity per g of strawberry N₂P was probably the most accurate method for comparison of PPO activity, but some variation, expressed as standard error, was observed for the different strawberry samples used and at least four different extractions were made using each of the adsorbents, alone or in combination. No electrophoretic analysis of the PPO isozyme pattern was attempted in these preliminary crude enzyme preparations.

**Extraction of PPO using XAD-4**

The results obtained using different levels of XAD-4 during the extraction of PPO are shown in Table 4. XAD-4 was used in the hydrated form, and the dilutions caused by this hydration were taken into account during the measurements of the PPO activity. An increase in the activity of PPO was noticed with an increase of the XAD-4 level used. Smith (1980) found that levels of XAD-4 higher than 1.0g XAD-4/g tissue were required to prevent browning of d'Anjou pear extracts. From visual observations, the extracts obtained at all the levels of XAD-4 tested were clear and colorless and no browning occurred after 8hrs. The control extract
The table below shows the characteristics of PPO extracts obtained using different levels of XAD-4.

<table>
<thead>
<tr>
<th>XAD-4 Level</th>
<th>PPO Activity</th>
<th>280_260</th>
<th>280_240</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>44±11</td>
<td>1.050</td>
<td>0.661</td>
</tr>
<tr>
<td>1</td>
<td>50±4</td>
<td>0.760</td>
<td>0.519</td>
</tr>
<tr>
<td>2</td>
<td>61±10</td>
<td>0.703</td>
<td>0.587</td>
</tr>
<tr>
<td>3</td>
<td>67±7</td>
<td>0.659</td>
<td>0.606</td>
</tr>
<tr>
<td>4</td>
<td>71±8</td>
<td>0.702</td>
<td>0.590</td>
</tr>
<tr>
<td>5</td>
<td>75±11</td>
<td>0.670</td>
<td>0.555</td>
</tr>
</tbody>
</table>

a 3.0g of strawberry nitrogen powder (N2P) were mixed with different levels of the resin.
b Activity of PPO as nmoles O2/min/gN2P. Volume of the extraction buffer used was 25ml. Results are the average of at least four different extractions, * standard error. Dilutions by the hydrated resin were taken into account.
Figure 5. UV-absorption spectra of strawberry PPO extracts obtained using different levels of Amberlite XAD-4: a, control without treatment 0.0g XAD-4/1g N₂P; b, 1.0g XAD-4/1g N₂P; c, 3.0g XAD-4/1g N₂P; d, 3.0g XAD-4/0g N₂P.
obtained without treatment with phenol adsorbents showed lower PPO activity, was dark pink in color when extracted and showed a red-brown color after 8hrs due to enzymatic browning. The UV scans of the XAD-4 treated extracts (Fig. 5) show the presence of a peak at 265nm and increasing absorbance at 230nm, indicating the presence of phenolics in the extracts. This is also seen in the low 280/260 and 280/240 absorption ratios (Table 4). Higher levels of the resin extracted more phenolics (Fig. 5), although the 280/260 and 280/240 absorption ratios remained approximately the same at all levels of XAD-4 used. The lower absorption values obtained at the higher XAD-4 levels (Fig. 5) seem to be due the dilution by the moisture content of the hydrated resin. A small peak at 240nm in the extraction buffer mixed with 3g of resin (Fig. 5) suggests a slight leaching of the resin during extraction. Interestingly, the control obtained using only strawberry N2P during the extraction showed a higher 280/260 absorption ratio (Table 4).

**Extraction of PPO using PVPP**

PVPP when used during the extraction of plant enzymes binds phenolics through the formation of hydrogen bonds thus preventing the protein-phenolic interactions (Loomis and Battaile, 1966; Rhodes, 1977). Different levels of PVPP were used during the extraction of strawberry PPO (Table 5). The PPO activity increased with increasing PVPP concentration
TABLE 5. Characteristics of PPO extracts obtained using different levels of PVPP.

<table>
<thead>
<tr>
<th>PVPP level</th>
<th>Extraction Buffer (ml)</th>
<th>PPO Activity</th>
<th>280</th>
<th>280</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>25</td>
<td>44±11</td>
<td>1.050</td>
<td>0.661</td>
</tr>
<tr>
<td>0.25</td>
<td>20</td>
<td>74±13</td>
<td>0.859</td>
<td>0.905</td>
</tr>
<tr>
<td>0.50</td>
<td>20</td>
<td>80±11</td>
<td>0.858</td>
<td>0.862</td>
</tr>
<tr>
<td>0.75</td>
<td>40</td>
<td>77±5</td>
<td>0.927</td>
<td>0.813</td>
</tr>
<tr>
<td>1.00</td>
<td>40</td>
<td>96±13</td>
<td>0.945</td>
<td>0.784</td>
</tr>
<tr>
<td>1.50</td>
<td>50</td>
<td>96±15</td>
<td>0.907</td>
<td>0.585</td>
</tr>
<tr>
<td>2.00</td>
<td>60</td>
<td>98±15</td>
<td>0.908</td>
<td>0.531</td>
</tr>
</tbody>
</table>

a 3.0 g of strawberry nitrogen powder were mixed with different levels of the polymer.
b Activity of PPO as nmoles $O_2/\text{min/gN}_2P$. Results are the average of at least four different extractions, $\pm$ standard error.
c Volume of the extraction buffer used.
Figure 6. UV-absorption spectra of strawberry PPO extracts obtained using different levels of polyvinylpoly- pyrrolidone (PVPP): a, control without treatment 0.0g PVPP/1g N2P; b, 0.5g PVPP/1g N2P; c, 1.0g PVPP/1g N2P; d, 1.0g PVPP/0g N2P.
and could be divided into two groups. At levels of 0.25g PVPP/g N\textsubscript{2}P, the extracts were pink in color and the levels of PPO activity were similar to those obtained using high levels of XAD-4. Some browning could be observed in the extracts containing less than 0.75g PVPP/g N\textsubscript{2}P after 8hrs. At levels of PVPP of 1.0g PVPP/g N\textsubscript{2}P and higher, the extracts obtained were clear and the activities higher, and no browning occurred after 8hrs.

Smith (1980) obtained similar results and suggested that the increase in activity with increasing levels of PVPP could be attributed to a concentration effect produced during the hydration of the dried PVPP. About 60% of the extraction buffer was adsorbed during the hydration of PVPP. Walker and Hulme (1965) found that PVPP could cause the inhibition of PPO; however, no inhibition was evident in the extracts obtained in this work. No inhibition of PPO by PVPP was also noticed by Halim and Montgomery (1978) and Montgomery and Sgarbieri (1975).

