THE UNIVERSITY OF MANITOBA

POLYPHENOL OXIDASE OF JERUSALEM ARTICHOKE (*HELIANTHUS TUBEROSUS* L.) TUBERS: ISOLATION, PURIFICATION AND PROPERTIES

by

JERZY ZAWISTOWSKI

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A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

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To my wife Urszula

and our children

Dorota and Robert

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ABSTRACT

An active and stable polyphenol oxidase system was isolated from Jerusalem artichoke (Helianthus tuberosus L.) tubers. The crude enzyme, extracted in 0.1 M phosphate buffer, pH 6.5, showed optimum activity using dihydroxyphenol substrates. In this respect, chlorogenic acid followed by catechol, and DL-dopa showed the highest reactivity. The enzyme exhibited an optimum pH of 6.0 using 0.01 M catechol as a substrate. The specific activity of the enzyme was approximately 13 and 3 times the level found in potatoes and sugar beets, respectively. Thermal inactivation data indicated an apparent activation energy of 54 kcal/mole. Over 84% of the total soluble enzyme was located in the tuber peels.

The crude enzyme was used to compare the efficacy of known plant PPO inhibitors in suppressing enzymatic browning which occurs during processing of artichoke tubers. Although all compounds inhibited PPO activity, the extent of inhibition was dependent on both the nature and concentration of the inhibitor. Using a 5 mM concentration, the order of effectiveness for the inhibitors in achieving 98-99% inhibition was: sodium and potassium metabisulfite > sodium bisulfite. At a 10 mM concentration the order of effectiveness for similar degree of inhibition was: L-cysteine > sodium bisulfite > potassium ethylxanthate. The

remaining compounds required higher concentrations in order to achieve either equal or lower levels of PPO inhibition. At pH values below 5.0, the pH alone was an effective inhibitor. The PPO activity in extracts stored at 4°C for 15 days was completely inhibited by 5 and 25 mM sodium metabisulfite and L-cysteine, respectively. Resumption of PPO activity in the presence of 10 mM L-cysteine increased progressively during storage. A linear relationship between remaining PPO activity and extract color was established.

Crude artichoke PPO was purified by metal-chelate affinity chromatography using copper conjugated to iminodiacetic acid Sepharose 6B. Four PPO fractions obtained after chromatography showed various specific activities. The highest degree of purification (160 X) was exhibited by a fraction showing no interaction with the gel. This fraction was used for all subsequent studies to characterize the Jerusalem artichoke PPO system.

The purified enzyme was primarily an o-dihydroxyphenoloxidase with apparent K_m values of 1.9, 3.5 and 3.9 mM for chlorogenic acid, 4-methylcatechol, and catechol, respectively. Metabisulfite was a highly effective mixed type of inhibitor for the enzyme (K_{ii}=80 μM and K_{is}=40 μM). Several forms were identified by gel filtration and SDS-gradient polyacrylamide gel electrophoresis: two aggregates with apparent MW of 120 K and 86 K and two monomeric subunits of 40-42 K and 32-34 K, respectively. Concentration dependant

association-dissociation phenomena most likely determine the multimeric state of this enzyme. While the aggregated forms exhibited specificity towards mono-, di- and polyhydroxyphenols, the low MW subunits were active only with o-dihydroxyphenols. The enzyme was found to contain appreciable amounts of associated carbohydrate material. The isoelectric points of the various enzyme species were within the range of 4.0 to 10.0; the predominant form being in the acidic pH region.

The acidic fraction of PPO was further purified by ion exchange chromatography. Isoelectric focusing analysis showed a cluster of activity bands (microheterogenity) in the pH region of 4.5. The amino acid composition of the enzyme was also determined. The pH optimum for oxidation of chlorogenic acid, 4-methylcatechol and catechol was 6.0, while the apparent K_m values for these substrates were 1.8, 3.6 and 4.0 mM, respectively. Substrate inhibition was observed by excess of chlorogenic acid. Potassium cyanide was an effective noncompetitive inhibitor with an apparent K_i value of 0.6 mM. Thermal inactivation data indicated an apparent activation energy of 26 kcal/mole. Kinetics of deactivation, upon copper removal by KCN treatment, and reconstitution reactions revealed that the former is a much slower process than reactivation.

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GENERAL INTRODUCTION

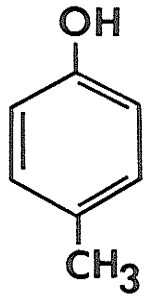
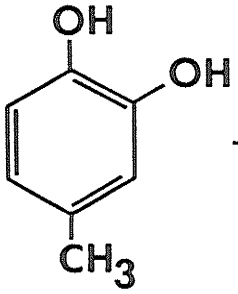
Browning reactions of fruits, tubers and vegetables, which occur after infliction of a mechanical injury to plant tissues, are initiated by the enzyme polyphenoloxidase (PPO, o-diphenol:O_2 oxidoreductase, EC 1.14.18.1). As such, the catalytic action and molecular properties of the enzyme have direct implications into the sensory attributes of both fresh and processed foods of plant origin. As a result, PPO has been a subject of numerous investigations. In two recent reviews by Mayer and Harel (1979) and Vámos-Vigyázó (1981), the research findings on various aspects of PPO action and molecular properties are discussed in a fairly comprehensive manner. Furthermore, the localization and functions of plant PPO's have been the subject of the reviews published by Vaughn and Duke (1984a) and Robb (1984). The presence of this enzyme was discovered over 90 years ago by Bertrand (1896). Since then it has featured in several pioneering studies (Robb, 1984), being the first enzyme to be purified by affinity chromatography (Lerman, 1953), one of the first enzymes to be shown to catalyse the incorporation of molecular oxygen into an organic molecule (Mason et al., 1955), and it provided an early example of what is now called suicide inactivation (Abeles and Maycock, 1976).

Recent cytochemical studies of PPO have provided evi-

dence that PPO is a plastid enzyme. The PPO has been localized in a diverse series of plastids: root plastids, potato tuber amyloplasts, epidermal plastids, carrot tissue culture plastids, etioplasts, chromoplasts as well as chloroplasts of many different species (Vaughn and Duke, 1984a). Moreover, studies on PPO in tentoxin (tetrapeptide that specifically affects plastid development, thus inducing severe chlorosis) treated plants revealed the lack of PPO activity by means of cytochemical, electrophoretic and spectrophotometric procedures (Vaughn and Duke, 1981a; 1984b; Duke and Vaughn, 1982).

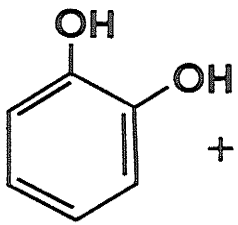
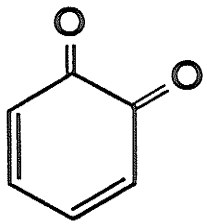
Polyphenol oxidase is a copper-containing enzyme which may catalyze two types of reaction (Fig. 1): the hydroxylation of monophenols to o-diphenols (Eq. a) and the dehydrogenation of o-diphenols to o-quinones (Eq. b). The latter products can subsequently undergo a series of nonenzymatic reactions to yield brown-black melanin pigments (Eskin et al., 1971; Whitaker, 1972; Matheis and Whitaker, 1984). The two enzymatic activities are commonly referred to as cresolase or monophenolase and catecholase or diphenolase activities, respectively (Nelson and Dawson, 1944). The ratio of diphenolase:monophenolase varies with the plant source, in most - the range was from 1:10 to 1:40 (Vámos-Vigyázo', 1981). There are, however plants such as mango (Joshi and Shilarkar, 1977), cherry (Lanzarini et al., 1972), banana (Palmer, 1963), and pear (Rivas and Whitaker, 1973) void of

Figure 1. Reactions catalysed by polyphenol oxidase.
a) hydroxylation of monophenol to o-diphenol
b) dehydrogenation of o-diphenol to o-quinone

a  + E-2Cu^{+} + O_2 + 2H^{+} $\xrightarrow{+2\bar{e}}$  + E-2Cu^{2+} + H_2O

p – Cresol

4 – Methylcatechol

b  + E-2Cu^{2+} + $\frac{1}{2}O_2$ $\xrightarrow{-2\bar{e}}$  + E-2Cu^{+} + H_2O

Catechol

o – Benzoquinone

of monophenolase activity. The hydroxylation reaction starts on a pure monohydroxyphenol substrate after a lag period which can be reduced or entirely eliminated by the addition of small amounts of o-dihydroxyphenols. In vivo, where diphenols more likely are always present, this induction phase cannot be observed. Most probably both hydroxylation of monophenols and dehydrogenation of o-dihydroxyphenols are catalyzed by the same PPO molecule (Vámos-Vigyázó, 1981). Although the mechanism of the reaction of plant PPO has not been fully elucidated, the overall mechanism of fungal PPO (Neurospora crassa) has been reported by Lerch (1981). Accordingly, the minimal functional molecular unit of PPO consists of a single-chain protein with two copper atoms per molecule, bound in part by histidine. The active site copper is binuclear and exists in different functional states: met, oxy, and deoxy. Monophenols bind to one of the Cu^{2+} atoms, while diphenols bind to both of them. The monophenolase activity is intimately coupled to the diphenolase activity yielding the two electrons required (Fig. 1, eq. 1) for the incorporation of one oxygen atom into the monophenol substrate (Lerch, 1981). The reaction mechanism appears to be similar to that of hemocyanin, and essentially follows the ideas of Kubowitz's (1938) from studies of PPO in potatoes.

The role of PPO in plant is ambiguous, because in healthy green tissues this enzyme exists in a latent form,

which hampers the study *in vivo*. However, several hypotheses on functions of PPO have been suggested. The most important is its role in the resistance of plants to infection by viruses, bacteria, fungi or mechanical injury. It has been reported that in case of infection or injury PPO increases in activity possibly due to inactivation of host latent enzyme, due to solubilization of this part of enzyme which is normally particulate, or even due to *de novo* synthesis (Mayer and Harel, 1979). This enhances quinone formation upon the action of the enzyme, and subsequently results in production of insoluble polymers. The plant tissues "impregnated" with these polymers act as barriers in the way of the spreading infection (Rubin and Artsikovskaya, 1960). According to another theory, intermediates of the oxidative polymerization of polyphenols might inactivate or bind some labile plant enzymes and viruses, respectively (e.g. cucumber mosaic virus) temporarily prevent or reduce infection (Pierpoint, 1966). Melanin and quinones have a bacteriostatic property and quinones have been shown to cause partial inactivation of a virus (Pierpoint et al., 1977). Production of quinones has been claimed to be essential for resistance of apple to infection by *Venturia inequalis* (Overeem, 1976) and helps to prevent spread of infection by *Botrytis cinerea* on broad bean (Deverall, 1961). In addition, the role of PPO in biosynthesis of plant phenolics, particularly phenylpropanoids and nondi-

rect involvement in lignin formation has been suggested (Robb, 1984). However, not enough conclusive results have been obtained to fully support this theory (Vaughan and Duke, 1984a).

Polyphenol oxidase is widely distributed in the plant kingdom being present in most, if not all plant tissues. It has been purified and studied in a number of plant including potatoes (Hyodo and Uritani, 1966; Weaver et al., 1968; Mondy and Koch, 1978), apples (Harel et al., 1965; Harel and Mayer, 1968), peaches (Harel and Mayer, 1970), bananas (Palmer, 1963), sugar beets and sugar cane (Gross and Coombs, 1976a,b) as well as avocados (Kahn, 1977). However, there is no information available on the artichoke PPO. Therefore, these studies were directed to identify, purify and characterize the properties of the PPO of Jerusalem artichoke tubers. This project had the following objectives: i) identification and characterization of PPO present in crude extract; ii) purification of this enzyme and isolation of the most predominant activity form; iii) study the properties of purified PPO.

CHAPTER ONE

PROPERTIES OF CRUDE POLYPHENOL OXIDASE IN JERUSALEM ARTICHOKE TUBERS

1.1 INTRODUCTION

Jerusalem artichoke (Helianthus tuberosus L.) tubers (hereafter called artichoke) contain a homologous series of glucofructans, consisting of linear chains of (2→1) linked β-D-fructofuranose residues terminated by a β-D-glucopyranose residue attached as in sucrose (Whistler and Smart, 1953). The degree of polymerization (DP) varies from 2 (in sucrose) to 35, inulin being defined as the fraction with a DP higher than 30 (Edelman and Jefford, 1968). The proportions of the different polyfructans vary according to the maturation state of the plant which depends on agronomic conditions, cultivation and harvest data (Chabbert et al., 1983). The polyfructan content of the tuber ranges from 75-85% of dry matter (Fleming and GrootWassink, 1979). Fructose, as a percentage of the polyfructans, varies from 75-98% according to growth and storage treatment of the tubers (Bacon and Loxley, 1952).

Artichoke is a plant of the Compositae family and is a native of North America. The main advantage of artichoke is its enormous carbohydrate yield (Table 1.1; Canadian data) ranging between 2.0 to 15.0 T/ha which out-performs corn and sugar beets having yields of 3.6-4.1 T/ha and 4.9-6.6 T/ha, respectively (Fleming and GrootWassink, 1979). In addition, a good growth of this crop can be maintained on poor land without competition for the good quality irrigated and fertilized land used to grow traditional crops

Table 1.1 Carbohydrate yield data for artichoke, corn, and sugar beet

Country	Carbohydrate yield (kg/ha)
Artichoke tubers[a]	
Canada	1,793 - 15,243
U.S.A.	3,844 - 7,173
Netherlands	3,584 - 6,052; 7,262
France	2,443 - 7,621
Germany	1,210 - 3,216
U.S.S.R.	1,597 - 2,398; 9,527[b]
Corn kernels[c]	
Canada	3,631 - 4,147
U.S.A.	3,373
Sugar beets	
Canada	4,920 - 6,612
U.S.S.R.	3,026

[a] Adapted using standard composition of 20% dry matter and 80% carbohydrate.

[b] Sunflower - artichoke hybrid.

[c] Adapted using standard composition of 82% dry matter and 75% carbohydrate.

Source: Fleming and GrootWassink, 1979

(Dorrell and Chubey, 1977). Artichoke has displayed remarkable resilience to frost and plant diseases (Stauffer et. al., 1975) which, in conjunction with its adaptability and carbohydrate yield, accounts for growing interest in this crop as a potential source of fructose.

Since the beginning of this century artichoke has been investigated in many countries and several systems on the pilot scale have been proposed for the production of fructose syrup (Eichinger et al., 1932; McGlumphy et al., 1932; Dykins and Englis, 1933; Englis et al., 1933; Englis and Fiess, 1942), crystaline fructose (Williaman, 1922; Yamazuki, 1954), and industrial alcohol (Underkofler et al., 1937). Unfortunately, these activities were discontinued, because agronomic technology and existing economics were unfavorable for commercial fructose production (Chubey and Dorrell, 1974). More recently, due to unpopularity of non-nutritive sweeteners and increased demands of the beverage industry for fructose syrup returned an interest in artichoke tubers as a fructose source. Consequently several procedures for preparing high fructose syrup were established (Fleming and GrootWassink, 1979; Kierstan, 1980; Hoehn et al., 1983). However, during processing of tubers, extensive discoloration was encountered (Bacon and Edelman, 1951; Fleming and GrootWassink, 1979; Kierstan, 1980; Guiraud and Galzy, 1981). Although the reasons were not elucidated, various methods of preventing dark syrup development

have been reported, such as adding sulfur dioxide (Conti, 1953), and potassium cyanide (Bacon and Edelman, 1951), boiling the tubers prior to extraction (Kierstan, 1983), or removing the outer layers of the tubers (Fleming and GrootWassink, 1979). Since the above treatments are recognized to inhibit a polyphenol oxidase one can assume that the underlying cause of the discoloration problem may be the presence of this enzyme which oxidizes the natural phenolic compounds upon comminution of the tubers. This hypothesis is supported by the fact that the presence of polyphenolics in artichoke tubers has been also reported (Paupardin, 1965).

In this chapter, preliminary evidence is reported to indicate the presence of a relatively active and stable artichoke polyphenol oxidase, as well as its involvement in discoloration of extracts during processing of artichoke tubers. In addition, some characteristics of the crude enzyme such as pH optima, heat stability, substrate specificity, localization within the tubers, as well as effect of inhibitors and their efficacy in suppressing enzymatic browning are also discussed.

1.2 MATERIALS AND METHODS

1.2.1 Materials

1.2.1.1 Jerusalem artichoke tubers

Artichoke tubers from the Columbia cultivar (Chubey and Dorrell, 1982) developed at the Agriculture Canada Research Station, Morden, Manitoba, were used throughout this investigation. The tubers (crop year 1976, 1982, and 1983) were harvested in late October and stored in plastic bags at $-30^{o}C$ (crop year 1976, 1982) or at $4^{o}C$ (crop year 1983) until used. The study of the effect of low temperature on PPO activity was carried out on tubers of the crop year 1976 and 1982. The other experiments were performed on tubers of the crop year 1983.

1.2.1.2 Other tubers

Sugar beets tubers cv. mono-HyD7 were supplied by Manitoba Sugar Company, while potato cultivars (Russet Burbank) were obtained locally. All tubers were harvested in the fall of 1983 and stored in plastic bags at $4^{o}C$ until used.

1.2.1.3 Chemicals

Unless otherwise indicated, all reagents were of analytical grade.

1.2.2 Methods

All preparation steps and analytical procedures, un-

less otherwise indicated, were carried out at 4°C. Analyses were performed in triplicate and results are expressed as means.

1.2.2.1 Extraction

Crude enzyme extracts were prepared from whole tubers that were washed in cold water, air dried and then sliced 0.5 cm thick. Fresh tuber slices (100 g) were homogenized for 1 min with 100 mL of 0.1 M sodium phosphate buffer, pH 6.5 (unless otherwise specified) using a commercial Oster blender. The homogenates were centrifuged (4,000 x g) at 4°C for 20 min. and the supernatants were filtered using a Whatman No. 4 filter paper. Approximately 150 ml of filtrate was obtained from each extraction trial. The crude enzyme was used for experiments immediately after extraction.

Distribution of PPO activity within the artichoke tubers was assessed by sectioning whole tubers into outer peels (ca. 0.5 mm), a secondary layer (50 mm) and the remaining middle core. Portions from each tuber section (100 g) were homogenized with phosphate buffer as described above and appropriate filtrate was assayed for PPO activity.

In order to compare the relative activity of PPO in artichoke tubers, to those of potato and sugar beet cultivars, 100 g of sliced tuber material was used from each source and prepared accordingly.

1.2.2.1.1 pH optimum

To determine the pH optimum for extraction of crude PPO from tubers, initial extractions were performed at pH 4.5-8.5 using 0.1 M sodium monobasic - dibasic phosphate buffer. All subsequent extractions were performed at pH 6.5.

1.2.2.1.2 Ionic strength optimum

To determine the ionic strength optimum for extraction, initial extractions of PPO were performed using sodium phosphate buffer, pH 6.5, in the range between 0.01 M (μ=0.22) and 0.5 M (μ=11.0). The ionic strength (μ) was calculated from the following formula:

$$\mu = \Sigma\, 1/2cZ^2$$

in which Σ indicates a summation of the term $1/2cZ^2$ for each kind of ion present in the solution, c being the molarity of the ion and Z, its valency.

1.2.2.2 Enzyme activity determination

The polyphenol oxidase catalyzes the oxidation of phenolic compounds (e.g. catechol) to quinones. The reaction is accompanied by uptake of oxygen, the rate of which is proportional to the enzyme concentration. Oxygen uptake was used as a measure of PPO activity according to the polarographic method of Yamaguchi et al. (1969). The biological oxygen monitor (model 53), standard bath assembly (model 5301) equipped with a reaction vessel, and oxygen

probe (model 5331), all from Yellow Springs Instrument Co., thermostat type 623 from Heto Birkerop, Denmark, and recorder B-5000 Omniscrib, Huston Instr., were used as a standard equipment for enzymatic assay. Three milliliters of substrate solution containing 0.01 M catechol (unless otherwise stated) dissolved in 0.1 M sodium phosphate buffer, pH 6.0, aerated at room temperature for 5 minutes, were placed in the reaction vessel and stirred for approximately 2 minutes to reach a constant temperature of 30°C. At time zero, 50 µl of enzyme solution was introduced into the reaction vessel using a calibrated microsyringe (Hamilton Co.). The time course of the reaction was plotted by a 100 mV recorder; the full scale represented 100% O_2 saturation. A tangent was drawn to the curve to estimate the initial reaction velocity. At 30°C the O_2 concentration in the air saturated solution is 0.23 mM. Final enzyme activity was calculated using the following formula:

$$U = \frac{0.23 \times a \times b}{100\%}$$

where: U is the activity units, a is the percentage of oxygen consumed during 1 minute of reaction, b is the conversion factor to express the enzyme activity per ml.

One unit of PPO activity was defined as the amount of oxygen (µmoles) consumed per minute at 30°C. Specific activity was expressed as units per mg of protein.

1.2.2.3 Protein determination

Protein was precipitated from undiluted extracts with trichloroacetic acid (TCA; final concentration 10% w/v) and the precipitate was washed twice with acetone in order to remove interfering polyphenolics (Anderson, 1968). The washed precipitate was solubilized with 0.1 N NaOH at room temperature for 2 hr and an aliquot used for estimating protein by the method of Lowry et al. (1951) using bovine serum albumin fraction V as a standard.

1.2.2.4 pH optimum

A pH profile (1.5 to 9.0) for crude PPO activity was determined using 0.1 M sodium phosphate buffer. Phosphoric acid (0.1 M) was used to adjust the pH of the reaction mixture below 4.5. Determination of PPO activity was carried out using catechol as a substrate. In addition, the autoxidation of catechol was monitored (reaction mixture without enzyme) and taken into account at each of the specified pH values.

1.2.2.5 Temperature stability

Temperature stability of PPO in the tubers was evaluated by drying tuber slices (0.5 cm) in a fluidized bed dryer (Model 150 Fitzpatrick) at 50, 60, 75 and 80^{o}C for 3 to 4 hours. Storage stability was evaluated at 1, 12 and 72 months at -30^{o}C. Activity was determined on portions equivalent to 100 g fresh matter and expressed as percent

residual activity. The temperature stability of the crude enzyme was determined using extracts (2 ml) that were heat treated at 60, 70, 75, 80 and 90^{o}C for varying time periods in a temperature controlled water bath. Following heat treatment portions of extracts were withdrawn, cooled in an ice bath and assayed for residual activity. Stability of the enzyme was expressed as log% activity remaining. The activation energy was then estimated from an Arrhenius plot of log reaction rate (LnK) versus the reciprocal of the absolute temperature.

1.2.2.6 Substrate specificity

Substrate specificity of the crude PPO was determined using 0.01 M of the following compounds in 0.1 M sodium phosphate buffer, pH 6.0; DL-tyrosine, p-cresol, chlorogenic acid, catechol, DL-dihydroxyphenylalanine (dopa), and caffeic acid. The reagents used were purchased from Sigma.