PVPP appeared to bind strawberry phenolics better than XAD-4 as seen from the higher 280/260 absorption ratios and from the lower 260nm absorption at levels of PVPP equal or higher than 1.0g PVPP/g N\textsubscript{2}P. However, the 280/260 ratios were still lower than 1.0, indicating the presence of some phenolics in the extracts. This is also shown by the low values obtained for the 280/240 absorption ratios. From the UV scans (Fig. 6) the 265nm peak is less evident than in the
XAD-4 treated extracts (Fig. 5), but the high 230nm absorption is still present. According to Lam and Shaw (1970) PVPP was not able to bind phenolics of molecular weight equal or lower than chlorogenic acid efficiently, and the presence of low molecular weight phenolics in the extracts could be the cause of the adsorption data obtained.

Extraction of PPO using Dowex 2

The Dowex anion exchange resins have been found to bind phenolics mainly through hydrophobic and ionic interactions (Lam and Shaw, 1970; Rhodes, 1977). Dowex 2 can probably bind phenolics through hydrogen bonding as well (Loomis et al., 1979). This combination of binding mechanisms makes Dowex 2 appear practical for the extraction of phenolics from the preparations. The results shown in Table 6 show a slight increase in the activity of PPO with increasing levels of Dowex 2. The activity levels obtained were lower than those obtained using PVPP or higher levels of XAD-4. According to Fassold (1961) at pH values from 5.0 to 5.8 or lower, non-specific binding of many proteins to ion-exchange resins was apparent. This could partially be the cause of the low activity levels obtained in Table 6, although Smith (1980) reported no change in activity, 280nm absorption and the 280/260 absorption ratios in PVPP extracts treated with Dowex 2. All the extracts obtained showed some brown color even at 1.5g Dowex 2/g N2P level. The 280/260 absorption ratios
**TABLE 6.** Characteristics of PPO extracts obtained using different levels of Dowex 2.

<table>
<thead>
<tr>
<th>Dowex 2 level&lt;sup&gt;a&lt;/sup&gt;</th>
<th>PPO Activity&lt;sup&gt;b&lt;/sup&gt;</th>
<th>280&lt;sup&gt;260&lt;/sup&gt;</th>
<th>280&lt;sup&gt;240&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>44±11</td>
<td>1.050</td>
<td>0.661</td>
</tr>
<tr>
<td>0.25</td>
<td>56±9</td>
<td>1.013</td>
<td>0.612</td>
</tr>
<tr>
<td>0.50</td>
<td>56±13</td>
<td>0.985</td>
<td>0.608</td>
</tr>
<tr>
<td>0.75</td>
<td>61±13</td>
<td>0.933</td>
<td>0.571</td>
</tr>
<tr>
<td>1.00</td>
<td>62±14</td>
<td>0.904</td>
<td>0.571</td>
</tr>
<tr>
<td>1.50</td>
<td>63±11</td>
<td>0.856</td>
<td>0.565</td>
</tr>
</tbody>
</table>

<sup>a</sup> 3.0g of strawberry nitrogen powder were mixed with different levels of the resin.

<sup>b</sup> Activity of PPO as nmoles O2/min/gN<sub>p</sub>. Volume of the extraction buffer used was 25ml. Results are the average of at least four different extractions, ± standard error. Dilutions by the hydrated resin were taken into account.
Figure 7. UV-absorption spectra of strawberry PPO extracts obtained using different levels of Dowex AG 2-X8 (Dowex 2): a, control without treatment 0.0g Dowex 2/1g N2P; b, 0.5g Dowex 2/1g N2P; c, 1.0g Dowex 2/1g N2P; d, 0.5g Dowex 2/0g N2P.
obtained were higher than those obtained using XAD-4 and similar to those obtained using PVPP. The 280/240 absorption ratios were low. This denoted the presence of phenolics in the extracts and from the UV scans (Fig. 7) it is apparent that less phenolics were removed with Dowex 2 than with either PVPP or XAD-4.

From the data obtained using PVPP, XAD-4 and Dowex 2 alone during the extractions of PPO, it is evident that the strawberry phenolic system is quite different than other systems treated similarly with these polymers. Previous work by Smith (1980) on pear PPO extraction gave higher values of 280nm absorption, and 280/260 and 280/240 absorption ratios which indicates better adsorption of pear phenolics using these polymers. In order to attempt to increase the effectiveness of the phenolic adsorption, combinations of the adsorbents were tried.

**Extraction of PPO using PVPP plus XAD-4**

Loomis (1974) and Loomis et al. (1979) were able to obtain crystal-clear protein-containing extracts from a variety of plant tissues including potato tubers and walnut hulls using a combination of PVPP and XAD-4. They noticed that neither of the polymers alone removed all of the phenolics. PVPP and XAD-4 used together provide a combination of hydrophobic interactions and hydrogen bonding of phenolics. The results obtained using combinations of PVPP:XAD-4 during
TABLE 7. Characteristics of PPO extracts obtained using different levels of XAD-4 and PVPP.

<table>
<thead>
<tr>
<th>Levels of XAD-4</th>
<th>Extraction Buffer (ml)</th>
<th>PPO Activity</th>
<th>280</th>
<th>280</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>25</td>
<td>$^{44}±^{11}$</td>
<td>1.050</td>
<td>0.661</td>
</tr>
<tr>
<td>0.50</td>
<td>25</td>
<td>$^{103}±^{20}$</td>
<td>0.639</td>
<td>0.574</td>
</tr>
<tr>
<td>1.00</td>
<td>25</td>
<td>$^{117}±^{20}$</td>
<td>0.648</td>
<td>0.541</td>
</tr>
<tr>
<td>1.50</td>
<td>25</td>
<td>$^{105}±^{14}$</td>
<td>0.683</td>
<td>0.471</td>
</tr>
<tr>
<td>2.00</td>
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<td>0.667</td>
<td>0.575</td>
</tr>
<tr>
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<td>$^{98}±^{12}$</td>
<td>0.638</td>
<td>0.601</td>
</tr>
<tr>
<td>1.50</td>
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<td>$^{98}±^{29}$</td>
<td>0.681</td>
<td>0.440</td>
</tr>
<tr>
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<td>0.738</td>
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<tr>
<td>2.00</td>
<td>25</td>
<td>$^{117}±^{10}$</td>
<td>0.768</td>
<td>0.598</td>
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</tbody>
</table>

a 3.0g of strawberry nitrogen powder were mixed with different levels of the polymers.
b Volume of the extraction buffer used.
c Activity of PPO as nmoles O$_2$/min/g N$_2$P. Results are the average of at least four different extractions, ± standard error.
Figure 8. UV-absorption spectra of strawberry PPO extracts obtained using different levels of PVPP and XAD-4: a, control without treatment 0.0g PVPP: 0g XAD-4/1g N₂P; b, 1.0g PVPP: 1g XAD-4/1g N₂P; c, 0.5g PVPP: 3g XAD-4/1g N₂P.
the extraction of strawberry PPO are shown in Table 7. The PPO activity levels obtained using any combination of PVPP and XAD-4 were similar or higher than those obtained using PVPP, XAD-4 or Dowex 2 alone, or in the combinations of PVPP:Dowex 2 (Table 8) and XAD-4:Dowex w (Table 9). The 280/260 absorption ratios were low, but from the UV scans shown in Fig. 8, it can be seen that more phenolics were extracted and that the increase in absorption at 230nm had disappeared. All extracts obtained were clear and no browning was noticed after 8hrs.

**Extraction of PPO using PVPP plus Dowex 2**

Phenolics bind to PVPP mainly through hydrogen bond formation and the combination of Dowex 2 with PVPP will increase the polar binding capacity, with the addition of hydrophobic binding of the phenolics to the Dowex 2 matrix. The characteristics of the extracts obtained using different combinations of PVPP:Dowex 2 are shown in Table 8 and Fig. 9. The PPO activity levels obtained were higher than those obtained using XAD-4 or Dowex 2 alone and similar to those obtained using Dowex 2 and XAD-4 combined (Table 9); but lower than those obtained using PVPP alone or in combination with XAD-4. The 280/260 and 280/240 absorption ratios were slightly lower than those obtained using either of the polymers alone during the extraction. There was an increase in the 280/260 absorption ratios with increasing PVPP to Dowex 2 levels. The UV spectra in Fig. 9 show that more
TABLE 8. Characteristics of PPO extracts obtained using different levels of PVPP and Dowex 2.

<table>
<thead>
<tr>
<th>Levels of PVPP</th>
<th>Extraction Buffer (ml)</th>
<th>PPO Activity&lt;sup&gt;c&lt;/sup&gt;</th>
<th>280&lt;sub&gt;260&lt;/sub&gt;</th>
<th>280&lt;sub&gt;240&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
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<td>0.00</td>
<td>25</td>
<td>1.050</td>
<td>0.661</td>
</tr>
<tr>
<td>0.50</td>
<td>0.50</td>
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<td>0.745</td>
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<td>0.814</td>
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<td>0.50</td>
<td>50</td>
<td>0.917</td>
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</tr>
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<td>1.50</td>
<td>0.75</td>
<td>50</td>
<td>0.901</td>
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<tr>
<td>1.50</td>
<td>1.00</td>
<td>50</td>
<td>0.909</td>
<td>0.438</td>
</tr>
</tbody>
</table>

<sup>a</sup> 3.0g of strawberry nitrogen powder were mixed with different levels of the polymers.

<sup>b</sup> Volume of the extraction buffer used.

<sup>c</sup> Activity of PPO as nmoles O₂/min/g N2P. Results are the average of at least four different extractions, ± standard error. Dilutions by hydrated resin were taken into account.
Figure 9. UV-absorption spectra of strawberry PPO extracts obtained using different levels of PVPP and Dowex 2: a, control without treatment 0.0g PVPP: 0g Dowex 2/1g N$_2$P; b, 1.5g PVPP: 1g Dowex 2/1g N$_2$P; c, 1.0g PVPP: 1g Dowex 2/1g N$_2$P.
phenolics were adsorbed using a combination of Dowex 2 and PVPP than using Dowex 2 alone and that the results were similar to those obtained using higher levels of PVPP alone. The increase in adsorption at 230nm disappeared.

From the values shown in Tables 7 and 8 and in Fig. 6, 8 and 9, PVPP appears to be the polymer mainly responsible for the adsorption of most of the phenolics from the extracts.

Extraction of PPO using XAD-4 plus Dowex 2

Both XAD-4 and Dowex 2 are polystyrene based polymers with Dowex 2 containing a quaternary ammonium derivative attached to the benzene rings. Thus combining XAD-4 with Dowex 2 increases the hydrophobic binding capacity during the extraction combined with the polarity of Dowex 2 which binds phenolics through both hydrogen bond formation and ionic interactions (Loomis et al., 1979). The results obtained using combinations of Dowex 2 and XAD-4 are shown in Table 9 and Fig. 10. The PPO activity levels were similar to those obtained using the Dowex 2:PVPP combination or using XAD-4 alone, and slightly higher than using Dowex 2 alone. But using PVPP alone or in combination with XAD-4 gave higher activity values. From the UV scans (Fig. 10) a peak at 265nm was present in the extracts. This peak was present in all the extracts treated with either XAD-4 or Dowex 2, and probable leaching of the resins could be the cause, although the UV spectra of the XAD-4 and Dowex 2 resins without strawberry N2P showed a peak at 245nm and not
TABLE 9. Characteristics of PPO extracts obtained using different levels of XAD-1 and Dowex 2.

<table>
<thead>
<tr>
<th>Levels of XAD-1</th>
<th>Dowex 2</th>
<th>PPO Activity</th>
<th>280/260</th>
<th>280/240</th>
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</thead>
<tbody>
<tr>
<td>0</td>
<td>0.00</td>
<td>44±11</td>
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</tr>
<tr>
<td>1</td>
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<td>68±16</td>
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<tr>
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<tr>
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</tr>
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</tr>
<tr>
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</tr>
<tr>
<td>3</td>
<td>1.00</td>
<td>85±19</td>
<td>0.595</td>
<td>0.663</td>
</tr>
</tbody>
</table>

a 3.0g of strawberry nitrogen powder were mixed with different levels of the polymers.

b Activity of PPO as nmoles O₂/min/g N₂P. Results are the average of at least four different extractions, ± standard error. Dilutions by hydrated resin were taken into account.
Figure 10. UV-absorption spectra of strawberry PPO extracts obtained using different levels of XAD-4 and Dowex 2: a, control without treatment 0g XAD-4: 0g Dowex 2/1g N₂P; b, 1g XAD-4: 1g Dowex 2/1g N₂P; c, 3g XAD-4: 1g Dowex 2/1g N₂P.
at 265nm (Fig. 5,7). The 280/260 and 280/240 absorption ratios were low, but there was a decrease in absorbance at 230nm.