1.2.2.7 Effect of inhibitors

The following compounds were tested as potential inhibitors for artichoke PPO: sodium diethyldithiocarbamate (DIECA), potassium ethylxanthate, sodium azide, disodium ethylenediaminetetraacetate (EDTA), 2,3-naphthalenediol, benzoic acid, 2-mercaptobenzothiazole, 2,3-dimercaptopropanol, thioglycollate, sodium and potassium metabisulfite, sodium hydrosulphite, sodium bisulfite, L-cysteine, sodium benzosulfonate, sodium borate and thiourea. These inhibi-

tors were also used to examine the relationship between the percent PPO activity remaining in artichoke extracts and their color (lightness). Each inhibitor was dissolved in sodium phosphate buffer (0.1 M, pH 6.5) and employed in final concentrations of 1.0, 5.0, 10.0 and 25.0 mM. Samples of artichoke tubers (100 g) were homogenized in 100 ml of phosphate buffer (as described in Section 1.2.2.3) containing each of the tested inhibitors in specified concentrations. The effect of pH on PPO inibitors was investigated using sodium metabisulfite and L-cysteine (2 mM) at pH 4.0-7.0. The efficacy of sodium metabisulfite (5 mM) and L-cysteine (10 mM) to inhibit PPO activity in crude extracts (pH 6.5) stored at 4^{o}C was also investigated. Incubation was carried out in sealed flasks containing air as the head space; sampling was performed every three days.

All inhibitor preparations were fresh when added to the enzyme extract at the start of incubation or storage. In addition, control enzymatic preparations (without inhibitor) were investigated for each experimental trial. Inhibition was evaluated by recording the percent PPO activity remaining and/or the resultant color (lightness) of the extracts.

1.2.2.7.1 Color measurements

Color development was measured using a Hunterlab colorimeter Model D25L-2. The colorimeter was standardized against a white tile C2-12418; the lightness (L) and chro-

maticity dimensions (a and b) were determined. Inhibitor treated extract solutions (100 ml) placed in a specimen dish (No 13851230) were used for determination of color. All extract solutions were standardized to contain 8% solids with phosphate buffer (0.1 M, pH 6.5) using an Abbe refractometer and then analyzed immediately.

1.3 RESULTS AND DISCUSSION

1.3.1 Effect of pH on extraction and activity

The effect of pH on extraction of crude PPO is presented in Table 1.2. Although optimum extraction was obtained at pH 6.5, relatively high specific activities were achieved for extractions at pH 7.0 and 5.5 (ca. 82 and 88% of the optimum value, respectively). Generally, higher activities were obtained using acidic rather than alkaline extractions. The results indicated a broad pH range for enzyme extraction and/or stability.

One of the general considerations regarding the isolation of PPO from plant tissues is the type of solution used for its extraction. This consideration is dependent partially on the nature and location of the enzyme in the tissue since PPO has been reported to exist both in soluble and particle bound forms (Mayer, 1987); the extent or ratio between the two forms is dependent on the maturity as well as the source of the plant tissue (Ivanov, 1966). Aqueous solutions of moderately high ionic strength and appropriate pH have been reported by several investigators, including Matthew and Parpia (1971), to be very successful in PPO extraction. In this investigation soluble crude PPO was extracted from artichoke tubers using 0.1 M phosphate buffer at an optimum pH of 6.5. Although 0.1 M phosphate buffer (ionic strength μ=2.2) was arbitrarily chosen, the buffer (pH 6.5) at the tested range of ionic strength

Table 1.2 Effect of pH on extraction of crude PPO[a]

pH	PPO activity[b] (U/ml)	Protein (mg/ml)	Specific activity (U/mg protein)
4.5	3.00 ± 0.05[c]	2.36 ± 0.02	1.27
5.0	3.56 ± 0.14	2.84 ± 0.04	1.25
5.5	5.72 ± 0.03	3.36 ± 0.02	1.70
6.0	3.36 ± 0.05	2.86 ± 0.02	1.18
6.5	6.08 ± 0.01	3.16 ± 0.01	1.92
7.0	5.64 ± 0.12	3.36 ± 0.07	1.68
7.5	3.40 ± 0.07	2.90 ± 0.02	1.17
8.0	4.00 ± 0.05	3.66 ± 0.09	1.09
8.5	3.64 ± 0.05	4.16 ± 0.14	0.88

[a] Whole tubers.

[b] Represents activity units (U); μM of oxygen consumed per min using catechol as substrate.

[c] Mean values ± SD based on triplicate determination of three extracts.

between 0.22 (0.01 M) and 11.00 (0.5 M) did not significantly affect an activity of extracted PPO (Table 1.3). Extractions at pH 7.0 and 5.5 also resulted in crude enzyme preparations with relatively high activities. The activities shown in these two extracts may represent a portion of the optimum pH extraction range, and/or indicate that other proteins are extracted under these pH conditions.

A pH profile is shown in Figure 1.1. Although optimum activity was observed at pH 6.0, the enzyme exhibited a broad activity range from pH 5.0 to 8.5. Many investigators have also reported a relatively wide pH range, in most cases between 4.0 and 7.0 for PPO activity from other plant sources (Vámos-Vigyázó, 1981). An optimum pH of 6.0 was also reported for peach and mushroom PPO using catechol as a substrate (Dawson and Mager, 1962; Jen and Kahler, 1974).

1.3.2 Temperature stability

The effect of temperature and time of exposure on stability of PPO in the tuber tissue is given in Table 1.4. These results suggested that both parameters influenced the amount of residual activity. The enzyme exhibited a certain degree of heat stability, retaining over 27% of its initial activity after heat treatment at 80°C for 4 hr. Temperatures used in this investigation were not sufficiently high to totally inactivate the enzyme. Storage at -30°C appeared to inactivate the enzyme rapidly during the first month of storage; thereafter a more gradual decrease in activity was

Table 1.3 Effect of ionic strength (μ) on extraction of crude PPO[a]

μ	PPO activity (U/ml)	Protein (mg/ml)	Specific activity (U/mg protein)
0.22	6.14 ± 0.08[b]	3.10 ± 0.04	1.98
0.55	5.68 ± 0.06	2.84 ± 0.02	2.00
1.10	6.24 ± 0.08	3.06 ± 0.04	2.04
2.20	6.66 ± 0.06	3.20 ± 0.02	2.08
4.40	6.00 ± 0.12	2.90 ± 0.03	2.07
6.60	6.32 ± 0.05	3.04 ± 0.00	2.08
8.80	6.14 ± 0.10	2.98 ± 0.05	2.06
11.00	6.55 ± 0.12	3.18 ± 0.08	2.06

[a] Sodium phosphate buffer (pH 6.5) was used for extractions.

[b] Mean values ± SD based on triplicate determination of three extracts.

Figure 1.1 pH optimum of crude PPO using catechol as a substrate.

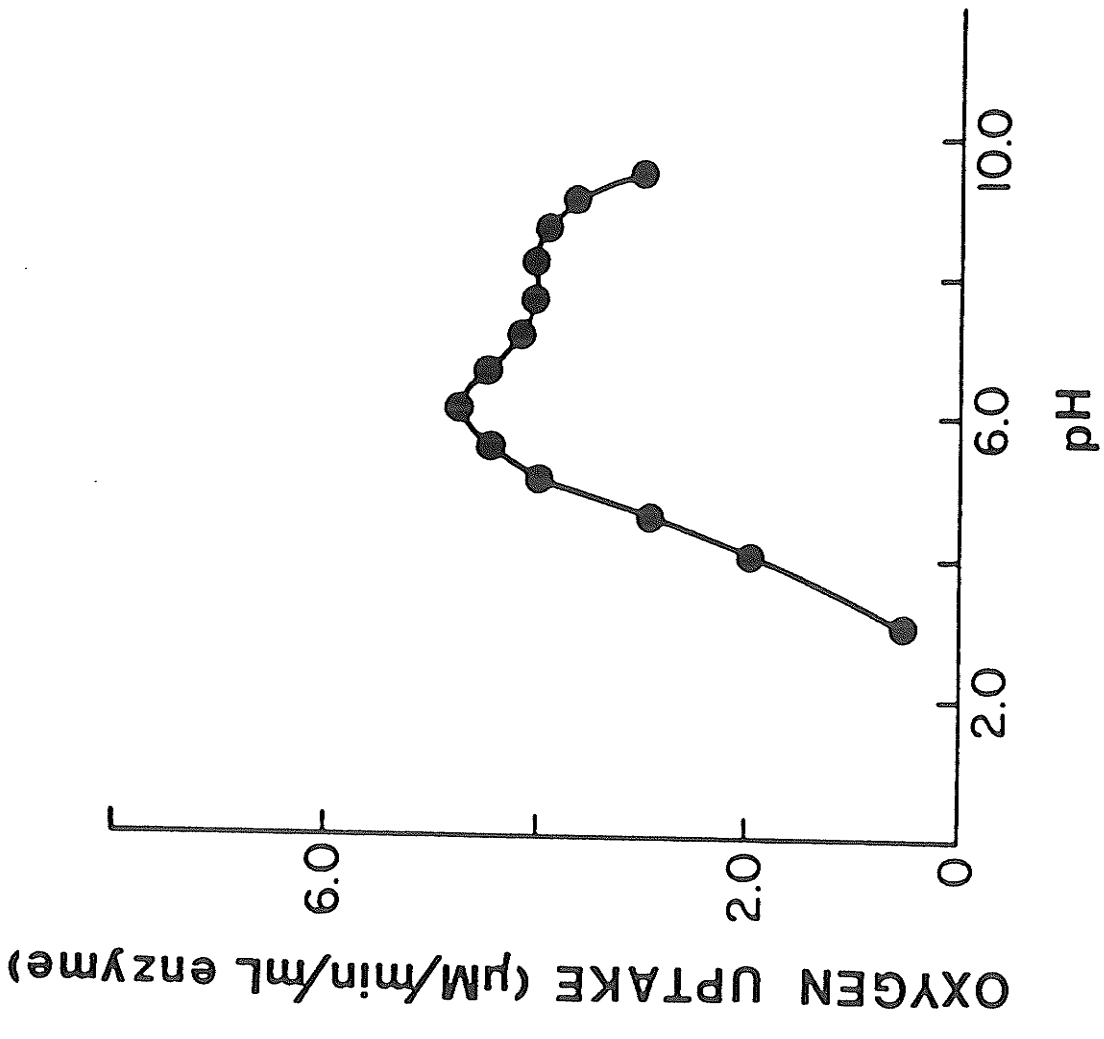

OXYGEN UPTAKE (μM/min/mL enzyme)
0
2.0
6.0
2.0
6.0
10.0
pH

Table 1.4 Effect of temperature on PPO activity

Treatment	Residual PPO activity (%)	Residual specific activity (%)
Fresh tuber (Control)	100.0	100.0
Drying[a]		
50°C for 4 hr	25.8	45.5
60°C for 3 hr	41.9	68.9
75°C for 3 hr	33.8	66.3
80°C for 4 hr	11.6	27.4
Freezing[b]		
-30°C for 1 mo	34.0	45.2
-30°C for 1 year	23.2	42.8
-30°C for 6 years	5.9	9.8

[a] Dried samples were prepared by processing tuber slices (5 mm) in a fluidized bed dryer.

[b] In sealed plastic bags.

observed.

Heat treatment of tubers slices at temperature extremes (80; -30°C) did not fully inactivate PPO. These residual levels would be expected to be higher in whole tubers because of a smaller specific area exposed to the temperature treatment. Since enzyme activities in tubers are known to change continuously during post harvest storage, it is difficult to predict the exact effects of time and temperature especially during protracted cold storage. PPO system from many plant sources, however, have been reported to be relatively stable to cold temperature storage (Pallavicini, 1969; Vámos-Vigyázó, 1981). This factor will undoubtedly be of importance if artichokes are harvested and stored commercially since even low residual levels of PPO activity may bring about discoloration following low temperature storage (Phillippon, 1975). Endogenous substrate release due to freeze - thaw cycles, which tubers may undergo upon storage, can further accentuate the problem.

The heat stability of crude PPO is presented in Fig. 1.2. Temperatures of 60 to 75°C for heating times up to 180 min appeared to have modest effects on enzyme activity when compared to temperatures of 80 and 90°C. An Arrhenius plot indicated an apparent activation energy of 54 kcal/mole (Figure 1.3). Although PPO does not belong to the extremely heat-stable enzyme group, results obtained in

Figure 1.2 Heat inactivation of crude PPO.

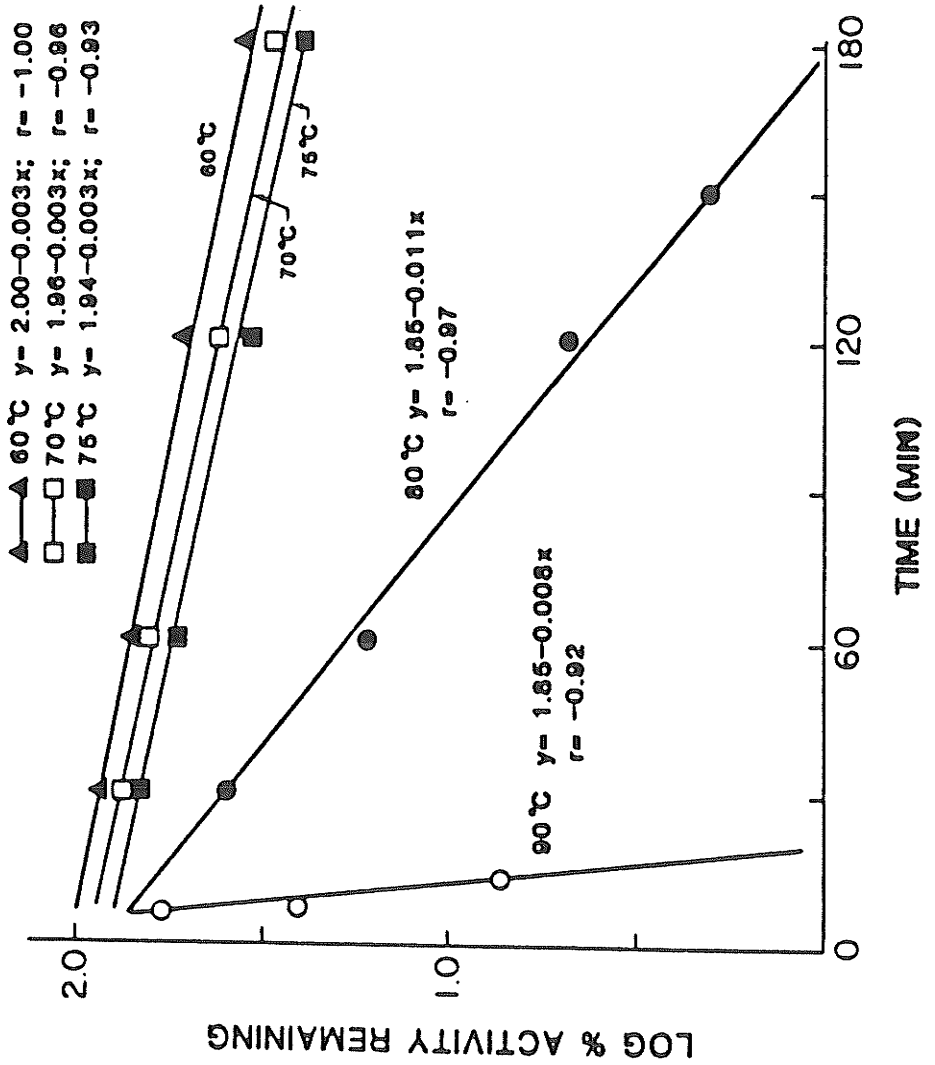
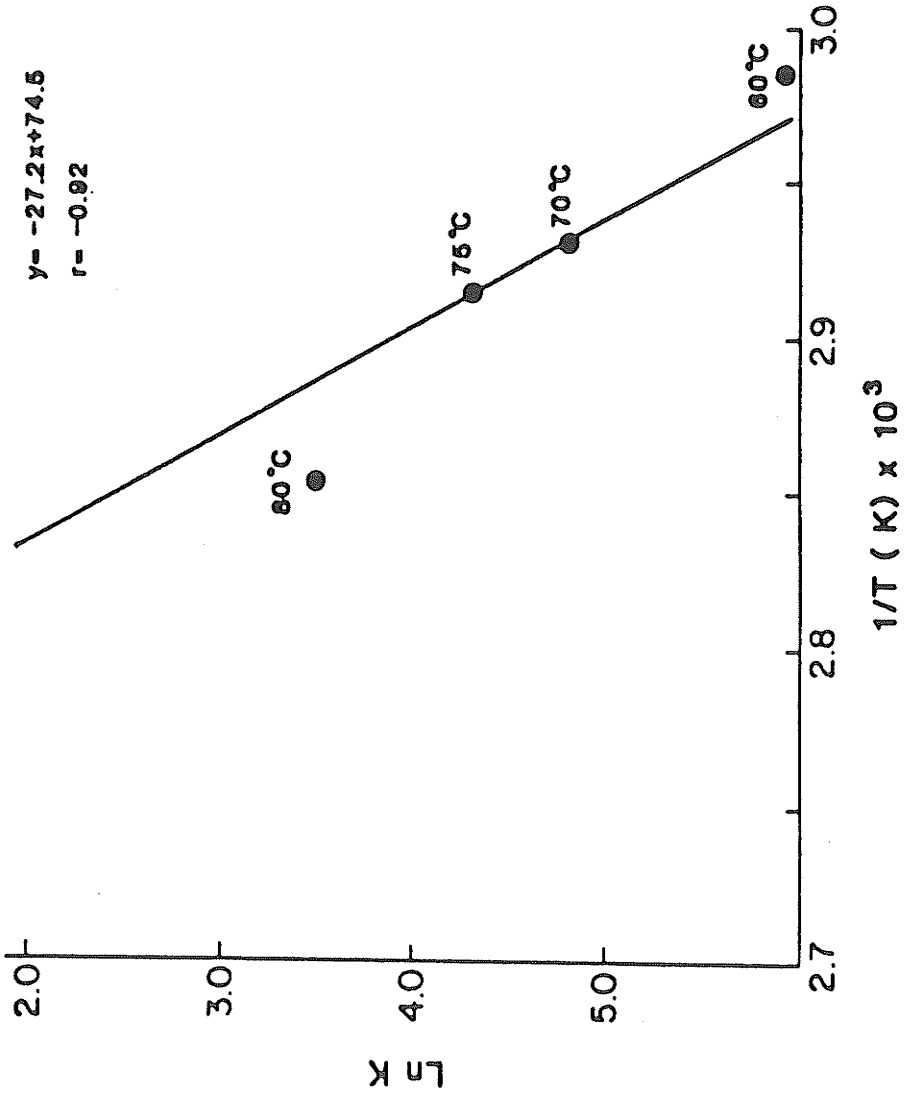

60℃ y= 2.00−0.003x; r= −1.00
70℃ y= 1.96−0.003x; r= −0.96
75℃ y= 1.94−0.003x; r= −0.93
60℃
70℃
75℃
80℃ y= 1.85−0.011x
r= −0.97
90℃ y= 1.85−0.008x
r= −0.92
LOG % ACTIVITY REMAINING
2.0
1.0
0
60
120
180
TIME (MIN)

Figure 1.3 Arrhenius plot for thermal inactivation data of Figure 1.2.


y= -27.2x+74.5
r= -0.92
80°C
75°C
70°C
60°C
2.0
3.0
4.0
5.0
ln K
2.7
2.8
2.9
3.0
1/T (K) x 10³

this study indicated that it is relatively thermotolerant. In comparison, crude PPO from bananas also using catechol as a substrate was reported to have an activation energy of 4.5 Kcal/mol (Padron et al., 1975). Unlike the PPO from strawberry, cherry, apple and pear (Mączyńska and Rembowski, 1966), the artichoke enzyme was quite stable at 75°C showing residual activity after 2.5 hr at 80°C. The linear time-course for the thermal inactivation of the crude PPO extract suggested a first order reaction. Generally thermotolerance of PPO is dependent on several factors including enzyme purity, pH and temperature optima as well as ripeness or maturity of tissue (Vámos-Vigyázó, 1981).

1.3.3 Substrate specificity

The PPO is known to catalyze two entirely different reactions: the hydroxylation of monophenols to the corresponding o-dihydroxyphenols and the oxidation of o-dihydroxyphenols to o-quinones (Robb, 1984). The ratios of hydroxylating:oxidizing activity have been shown to vary with the plant source and are influenced by the methodology used during their isolation and purification (Walker, 1975). In this study, the crude enzyme showed highest specificity towards dihydroxyphenolic substrates (Table 1.5); in this respect, chlorogenic acid followed by catechol and dopa were the most reactive, while activity towards monohydroxyphenols was 50 times lower than that for chlorogenic acid.

Table 1.5 Substrate specificity of crude PPO

Substrate[a]	PPO activity (U/ml)	Protein (mg/ml)	Specific activity (U/mg protein)
Monohydroxyphenols			
DL-Tyrosine	0.25 ± 0.06[b]	3.16 ± 0.04	0.08
p-Cresol	0.23 ± 0.04	2.94 ± 0.05	0.08
Dihydroxyphenols			
Chlorogenic acid	11.50 ± 0.19	2.94 ± 0.05	3.91
Catechol	5.01 ± 0.02	2.94 ± 0.05	1.70
DL-dopa[c]	4.96 ± 0.08	3.16 ± 0.04	1.57
Caffeic acid	1.89 ± 0.07	2.54 ± 0.02	0.74

[a] DL-Tyrosine was used at concentration of 1.6mM, all other substrate concentration levels were 10mM.

[b] Mean values ± SD based on triplicate determination of three extracts.

[c] 3,4-dihydroxyphenylalanine.

The reactivity of chlorogenic acid was about four fold higher than of catechol. Similar observations have been reported for PPO from pears (Halim and Montgomery, 1978). Walker (1975) reported that the site of the substitution of mono and dihydroxyphenols was an important factor with respect to PPO activity. Monophenols were hydroxylated only if they had a para-substituted group greater than CH_2 while p-substituted 3,4-dihydroxyphenols were oxidized at higher rates than 2,3-dihydroxyphenols. In the present investigation all the dihydroxyphenolic substrates contained a 3,4-dihydroxyphenol structure with a carbon side chain or ring attached to the number one position of the benzene ring. The presence of an electron-donating group in position four (as in chlorogenic acid) has been reported to increase the reactivity of the substrate (Lanzarini et al., 1972).

1.3.4 Distribution of enzyme

Analysis of the artichoke tuber indicated that the outer peels contained the majority of PPO activity followed by the secondary inner layer. Over 84% of the activity was confined to the peels, while the middle core exhibited approximately 11% of total activity (Table 1.6). Although similar localization of enzyme activity has been reported for potatoes (Craft, 1966) and grapes (Ivanov, 1966) the distribution of PPO in plants varies with species, age and maturity (Walker, 1975). For example, Ivanov (1966) repor-

Table 1.6 Distribution of PPO within the artichoke tuber

Tuber portion[a]	PPO activity (U/ml)	Protein (mg/ml)	Specific activity (U/mg protein)
Outer peel	13.80 ± 0.16[b]	5.36 ± 0.01	2.57
Secondary layer	1.74 ± 0.05	2.80 ± 0.06	0.62
Middle core	0.88 ± 0.00	2.26 ± 0.05	0.39

[a] Outer peel: ca. 0.5 mm thick; secondary layer: 50 mm thick; middle core: remaining tuber.

[b] Mean values ± SD based on triplicate determination of three extracts.

ted that the PPO activity in grape skins decreased during ripening.