The protein concentration of the extracts was measured using the Warburg-Christian method from the UV absorption data obtained for the different extracts (Layne, 1957). Protein concentration values were obtained in a range from 0.4mg of protein for the untreated extracts to 0.03 mg of protein per ml of extract for the XAD-4:Dowex 2 treated extracts. These protein concentration values were below the turbidity protein limits of 0.5mg protein/ml using potassium ferrocyanide to precipitate the protein. These results show the presence of very low levels of protein in the extracts, and the UV spectra obtained are not UV-protein spectra, but spectra of the phenolic compounds remaining in the extracts. It should also be noted that there is an increase absorption by proteins at wavelengths lower than 230nm.

Effects of Dialysis, Lyophilization and Electrophoretic Studies of the Extracts

To further evaluate the effectiveness of the extraction methods, the extracts that showed the highest activities were subjected to additional analysis (Table 10). The first
TABLE 10. Effect of the different treatments on strawberry PPO. Extracts obtained varying levels of the polymeric polyphenols adsorbents.

**CRUDE ENZYME EXTRACTS**

<table>
<thead>
<tr>
<th>Levels of XAD-4</th>
<th>Levels of PVPP</th>
<th>Levels of Dowex 2&lt;sup&gt;a&lt;/sup&gt;</th>
<th>PPO Activity&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Polyphenols (G.E.)&lt;sup&gt;c&lt;/sup&gt;</th>
<th>280</th>
<th>260</th>
<th>240</th>
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<tbody>
<tr>
<td>A</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>35</td>
<td>252.7</td>
<td>1.067</td>
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<tr>
<td>B</td>
<td>2.0</td>
<td>-</td>
<td>-</td>
<td>75</td>
<td>90.6</td>
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<td>0.818</td>
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<td>C</td>
<td>4.0</td>
<td>-</td>
<td>-</td>
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<td>0.815</td>
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<td>-</td>
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<td>-</td>
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<tr>
<td>E</td>
<td>-</td>
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<td>81</td>
<td>81.3</td>
<td>0.900</td>
<td>0.686</td>
</tr>
<tr>
<td>F</td>
<td>-</td>
<td>-</td>
<td>1.00</td>
<td>62</td>
<td>149.9</td>
<td>0.927</td>
<td>0.617</td>
</tr>
<tr>
<td>G</td>
<td>1.0</td>
<td>1.0</td>
<td>-</td>
<td>67</td>
<td>73.4</td>
<td>0.717</td>
<td>0.595</td>
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<tr>
<td>H</td>
<td>2.0</td>
<td>-</td>
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<td>84.5</td>
<td>0.624</td>
<td>0.806</td>
</tr>
<tr>
<td>I</td>
<td>-</td>
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<td>1.00</td>
<td>60</td>
<td>76.3</td>
<td>0.745</td>
<td>0.538</td>
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</table>

<sup>a</sup> Levels of the polymers mixed with 6.0g of strawberry nitrogen powder.

<sup>b</sup> Activity of PPO as nmoles O<sub>2</sub>/min/g N<sub>2</sub>P. Dilutions by the hydrated resin taken into account.

<sup>c</sup> Concentration of polyphenols expressed as gallate equivalents. 1 G.E.=1 μg gallic acid/ml extract.
study of the extracts involved the evaluation of the isozyme patterns of PPO on polyacrylamide gel electrophoresis. The extracts were prepared as described in Fig. 4 and 300 μL of the extracts were subjected to electrophoresis. PPO activity and protein were detected on the gels using catechol and amido black, respectively. Since no visible bands were observed using either of the detection methods, concentration of the extracts was necessary.

Extraction of PPO

Crude enzyme extracts were prepared as described in Fig. 4, but using twice the quantities of buffer, resins and N2P. To compare the effectiveness of the phenol extraction, the total phenolic content of each of the extracts was measured spectrophotometrically as gallate equivalents as described by Singleton and Rossi (1965) (Table 10). Approximately 60 to 70% of the polyphenols originally present in the extract without treatment with adsorbents, could be adsorbed in most of the preparations. The one exception was preparation F (Dowex 2) where only 40% of the phenolics could be adsorbed.

The PPO activity levels obtained were somewhat lower perhaps due to a higher dilution of the enzyme by using twice the quantities used in the preliminary extractions. The highest PPO activity levels were obtained using higher levels of PVPP or XAD-14 alone, while the remainder of the extracts showed almost the same PPO activity levels. The
280/260 absorption ratios were highest in extracts treated with PVPP (E) and Dowex 2 (F). It is interesting to note that the Dowex 2 treated extract (F) had the highest 280/260 absorption ratio among the treated extracts, and at the same time the highest level of residual phenolics after extraction. This may have been due to some phenolics absorbing strongly at 280nm (i.e., D-catechin) present in the extract. The 280/240 absorption ratios were correspondingly low.

**Dialysis of the Extracts**

The high molarity of the extraction buffer and the concentration of the extracts after the addition of the polymeric adsorbents made the ionic strength of the extracts unacceptably high for concentration. To lower the ionic strength the extracts were dialyzed against 0.05M acetate at pH 5.0. The results are shown in Table 11. Measurement of the total phenolic content of the dialyzed extracts revealed levels too low to be accurately detected with the method used. Values of approximately 35 gallate equivalents were obtained for all the extracts except the untreated extract which had a value of 43 gallate equivalents. These results suggest that most of the phenolics that remained in the extracts were removed during dialysis. Similar results were obtained by Benjamin and Montgomery (1973) during the dialysis of cherry PPO extracts. However, from the 280/260 and 280/240 absorption ratios it was evident that some phenolics remained in the extracts.
TABLE 11. Effects of dialysis and lyophilization on strawberry PPO.

<table>
<thead>
<tr>
<th></th>
<th>Levels of XAD-4</th>
<th>PVPP</th>
<th>Dowex 2&lt;sup&gt;a&lt;/sup&gt;</th>
<th>PPO Activity&lt;sup&gt;b&lt;/sup&gt; 280</th>
<th>260</th>
<th>240</th>
<th>PPO Activity&lt;sup&gt;b&lt;/sup&gt; 280</th>
<th>260</th>
<th>240</th>
<th>Pectin Content&lt;sup&gt;c&lt;/sup&gt;</th>
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<tr>
<td>A</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>28</td>
<td>0.874</td>
<td>0.450</td>
<td>8</td>
<td>0.957</td>
<td>0.499</td>
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<tr>
<td>B</td>
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<td>-</td>
<td>-</td>
<td>53</td>
<td>0.800</td>
<td>0.409</td>
<td>21</td>
<td>0.772</td>
<td>0.394</td>
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<tr>
<td>C</td>
<td>4.0</td>
<td>-</td>
<td>-</td>
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<tr>
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<td>0.769</td>
<td>0.353</td>
<td>63</td>
<td>0.930</td>
<td>0.395</td>
<td>++</td>
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</table>

<sup>a</sup> Levels of the polymers mixed with 6.0g of strawberry nitrogen powder during the preparation of the crude enzyme extract (see Table 10).