In this study a relatively active and stable PPO enzyme was shown to exist in artichoke tubers. A comparison of PPO activity in artichoke, sugar beet and potato is presented in Table 1.7. The artichoke tuber contained the highest level of PPO activity, approximately 3.5 and 13 times higher than in sugar beets and potatoes, respectively. Although enzyme activity was highest in the artichoke tuber it should be recognized that PPO (particulate bound and soluble) levels in plants vary and therefore the values cited are only approximate. In addition, the PPO activity will vary depending on the reactivity of the substrate. For example, sugar beet PPO while ineffective on tyrosine, was found to oxidize dopa (Gross and Coombs, 1976a).

1.3.5 Effect of inhibitors

It is highly recommended that PPO extraction from plant sources be performed in buffer containing reductants in order to minimize reactions with endogenous phenols (Anderson and Rowan, 1967). This precaution, however, was not taken in order that the data presented more aptly reflect enzyme inhibition under normal processing conditions.

In these studies, a number of chemical compounds has been investigated as potential inhibitors of PPO. These chemicals may be classified according to their mode of action into two main types (Walker, 1975; Mayer and Harel,

Table 1.7 Comparison of PPO activity in various tubers

Tuber[a]	PPO activity (U/ml)	Protein (mg/ml)	Specific activity (U/mg protein)
Artichoke	6.36 ± 0.04[b]	3.01 ± 0.05	2.11
Sugar beet	1.66 ± 0.09	2.40 ± 0.09	0.69
Potato	1.06 ± 0.03	6.50 ± 0.01	0.16

[a] Fresh tubers (1983 fall harvest; stored at 4°C).

[b] Mean values ± SD based on triplicate determination of three extracts.

1979; Vámos-Vigyázó, 1981). Inhibitors acting primarily on PPO may interact with the enzyme via:

- copper site
- phenolic substrate site

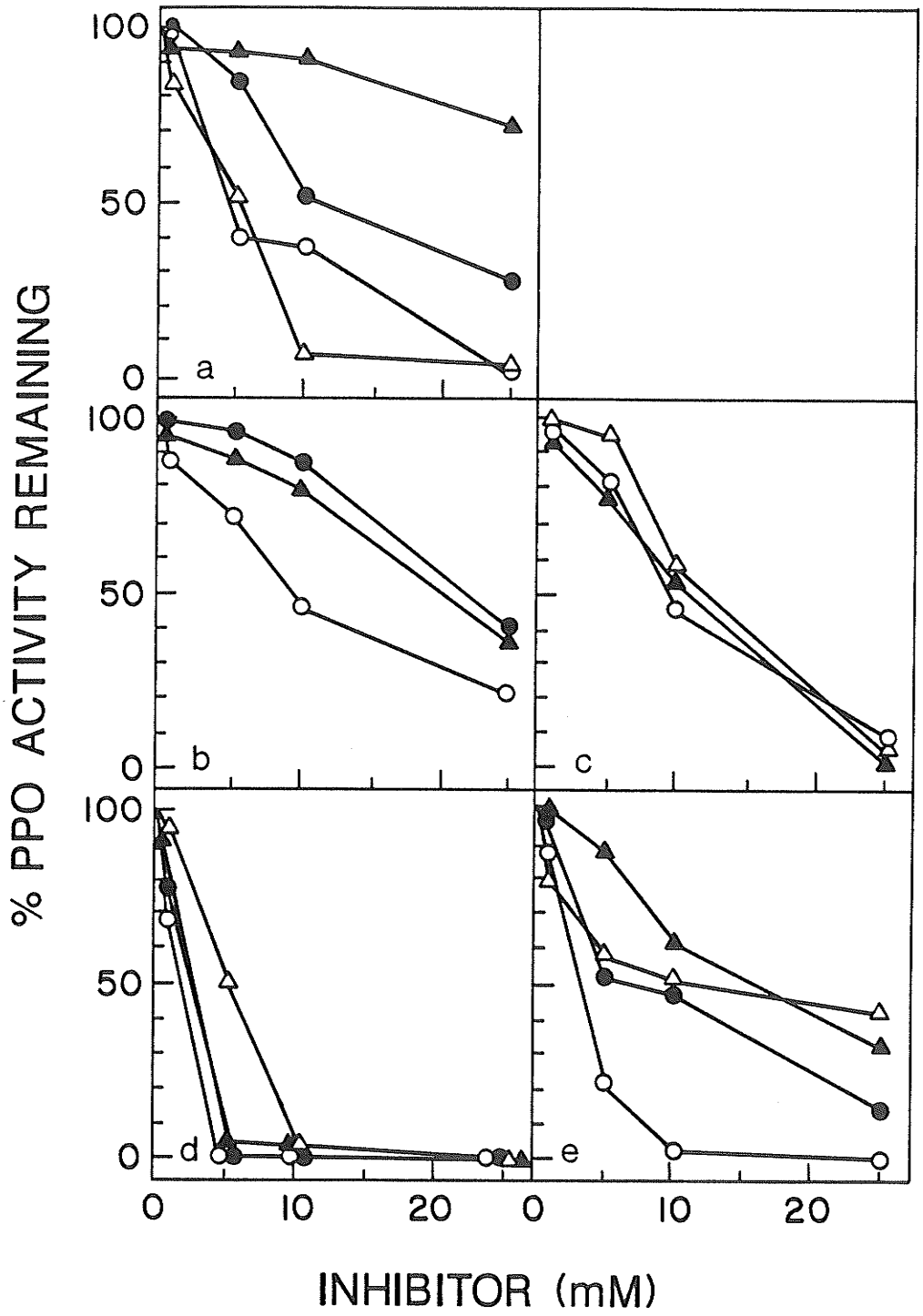
Inhibitors may also react with the final product of enzymatic oxidation; quinones by:

- reducing them to o-diphenols
- forming with them colorless complexes

1.3.5.1 Inhibition of enzymatic browning

Polyphenol oxidase is a metalloprotein with copper as the prosthetic group (Vámos-Vigyázó, 1981). In this respect four chelating compounds - DIECA, sodium azide, potassium ethylxanthate and EDTA were initially evaluated as PPO inhibitors (Fig. 1.4 a). Both DIECA and potassium ethylxanthate inhibited PPO activity by at least 50% in the 2-5 mM range. Higher concentrations of sodium azide (10 mM) were required for similar levels of inhibition; EDTA was relatively ineffective at all chosen concentrations. Although sodium azide and EDTA inhibit PPO, they are considered to be less specific chelators than DIECA or potassium ethylxanthate for copper (Abukharma and Woolhouse, 1966; Wong et al. 1971). In addition, inorganic ions (such as azide) inhibit PPO activity mainly in acidic media when dissociation is incomplete (Robb et al., 1966). According to Anderson (1968) DIECA and potassium ethylxanthate not only chelate copper but also combine with quinones formed by PPO.

Figure 1.4 The effect of various inhibitors on artichoke PPO activity at 30°C using catechol as a substrate. Reaction mixtures were adjusted to an initial pH of 6.5.
a. Chelators: DIECA (○); sodium azide (●); potassium ethylxanthate (□); EDTA (▲).
b. Competitive inhibitors: 2,3-naphthalenediol (○); benzoic acid (●); salicylic acid (▲). c. Reducing thiols: 2-mercaptobenzothiazole (○); 2,3-dimercaptopropanol (▲); thioglycollate (□). d. Sulfites: sodium metabisulfite (○); potassium metabisulfite (●); sodium hydrosulfite (▲); sodium bisulfite (□). e. Quinone couplers: L-cysteine (○); sodium benzosulfonate (●); sodium borate (△); thiourea (□). The SD of all values is less than ± 9%.


% PPO ACTIVITY REMAINING
100
50
0
a
b
c
d
e
0
10
20
INHIBITOR (mM)

The inhibition of PPO by 2,3-naphthalenediol, benzoic and salicylic acids is shown in Fig. 1.4b. Within this group of compounds which act mainly as competitive inhibitors (Walker, 1975) only 2,3-naphthalenediol inhibited 50% enzyme activity in the 10 mM range. Benzoic and salicylic acids were less effective as inhibitors each requiring a concentration of 25 mM for 50% inhibition. The higher concentration required for these two compounds may also reflect pH dependant reactions (Pifferi et al., 1974). Although 2,3-naphthalenediol was recognized as a highly specific inhibitor of apple PPO (Mayer et al., 1964) the maximum inhibition obtained (78%) in this study resulted in unacceptable color formation.

The next group of inhibitors, 2-mercaptobenzothiazole, dimercaptopropanol and thioglycollate, all exhibited similar levels of PPO inhibition (Fig. 1.4c). At a concentration of 25 mM approximately 95% PPO inhibition was reached. The objectionable odor imparted to the sample using this concentration, however, precludes their use. Compounds like SO_2 and metabisulfite have been shown to prevent browning by reducing the enzymatically-formed quinones back to o-diphenols (Anderson and Rowan, 1967). Some thiol compounds including 2-mercaptobenzothiazole also appear to react with enzyme prosthetic groups (Palmer and Roberts, 1967).

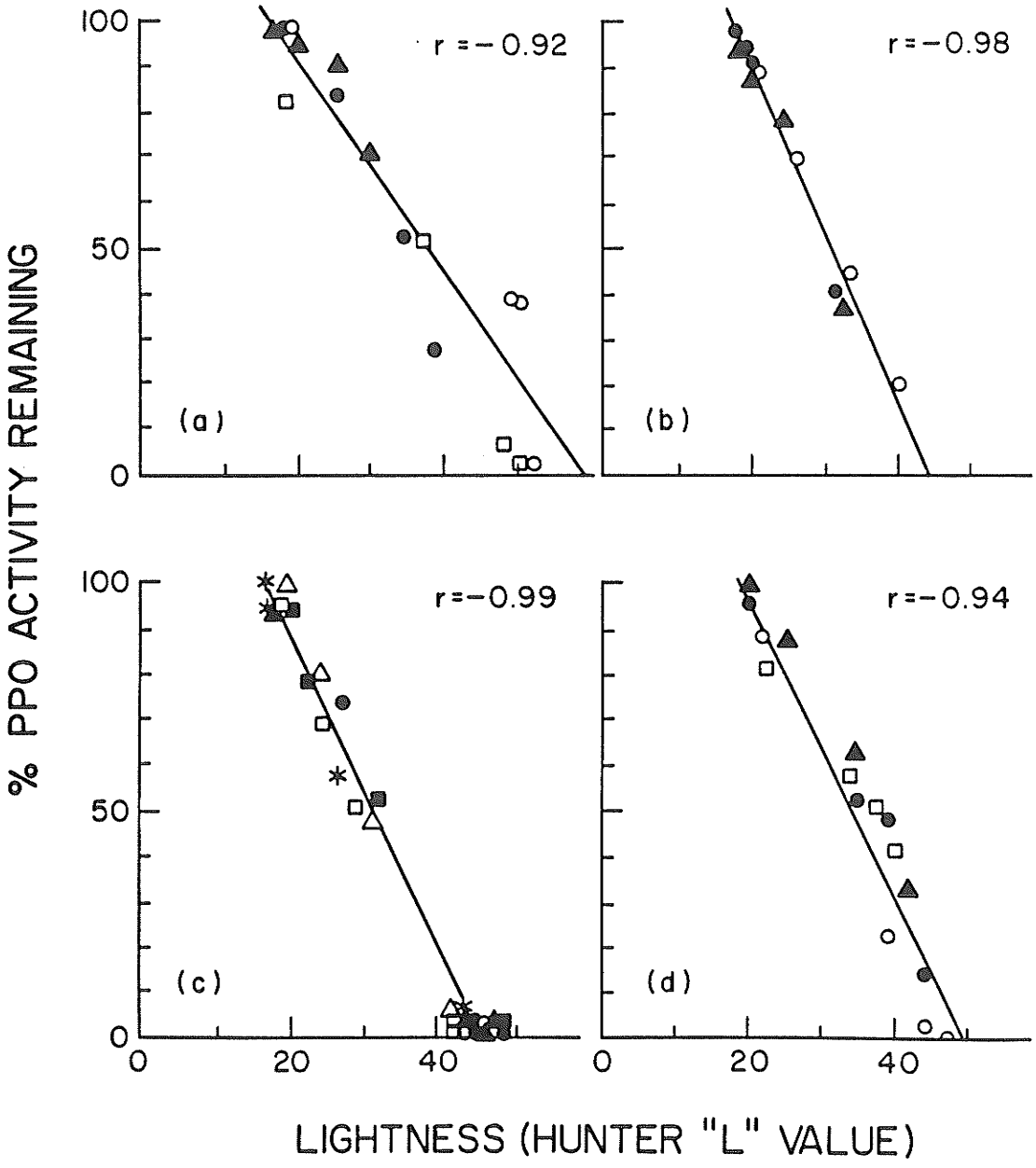
Figure 1.4d shows the inhibitory effects of sulphites represented by potassium and sodium metabisulfite, sodium

hydrosulfite and sodium bisulfite on PPO artichoke activity. Even though these compounds are generally considered as reducing agents (Anderson, 1968; Walker, 1975) their mechanism of action remains obscure. They have been considered to act as quinone couplers (Walker, 1975; Loomis, 1974) and as direct inhibitors of PPO (Golan-Goldhirsh and Whitaker, 1984). At a 5 mM concentration, sodium and potassium metabisulfite and sodium hydrosulfite each inhibited PPO activity by 98-99%. A similar level of inhibition was attained by using 10 mM sodium bisulfite. In addition, the univalent cations, Na^+ and K^+ showed no effect on the inhibitory action of PPO by metabisulfite.

The effects of several quinone couplers (L-cysteine, benzosulfonate, borate and thiourea) on PPO activity are shown in Fig. 1.4e. Within this group of compounds, which react with quinones to form colorless complexes (Pierpoint, 1966) L-cysteine, which also acts as a reducing agent, showed the greatest inhibitory effect. At a concentration of 10 mM, L-cysteine inhibited PPO activity by 99%. L-cysteine was also shown to be the most effective inhibitor for mushroom, avocado and banana PPO (Kahn, 1985a). Two additional compounds, sorbic and ascorbic acids (25 mM) inhibited PPO activity by ca. 50% (data not shown). These compounds were not further evaluated.

Figure 1.5 shows the relationship between remaining PPO activity and resultant extract color (represented by light-

Figure 1.5 The relationship of color (lightness) to PPO activity in artichoke extracts containing various inhibitors.
a. Chelators: DIECA (○); sodium azide (●); potassium ethylxanthate (□), EDTA (▲). b. Competitive inhibitors: 2,3-naphthalenediol (○); benzoic acid (●); salicylic acid (▲). c. Sulfites and reducing thiols: sodium metabisulfite (○); potassium metabisulfite (●); sodium hydrosulfite (▲); sodium bisulfite (□); 2-mercaptobenzothiazole (△); 2,3-dimercaptopropanol (■); thioglycollate (*). d. Quinone couplers: L-cysteine (○); sodium benzosulfonate (●); thiourea (□); sodium borate (▲). For all inhibitors a series of concentrations was used (1, 5, 10, and 25 mM); data points are presented in ascending order of inhibitor concentration, from top to bottom. The SD of all values is less than ±9% in respect to PPO activity remaining and less than ±5% in respect to L-value.


% PPO ACTIVITY REMAINING
LIGHTNESS (HUNTER "L" VALUE)
r = −0.92
r = −0.98
r = −0.99
r = −0.94
(a)
(b)
(c)
(d)
100
50
0
0
20
40

ness; value L) when treated with various inhibitor groups. Correlation coefficient values for all these composite plots ranged from 0.92 to 0.99.

1.3.5.2 Effect of pH

The effect of pH on PPO inhibition by sodium metabisulfite and L-cysteine is shown in Table 1.8. At pH levels of 5.0 or lower, the pH alone was an effective inhibitor. At a pH greater than 5.0, however, L-cysteine and metabisulfite were mainly responsible for PPO inhibition. Metabisulfite appeared more effective as an inhibitor when compared to L-cysteine; the level of inhibition was pH dependent and increased with decreasing pH. Inhibition by cysteine did not appear to be pH dependent, especially between 5.0-6.5.

1.3.5.3 Inhibitor stability

The effect of metabisulfite and L-cysteine on PPO activity in artichokes stored at 4^{o}C is shown in Fig. 1.6a. Metabisulfite and L-cysteine (25 mM) completely inhibited PPO activity over a 15-day period. A 5 mM concentration of L-cysteine, however, only inhibited PPO activity during the first three days of storage. Resumption of PPO activity in these extracts gradually increased to 55% at 15 days. This resumption of enzyme activity can be attributed to inhibitor consumption during subsequent quinone-coupled reactions (Pierpoint, 1969). Montgomery (1983) reported simi-

Table 1.8 Effect of pH and inhibitors on artichoke PPO activity[a] at 30°C.

pH	Control	L-Cysteine[b]	Sodium metabisulfite[b]
4.0	1.07 ± 0.06	1.05 ± 0.04	0.90 ± 0.04
4.5	1.47 ± 0.03	1.41 ± 0.01	1.08 ± 0.04
5.0	1.77 ± 0.05	1.62 ± 0.05	1.28 ± 0.06
5.5	1.80 ± 0.09	1.63 ± 0.01	1.36 ± 0.03
6.0	1.88 ± 0.05	1.64 ± 0.08	1.37 ± 0.01
6.5	1.92 ± 0.07	1.65 ± 0.09	1.44 ± 0.09
7.0	1.88 ± 0.01	1.76 ± 0.07	1.52 ± 0.02

[a] Data are expressed as specific activity (units/mg protein ± SD, n=3).

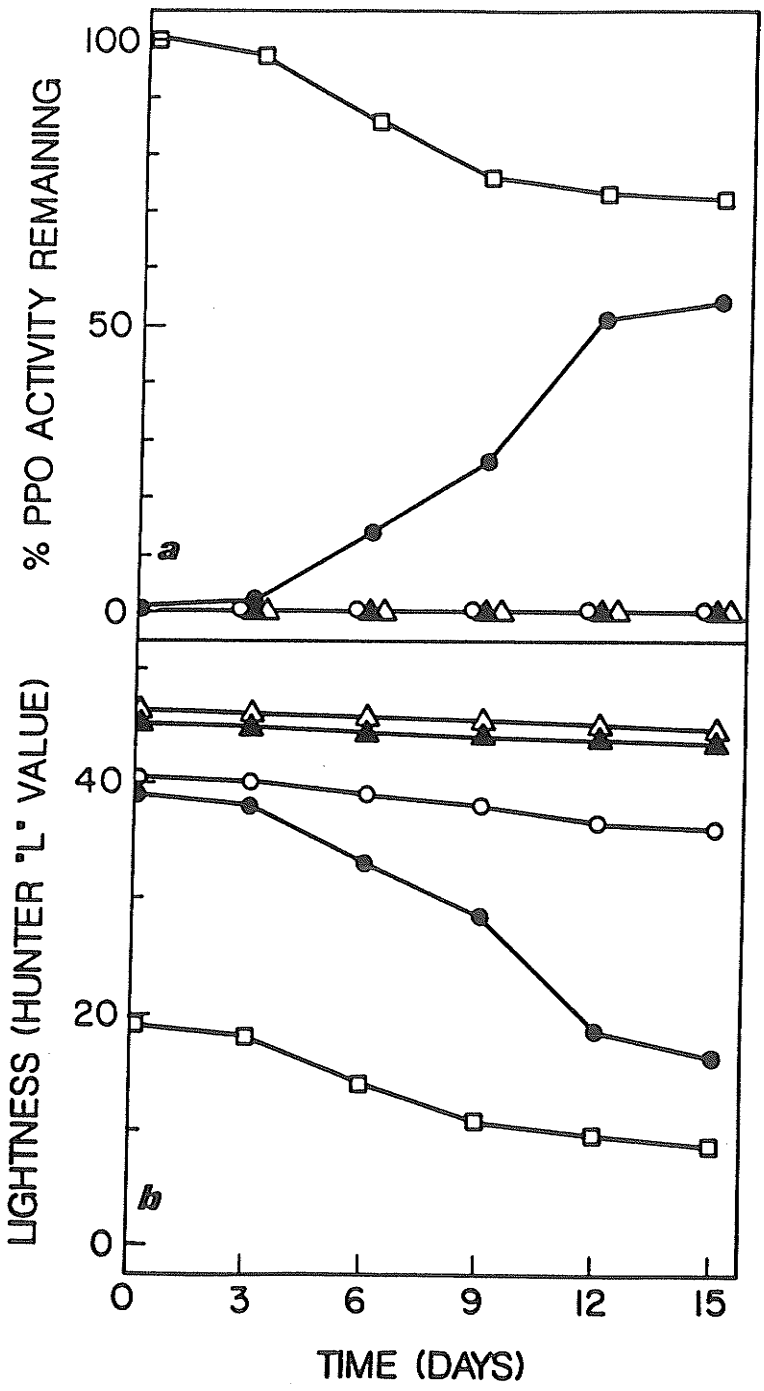
[b] 2 mM.

lar results with L-cysteine in pear juice concentrates.

The color development in artichoke extracts stored at 4°C is shown in Fig. 1.6b. Extracts containing either 10 or 5 mM sodium metabisulfite did not darken on storage. The cysteine treated extracts, expecially those containing 10 mM darkened progressively with time. Since the extract containing 25 mM cysteine did not contain any residual PPO activity (Fig. 1.6a), the slight darkening observed may be due to non-enzymatic melanization. Although residual cysteine levels were not determined in this study, Montgomery (1983) reported that darkness of pear juice concentrates increased with decreasing residual levels of cysteine.

All compounds examined in this study were shown to inhibit artichoke PPO activity. The degree of inhibition depended upon both the nature and concentration of the inhibitor. Since many of these compounds can impart off-flavor, off-odor and toxicity, their effective use in foods, discounting economic feasibility, may be limited. Alternatively, the application of high temperature treatment may only prove partially effective in controlling PPO activity and may also contribute to changes in flavor and texture. A combination of treatments involving heat and chemical inhibitors should therefore be considered. This could not only effectively reduce problems encountered in each treatment method but could also reduce processing costs. Although a pH of 5.0 or lower was shown to be an

Figure 1.6 Effect of inhibitors on PPO activity (a) and color development-lightness (b) in artichoke extracts stored at 4° C. Extracts contain L-cysteine at concentration of: 10 mM (●); 25 mM (○), or sodium metabisulfite at concentration of: 5 mM (▲); 10 mM (△), or no inhibitor (□). The SD of all values is less than ±5%.


100
50
0
% PPO ACTIVITY REMAINING
a
40
20
0
LIGHTNESS (HUNTER "L" VALUE)
b
0
3
6
9
12
15
TIME (DAYS)

effective inhibitor of PPO, the use of acids may prove deleterious. In particular, partial hydrolysis of the crude inulin syrup may not only lead to fructose decomposition - (Fleming and GrootWassink, 1979) but may also incur product loss in the ultrafiltration step (Hoehn et al., 1983).

CHAPTER TWO
PURIFICATION OF ARTICHOKE POLYPHENOL OXIDASE

2.1 INTRODUCTION

The isolation and purification of polyphenol oxidase of plant origin is associated with many problems making the purification of this enzyme extremely difficult (Mathew and Parpia, 1971). There are other enzymes of similar molecular properties such as laccase and peroxidase that exist in plant tissue. Furthermore, these enzymes show overlapping substrate specificities by being able to convert phenols to quinones (Harel and Mayer, 1970; Robb, 1984). In addition, plant PPO is present in relatively small amounts. Kertesz and Zito (1962) estimated that even in mushroom samples (regarded as the best source of polyphenol oxidase) the concentration of the enzyme is of the order of 0.004% (40 mg/kg).

However, the main drawback to the isolation of PPO is the occurrence of both, the enzyme and phenolic substrate, side by side in intact cells. The PPO is located exclusively in the plastids of healthy tissues and is apparently not even activated until it crosses the plastid envelope. The vast majority of phenolic compounds in higher plant cells are located in the vacuole - a cellular location isolated from PPO (Czaninski and Catesson, 1972; Vaughn and Duke, 1981b; Vaughn and Duke, 1984a). However, as soon as the plastid and vacuole contents are mixed upon cellular disruption, internal organization is damaged. The enzyme and substrate interact to yield quinones which subsequently

react with PPO and other proteins/enzymes forming very strong noncovalent and covalent complexes (Loomis, 1974; McManus et al., 1985). It has been reported that phenolics may inactivate the PPO (Sanderson, 1965), or in contrast, reacting with proteins may produce additional multiple forms which are not present in the intact tissue (Smith and Montgomery, 1985). Thus, properly chosen methods of extraction and isolation of PPO are of necessity to prevent any changes of the enzyme during the course of its purification. Many attempts to minimize phenolic - protein interactions have been taken. Comminution and homogenization are often performed in liquid a nitrogen or in nitrogen atmosphere (Balasingam and Ferdinand, 1970; Thomas and Janave, 1973; Benjamin and Montgomery, 1973). Extraction steps were carried out in the cold, at -20 to -30^{o}C (Vámos Vigyázó, 1981). In the early research lead acetate has been widely used as a precipitant for polyphenols (Nelson and Dawson, 1944), but a disadvantage is that it does not precipitate some plant phenols (Loomis and Battaile, 1966) and results in considerable loss of enzyme itself (Mathew and Parpia, 1971). More recently, insoluble polyvinyl pyrrolidone (PVP) has been used for the removal of phenols during separation of subcellular fractions and in the initial steps of the purification of the enzyme from plant tissues (Chan and Yang, 1971; Cash et al., 1976; Galeazzi et al., 1981). However, also this technique is not free of

disadvantages. It requires a large amount of PVP (1g PVP/1g plant tissue), acidic pH of extraction (maximum bonding of plant phenols occurred at 3.5), as well as a time consuming pretreatment procedure prior to extraction (Andersen and Sowers, 1968; Smith and Montgomery, 1985). Moreover, PVP was found to inhibit PPO activity to some extent (Anderson, 1968). Other polymers have also been successfully used for the removal of phenols, e.g. ion exchange resins (Cornwell and Wrolstad, 1981; Smith and Montgomery, 1985), polycaprolactam (Sanderson,1964), and polyethylene glycol (Badran and Jones, 1965; Benjamin and Montgomery, 1973; Park and Luh, 1985). Polyethylene glycol (PEG) because of its solubility in acetone is often used in combination with the latter for the preparation of acetone powder (Loomis, 1968). Acetone precipitation followed by buffer extraction, is one of the methods most often used as the initial step in isolation of PPO (Clayton, 1959; Palmer, 1963; Walker, 1964; Dizik and Knapp,1970; Wong et al., 1971; Padron at el., 1975; Gross and Coombs, 1976a; Satjawatcharaphong et al., 1983). This method presents certain advantages in obtaining a crude enzyme preparation with high yield of activity (Flurkey and Jen, 1978) and free of artifacts (Kahn, 1975). To prevent the enzyme inactivation during buffer extraction, reducing agents or quinone couplers such as ascorbic acid (Abukharma and Woolhouse, 1966), cysteine (Stelzig et al., 1972), sodium matabisulfite (Stokes et

al., 1968), sodium diethyldithiocarbamate (Pierpoint, 1966), or thiourea (van Driessche at el., 1984) have been used.

After preparation of a crude extract, most published procedures follow conventional methodology. The methods have been varied according to the enzyme source and the degree of purity to be attained. Most often the first step of purification includes removal of inactive protein by precipitation with protamine sulphate (Balasingam and Ferdinand, 1970), calcium acetate (Patil and Zucker,1965), or fractionation with ammonium sulphate (being the most widely used) preceding dialysis, or Sephadex G-25 chromatography (Vámos Vigyázó, 1981).

For further purification: adsorption, ion exchange, and gel filtration chromatography, or combinations of some of these methods have been frequently used, but hydrophobic chromatography (Mayer and Harel, 1979), and preparative isoelectrofocusing have been also employed in some cases (Interesse et al., 1983).

Adsorption chromatography with celite has been found to be selective for PPO, as the adsorbent is a relatively specific binder of copper proteins (Mathwew and Parpia, 1971). Adsorption on calcium phosphate has been applied to apple (Stelzig et al., 1972), and wheat PPO (Interesse et al., 1983), while hydroxylapatite chromatography by itself (Kidron et al., 1977), or following elution from carboxyme-

thyl (CM) - cellulose (Vaughan et al., 1975), or diethylaminoethyl (DEAE) - cellulose (Rivas and Whitaker, 1973; Flurkey and Jen, 1980) columns were used for purification of spinach beet, pear, and peach PPO, respectively.

Ion exchange chromatography has been the most favored method in the literature, especially in combination with gel filtration chromatography on Sephadex (Mayer and Harel, 1979), or more recently on Sephacryl S-200 (Hsu et al., 1984), and Ultrogel AcA 34 (Flurkey and Jen, 1980) columns.

Several attempts have been made to purify PPO by affinity chromatography from different sources including mushroom (Gutteridge and Robb, 1973; O'Neill et al., 1973; Menon and Haberman, 1975), larvae of housefly (Shimoda et al., 1975), and mammalian tissue (Jimbow et al., 1975; Jimbow et al., 1981). However, its use has not been extended for purification of the plant enzyme. The affinity chromatography was for the first time introduced by Lerman (1953) and used for purification of mushroom PPO. It is difficult, however, to conclude whether true affinity chromatography has been observed. Moreover, the low capacity of design adsorbents, and oxidation of ligands (phenolic substrates) during the operation of the column probably precludes its widespread use (Robb, 1984).

Purification of PPO has been recently reported to be achieved by hydrophobic chromatography on Phenyl Sepharose CL-4B (Mayer and Harel, 1979). This method is particularly

attractive since it is possible that the active site of PPO has a hydrophobic character. Certainly PPO binds well to hydrophobic chromatography media, such as Phenyl-Sepharose, at high ionic strength (Robb, 1984). However it is difficult to evaluate this method since only a few applications have been published (Flurkey and Jen, 1978; Wissemann and Lee, 1980).

Conventional methods of PPO purification are generally based on relatively small differences in the physicochemical properties of the proteins in the mixture. They are frequently laborious and incomplete, and the yields are often low (Mathew and Parpia, 1971). Thus, there is always need for alternative separation procedures based on new principles. A relatively new method, referred to as metal chelate or immobilized metal affinity chromatography, introduced by Porath et al. (1975) is now becoming widely accepted for the isolation of nonenzymatic proteins (Porath and Belew, 1983). Nevertheless, some attempts of enzyme purification from various sources have been reported (Ohkubo et al., 1980; Horlein et al., 1982; ; Krishnan and Vijayalakshmi, 1985; Redinbaugh and Campbell, 1985; Weselake et al., 1986). Immobilized metal affinity chromatography (IMAC) could be defined as a separation technique based on specific and reversible interaction between immobilized metal on an insoluble matrix and the protein molecule. The binding of protein occurs via the amino acid residues

exposed on its surface, which are able to participate in coordination bonding (Porath and Olin, 1983). Amino acid residues that possess an ionizable proton can display ligand properties. Nitrogen donors include the imidazole group of histidine, the ε-amino group of lysine and the guanidinium group of arginine. The carboxyl groups of aspartate and glutamate and the hydroxyl group of tyrosine can function as oxygen donors, and the thiol group of cysteine can act as a sulphur donor for coordination with metal ions. The nitrogen atom of the peptide linkage and the N-terminal amino group can also co-ordinate to metal ions (Conlon and Murphy, 1976; Hemdan and Porath, 1985). The coordination binding between proteins and the immobilized metal is pH dependent and proteins are usually desorbed simply by reducing the pH, or decreasing the salt concentration of the eluant (Lonnerdal and Keen, 1982). More selective desorption is achieved by including competing displacers (ligands) in the eluent buffer, such as glycine, histamine, histidine and imidazole (Kikuchi and Watanabe, 1981; Fanou-Ayi and Vijayalakshmi, 1983; Porath et al., 1983; Andersson, 1984).

The present study describes the purification of PPO from artichoke tubers by immobilized copper affinity chromatography (ICAC). To our knowledge this is the first application of this method for the purification of polyphenol oxidase. Although several attempts to purify this

enzyme by means of other methods such as DEAE-cellulose ion exchange, gel filtration, affinity and hydrophobic chromatography were unsuccessful, all of these methods are described in this chapter. In addition, the fractionation of the purified artichoke PPO by DEAE-Sepharose is also described in here.

2.2 MATERIALS AND METHODS

2.2.1 Materials

2.2.1.1 Jerusalem artichoke tubers

Artichoke tubers from the Columbia cultivar (crop of 1983) were obtained from the Agriculture Canada Research Station, Morden, Manitoba. Part of the tubers was used for acetone powder preparation immediately after harvest, while the other part was stored in plastic bag at 4^{o}C until used.

2.2.1.2 Chemicals

Disodium salt of iminodiacetic acid (IDA), 1,4-butanediol diglycidyl ether, cyanogen bromide (CNBr), insoluble polyvinylpyrrolidone (PVP), L-dopa, glycine, L-histidine, ammonium persulfate, riboflavin sulfate, and Coomassie Brilliant Blue R-250 were purchased from Sigma (St. Louis, Mo, USA). Sepharose 4B, Sepharose 6B, DEAE-Sepharose CL-4B, phenyl Sepharose CL-4B, Sephadex G-25, Sephadex G-100, and Broad pI Calibration kit (pH 3-10) were obtained from Pharmacia (Uppsala, Sweden). Polyethylene glycol (Carbowax PEG 8,000; MW 7,000-9,000), catechol, p-cresol, p-phenylenediamine from Fisher (Fisher-Kendall Sci. Co.); acrylamide, N,N'-Methylene-bis-acrylamide (BIS), N,N,N',N'-tetramethylethylenediamine (TEMED) from BIO-RAD (Richmond Calif.); DEAE-cellulose from Whatman Ltd. (England), ampholine polyacrylamide gel plates (pH 3.5-9.5) were purchased from LKB-Produkter AB (Bromma, Sweden). Other reagents were

of analytical grade. Isoelectric point data are from information provided by the supplier.

2.2.2 Methods

All preparation steps and analytical procedures, unless otherwise indicated, were carried out at 4°C.

2.2.2.1 Extraction

Crude enzyme extracts were prepared as described in Chapter one using either deionized water (pH 4.4) or 0.1 M sodium phosphate buffer, pH 6.5. Extraction with PVP, or from acetone powder were carried out as outlined in the general extraction procedure (Chapter one). The PVP (10 g), prepared according to the method of Loomis (1974), was added at the beginning of extraction. The reagent was boiled for 10 minutes in 10% HCl (solid phase/liquid phase ca.1:4), then neutralized with 10% KOH, washed with deionized water, and air dried at 60°C. Crude PPO was extracted from acetone powder using 0.1 M sodium phosphate buffer (1:30 w/v), pH 6.5 containing 20 mM L-ascorbic acid. After stirring for 1 hr, the slurry was centrifuged at 4,000 x g for 20 minutes and the supernatant was filtered.

2.2.2.2 Acetone powder preparation

An acetone powder was prepared from fresh prechilled artichoke tuber (10 kg) slices as described by Wong et al. (1971). Portions of 1 kg of artichoke were homogenized

in a large Waring Blendor with 2 liters of cold acetone (-20°C) and 6.7 g of polyethylene glycol (PEG) for 1 min. The slurry was filtered under suction through fiberglass filter paper. The above step was repeated on the pellet (ca. 220 g) using approximately three volumes of cold acetone without PEG. The wet cake was washed repeatedly with cold acetone (ca. 300 ml) until the filtrate became colorless. The material was allowed to dry at room temperature overnight to give about 56 g total weight of powder. The powder was stored under vacuum in a desiccator at 4°C until use.

2.2.2.3 Initial enzyme purification

The filtrate obtained from extraction of acetone powder with buffer was brought to 20% saturation with solid ammonium sulphate. After standing for 2 hr in the cold the precipitate was removed by centrifugation at 4,000 x g for 20 minutes and discarded. The supernatant was brought to 80% saturation with ammonium sulphate; after standing overnight in the cold the precipitate which contained most of the enzyme activity was separated by centrifugation at 4,000 x g for 20 minutes. The precipitate was dissolved in approximately 40 to 50 ml 0.1 M sodium phosphate buffer (pH 6.5) and then loaded on a Sephadex G-25 column (2.6 x 70 cm) equilibrated with the same buffer. Fractions were assessed for PPO activity, protein, and ammonium sulphate content. The latter analysis was performed using the

conductivity meter. Fractions having PPO activity and being free of $(NH_4)_2SO_4$ were pooled and concentrated by diafiltration using an Amicon cell with a membrane having a molecular weight cut-off of 10,000 (Diaflo membrane, UM10, Amicon Corp., Lexington, Mas.). Unless otherwise indicated, the concentrated crude enzyme solution was used for further purification by means of column chromatographic techniques described below.

2.2.2.4 Immobilized copper affinity chromatography

The IDA was coupled to epoxy-activated Sepharose 6B, as described by Porath et al. (1975). Chromatography on a small scale was performed to evaluate efficiency, while a preparative column was employed to collect enzymatic material for further characterization. The gel was packed into two columns: a separation (1 x 20 cm) and a guard (1 x 6 cm) column. On the large scale, a separation column of 2.5 x 40 cm was used. The first column was equilibrated with aqueous solution of $CuSO_4.5H_2O$ (6 mg/ml) until the entire matrix became blue (i.e. fully saturated). Excess copper ions were removed by washing the column with deionized water. The second column, free of copper, was coupled in sequence with the separation column and served to adsorb any metal ions leaching out during chromatography. Both columns were equilibrated and eluted with 50 mM TRIS-HCl, pH 7.5, containing 0.15 M NaCl, and eluted with

the same buffer containing 20 mM glycine, and then 10 mM L-histidine in stepwise manner. Columns were regenerated after each run with 50 mM EDTA containing 0.5 M NaCl in 20 mM sodium phosphate buffer.

2.2.2.4.1 Preparation of IDA-Sepharose

A two step procedure was employed for preparation of gel: oxirane activation of Sepharose 6B (Sundberg and Porath, 1974), and attachment of IDA to the activated gel matrix (Porath et al., 1975). The 10 g of suction dried Sepharose 6B, 11 ml of 1,4-butanediol diglycidyl ether, 11 ml of 0.6 N sodium hydroxide, and 22 mg of sodium borohydride were placed in a conical flask. The flask was sealed and shaken for 8 hr at room temperature and the slurry was then suction dried using a sintered glass funnel. Disodium salt of iminodiacetic acid (1.3 g) was dissolved in 7 ml of 2 M sodium carbonate and added to 10 g of suction-dried oxirane-activated Sepharose 6B in a conical flask. The suspension was heated to 65^{o}C and shaken for 24 hr. The gel was collected on a sintered glass funnel and washed with dionized water.

2.2.2.5 Ion exchange chromatography

Ion exchange chromatography was performed on the purified PPO (ICAC) using a DEAE - Sepharose CL 6B column. The column (1 x 20 cm) was equilibrated overnight with 0.04 M TRIS-HCl buffer, pH 6.0. After the sample (2 ml; 144 enzyme

units, 1.2 mg of protein) was applied to the column, five void volumes of the starting buffer were allowed to flow through the ion exchange gel. Subsequently, a linear gradient between 0 and 1.0 M NaCl in the starting buffer was used for elution of the enzyme bound to the column.

2.2.2.6 Other column chromatographies

Several column chromatographic techniques were employed to establish an optimal procedure for further purification of PPO. During the course of these experiments the columns were eluted with a flow rate of 15 ml per hr, and fractions of 4 ml were collected.

2.2.2.6.1 DEAE-cellulose chromatography

The chromatographic procedure was essentially as described by Wong et al. (1971). Before use, DEAE-cellulose was prepared as recommended by Whatman Ltd. (Publication 607A, 1980). Cellulose powder (50 g) was stirred with 750 ml of 0.5 N HCl for 30 minutes and then washed with deionized water under suction until the filtrate was of neutral pH. The above step was repeated using 750 ml of 0.5 N NaOH. After removal of fine particles and adjusting the pH to 6.5 by washing the cellulose with 0.05 M sodium phosphate buffer, pH 6.5, the cellulose was packed into the column (1 x 40 cm). The column was equilibrated overnight with the above buffer before use. After the sample was applied, the column was eluted with five volumes of the equilibration

buffer, and subsequently with a linear gradient of ionic strength (between 0 to 0.3 M NaCl), in the equilibrating buffer, and then a pH gradient from 7.0 to 4.5 using 0.05 M sodium phosphate buffer containing 0.3 M NaCl.

2.2.2.6.2 Gel filtration chromatography

Gel filtration chromatography was performed on a Sephadex G-100 column (2.5 x 100 cm). The column was equilibrated and subsequently eluted with 0.05 M sodium phosphate buffer, pH 6.5. The void volume of 128 ml was determined using Blue Dextran 2000 for calibration the column according to Andrews (1970).

2.2.2.6.3 Affinity chromatography

Affinity chromatography was performed using Sepharose 4B coupled to L-dopa, a substrate of PPO. The gel was packed into the column (1 x 20 cm) and washed with 0.05 M sodium phosphate buffer, pH 6.0. After the sample was applied, the column was equilibrated with the same buffer, until no protein was detected from the column. Subsequently, the gel was eluted with a pH gradient between 6.0 and 4.5 using 0.05 M phosphate buffer.

2.2.2.6.3.1 Preparation of L-dopa Sepharose 4B

The CNBr activation of Sepharose 4B was performed according to Cuatrecasas (1970) in the following way: The 10 g of suction dried Sepharose 4B was washed several times with distilled water on a sintered glass funnel. Distilled

water (10 ml) was then added to the wet product and matrix activation was effected by CNBr (1.5 g) dissolved in distilled water (20 ml). The solution of CNBr was added to Sepharose under stirring and the pH of the mixture was maintained at 11 by 5 M NaOH for 6 to 7 minutes. Sepharose was subsequently washed with 1 l of ice cold distilled water, and then with 1 l of ice cold 1 M KH_2PO_4, pH 7.0. The coupling reaction was performed according to Jimbow et al. (1981). Usually 15 g of the activated Sepharose cake was mixed (rotary mixer) with 50 ml of 1 M KH_2PO_4 (pH 7.0) containing 1.22 g of DL-dopa in the cold overnight. The substituted sepharose was then washed with 1 l of distilled water and 1 l of 0.05 M sodium phosphate buffer, pH 6.0, and was used for chromatography. The amount of dopa bound to Sepharose was determined spectrophotometrically by the following formula:

$$1 - \frac{D(B + D)}{AB} \times \frac{E}{C} \times \frac{10^6}{F} = \mu\text{moles bound/mg of Sepharose}$$

where A is A_{280} of dopa solution before addition of Sepharose; B is the ml of dopa solution before addition of Sepharose; C is the g of Sepharose added to the dopa solution; D is A_{280} of the supernatant of the dopa-Sepharose mixture after reaction overnight; E is the g of dopa before the addition of Sepharose; F is the moles of dopa added. By this procedure, it was found that approximately 10 μmoles of dopa per g of Sepharose were coupled.

2.2.2.6.4 Hydrophobic chromatography

Hydrophobic chromatography was performed according to the procedure of Wissemann and Lee (1980) using phenyl Sepharose CL-4B. Pre-swollen commercial gel was packed into a column (1 x 20 cm) and equilibrated overnight with 0.05 M sodium phosphate buffer, pH 6.5, containing 1 M ammonium sulphate, and 1 M NaCl. After the sample was applied, the column was eluted using the following buffers in a gradient stepwise manner: a) gradient between 50 ml of equilibrating buffer and 50 ml of 0.05 M phosphate buffer, pH 6.5 b) 50 ml of 0.05 M phosphate buffer - 50 ml of distilled water c) 50 ml water - 50 ml of 50% solution of ethylene glycol.

2.2.2.7 Electrophoretic techniques

2.2.2.7.1 Analytical polyacrylamide gel electrophoresis

Gradient (6-12%) polyacrylamide gel electrophoresis (PAGE) was performed on an LKB 2001 Vertical Electrophoresis Unit using the modified method of Davis (1964). The stacking gel consisted of 5.9% acrylamide and 0.1% BIS and was prepared in 0.16 M Tris-0.25 Na H_3PO_4 buffer (pH 6.9). The solutions were polymerized under UV light by the addition of 2.5 ml catalyst (0.06% ammonium persulfate and 0.002% riboflavin) and 5 μl TEMED per 15 ml of gel solution. The separating gel consisted of 0.95 M Tris-HCl buffer (pH 8.5) and a gradient of acrylamide, BIS and glycerol ranging from 5.9 to 11.4, 0.2 to 0.6 and 0.5 to 5.0%,

respectively. This gel was polymerized under UV light by the addition of 1.9 ml of catalyst and 30 µl TEMED per 15 ml of gel solution. The electrode compartments were filled with two buffer systems. The upper tank contained 0.04 M Tris-0.04 M glycine buffer (pH 8.9), while the lower tank contained 0.06 M Tris-HCl buffer (pH 7.5). Each gel was run for 3 hr at 20°C with a constant current of 30 mA.

2.2.2.7.2 Analytical isoelectric focusing

Isoelectric focusing (IEF) was performed on a LKB 2117 Multiphor horizontal unit according to the LKB instruction manual, 1804-101 (1986) using commercial LKB ampholine polyacrylamide gel plates, pH 3.5 - 9.5. To determine the pH gradient, the Broad pI Calibration Kit (Pharmacia) was used. Each gel was run for 1.5 hr at 10°C using 1 M H_3PO_4 as an anode-electrode solution, and 1 M NaOH as a cathode-electrode solution with the following settings of power supply: P=30 W; U=1500 V; I-50 mV. Sample application was made directly with a micropipetor at a point located 2 cm from the anode.

After electrophoresis (PAGE, IEF) the gels were washed 3x (30 minutes each) with distilled water and the PPO bands were detected according to Benjamin end Montgomery (1973) using 10 mM catechol (unless otherwise specified) dissolved in 0.1 M sodium phosphate buffer, pH 6.0 containing 0.05% p-phenylenediamine. The gels were immersed into the aerated substrate solution, incubated for 10 minutes, and

then rinsed with water. After IEF a portion of the gel was stained for protein as follows. The gel was incubated in the fixing solution (aqueous solution of 11.5% TCA and 3.5% sulphosalicylic acid) for 1 hr. This solution precipitates the proteins and allows the Ampholines to diffuse out. After fixing, the gel was washed in destaining solution (aqueous solution of 25% ethanol and 8% acetic acid) for 5 minutes, and stained with 0.1% Coomassie Brilliant Blue R-250 (CBB) in destaining solution for 10 minutes. The gel was then washed with several changes of the destaining solution until the background was clear. After electrophoresis the gels were photographed on a commercial light box using Kodak Technical PAN ASA 32 film. All the above procedures were performed at room temperature.