<sup>b</sup> Activity of PPO as nmoles O<sub>2</sub>/min/g N<sub>2</sub>P. Dilutions by hydrated resins and during dialysis taken into account.

<sup>c</sup> Designates the presence and level of pectin in the resolubilized lyophilized extracts; 100% gelatinized, ++++, 25% gelatinized, +.
PPO activity levels unexpectedly decreased in some of the extracts. Extracts treated with PVPP showed the least changes in PPO activity.

Concentration of the extracts

After dialysis, the extracts were frozen in liquid nitrogen and were lyophilized. The lyophilized samples were resolubilized in a minimum volume of 0.05M Na-citrate buffer, pH 5.2. The results for the resolubilized extracts are shown in Table 1. Difficulty was experienced in resolubilizing the lyophilized extracts and some of them could not be completely solubilized. This is reflected in the large losses in PPO activity for some of the extracts. Again, those extracts treated with PVPP lost less activity. The untreated control extract was a brown-red powder after lyophilization and the Dowex 2 (F) treated sample was a pink powder. This was evidence for the presence of phenolics in the extracts. The remainder of the extracts were white powders after lyophilization. UV spectral analysis of the resolubilized samples showed a slight increase in the 280/260 adsorption ratios, while the 280/240 absorption ratios remained almost the same. High absorbence in the 230nm region was present in all extracts.

Disc-electrophoresis

After the concentration of the extracts through lyophilization, samples of 300 μl of each extract were subjected to disc-electrophoresis. The gels used consisted of 5% stacking
gel and 7% (w/v) running gel. During electrophoresis of the extracts, the samples required a very long time to penetrate the stacking gel and the end of the stacking gel collapsed in most instances. After completion of the run, the gels were immersed in a 10mM solution of catechol for PPO activity detection. PPO activity was found only in the first few mm of the running gels, often as a smear. No PPO isozymes were noticed in the remainder of the gel and no PPO activity was detected in the untreated and Dowex 2 treated samples. The procedure was repeated obtaining similar results. A third run was made using a 5% (w/v) running gel instead of the 7% (w/v) gel, obtaining the same type of results.

By concentration of the extracts, PPO activity was detected in the gels after electrophoresis. After the resolubilized extracts had been stored at 4°C for a longer period of time, gelation of the extracts was noticed. The level of gelation varied from total gelation to approximately 25% gelation (See Table 11). For pectin gel formation, an acid pH is required, which shifts the equilibrium towards the un-ionized carboxyl groups of the polygalacturonic acid moieties. This decreases the attraction between the water and the pectin molecules, increases the attraction between the carboxyl groups, and this promotes the precipitation of the pectin (Hodge and Osman, 1976). Compounds, such as sugars, further decrease the hydration of the pectin
promoting a faster precipitation. Thus, the adsorption of water by the resins and the lyophilization of the extracts may have promoted the pectin precipitation. High levels of pectin in PPO extracts were also noticed by Cash et al. (1976) and Cash (1978) in Concord grapes and strawberry extracts, and by Wong et al. (1971) in Clingstone peach extracts. Cash (1978) suggested the use of calcium chloride to precipitate the pectin from strawberry extracts. Wong et al. (1971) suggested the use of calcium acetate for the precipitation of the pectin. Montgomery et al. (1968) used protamine sulfate for the precipitation of anionic polymers present in pea extracts during the purification of carboxyl esterases from this seed. They noticed that the extraction of dried peas with PVPP did not appreciably lower the 260nm absorbence and that the absorbence at 260nm was probably due to nucleic acid substances rather than phenolic compounds.

The composition of the gel formed in the concentrated extracts is not known, however, pectin appears most probable. The precipitation of pectin using calcium chloride was attempted on PVPP treated extracts, but while a white sticky colloid was formed and a great loss in PPO activity occurred. From these findings, a careful and in depth study of the pectin problem during PPO extraction from strawberries seems necessary. Later work will be involved in the design of a purification procedure of PPO in which several methods of pectin extraction will be analyzed.
The presence of the large amounts of pectin in the extracts may have interfered in the extraction of phenolics; in protein determination; in UV spectral analysis; and during electrophoresis. A probable cause for these interferences could have been the adsorption of phenolics and protein, including PPO, on the pectin polymer through non-covalent binding.

Characterization of the Strawberry PPO

After the evaluation of the extracts, those obtained using 2.0g PVPP/g N₂P and 1.0g PVPP+1.0g XAD-4/g N₂P yielded consistently the highest levels of activity of PPO. The use of the UV data did not seem appropriate for the final choice of a resin or resin combination to be used for further analysis. Strawberries have a very high content of anthocyanins as well as catechin and epicatechin (Stöhr and Herrmann, 1971). Gray (1978) noticed high affinity of PVPP towards these compounds, but noticed low affinity of PVPP towards chlorogenic acid. Lam and Shaw (1970) suggested that PVPP could bind possibly only those compounds with molecular weights higher than chlorogenic acid. Loomis et al. (1979) noticed that PVPP removed very little of the browning precursors from potato tuber extracts, while XAD-4 removed nearly all of these compounds. In the case of strawberries, compounds naturally present in the fruit, such as caffeic acid, protocatechuic acid, p-coumaric acid, gallic acid and 4-hydroxybenzoic acid, all with
TABLE 12. Effect of pH on the activity of strawberry PPO.

SUBSTRATES

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<th>Activity</th>
<th>Percent Max. Act.</th>
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</table>

a Activity of PPO as nmoles O₂/min/g N₂P.  
PPQ extracts obtained using 1:1 level of PVPP and XAD-₄.
Figure 11. pH optima for activity of strawberry PPO using A, catechol and B, 4-methyl catechol as substrates. PPO extracts prepared using 1:1 level of XAD-4 and PVPP.
molecular weights equal or less than chlorogenic acid might not be removed completely using PVPP alone. The combination of PVPP and XAD-4 should extract a larger fraction of the phenolics (See Fig. 8), and this combination gave consistently the highest levels of extracted PPO activity (Table 7). The combination of PVPP and XAD-4 at a 1:1 dry weight level was, therefore, chosen for the preparation of the extracts during the characterization of the PPO.