2.2.2.8 Enzyme and protein determination

Activity of PPO was determined as described in the previous chapter (section 1.2.2.2) using 50 µl (crude extract) or 10 µl (purified PPO) enzyme solution. Enzymatic activity of the eluates collected during chromatography was estimated semi-quantitatively according to the modified method of Montgomery and Sgarbieri (1975). The 0.1 ml eluate was added to 2.9 ml of aerated 10 mM catechol in 0.1 M sodium phosphate buffer (pH 6.0). The mixture was incubated for 10 min at room temperature and the intensity of the color was measured spectrophotometrically at 415 nm.

The absorbance was taken as measure of activity. Protein was determined as described previously (section 1.2.2.3) or by measuring absorbance at 280 nm. All PPO preparations from various chromatographic procedures were ultrafiltrated (Diaflo membrane, UM10, Amicon Corp., Lexington, Mas.) in order to exchange the buffer with 0.1 M sodium phosphate, pH 6.0, before enzyme activity and protein measurment was performed.

2.3 RESULTS AND DISCUSSION

2.3.1 Extraction

A problem often associated with purification of plant PPO is enzyme action on endogenous phenolic compounds upon comminution of plant tissue. This can result in pigment formation which may cause inactivation of PPO (Walker, 1964). In addition, endogenous phenolic substrates, if not removed during extraction of PPO, can react with the protein to produce additional multiple forms which were not present in the intact tissue (Smith and Montgomery, 1985). Therefore, several different extraction methods for artichoke PPO were investigated to establish a procedure yielding an active and colorless enzyme preparation. A comparison of extraction procedures (Table 2.1) revealed that the highest PPO specific activity was obtained using phosphate buffer extraction with or without PVP. However, both methods yielded dark brown extracts (L value was approximately 18, and 16, respectively). It is worthwhile to note that addition of PVP to the buffer had little effect on the PPO activity as well as color of the final enzyme preparation. This could be due to an insufficient amount of PVP used for extraction (Ben-Shalom et. al., 1977) or inadequate pH for adsorption of phenolics (Andersen and Sowers, 1968). In contrast, an almost colorless (a straw-like) extract was obtained by the PEG - acetone treatment following extraction with the buffer (L value was ca. 40). Similar

Table 2.1 Extraction of crude PPO by various methods

Extraction method	Activity (U/ml)	Protein (mg/ml)	Specific activity (U/mg protein)	Lightness[a] (L)
water[b]	3.8 ± 0.1[c]	3.6 ± 0.1	1.1	15.8 ± 0.8
buffer[d]	4.5 ± 0.1	3.4 ± 0.0	1.3	16.0 ± 0.5
buffer + PVP	4.2 ± 0.1	3.5 ± 0.1	1.2	18.4 ± 0.3
PEG-acetone + buffer	2.8 ± 0.1	3.1 ± 0.1	0.9	39.9 ± 0.8

[a] Determined by Hunter lab Colorimeter; L value for distilled water was ca. 54.

[b] Deionized water, pH 4.4; maintained as a control

[c] Mean values ± SD based on triplicate determination of three extract.

[d] Sodium phosphate buffer, pH 6.5.

observation was noticed by Benjamin and Montgomery (1973) who reported that the PEG-acetone treatment was the best method for removal of phenolics during extraction of cherry PPO. Though the recovery of the enzyme activity was about 70% of that obtained by buffer extraction, this procedure was adopted as the method of choice for removing the phenolic constituents and reducing the discoloration during the extraction of PPO. In addition, buffer extraction followed acetone powder preparation was carried out in the presence of ascorbic acid which is frequently incorporated into the extracting media (Mayer, 1966). This compound reduces quinones back to phenols, thus protecting PPO against inhibition. In contrast to the results reported by Rivas and Whitaker (1973) it has been found that addition of ascorbic acid retarded color changes in the artichoke PPO extract stored for several days in the cold, while the preparation without it was more susceptible to darkening. Nevertheless, the buffer extract obtained using the acetone powder was immediately submitted for further purification.

2.3.2 Initial purification

Table 2.2 shows PPO activity in various fractions precipitated with ammonium sulphate. The enzyme activity recovered was dependant on the amount of ammonium sulphate used for saturation. The specific activity of fractions precipitated between 0 and 20%, and between 80 and 90% of saturation were 0.21 and 0.12 U/mg protein, respectively

Table 2.2 PPO activity of various ammonium sulphate (AS) fractions[a]

AS fractions[b] (% of saturation)	Activity (U/ml)	Protein (mg/ml)	Specific activity (U/mg protein)
0 - 10	trace	3.20 ± 0.05[c]	-
10 - 20	0.64 ± 0.04	3.10 ± 0.05	0.21
20 - 30	4.37 ± 0.20	2.05 ± 0.08	2.13
30 - 40	3.60 ± 0.12	1.80 ± 0.01	2.00
40 - 50	4.40 ± 0.10	3.48 ± 0.20	1.26
50 - 60	3.13 ± 0.09	0.92 ± 0.30	3.40
60 - 70	2.80 ± 0.06	0.94 ± 0.05	2.98
70 - 80	2.56 ± 0.08	1.42 ± 0.05	1.80
80 - 90	0.25 ± 0.10	2.10 + 0.03	0.12

[a] Acetone powder buffer extract was used for AS fractionations.

[b] Pellets precipitated between the various degrees of % AS saturation were dissolved in 0.1 M sodium phosphate buffer, pH 6.0, and subsequently used for determination of PPO activity and protein content.

[c] Mean values ± SD of two determinations.

and were significantly lower than that of the other fractions. Thus, for further purification of PPO, the fraction precipitated from the acetone powder buffer extract between 20 and 80% of ammonium sulphate saturation was used. Approximately 98% of the initial enzyme activity was recovered and almost 90% of the contaminating proteins were eliminated (Table 2.4). A resulting eight fold increase in purification was thus obtained. In addition, desalting on a Sephadex G-25 column resulted in a further 11 fold increase in purification (Table 2.4). This method was found to be superior to other methods of desalting (e.g. dialysis, ultrafiltration), because it enabled the additional removal of nonenzymatic proteins. During this step, about 4% of the initial amount of protein was eliminated and 72% of enzymatic activity, free of ammonium sulphate, was retained in pooled, concentrated fractions (Fig. 2.1).

2.3.3 Purification of PPO by various chromatographic techniques

Several chromatographic techniques were employed for purification of artichoke PPO (Fig. 2.2). The elution profile of PPO from DEAE-cellulose column (Fig. 2.2A) revealed three fractions. The first, flow-through fraction (F_1), was obtained with the equilibrating buffer. Subsequent gradient elution, first with NaCl, and then with pH disclosed F_2, and F_3 fractions, respectively. Gel filtration on Sephadex G-100 (Fig. 2.2B), and hydrophobic chromatography on phenyl

Figure 2.1 Sephadex G-25 column chromatography of PPO. Salted out enzyme (40 ml, 67 PPO units, 70 mg protein) by ammonium sulphate (20-80%), dissolved in 0.1 M sodium phosphate buffer, pH 6.5, was applied to a column (2.6 x 70 cm). The column was eluted with the same buffer at a flow rate of 26 ml/hr. Fractions of 4 ml each were collected. Column fractions 18 to 32 were pooled, concentrated and used for further purification.

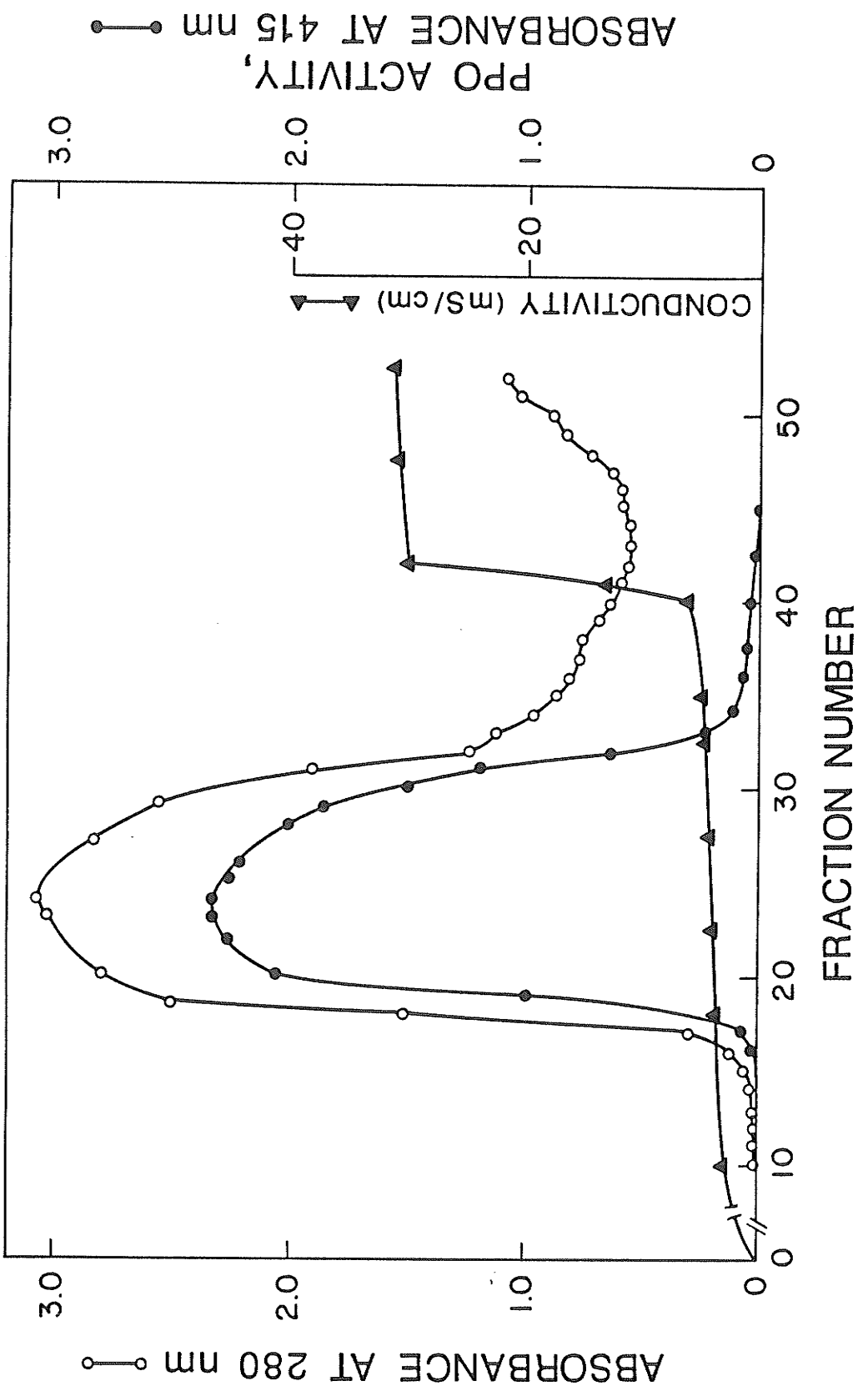
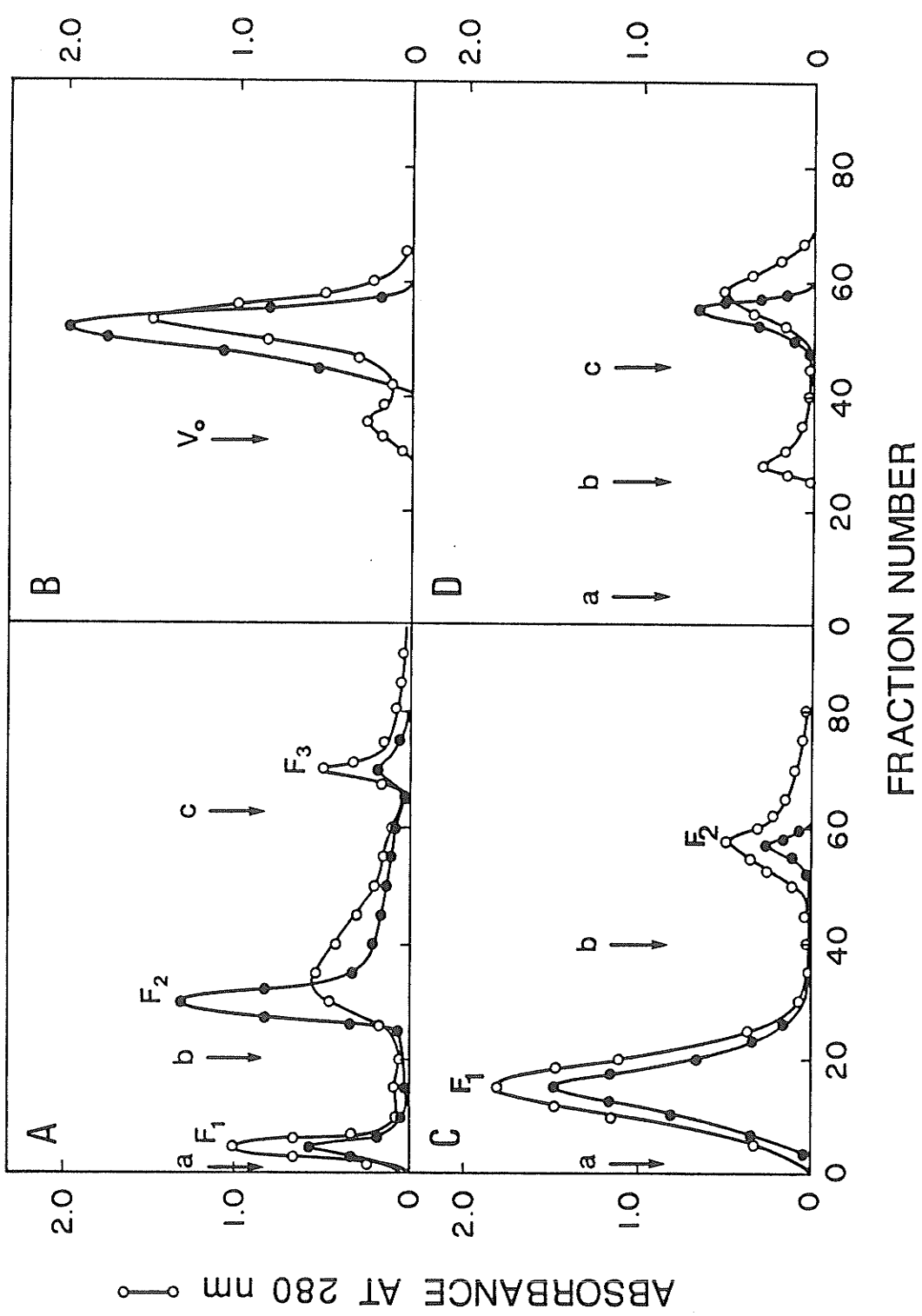

ABSORBANCE AT 280 nm
PPO ACTIVITY,
ABSORBANCE AT 415 nm
CONDUCTIVITY (mS/cm)
FRACTION NUMBER
0
10
20
30
40
50
1.0
2.0
3.0
20
40

Figure 2.2 Various column chromatographic methods of PPO purification:
A. DEAE-cellulose chromatography; the enzyme sample (6.5 ml, 380 PPO units, 40 mg protein) was applied to a column (1 x 40 cm). The column was eluted with: a) 0.05 M sodium phosphate buffer, pH 6.5, and subsequently with b) linear gradient of NaCl (0 to 0.3 M) in the same buffer, and c) pH gradient from 7.0 to 4.5 using 0.05 M sodium phosphate buffer containing 0.3 M NaCl.
B. Sephadex G-100 chromatography; the enzyme sample (2.0 ml, 190 PPO units, 20 mg protein) was applied to a column (2.5 x 100 cm). The column was eluted with 0.05 M sodium phosphate buffer, pH 6.5.
C. DL-dopa Sepharose 4B chromatography; the enzyme sample (15 ml, 570 PPO units, 60 mg protein) was applied to a column (1 x 20 cm) The column was eluted with: a) 0.05 M sodium phosphate buffer, pH 6.0, and subsequently with b) the gradient pH from 6.0 to 4.5 using the same buffer.
D. Phenyl Sepharose CL-4B chromatography; the enzyme sample (5 ml, 240 PPO units, 25 mg protein) was applied to a column (1 x 20 cm). After equilibrating with 0.05 M sodium posphate buffer, pH 6.5, containing 1 M ammonium sulphate, and 1 M NaCl, the column was eluted in a gradient-stepwise manner using following buffer systems: a) 50 ml of equlibrating buffer - 50 ml 0.05 M phosphate buffer, b) 50 ml 0.05 M phosphate buffer - 50 ml distilled water, c) 50 ml water - 50 ml 50% solution of ethylene glycol.
Partially purified PPO was used as the enzyme sample. The columns were eluted with a flow rate of 15 ml per hr, and fractions of 4 ml were colected.


ABSORBANCE AT 280 nm
PPO ACTIVITY,
ABSORBANCE AT 415 nm
FRACTION NUMBER
A
B
C
D
a
b
c
F1
F2
F3
Vo
0
1.0
2.0
0
20
40
60
80

Sepharose CL-4B (Fig. 2.2D) revealed one fraction with PPO activity, while with affinity chromatography on L-dopa Sepharose 4B (Fig. 2.2C) two fractions F_1 (flow-through) and F_2 (pH gradient) were obtained. The F_1 fraction could be the result of column overload, or might reflect the presence of a PPO component with negligible affinity towards the immobilized ligand (L-dopa); in contrast, the F_2 fraction showed affinity towards L-dopa. Attempts to purify PPO by the above chromatographies were not successful, because of a low degree of purification, and in most cases low yield of activity, as shown in Table 2.3. The best preparation of PPO was obtained with DEAE-cellulose which resulted in a two-fold purification, while the highest yield of activity (83%) was obtained by gel filtration. The latter chromatography resulted in negligible purification of the enzyme. Extremely low enzyme activity was achieved by affinity and hydrophobic chromatographies; i.e., 5 and 3% over the initial PPO activity, respectively. In both cases PPO was bound to the attached ligand (L-dopa, or phenyl, respectively) so strongly that several methods employed to elute the enzyme were unsuccessful. It is worthwhile to note that the choice of eluents for affinity chromatography was limited. For example, when the column was eluted with an ascending pH gradient between 6.0 and 8.5 a rapid darkening of the gel was observed due to autooxidation of L-dopa.

Table 2.3 Purification of PPO by various column chromatographic techniques

Chromatography	Specific activity (U/mg protein)	Yield in activity (%)	Purification (fold)
Initial sample[a]	10.0	100.0	1.0
DEAE-cellulose:			
F_1	3.2	5.1	0
F_2	20.0	16.4	2.0
F_3	4.6	5.1	0
Sephadex G-100	11.0	83.0	1.1
DL-dopa Sepharose 4B:			
F_1	10.0	72.0	0
F_2	12.0	5.0	1.2
phenyl Sepharose 4B-CL	16.0	3.2	1.6

[a] PPO sample after ammonium sulphate fractionation, and desalting on Sephadex G-25 column was applied to the columns.

An alternative method which yielded an enzyme preparation with high degree of purity was immmobilized copper affinity chromatography (ICAC). This method was consequently used for further purification and characterization of PPO.

2.3.4 Immobilized copper affinity chromatography

A typical elution profile of PPO obtained with a small scale ICAC is shown in Fig. 2.3. When the column was eluted with the equilibration buffer, two flow-through fractions of activity, P_1 and P_2 were obtained. To ensure that these fractions were not a result of column overloading, the P_1 fraction was rechromatographed. Approximately 90% of the reapplied activity was recovered as a flow-through fraction. Stepwise elution, first with glycine and then histidine, revealed two additional fractions (P_3 and P_4 respectively). The majority of the PPO activity was present in the P_1 and P_3 fractions which contained approximately 91 and 76 activity units, respectively; fractions P_2 and P_4 contained 23 and 18 activity units, respectively (Table 2.4). Approximately 55% of the initial activity applied to the column was recovered. The overall recovery of activity was ca. 40% when compared to the initial extract.

In this study artichoke PPO was separated into four fractions of activity using ICAC. This type of chromatography is based on the ability of proteins to form coordination bonds with a metal ions bound to the gel matrix

Figure 2.3 Immobilized copper affinity chromatography of PPO (small scale).
Partially purified enzyme (5 ml, 381 PPO units, 38 mg protein) was applied to an ICAC column (1 x 20 cm). The column was eluted with 0.05 M TRIS-HCl buffer, pH 7.5, containing 0.15 M NaCl, and subsequently with the same buffer, containing 20 mM glycine or 10 mM histidine, as indicated on the chromatographic profile, at a flow rate of 15 ml/hr. Fractions of 4 ml each were collected.