pH Optima of Strawberry PPO

The first characteristic analyzed was the effect of pH on the PPO activity. The results are shown in Table 12 and Fig. 11. Using catechol as a substrate, a bell-shaped curve was obtained with a pH of 5.5 determined as optimum. At pH above 6.5 it was necessary to include a blank due to auto-oxidation of the substrate. PPO extracts with the enzyme inactivated by boiling were used as controls. From pH 2.5 to 7.5 the PPO activity was higher than the auto-oxidation rate, but at pH values higher than 7.5 auto-oxidation occurred faster. From substrate specificity studies, 4-methyl catechol was found to be much better a substrate than catechol for strawberry PPO. Using 4-methyl catechol as a substrate, a pH optimum of 4.5 was observed for strawberry PPO. A rapid decrease in activity was found at acid pH values and to a lesser degree at alkaline values of pH. At pH of 3.5 or lower, there was less than
10% of the maximum activity of PPO using 4-methyl catechol as a substrate as compared to approximately 50% maximum activity when catechol was used as a substrate. A difference in pH optima with different substrates has also been noticed by other workers (Abukharma and Woolhouse, 1966; Gregory and Bendall, 1966). Mihalyi et al. (1978) noted that the effect of pH on the activity of PPO of a given fruit was found to be highly substrate dependent. The different pKa values of the substrates, pKa 9.37 for catechol and 9.67 for 4-methyl catechol (Serjeant and Dempsey, 1979), and the pH of the buffer system used may also affect the levels of affinity of PPO towards the substrates. The pH affects also the conformation of the enzyme and probably its specificity towards the different substrates.

**Effect of pH during PPO Extraction**

The pH stability of strawberry PPO was measured by extracting the enzyme in buffers ranging in pH from 2.5 to 9.5. The values obtained using catechol and 4-methyl catechol are shown in Table 13 and Fig. 12. Using catechol, two regions of extracted PPO activity were apparent at the different pH levels used. At pH values from pH 2.5 to 6.0 a maximum activity was extracted at pH 4.5. No auto-oxidation of the substrate was noticed. A bell-shaped curve was obtained with a plateau extending from pH 5.0 to 6.5. At pH values higher than 6.5 auto-oxidation of the substrate
TABLE 13. Effect of pH during the extraction of strawberry PPO. Extraction done using 1:1 levels of XAD-4 and PVPP.

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</tr>
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<td>86</td>
<td>5.0</td>
<td>255</td>
<td>35</td>
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<tr>
<td>5.5</td>
<td>62</td>
<td>86</td>
<td>5.5</td>
<td>168</td>
<td>23</td>
</tr>
<tr>
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<td>64</td>
<td>89</td>
<td>6.5</td>
<td>146</td>
<td>20</td>
</tr>
<tr>
<td>7.0</td>
<td>100</td>
<td>*</td>
<td>7.5</td>
<td>103</td>
<td>*</td>
</tr>
<tr>
<td>8.5</td>
<td>35</td>
<td>*</td>
<td>9.5</td>
<td>29</td>
<td>*</td>
</tr>
</tbody>
</table>

a Activity of PPO as nmoles O₂/min/g N₂P.

(*) At pH equal or higher than 7.0 auto-oxidation occurred during the extraction and assay.
The enzyme extracts were prepared using 1:1 level of PVPP and XAD-4.
Figure 12. Effect of pH on the extraction of strawberry PPO using A, catechol, and B, 4-methyl catechol as substrates. PPO extracts prepared using 1:1 level of XAD-4 and PVPP.
occurred. Another peak was present at pH 7.5 after subtracting the auto-oxidation values from the PPO activity values obtained using oxygen electrode. The presence of this second peak could be explained by the auto-oxidation of the natural phenolics in the strawberry extracts occurring during the extraction of the enzyme at high pH levels, i.e. above pH 6.5. Additional polymerization and oxidation reactions of the phenolic intermediates remaining in the extracts could have occurred during the PPO activity measurements. These secondary reactions, together with the oxidation of the added substrate, catechol, catalyzed by PPO, both occurring during the PPO activity measurements, could account for the presence of the extracted activity peak at higher pH values. The decrease in activity at basic pH levels could have been due to the inactivation of PPO. Some oxygen uptake could still be measured at a pH of 9.5 where no PPO activity would be expected, and was probably due to secondary oxidation reactions. Such a decrease in extraction of PPO activity at basic pH values was also noticed by Halim and Montgomery (1978) and Benjamin and Montgomery (1973).

When 4-methyl catechol was used as a substrate during the PPO activity measurements, a bell-shaped curve was obtained with the maximum activity extracted also at pH 4.5 (Table 13). No extraction at pH values higher than 6.5 were attempted, but a decrease in activity was noticed at
pH values higher or lower than pH 4.5. The shape of the curve obtained using 4-methyl catechol as a substrate during the pH stability measurements is very similar to that obtained during the measurement of the pH optimum for PPO activity using the same substrate. Rhodes (1977) reported that the pH for optimum activity was often the pH of greatest stability.

Heat Stability of Strawberry PPO

The PPO extracts obtained were subjected to heat treatments at constant temperatures of 70, 80, 90, 95 and 100°C. The results obtained are shown in Table 14 and Fig. 13 and 14. The time to attain 50% inactivation of the enzyme using catechol as a substrate at 70, 80, 90, 95 and 100°C were 2.78, 0.92, 0.75, 0.65 and 0.32 min, respectively. When 4-methyl catechol was used as a substrate, the half-lives of PPO activity at 70, 80, 90, 95 and 100°C were 1.72, 0.62, 0.25, 0.23 and 0.10 min, respectively. These data show that inactivation of PPO occurred faster when the activity was measured using 4-methyl catechol than with catechol. The rate of heat inactivation for each of the assays was greatest with increasing temperatures and followed first order kinetics. A deviation from linearity was noticed for the 80°C treatment using catechol as a substrate after 5 min.

The values obtained for heat inactivation vary markedly from those obtained by other workers. Half-lives of
TABLE 14. Heat inactivation of strawberry PPO. Extracts obtained using 1:1 levels of XAD-4 and PVPP.