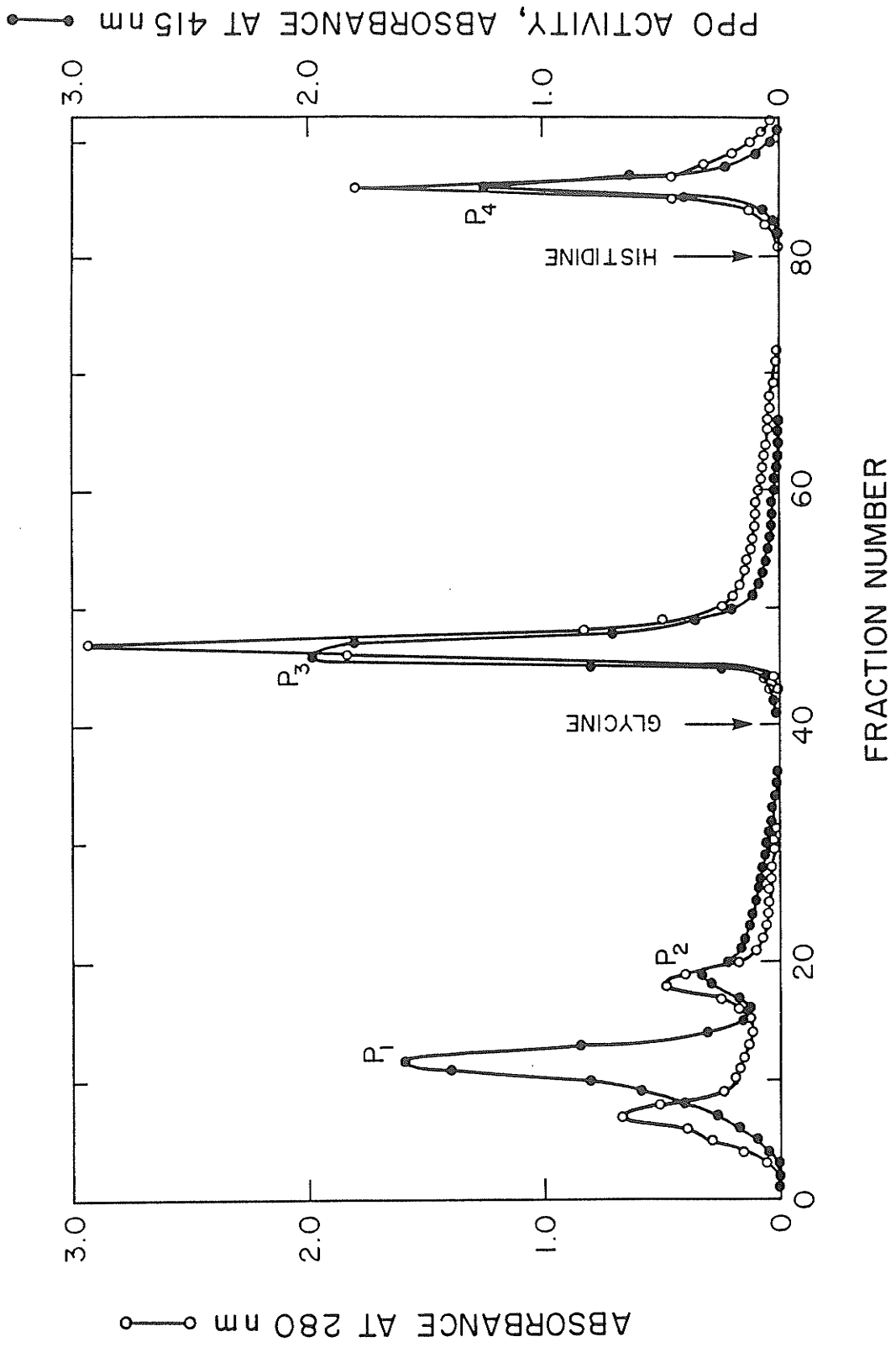

PPO ACTIVITY, ABSORBANCE AT 415 nm
ABSORBANCE AT 280 nm
FRACTION NUMBER
HISTIDINE
GLYCINE
P1
P2
P3
P4
0
1.0
2.0
3.0
20
40
60
80

Table 2.4 Purification of PPO by the small scale immobilized copper affinity chromatography

Steps	Activity (units)	Protein (mg)	Specific activity (U/mg protein)	Yield in activity (%)	Purification (fold)
Acetone powder buffer extraction	528.0	557.0	0.9	100.0	1.0
20-80% ammonium sulfate saturation	519.0	72.0	7.2	98.0	8.0
Sephadex G-25	381.0	38.5	9.9	72.0	11.0
ICAC fractions					
P_1	90.8	0.6	144.0	17.2	160.0
P_2	23.4	1.4	16.7	4.5	18.6
P_3	76.4	4.6	16.6	14.6	18.4
P_4	17.6	4.5	3.9	3.3	4.3

through the presence of surface chelating amino acids (i.e. metal binding sites). The differences in affinity among the PPO fractions for the gel may reflect differences in the number or density of copper chelating amino acid groups exposed on the surface of the enzyme molecules (Porath and Olin, 1983). The fact that P_1 and P_2 fractions were obtained as flow-through fractions may indicate that the enzyme components present do not have effective copper ion binding sites on their molecular surfaces (Lonnderdal and Keen, 1982). In contrast, the P_3 and P_4 fractions were adsorbed strongly to the column. Lowering the pH from 7.5 to 4.5 or increasing the ionic strength from 0 to 0.3 M NaCl did not desorb these fractions. A stepwise elution, however, with buffer first containing glycine and then, histidine in order of their stability constants to copper (Kikuchi and Watanabe, 1981), resulted in their desorption. These results suggest that the enzyme components of P_3 and P_4 fractions bind to the metal-chelated gel via structural sites possibly composed of either glycine or histidine residues.

The four enzymatic fractions obtained after ICAC were indicative of the enzyme heterogeniety based on differences in affinity for immobilized copper. This is in agreement with reports on heterogeniety of plant PPO, although multiple forms of this enzyme from various plant sources have been distinguished on the basis of their charge (Harel et

al., 1965; Park and Luh, 1985), molecular weight (Arnoud, 1968; Pruidze et al., 1983) and electrophoretic mobility (Constantinides and Bedford, 1967; Harel et al., 1973). Moreover, all four fractions exhibited varying enzymatic activity towards p-cresol and L-tyrosine (Table 2.5), catechol and DL-dopa (Table 2.6), indicating hydroxylase and oxidase activities, respectively. Although both activities were highest in the P_1 fraction, this fraction showed a significant hydroxylase activity approximately 20 fold higher than other ICAC fractions.

The above results reflect a small scale purification procedure. The chromatography was repeated on a larger scale. The column (2.5 x 40 cm) and enzymatic sample (49.5 ml) used for purification were approximately 10 times bigger than in the previous experiment. The data in Table 2.7 reveal the effectiveness of the large scale ICAC chromatography, while the ICAC chromatographic profile of PPO is illustrated in Fig. 2.4. The results obtained were similar to that of the small scale procedure, although some minor differences were observed. Four fractions with an increase in specific activity of 140 for P_1, 9 for P_2 and P_3, and 5 fold for P_4 (Table 2.7) were detected. Furthermore, the total yield of all fractions accounted for approximately 33% of the total activity of the acetone powder. Although this was lower by 7% than the total yield for the small scale preparation, the enzyme recovery only from ICAC co-

Table 2.5 Hydroxylase activity of ICAC fractions

Fractions	Activity (U/ml)	Protein (mg/ml)	Specific activity (U/mg protein)
p-cresol[a]			
P_1	2.24 ± 0.04[b]	0.32 ± 0.02	7.00
P_2	0.38 ± 0.00	0.84 + 0.07	0.45
P_3	1.24 ± 0.10	2.16 ± 0.08	0.57
P_4	0.84 ± 0.02	2.05 + 0.08	0.41
L-tyrosine[c]			
P_1	1.61 ± 0.02	0.32 ± 0.02	5.03
P_2	0.20 ± 0.02	0.84 ± 0.07	0.24
P_3	0.82 + 0.06	2.16 ± 0.08	0.38
P_4	0.38 + 0.02	2.05 ± 0.08	0.18

[a] 10 mM p-cresol was used as a substrate for determination of activity.

[b] Mean values ± SD of two determination.

[c] 1.6 mM L-tyrosine was used as a substrate for determination of activity.

Table 2.6 Oxidase activity of ICAC fractions

Fractions	Activity (U/ml)	Protein (mg/ml)	Specific activity (U/mg protein)
catechol[a]			
P_1	48.37 ± 0.12[b]	0.32 ± 0.02	151.16
P_2	13.94 ± 0.08	0.84 ± 0.07	16.59
P_3	35.38 ± 0.06	2.16 ± 0.08	16.38
P_4	7.92 + 0.10	2.05 ± 0.08	3.86
DL-dopa[c]			
P_1	37.35 ± 0.18	0.32 ± 0.02	116.72
P_2	7.86 ± 0.12	0.84 ± 0.07	9.36
P_3	21.63 ± 0.12	2.16 ± 0.08	10.01
P_4	16.50 ± 0.08	2.05 ± 0.08	8.05

[a] 10 mM catechol was used as a substrate for determination of activity.

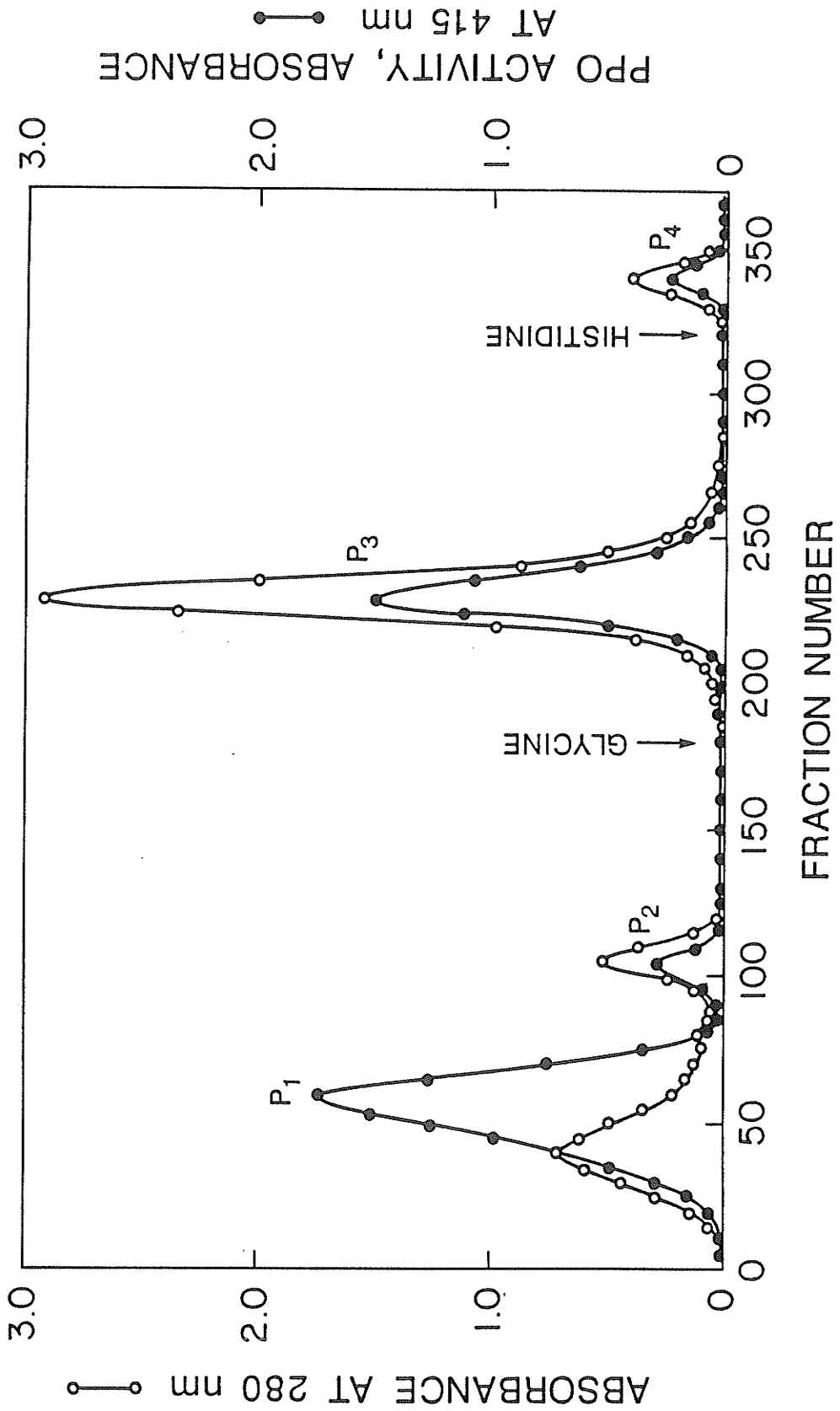
[b] Mean values ± SD of two determinations.

[c] 10 mM dopa was used as a substrate for determination of activity.

Table 2.7 Purification of PPO by the large scale immobilized copper affinity chromatography

Steps	Activity (units)	Protein (mg)	Specific activity (U/mg protein)	Yield in activity (%)	Purification (fold)
Acetone powder buffer extraction	6981.0	7388.0	0.9	100.0	1.0
20-80% ammonium sulfate saturation	4935.0	723.0	6.8	70.7	8.0
Sephadex G-25	3843.0	391.0	9.8	55.0	11.0
ICAC fractions					
P_1	1338.0	10.6	126.2	19.2	140.0
P_2	66.0	8.5	7.8	0.9	9.0
P_3	843.0	102.0	8.3	12.1	9.0
P_4	21.0	5.0	4.2	0.3	5.0

Figure 2.4 Immobilized copper affinity chromatography of PPO (large scale).
Partially purified enzyme (49.5 ml, 3843 PPO units, 391 mg protein) was applied to an ICAC column (2.5 x 40 cm). Column elution was as described for Fig. 2.3.


PPO ACTIVITY, ABSORBANCE
AT 415 nm
ABSORBANCE AT 280 nm
FRACTION NUMBER
HISTIDINE
GLYCINE
P1
P2
P3
P4
0
50
100
150
200
250
300
350
1.0
2.0
3.0

lumn was improved by 5%, mainly because the P_1 fraction was isolated with overall yield higher by 2%. In addition, P_1 represented 59% of the total recovered activity and was the most highly purified fraction. Thus, P_1 was used in all subsequent studies for the characterization of the Jerusalem artichoke PPO system.

2.3.5 Electrophoresis

Fractions P_1 to P_4, from ICAC, were analyzed by gradient polyacrylamide gel electrophoresis (PAGE, Fig. 2.5), as well as by isoelectric focusing (IEF, Fig. 2.6) coupled with enzymatic staining for oxidase activity using catechol as a substrate (PAGE, IEF), as well as for hydroxylase activity with p-cresol (PAGE, Fig 2.5b).

The analysis of PPO fractions by PAGE stained with catechol (Fig. 2.5a) yielded an electrophoretic pattern in which three groups of bands, referred to as A, B and C predominated. A comparison of the four activity peaks indicated that the group A bands, of low mobility, were present only in the P_1 fraction. Group B, represented by two bands, was most apparent in the P_3 fraction. The group C bands of high mobility were found in fractions P_1 - P_3. Fraction P_4 from ICAC showed faint activity bands; two of which appeared co-terminous with sub-species of group B bands, and another band intermediate to groups B and C. In addition, Fig. 2.5b shows the electrophoregram of the PPO fractions stained with p-cresol. The P_1 was the only one which exhi-

Figure 2.5 Gradient (6-12%) polyacrylamide gel electrophoresis patterns of ICAC PPO fractions
A. The gel stained for oxidase activity with catechol as a substrate.
B. The gel stained for hydroxylase activity with p-cresol as a substrate.
1) Crude PPO extracted from acetone powder, 2-5) Fractions after ICAC chromatography, P_1-P_4 respectively. Three units of activity were applied in each well.

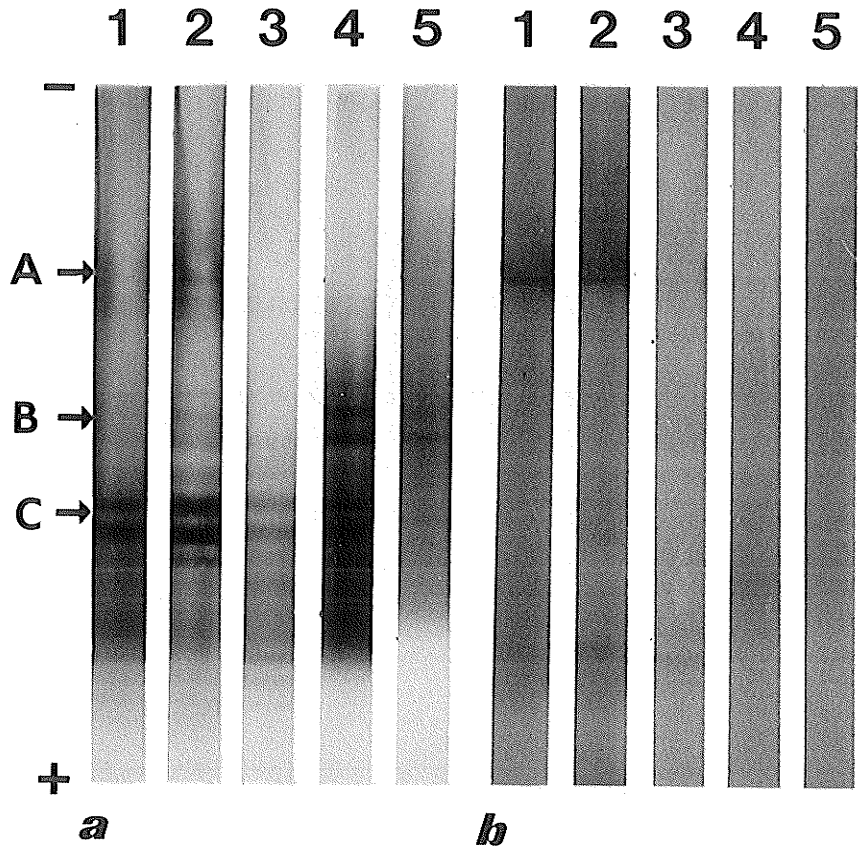

1 2 3 4 5 1 2 3 4 5
−
A
B
C
+
a
b

Figure 2.6 Analytical isoelectric focusing patterns of ICAC PPO fractions
1) Crude PPO extracted from acetone powder, 2-5) Fractions after ICAC chromatography, P_1-P_4 respectively. One unit of activity was applied in each well. The gel after electrophoresis was stained with catechol as a substrate. 6) Broad pI calibration kit, Pharmacia (5 μg protein stained with CBB): trypsinogen (pI-9.3), lentil lectin-basic band (8.6), lentil lectin-middle band (8.4), lentil lectin-acidic band (8.1), myoglobin-basic band (7.3), myoglobin-acidic band (6.8), human carbonic anhydrase B (6.5), bovine carbonic anhydrase B (5.8), β-lactoglobulin A (5.2), soyabean trypsin inhibitor (4.5), amyloglucosidase (3.5).

1
2
3
4
5
6
pH
9.3
8.6
8.4
8.1
7.3
6.8
6.5
5.8
5.2
4.5
3.5

bited an electrophoretically detectable hydroxylase activity in the low mobility region corresponding to group A bands.

Fig. 2.6 shows IEF patterns of ICAC fractions on ampholine (pH 3.5-9.5) polyacrylamide gel stained with catechol as substrate. All fractions exhibited a broad range of isoelectric points with predominant species in the acidic region of pH between 4 and 5. Therefore, the artichoke PPO was further fractionated to isolate an acidic fraction by ion exchange chromatography.

2.3.6 DEAE - Sepharose CL-4B chromatography

A typical chromatographic profile for the elution of PPO from the DEAE - Sepharose CL-6B column is shown in Fig. 2.7. The elution pattern revealed three peaks exhibiting PPO activity. The first peak was obtained with the void volume while the second major, and the third minor fractions were eluted with the NaCl gradient. These fractions had specific activities of 20, 130, and 30 units per mg of protein, respectively, and contained about 84% of the activity applied to the column. Furthermore, the PPO activity in the second fraction accounted for 89% of total activity recovered.

Isoelectric focusing between pH 3.5 and 9.5 of the PPO before and after the ion exchange chromatography (Fig. 2.8) demonstrated the effectiveness of the above chromatographic system to fractionate the various enzymatic forms. The

Figure 2.7 DEAE-Sepharose CL-6B column chromatography. The purified enzyme (P_1), after ICAC chromatography (2 ml, 144 PPO units, 1.2 mg protein) was applied in a column (1 x 20 cm). The column was eluted with 0.04 M TRIS-HCl buffer, pH 6.0, and subsequently with the same buffer containing a linear gradient of 0 to 1.0 M NaCl, at flow rate of 15 ml/hr. Fractions of 4 ml each were collected.

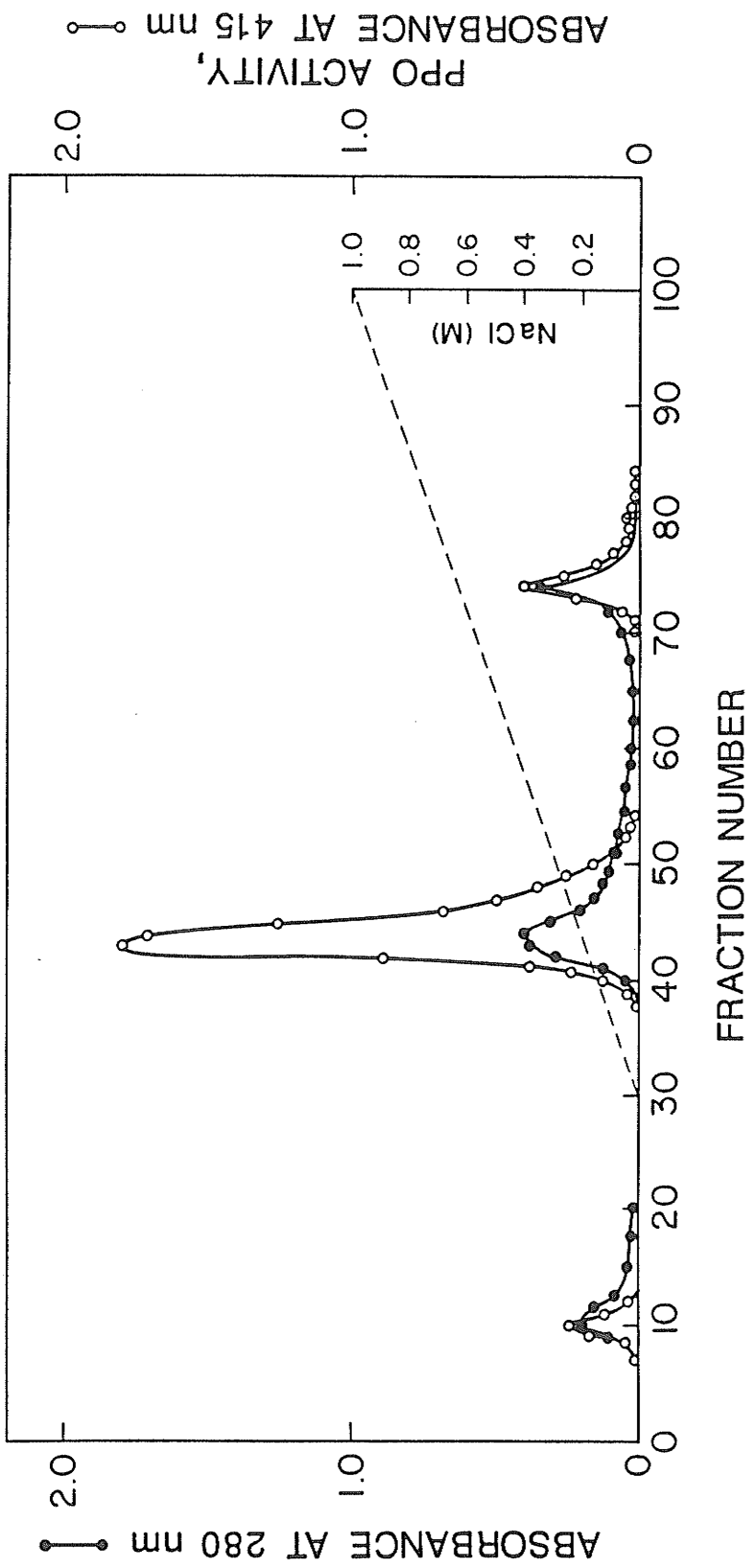
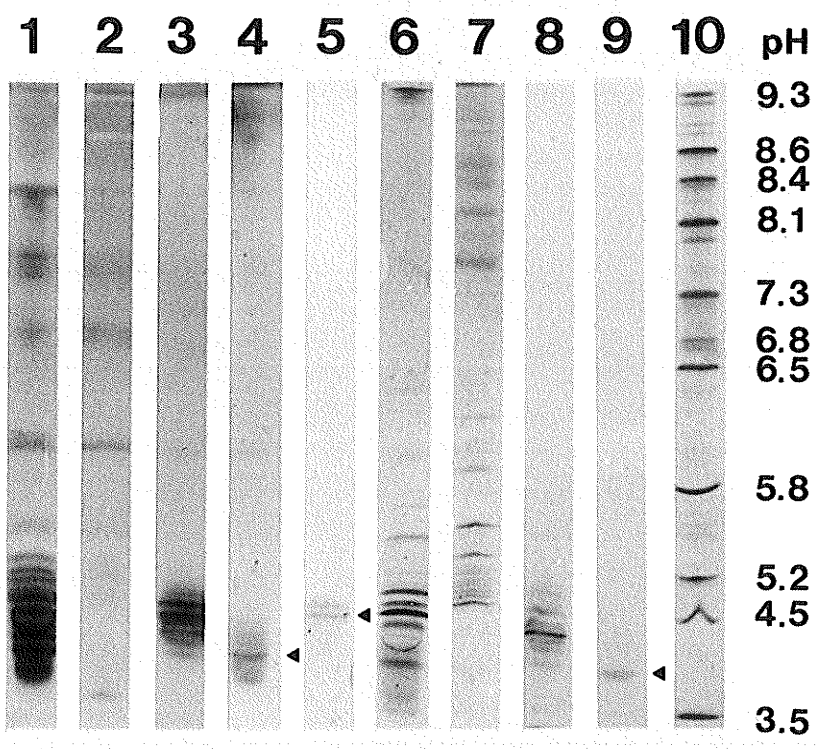

ABSORBANCE AT 280 nm
PPO ACTIVITY, ABSORBANCE AT 415 nm
FRACTION NUMBER
NaCl (M)
0
1.0
2.0
10
20
30
40
50
60
70
80
90
100
0.2
0.4
0.6
0.8
1.0

Figure 2.8 Analytical isoelectric focusing patterns of PPO fractionated by DEAE-Sepharose CL-6B ion exchange chromatography: 1 and 6) PPO before chromatography; 2 and 7) PPO eluted with the equilibrating buffer (0.04 M TRIS-HCl buffer); 3, 4 and 8) PPO eluted with a gradient of 0 to 0.4 M NaCl; 5 and 9) PPO eluted with a gradient of 0.4 to 1M NaCl. Lanes 1-3 (0.4 units/well) and 5 (0.2 units/well) PPO stained with catechol; lane 4 (0.4 units/well) PPO stained with p-cresol; lanes 5-8, (10 μg protein/well) and 9 (5 μg protein/well stained with CBB. Broad pI calibration kit, Pharmacia (5 μg protein stained with CBB; lane 10): trypsinogen (pI-9.3), lentil lectin-basic band (8.6), lentil lectin-middle band (8.4), lentil lectin-acidic band (8.1), myoglobin-basic band (7.3), myoglobin-acidic band (6.8), human carbonic anhydrase B (6.5), bovine carbonic anhydrase B (5.8), β-lactoglobulin A (5.2), soyabean trypsin inhibitor (4.5), amyloglucosidase (3.5).