<table>
<thead>
<tr>
<th>TIME (min)</th>
<th>CATECHOL</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>70°C</td>
<td>80°C</td>
<td>90°C</td>
<td>95°C</td>
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<td>70°C</td>
<td>80°C</td>
<td>90°C</td>
<td>95°C</td>
<td>100°C</td>
</tr>
<tr>
<td></td>
<td>(Percent Activity)a</td>
<td>(Percent Activity)a</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.00</td>
<td>100</td>
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<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>0.50</td>
<td>-</td>
<td>79</td>
<td>69</td>
<td>57</td>
<td>36</td>
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<td>84</td>
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<td>29</td>
<td>34</td>
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<td>83</td>
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<td>6</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>1.50</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>0</td>
</tr>
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<td>3.00</td>
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<td>15</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>25</td>
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<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5.00</td>
<td>29</td>
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<td>0</td>
<td>0</td>
<td>0</td>
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<td>1</td>
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<td>8.00</td>
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<td>13</td>
<td>2</td>
<td>0</td>
<td>0</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>12.00</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Half life (min)b</td>
<td>2.78 0.92 0.75 0.65 0.32</td>
<td>1.72 0.62 0.25 0.23 0.10</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Percent remaining activity of PPO.
b Half lives of PPO at the different temperature treatments; time at which 50% of the activity is lost.
The enzyme extracts were prepared using 1:1 levels of PVPP and XAD-4.
Figure 13. Heat inactivation of strawberry PPO; catechol used as a substrate.
Figure 14. Heat inactivation of strawberry PPO; 4-methyl catechol used as a substrate.
PPO activity at 70°C for avocado PPO was 8min (Kahn, 1977), for green olive PPO 15min (Ben-Shalom et al., 1977a) and for d'Anjou pear PPO 11.7min (Halim and Montgomery, 1978). These values are much larger than those obtained for strawberry PPO. Mihalyi et al. (1978) noticed also a different behavior of the PPO extracted from Jonathan apples assayed at different temperatures using pyrogallol and chlorogenic acid as substrates. With pyrogallol, PPO activity increased constantly from 5 to 35°C while with chlorogenic acid a maximum activity of PPO occurred at 30°C while at lower or higher temperatures the activity decreased. Analyzing PPO extracted from other sources they concluded that the action of temperature on the PPO activity was dependent on the substrate used. These data suggest that total heat inactivation of PPO towards one substrate does not necessarily mean inactivation towards other substrates.

Substrate Specificity of Strawberry PPO

Several o-diphenols and monophenols were tested as substrates for strawberry PPO. The results listed in Table 15 show that strawberry PPO was not active towards the monophenols, L-tyrosine or p-coumaric acid, and showed activity towards all the o-diphenols investigated. The highest activity levels were towards (4)-catechin followed by 4-methyl catechol and pyrogallol.

A second category of o-diphenol substrates showing
### TABLE 15. Substrate specificity of strawberry PPO. Extracts obtained using 1:1 levels of XAD-4 and PVPP.

<table>
<thead>
<tr>
<th>SUBSTRATE</th>
<th>Concentration (mM)</th>
<th>PPO Activitya</th>
</tr>
</thead>
<tbody>
<tr>
<td>(+)-D-Catechin</td>
<td>10</td>
<td>545</td>
</tr>
<tr>
<td>4-Methyl catechol</td>
<td>10</td>
<td>435</td>
</tr>
<tr>
<td>Pyrogallol</td>
<td>10</td>
<td>340</td>
</tr>
<tr>
<td>Chlorogenic Acid</td>
<td>5</td>
<td>61</td>
</tr>
<tr>
<td>Caffeic Acid</td>
<td>10</td>
<td>70</td>
</tr>
<tr>
<td>Catechol.</td>
<td>10</td>
<td>49</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>72</td>
</tr>
<tr>
<td>Protocatechuic Acid</td>
<td>10</td>
<td>22</td>
</tr>
<tr>
<td>Dihydroxyphenylalanine</td>
<td>10</td>
<td>13</td>
</tr>
<tr>
<td>o-coumaric Acid</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>L-tyrosine</td>
<td>10</td>
<td>0</td>
</tr>
</tbody>
</table>

a. Activity of PPO as nmoles O_{2}/min/g NBP. The enzyme extracts were obtained using 1:1 level of PVPP and XAD-4.
approximately only 1/10 as much activity is composed of chlorogenic acid, caffeic acid and catechol in decreasing order of activity. Chlorogenic acid presented problems of solubility, and only levels of 5mM or lower could be used. Similar problems in solubility were found with catechin and caffeic acid. Much lower levels of PPO activity were detected using protocatechuic acid and dihydroxyphenylalanine (DOPA).

Of all the substrates tested, (±)-catechin, caffeic acid, chlorogenic acid, protocatechuic acid and DOPA are naturally present in strawberries. Therefore, strawberry preparations have a great potential for enzymic browning to occur. The structural similarity between D-catechin and the anthocyanin pigments present in strawberries suggest that these pigments could serve as substrates for strawberry PPO, at least in the case of cyanidin glycosides.

Several phenolics that do not serve as substrates for PPO act as synergists or inhibitors of PPO (Golan et al., 1977). PPO obtained from other sources that also showed a high specificity towards (±)-catechin, 4-methyl catechol and pyrogallol are those from green olive PPO (Ben-Shalom et al., 1977a), Concord grape PPO (Cash et al., 1976) and pear PPO (Rivas and Whitaker, 1973).

Effect of Inhibitors on Strawberry PPO

The effect of several types of PPO inhibitors were studied and the results are shown in Table 16. Cysteine
TABLE 16. Effect of inhibitors on strawberry PPO. 
Extracts obtained using 1:1 levels of XAD-4 and PVPP.

<table>
<thead>
<tr>
<th>INHIBITOR</th>
<th>Concentration (mM)</th>
<th>Percent Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diethyldithiocarbamate</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>100</td>
</tr>
<tr>
<td>Potassium metabisulfite</td>
<td>1</td>
<td>74</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>100</td>
</tr>
<tr>
<td>Potassium cyanide</td>
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<td>90</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>96</td>
</tr>
<tr>
<td>Dithiothreitol</td>
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<td>81</td>
</tr>
<tr>
<td></td>
<td>10</td>
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<td>8</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>10</td>
</tr>
<tr>
<td>Benzenesulfinic Acid</td>
<td>10</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>18</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>10</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>54</td>
</tr>
<tr>
<td>Ascorbic Acid*</td>
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<td>0</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0</td>
</tr>
</tbody>
</table>

(*) The consumption of oxygen increased when ascorbic acid was added to the extract.
(a) The substrate used was 4-methyl catechol.
The enzyme extracts were obtained using 1:1 level of PVPP and XAD-4. PPO activity was measured using the oxygen electrode and only the initial portion of the curves were considered.
(Cys) and benzene sulfinic acid (Bsu) are known to react covalently with the quinones formed by the PPO activity and, therefore, inhibit the formation of dark polymers (Davies and Pierpoint, 1975; Kahn, 1977). Through the use of the oxygen electrode for the PPO activity measurements, it is possible to detect both the oxygen uptake due to the PPO activity and the uptake due to the secondary oxidation and polymerization reactions of the products of the PPO oxidation. Thus the activity measured is the sum of both oxidations. Cys or Bsu will inhibit the secondary reactions but will not inhibit PPO and the low inhibition reported in Table 16 could be the absence of oxygen uptake due to the secondary reactions. In practice, Cys (or Bsu) serve as good inhibitors of the browning reactions (Montgomery, 1976; Pierpoint, 1970). Cys as a thiol can also reduce quinones, but the reaction of Cys with the quinones will depend upon relative concentrations of Cys and the quinones (Walker, 1977).