1
2
3
4
5
6
7
8
9
10
pH
9.3
8.6
8.4
8.1
7.3
6.8
6.5
5.8
5.2
4.5
3.5

predominant enzyme species displayed isoelectric points in the region of pH 4.5 (Fig. 2.8; lane 1 and 3) and corresponded with the bulk activity of artichoke PPO eluting in the second major peak of Fig. 2.7. In addition, only this fraction exhibited hydroxylase activity, when the gel was stained with p-cresol (Fig. 2.8; lane 5), although it was too low to be detected by the polarographic test. Furthermore, the electrophoretic data of this fraction showed no additional protein contaminants (Fig. 2.8; lane 6). Consequently, this fraction, referred herein as PPO acidic fraction, along with P_1 fraction obtained from ICAC chromatography, were used in all subsequent studies for further characterization of the artichoke PPO system. It is of interest here to note that similar acidic pI values have been also reported for polyphenol oxidases from potato tubers (Matheis and Belitz, 1975) and olives (Ben-Shalom et al., 1977).

CHAPTER THREE
CHARACTERIZATION OF PURIFIED ARTICHOKE POLYPHENOL OXIDASE SYSTEM

3.1 INTRODUCTION

Although extensive basic research has been carried out on various aspects of catalytic as well as molecular properties of polyphenol oxidase, its behaviour in foods is still not fully understood for a number of reasons. The PPO can catalyse more than one reaction and displays activity towards a great range of substrates, as indicated by the various names by which it is commonly known: catecholase, tyrosinase, cresolase, polyphenoloxidase, phenolase, etc. There are other plant enzymes such as laccase and peroxidase which show overlapping substrate specificities with PPO. Moreover, the enzyme apparently occurs in multiple forms in each plant species (Vámos-Vigyázó, 1981).

Catechol and cresol are commonly used for estimation of the PPO activity, but natural phenolic compounds existing in a particular plant tissue are the preferred substrates of the respective PPO. The most important natural substrates of PPO in fruits and vegetables are cinnamic acid esters, catechins, 3,4-dihydroxyphenylalanine (dopa), and tyrosine (Walker, 1975). Chlorogenic acid (3:4-dihydroxycinnamoyl-quinic acid) is one of the o-diphenols most widespread in plant tissues. It appears to be the main substrate for PPO from apples (Walker, 1962) and pears (Walker, 1964). Alberghina (1964) found that PPO from potato tubers had an affinity much higher for this compound than for catechol or dopa. In the vast majority of cases

chlorogenic acid, 4-methylcatechol, caffeic and catechin were readily oxidized but the rate of oxidation was much reduced with dopa (Walker, 1975). The affinity of plant PPO for the phenolic substrates is relatively low. The K_m value is high usually about 1 mM (Mayer and Harel, 1979). It is worthwhile to point out that much higher substrate affinity for phenolics with K_m value of 0.01 mM was reported for the enzyme isolated from potato tubers (Batistuti and Lourenço, 1985).

The optimum pH for maximum activity of the enzyme appears to vary between preparations and substrates. In general, PPO is active between pH 4.0 and 7.0 and does not have a very sharp pH optimum. At approximately pH 3, the enzyme is irreversibly inactivated (Vámos-Vigyázó, 1981). It is difficult to measure the activity of the enzyme directly, owing to the large number of secondary reactions which follow the initial enzymatic oxidation. Various assay methods of PPO activity have been compared critically (Mayer et al., 1966). Polarographic measurement of the initial oxygen uptake appears to give the most sensitive and reliable estimate the enzyme activity.

As stated earlier, many reagents inhibit PPO activity that could be divided into two main categories of PPO inhibitors: reagents which interact with the copper in the enzyme and compounds which affect the site where the phenolic substrate binds. Furthermore, some inhibitor studies

have provided valuable insights into the mode of enzymatic action, while other inhibitors such as 2,3-naphthalenediol (Mayer et al., 1964), or tropolone (Kahn, 1985b) may be used to differentiate PPO from laccase or peroxidase, respectively. In addition to the number of different types of PPO inhibitors (discussed in previous chapter), there are several natural inhibitors isolated from various tissues (Walker, 1975; Mayer and Harel, 1979). A low MW peptide, which inhibits PPO from apple and mushroom, was isolated from Dactylium dendroides by Harel et al. (1967). Walker (1970) has reported the isolation of a low MW compound from Penicillium expansum which inhibited apple and tobacco PPO. This compound is believed to play an important role in overcoming the fruit's defence mechanism and thus facilitating infection by the phytopathogen.

The heterogeneity of plant polyphenol oxidases has been reported for a broad range of enzymes of fruit and vegetable origin. The molecular forms referred to as isoenzymes or multiple forms were distinguished on the basis of electrophoretic mobilities or isoelectric points, heat resistance, substrate specificity, and molecular weight (Constantinides and Bedford, 1967). The number of multiple forms of PPO within a particular species was found to be dependent on the methods used for their separation and detection. In some cases, multiple forms of this enzyme may be the results of proteolytic degradation (Flurkey and Jen,

1980) or interaction between enzyme and nonezymatic proteins (Smith and Montgomery, 1985). In addition, some enzyme preparations show multiplicity of forms which results from association-dissociation phenomena of different molecular weight forms. Thus, Harel and Mayer (1968) have reported three forms of PPO from apple, having MWs of 30-40, 60-70 and 120-130 K. These were shown to undergo interconversions. Interconversion can be influenced by changes in pH, ionic strength, protein concentration as well as by protein dissociating agents. Often the pattern of multiple forms was found to vary within the same cultivar depending on the time of harvest, storage conditions etc. Multiplicity of MW has been observed for PPO from potato tubers, sugar beet, sugar cane, avocado and banana (Mayer and Harel, 1979). The MW of the subunits (monomer) of PPO from higher plants has frequently been reported to be about 30-40 K (Mayer and Harel, 1979), although MW values as low as 10 and 12 K have been also reported for the enzyme from banana (Palmer, 1963) and sugar beet chloroplasts (Mayer, 1966), respectively.

Many reports suggest that plant PPO is a metalloprotein with copper as the prosthetic group (Vámos-Vigyázó, 1981). In his pioneering work on PPO from potato peels, Kubowitz (1938) concluded that the enzyme contained two copper atoms at its active site. Numerous reports on enzymes from various sources, which appeared later, suggested

a copper content of one atom per polypeptide chain or subunit in PPO (Robb et al., 1965; Balasingam and Ferdinand, 1970). For example, Interesse et al. (1983) have reported one atom of copper per mole of monomer of MW 30 K in wheat PPO. However, recent studies with Agaricus bispora and Neurospora crassa PPO have been shown that the functional unit of the enzyme contains a pair of copper ions. These findings are in agreement with the studies on spinach beet (Beta vulgaris) PPO revealing the presence of two copper ions per functional unit of the 40 K enzyme species (Vaghan et al., 1975). Some studies have been carried out in order to identify the amino acid residues involved in the binding of the copper atoms to the apoenzyme at the active site. The experimental evidence for mammalian, mushroom and fungal PPOs suggests the involevment of histidyl residues as metal ligands of the active site copper (Lerch, 1981). However, no data on the active site of plant PPO have been reported. Studies on this subject have been hampered by the difficulties associated with purification of PPO, its molecular heterogeneity as well as the apparent loss of copper from the enzyme during its purification.

For similar reasons, there are only a few reports on the amino acid composition of PPO from higher plants. Wheat (Interesse et al., 1983), grape (Kidron et al., 1977), spinach (Vaughan et al., 1975), and potato tuber (Balasingam and Ferdinand, 1970) PPO show considerable similarity

in amino acid composition, although some differences have been observed. Similarities were found in the basic amino acids and in the content of threonine, serine, glycine alanine and isoleucine, whereas the number of hydrophobic residues were different. Furthermore, the content of sulphur containing amino acids was relatively low; the lowest being with the grape enzyme.

The molecular properties of plant PPO are rather complex. A systematic study of the properties of this enzyme from a variety of plant sources, might help to understand its biological function in plant, and its contribution to changes encountered during processing of foods of plant origin. In this chapter, the systematic characterization of the purified artichoke PPO is described. The characteristics of this enzyme with respect to pH optima, substrate specificity, kinetic properties, behaviour towards inhibitors, kinetics of thermal inactivation, metallo-properties, amino acid composition as well as some insights into the nature of PPO multiplicity were the subject of these studies.

3.2 MATERIALS AND METHODS

3.2.1 Materials

3.2.1.1 Enzyme

Artichoke PPO was isolated and purified using immobilized copper affinity chromatography. The P_1 fraction of highest activity was further fractionated with DEAE - Sepharose CL-6B chromatography to give a PPO acidic fraction. All procedures for isolation and purification of the artichoke PPO are described in the previous chapter, section 2.2.2.4, and 2.2.2.5, respectively. Both enzyme preparations were used in all subsequent studies on characterization of the artichoke PPO system.

3.2.1.2 Chemicals

Molecular weight (MW) standards for SDS-PAGE: phosphorylase B (92.5K), bovine serum albumin (66.2K), ovalbumin (45K), carbonic anhydrase (31K), soybean trypsin inhibitor (21.5K), and lysozyme (14.4K); sodium dodecyl sulfate (SDS), and ammonium persulfate were obtained from Bio-Rad (Richmond Calif.). The MW standards for gel filtration: Blue dextran, β-galactosidase (116K), bovine albumin (66K), egg albumin (45K), trypsinogen (24K), lysozyme (14.3K), and L-tryptophan as well as basic fuchsin, periodic acid, succinic anhydride, 2-mercaptoethanol, ferrulic, coumaric, caffeic, procatechuic, chlorogenic and gallic acids, rutin,

quercetin, DL-tyrosine, 2,3-naphthalenediol and thioglycollate were purchased from Sigma (St. Louis, Mo, USA). Ultrogel AcA-44 was a product of LKB-Produkter AB (Bromma, Sweden) while triethylamine, phenylisothiocyanate (Pico.tag reagents) were supplied by Waters Assoc. (Clifton, N.J., USA). Urea, sodium metabisulfite, sodium diethyldithiocarbamate, phenol, potassium cyanide, p-cresol, catechol, 4-methylcatechol, catechin, hydroquinone were obtained from Fisher Scientific. Other reagents were of analytical grade or as described in previous chapters.

3.2.3 Methods

In the following studies PPO activity and protein content were determined as described previously (section 1.2.2.2, and 1.2.2.5). Unless otherwise indicated, all experimental steps were carried out at $4^{o}C$.

3.2.3.1 Electrophoretic techniques

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed at $20^{o}C$ on an LKB 2001 vertical electrophoresis unit using 6-12% gradient gels and the discontinuous buffer system (Laemmli, 1979). The stacking gel consisted of 4% acrylamide and 0.1% N,N'-methylene-bisacrylamide (BIS) while the separating gel was prepared with solutions of acrylamide, BIS, and glycerol ranging from 6 to 12, 0.2 to 0.6, and 0.5 to 5%, respectively. Both gels contained 0.1% SDS and were polymerized by the

addition of 5 µl N,N,N',N'-tetramethylethylenediamine (TEMED) and 50 µl 10% ammonium persulfate (AP) solution (stacking gel), or 3.3 µl TEMED and 50 µl 10% AP (separation gel) per 10 ml of gel solution. The gels were run for 3.5 hr at a constant current of 30 mA per gel. The electrode buffer contained 0.025 M TRIS, 0.192 M glycine and 0.1% SDS at pH 8.3. Phosphorylase B (92.5K), bovine serum albumin (66.2K), ovalbumin (45K), carbonic anhydrase (31K), soybean trypsin inhibitor (21.5K), and lysozyme (14.4K) were used as molecular weight (MW) markers. For electrophoresis under non-reducing conditions (to permit substrate staining), the enzyme was dissolved in 62.5 mM TRIS-HCl buffer, pH 6.8, containing 2% SDS and 10% glycerol, while under reducing conditions (protein staining), the buffer contained 5% 2-mercaptoethanol (ME). After reduction the solutions were heated for 1.5 min in a boiling water bath, cooled on ice and applied to the gel.

Isoelectric focusing (IEF) was performed as described in chapter two, section 2.2.2.7.2.

Gels after SDS and IEF were stained for carbohydrates using the periodic acid-Schiff (PAS) procedure. Because high concentrations of SDS produced an intense background, the SDS was removed before staining for carbohydrates according to the method of Fairbanks et al. (1971). The gels were washed at room temperature for the stated time using the following solutions: 1) 25% isopropyl alcohol, 10% ace-

tic acid; overnight; 2) 10% isopropyl alcochol 10% acetic acid;6-9 hr; 3) 10% acetic acid; overnight. The PAS procedure was performed as described by Pharmacia (1983). After washing the gel in 10% acetic acid at room temperature for at least 1 hr, it was put in 0.2% aqueous periodic acid, and held at 4°C for 45 minutes. The gel was then transfered immediately into the Schiff's reagent prepared as described below, and refrigerated for 45 minutes. Destaining of the gel slab was carried out at room temperature with two or three changes of 10% acetic acid until the background was clear. The Schiff's reagent was prepared as follows: basic fuchsin (1 g) was dissolved in 200 ml of boiling distilled water, stirred for 5 minutes, and cooled to 50°C. The solution was filtered, 20 ml of 1 N NaCl were added to the filtrate, and cooled to 25°C. Next, 1 g of sodium metabisulphite was added and the solution was left in the dark for 24 hr. After that, 2g of activated charcoal was added and the reagent was shaken for 1 minute, filtered and stored at room temperature until use.

To ascertain the existence of aggregated multisubunit forms of PPO, as described by other researchers (Jolley et al. 1969), several bond breaking agents such as 2-mercaptoethanol (0.1-3%), SDS (0.1-5%), urea (2-8 M) and succinic anhydride (10-30 mg/ml of enzyme) for acylation of amino groups, were used to treat PPO solutions. A 0.5 ml sample of the P_1 enzyme preparation (0.4 mg protein) in

0.1 M sodium phosphate buffer, pH 6.5, was incubated with each reagent (except succinic anhydride) at a specific concentration for various periods of time (2-300 min) prior to enzyme activity measurements and electrophoresis. Succinylation was carried out at room temperature according to Jolley et al. (1969), by the slow addition of a specified amount of succinic anhydride (divided into three portions) to 1 ml of enzyme (0.8 mg protein). The pH was maintained between 6.5 and 7.5 by the addition of 1 N NaOH. After succinylation the enzyme solution was dialyzed against deionized water for 12 h and lyophilized. The effects of pretreatments with the various agents on the artichoke PPO were monitored by SDS-PAGE. Electrophoretic procedures were carried out as described above, except that for the urea treated samples, 2 M urea was included in the electrode buffer.

3.2.3.2 Ultrogel AcA-44 chromatography

In addition to SDS-PAGE, molecular weight determinations of PPO were also performed on an Ultrogel AcA-44 column (1.6 x 90 cm) according to Andrews (1970). The column was calibrated using Blue dextran (void volume), β-galactosidase (116K), bovine albumin (66K), egg albumin (45K), trypsinogen (24K), lysozyme (14.3K), and L-tryptophan. Elution positions are reported as K_d, the distribution coefficient was calculated as follows:

$$K_d = (V_e - V_o)/V_s$$

where: V_o is the void volume (considered here as the elution volume of Blue dextran), V_e is the elution volume of the species of interest and V_s is the volume of solvent inside the gel and available to very small molecules ($V_s=V_o-V_i$) where V_i is elution for tryptophan.

The proteins were eluted with 20 mM sodium phosphate buffer, pH 6.5, including 0.15 M NaCl (some runs were carried out without NaCl) at a rate of 15 ml/h. Fractions of 4 ml were collected and monitored for protein, catecholase and cresolase activity. Cresolase activity of PPO was measured essentially as described by Montgomery and Sgarbieri (1975) using p-cresol as substrate and incubating the reaction mixture for 1 hr at 30°C prior to spectrophotometric analysis. Fractions of the two peaks bearing PPO activity (Fig. 3.5) were collected separately, lyophilized and used for protein and carbohydrate analysis. Total carbohydrate content was determined by the orcinol-sulfuric method, using glucose as standard (Miller et al. 1960). Protein was determined by the method of Lowry et al. (1951), while PPO activity was measured polarographically.

3.2.3.3 Effect of pH

A pH profile (3.0 to 8.5) for PPO activity was determined using 10 mM of 4-methylcatechol, catechol, and chlorogenic acid in 0.1 M sodium phosphate buffer. In the range of pH 3.0-4.0, buffers were prepared by mixing

solutions of sodium phosphate monobasic and phosphoric acid while at pH > 4.0 mixtures of monobasic and dibasic phosphate salts were used. In addition, the autooxidation of phenolic substrates was monitored and taken into account at each of the specified pH values.

3.2.3.4 Substrate Specificity

Substrate specificity of PPO was determined using 10 mM of the following compounds in 0.1 M sodium phosphate buffer, pH 6.0: phenol, p-cresol, catechol, 4-methylcatechol, DL-3,4 dihydroxyphenylalanine (dopa), (+) catechin, hydroquinone, p-phenylenediamine, ferrulic, coumaric, caffeic, procatechuic, chlorogenic and gallic acids, as well as 1.0 mM of rutin and quarcetin, and 1.6 mM DL-tyrosine. Enzymatic activity was expressed as a % of relative activity as compared to catechol (100%).

3.2.3.5 Effect of Inhibitors

The following compounds were used for the inhibition studies: sodium diethyldithiocarbamate, 2,3-naphthalenediol, thioglycollate and sodium metabisulfite. Inhibition of PPO activity was tested polarographically in a reaction mixture (3 ml) consisting of 10 mM catechol, 10 mM of inhibitor in sodium phosphate buffer, pH 6.0.

3.2.3.6 Kinetic studies

Michaelis constant (K_m) and maximum velocity (V_{max}) values were determined by a least-squares fit of the data

on double reciprocal plots according to Lineweaver and Burk (1934). A 10 µl sample of constant amounts of PPO (P_1 or the acidic fraction containing 8 µg, and 4 µg of protein were used, respectively) were incubated with various (at least seven) substrate (catechol, 4-methylcatechol, chlorogenic acid) concentrations (0.2-50 mM) at 30°C and the enzymatic reaction was followed polarographically.

The dissociation constant (K') from inhibition of the PPO - catalysed oxidation of chlorogenic acid by excess of this substrate was quantitated using a plot of 1/V vs. substrate concentration (Dixon and Webb, 1960). Inhibition of PPO activity by potassium cyanide was performed using 1 and 5 mM concentrations of 4-methylcatechol as substrate, and variable inhibitor concentrations ranging from 0 to 2 mM. The K_i value and the inhibition type were assessed from the corresponding Dixon plot (Dixon, 1953).

Lineweaver-Burk plots were used to evaluate the type of inhibition and the apparent K_i value for 2,3-naphthalenediol. Six substrate concentrations were used within the range of 1 to 20 mM.

3.2.3.7 Amino acid analysis

Amino acid analysis of the PPO acidic fraction according to the Pico - Tag technique was carried out as described in the Waters instruction manual (No.88140; 1984). The sample (85 ug protein/ml) was hydrolyzed in the Work-

station at 105°C for 24 h using 6N HCl containing 1% v/v phenol. Next, the sample was derivatized with a mixture of ethanol, distilled water, triethylamine, and phenylisothiocyanate (7:1:1:1) for 20 minutes at room temperature. After derivatization, analysis of the enzyme hydrolysate (phenylthiocarbamyl derivatives of the amino acids), was performed by high-pressure liquid chromatography (HPLC) using a reversed phase μBONDAPAK C_{18} column in the Waters HPLC system.

3.2.3.8 Heat inactivation

Heat treatments of PPO (acidic fraction) were carried out at 60, 65, 70, 75, and 80° C for varying periods of time in a temperature controlled water bath. The enzyme (250 μl; 100 μg of protein) in 0.05 M sodium phosphate buffer, pH 6.0, was placed in a prewarmed capillary tube at the specified temperature and portions were withdrawn at various time intervals, cooled immediately in an ice bath and assayed for residual activity. Stability of the enzyme was expressed as log % activity remaining. The activation energy was then determined by an Arrhenius plot of log reaction rate (lnK) vs. the reciprocal of the absolute temperature.

3.2.3.9 Preparation of apoform and enzyme reconstitution

A 0.5 ml (200 μg of protein) of the PPO acidic fraction placed in a micro - dialysis cell, as described by

Rodriguez and Tait (1983), was used for this study. Small portions of the enzyme were withdrawn at various time intervals and assayed for PPO activity. The apoenzyme was prepared by dialysis of PPO against 0.01 M KCN, pH 7.0, for 28 hours, followed by dialysis against distilled water (Kertesz et al., 1972). The enzyme was reconstituted by dialysis of its apoform against 1 mM $CuSO_4$ in 0.1 M sodium phosphate buffer, pH 6.5, for 4 hours. Excess copper was subsequently removed by affinity chromatography using an iminodiacetic acid - Sepharose 6B column (1 x 10 cm) as described by Porath et al. (1975). The reconstituted enzyme was collected and concentrated by diafiltration using an Amicon cell with a membrane having an MW cut-off of 10,000 (Diaflo membrane, UM 10, Amicon Corp., Lexington, Mas.).