Potassium metabisulfite or SO₂ inhibits PPO effectively. The actual mode of inhibition is not well understood, but reactions with the quinones (Kelley and Adams, 1977), with the intermediates of the reaction or with the substrates (Embs and Markakis, 1965) and direct inhibition of PPO (Haisman, 1974) are suggested. Loomis (1974) reports levels of 1mM SO₂ as ineffective for total inhibition of PPO. Both diethyldithiocarbamate and dithiothreitol inhibited PPO completely at 5 and 10mM concentrations, respectively. At
1mM concentrations high inhibition of PPO was obtained using dithiothreitol while almost no inhibition was noticed using diethylidithiocarbamate at the same level. Gregory and Bendall (1966) needed levels of diethylidithiocarbamate equal or higher than 5mM for total inhibition of PPO.

Potassium cyanide is also an effective PPO inhibitor, either through combining with the quinones (Anderson, 1968) or by reacting with the copper in the active site of PPO (Mayer and Harel et al, 1979). Abukharma and Woolhouse (1966) obtained 60% inhibition of PPO using 0.1mM potassium cyanide while Gregory and Bendall (1966) required more than 1mM for total inhibition.

Ascorbic Acid inhibits oxidative browning of o-diphenols by reducing the o-quinones formed back to o-diphenols (Anderson, 1968). The rate of reduction depends upon the concentrations of o-diphenols, ascorbate and enzyme present. At high levels, browning can be inhibited for long periods until the ascorbate is consumed, after which accumulation and condensation of quinones occur (Pierpoint, 1966). At both levels of ascorbate used for the experiments, no direct inhibition of O₂ uptake by PPO was observed and the controls showed very high auto-oxidation levels. A disadvantage of the use of ascorbic acid for the practical inhibition of browning in the case of strawberries is that ascorbic acid can promote the bleaching of the anthocyanin pigments (Poei, 1980).
Another compound that has a practical use in the inhibition of PPO is sodium chloride (NaCl). Ben-Shalom et al. (1977a) noticed that inhibition of PPO by NaCl was pH dependent, with higher concentrations of NaCl needed for inhibition at higher pH. Luh and Phithakpol (1972) reported 30% inhibition using 3.0% NaCl and 16% inhibition at 0.5% NaCl. At 0.1M level of NaCl, i.e. 0.6% NaCl, about 50% inhibition of PPO was possible while lower levels (0.01M, i.e. 0.06% NaCl) only 13.3% inhibition was obtained.

Interesting observations can be made with the use of the oxygen electrode for inhibition studies. Accurate measurements of PPO activity, even at low levels, are obtained and differentiation between inhibition of the enzyme and of the polymerization or secondary reactions is possible. The use of spectrophotometric measurements of PPO activity depends upon the formation of brown pigments due to the secondary reactions and the inhibition of these reactions would be taken as the inhibition of the PPO reaction. But from the results obtained using Cys, Bsu and ascorbic acid, it can be seen that this is not the case. The secondary reactions did not occur as long as these inhibitors were present, but PPO was active as shown by oxygen uptake.
SUMMARY

Different phenol adsorbents were used during the extraction of strawberry PPO to prevent possible modifications or inhibition of the enzyme due to protein-phenolic interactions. Special care was also taken during the storage and preparation of the extracts in order to minimize the PPO modification or inactivation in these steps. The most efficient extraction procedure was used to extract PPO for the partial characterization of this enzyme.

This experiment resulted in the following:

1. Adequate storage of the fresh strawberries was by quick freezing the washed fruits in liquid nitrogen, sealing in oxygen-impermeable bags under vacuum and storage at -40°C.

2. By homogenizing the frozen strawberries under liquid nitrogen in a Waring Blender and storing the nitrogen powder (N₂P) under liquid nitrogen it was possible to prevent chemical reactions in the homogenized tissues. No loss in PPO activity was noted after 95 days of storage.

3. The buffer-extracted PPO showed low levels of activity and quick onset of browning with a consequent loss of PPO activity with time.
4. In order of decreasing efficiency, PVPP:XAD-4; PVPP; PVPP:Dowex 2; XAD-4:Dowex; and XAD-4, improved the extraction of PPO by binding the phenolic compounds in the extracts. Dowex 2 alone and at all the levels tried could not remove enough phenolics, and browning of the extracts occurred.

5. Electrophoretic studies showed the necessity of concentrating the extracts. PPO activity could only be detected in the initial portion of the gels, except for the Dowex 2 and untreated extracts, for which no PPO activity could be detected. The presence of pectin in the extracts was suggested as responsible for the interference during electrophoresis.

6. The extracts obtained using PVPP alone or in combination showed the highest efficiency during extraction and the best stability. The combination of PVPP and XAD-4 was suggested for the extraction of PPO since the combination of the phenol-binding characteristics of both adsorbents appeared to complement each other.

7. A pH optimum of 5.5 was obtained using catechol as a substrate while with 4-methyl catechol the pH optimum for activity measured was 4.5.

8. Maximum activity of PPO was extracted at pH 4.5 using either catechol or 4-methyl catechol during the assays.
The temperature stability of PPO varied depending on the substrate used for the assays. Inactivation followed first order kinetics and the half-lives reported when catechol was used as a substrate were 2.78, 0.92, 0.75, 0.65 and 0.32 min at 70, 80, 90, 95 and 100°C, respectively. When 4-methyl catechol was used, the half-lives were 1.72, 0.62, 0.25, 0.23 and 0.10 min at 70, 80, 90, 95 and 100°C, respectively.

No cresolase activity was detected. The PPO showed activity towards all the o-diphenols tried. The highest activities measured were using D-catechin, 4-methyl catechol and pyrogallol as substrates.

Diethyldithiocarbamate, potassium metabisulfite, potassium cyanide and dithiothreitol showed direct and effective inhibition of PPO. Partial inhibition of PPO was possible using high concentrations of sodium chloride. Cysteine, benzene sulfinic acid and ascorbic acid did not inhibit PPO directly but were efficient in inhibiting the secondary reactions of the o-quinones.

With the use of the polarographic assay method, it was possible to differentiate between direct inhibition of PPO and inhibition of the secondary reactions of the o-quinones.
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