3.3 RESULTS AND DISCUSSION

Artichoke PPO was purified by ICAC chromatography. As a result of this purification step the P_1 fraction was obtained. However, during the course of its characterization, the enzyme was found to exhibit a multiplicity of forms with a broad range of isoelectric points; the predominant species being in the acidic pH region. Therefore, the P_1 enzyme was further fractionated by ion exchange chromatography which yielded the PPO acid fraction. Consequently, both enzymatic preparations were used for characterization of the molecular properties of the artichoke PPO system.

3.3.1 Characteristics of P_1 fraction

3.3.1.1 pH optimum

Purified PPO (P_1 fraction) had a pH optimum of 6.0, when catalyzing the oxidation of catechol (Fig. 3.1). This optimum pH region coincides with that of the crude PPO extract from tubers (Fig. 1.1) and it further implies that P_1 is a representative fraction of the total endogenous PPO enzymatic system.

3.3.1.2 Substrate specificity

A number of compounds was tested as substrates for the purified enzyme preparation and the corresponding relative activities are summarized in Table 3.1. The enzyme fun-

Figure 3.1 Effect of pH on the activity of PPO - P_1 fraction (catechol as a substrate).

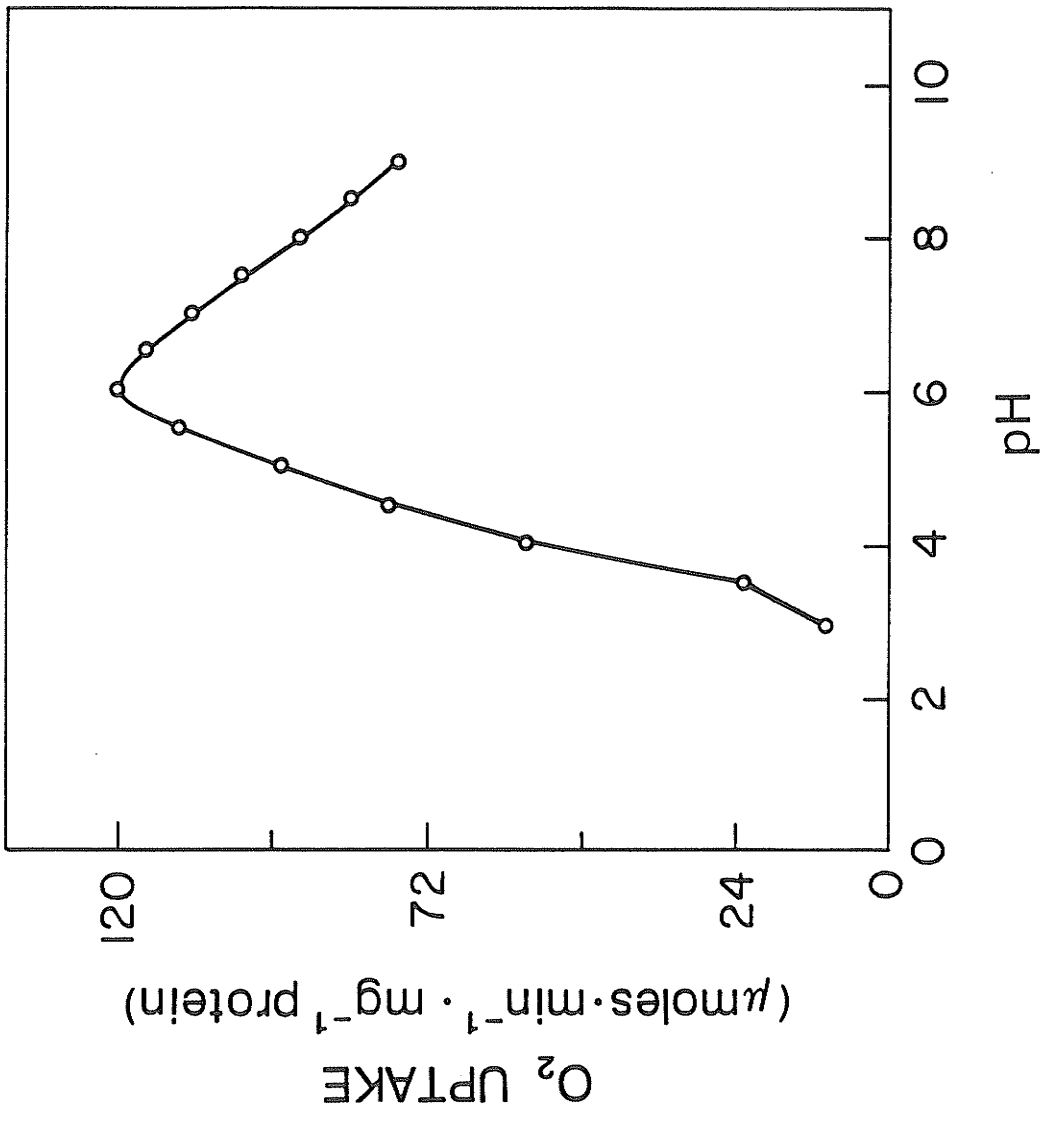

O2 UPTAKE
(μmoles·min⁻¹· mg⁻¹ protein)
120
72
24
0
0
2
4
6
8
10
pH

Table 3.1 Substrate and inhibitor properties of various compounds on the artichoke PPO

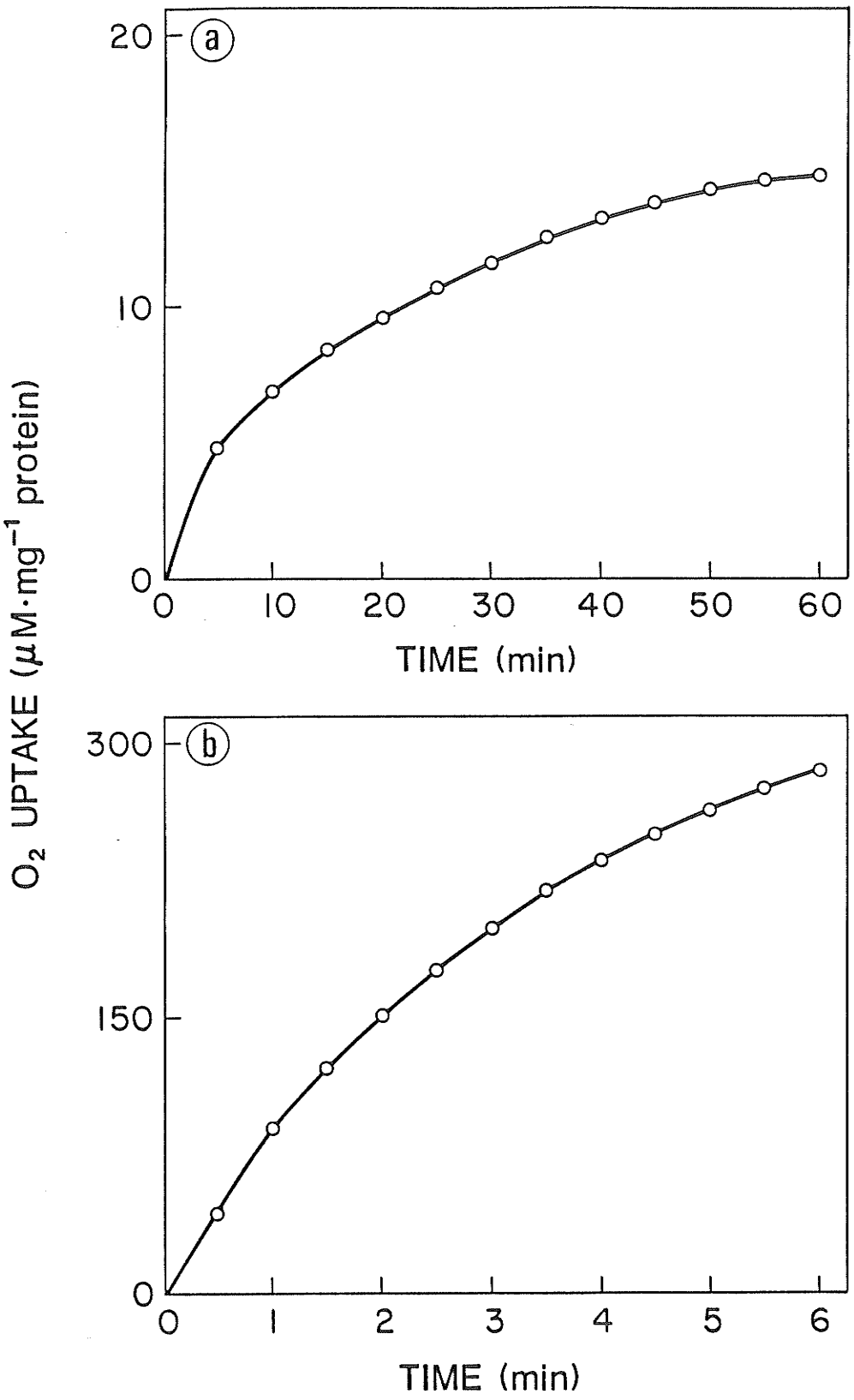
Compounds[a]	Relative activity (%)[c]	Km (mM)	V_m $\mu moles O_2 . min^{-1} . mg^{-1}$ protein	K_i (mM) type of inhibition
SUBSTRATES				
Monophenols				
p-cresol	2.5			
DL-tyrosine[b]	3.5			
o-Diphenols				
catechol	100.0	3.9	80	
4-methylcatechol	157.0	3.5	161	
chlorogenic acid	180.0	1.9	193	
DL-dopa	67.0			
Polyphenols				
gallic acid	7.5			
+catechin	37.0			
Others				
hydroquinone	0			
p-phenylenodiamine	0			
INHIBITORS				
sodium metabisulfite	0			K_{ii}:0.08; K_{is}:0.04 mixed[d]
sodium diethyldithiocarbamate	6.0			1.6 non-competitive
2,3-naphthalenediol	25.0			3.6 competitive
thioglycollate	53.0			-

[a] concentration of 10 mM; [b] concentration of 1.6 mM; [c] compared to catechol; [d] K_{ii} - K_i intercept, K_{is} - K_i slope

ctions, primarily as an o-diphenol oxidase, but also has the ability to hydroxylate monophenols. In this respect, chlorogenic acid followed by 4-methylcatechol, and catechol were the most reactive substrates, while activities toward p-cresol and DL-tyrosine were 40 and 25 fold lower, respectively, than for catechol. Similar observations have been reported for potato PPO (Abukharma and Woolhouse, 1966), except that the ratio of cresolase to catecholase activity was much higher. In addition, Figure 3.2 shows that the rate of p-cresol hydroxylation (monophenol activity, a) was much lower than oxidation of catechol (catecholase activity, b), as can be jugged by oxygen consumption.

Monophenol oxidase activity of PPO is often lost during purification (Mason, 1955; Sharma and Ali, 1980; Batistuti and Lourenco, 1985), probably as a result of changes in the structure of protein (Walter and Purcell, 1980). In addition, there is a lag phase, characteristic of monophenol hydroxylation (Robb, 1984), and thus it is difficult to assess the true hydroxylase activity of PPO preparations. The low hydroxylase values obtained for the purified enzyme, however, do not reflect any inactivation during purification, since similar data were also found for the crude extract (chapter one). Furthermore, addition of small amounts of 4-methylcatechol (20 μM) or DOPA (16 μM) to substrate solutions of p-cresol, or DL-tyrosine, respectively, which reduced the lag period, did not alter the above

Figure 3.2 Oxidation of p-cresol (a), and catechol (b) by PPO.

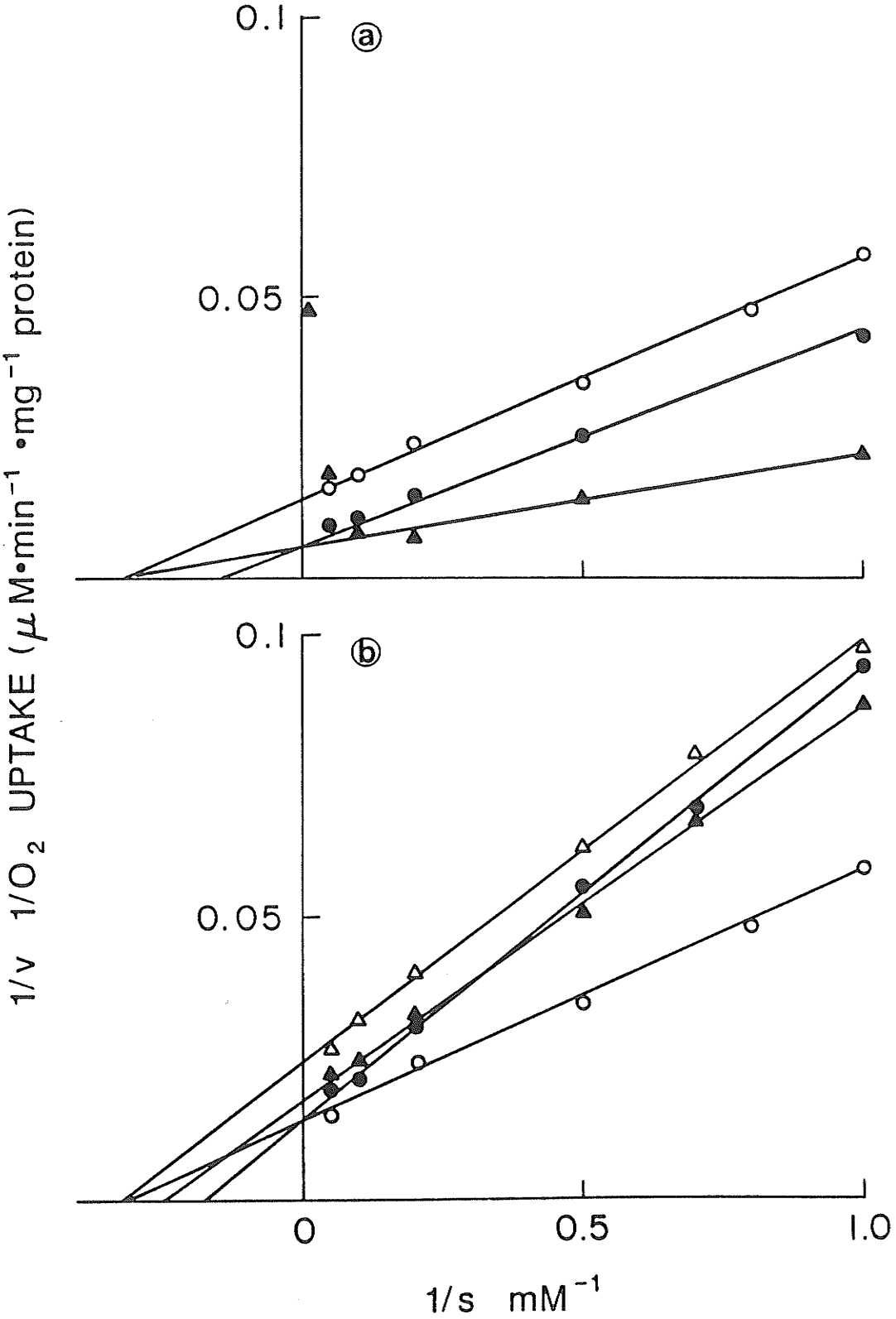

a
20
10
0
0
10
20
30
40
50
60
TIME (min)
b
300
150
0
0
1
2
3
4
5
6
TIME (min)
O2 UPTAKE (μM·mg-1 protein)

trends in relative monophenol oxidase activity.

The PPO was devoid of laccase activity, as indicated by its ability to react with tyrosine, the lack of reaction with p-dihydroxyphenol and p-phenylenediamine as well as by its inhibition with 2,3-naphthalendiol (Table 3.1). The presence of peroxidase was also excluded by the fact that PPO activity was not affected by the addition of catalase and ethanol to the reaction mixture (Mayer and Harel, 1979).

Double-reciprocal Lineweaver-Burk plots for the three most effective substrates of PPO are presented in Fig. 3.3a. Chlorogenic acid, 4-methylcatechol and catechol had apparent K_m values of 1.9, 3.5 and 3.9 mM, respectively. The K_m values are of similar order of magnitude with those reported for the oxidation of these substrates by the potato enzyme (Abukharma and Woolhouse, 1966). The oxidation reaction followed Michaelis-Menten kinetics in the range of 0.2 to 50 mM for catechol and 4-methylcatechol. On the other hand, chlorogenic acid was found to inhibit PPO at concentrations greater than 10 mM. Enzyme inhibition by excess of substrate has been previously reported for the oxidation of chlorogenic acid by potato (Abukharma and Woolhouse, 1966) and black poplar PPO (Tremolieres and Bieth, 1984). The black poplar PPO is considered to possess an extended substrate-binding site (Tremolieres and Bieth, 1984). Substrate inhibition is often rationalized by as-

Figure 3.3 Lineweaver-Burk plots for PPO of various substrates and inhibitors.
a. Effect of substrate concentration: catechol (○); 4-methylcatechol (●); chlorogenic acid (▲).
b. Inhibition of PPO catalyzed oxidation of catechol. Inhibitors: 1 mM sodium diethyldithiocarbamate (△); 0.02 mM sodium metabisulfite (▲); 2.5 mM 2,3-naphthalenediol (●); no inhibitor (○).


0.1
ⓐ
0.05
0.1
ⓑ
0.05
0
0.5
1.0
1/v 1/O2 UPTAKE (μM•min-1 •mg-1 protein)
1/s mM-1

suming that at high substrate concentrations, a second molecule could bind ineffectively near the active center of the enzyme and thus retard the catalytic transformation of a substrate molecule resting at the active center (Trowbridge et al. 1963, Tremolieres and Bieth, 1984). Among the three substrates examined, chlorogenic acid supported the highest rate of PPO catalyzed O_2 consumption (V_{max}: 193 μM $O_2.min^{-1}.mg^{-1}$.protein) and exhibited the greatest affinity (K_m: 1.9mM). The high affinity of chlorogenic acid has also been reported for PPO from pears (Rivas and Whitaker, 1973), sugar cane leaves (Coombs et al., 1974) and potato (Batistuti and Lourenco, 1985) and it seems to be related with the presence of the unsaturated -CH=CH-R group in the para position (Walker and Wilson, 1975). Catechol and 4-methylcatechol had similar K_m values but were oxidized at significantly different maximum rates. The V_{max} for 4-methylcatechol (161 μM $O_2.min^{-1}.mg^{-1}$ protein) was about two times higher than that for catechol(80 uM $O_2.min^{-1}.mg^{-1}$ protein). This is in agreement with the view that the presence of an electron-donating group at the para position increases the reactivity of the substrate (Vámos-Vigyázó, 1981).

3.3.1.3 Effect of inhibitors

The effect of inhibitors on the artichoke PPO was investigated using a number of chemical compounds which exhibit a different mode of inhibitory action (Table 3.1).

Under identical inhibitor concentrations, the order of effectiveness was: sodium metabisulfite (MBS) > sodium diethyldithiocarbamate (DIECA) > 2,3-naphthalenediol > thioglycollate.

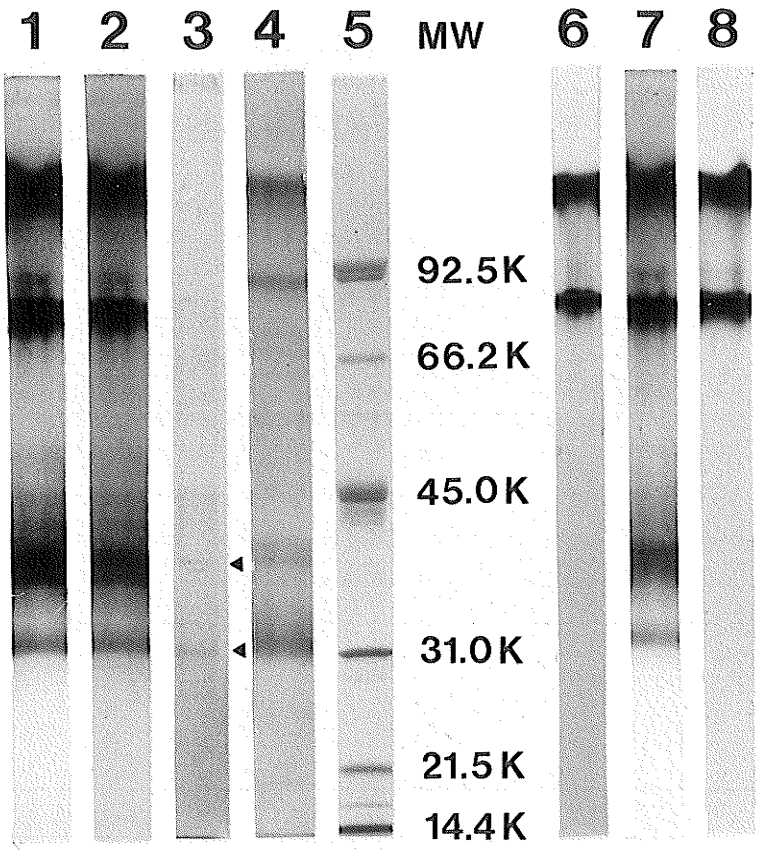
The MBS showed the highest inhibitory effect as evidenced by the relatively low K_i values derived from an apparently mixed type of inhibition pattern (K_{ii}: 0.08 mM, K_{is}: 0.04 mM; Fig. 3b.). The effect of MBS on PPO is very complex. Along with other sulfites, it is generally considered as a reducing agent for quinones derived by enzymatic browning (Ponting et al., 1972; Walker, 1975) as well as a quinone coupler (Embs and Markakis, 1965; Loomis, 1974). In both cases, it is rather the inhibition of the formation of coloured compounds produced in secondary non-enzymatic reactions than inhibition of PPO per se (Vámos-Vigyázó, 1981). However, since artichoke PPO was assayed by a polarographic method, which is based on O_2 consumption, it is possible that MBS directly inhibited the enzyme. This is in agreement with several reports which suggested a direct inhibitory effect of this compound on plant polyphenol oxidases (Golan-Goldhirsh and Whitaker, 1984; Sayavedra-Soto and Montgomery, 1986). The inhibitory action of DIECA implies that artichoke PPO most likely is a metalloprotein, as this inhibitor has been shown to complex the copper prosthetic group of PPO from a variety of plant sources (Swain, 1966; Grucarevic, 1971; Wong et al., 1971; Benjamin

and Montgomery, 1973; Roudsari et al. 1981). The DIECA showed non-competitive inhibition with catechol having an apparent K_i value of 1.6 mM (Fig. 3.3b). Competitive inhibition of the artichoke PPO was exhibited by 2,3-naphthalenediol (Fig. 3.3b). Inhibition of PPO by this compound also indicates that the enzyme is free of laccase activity (Mayer et al. 1964). Thioglycollate inhibited artichoke PPO by 50%. The mode of action of this inhibitor is controversial. Some researchers consider it as a reducing agent of the enzymatically generated quinones (Pierpoint, 1966), while others have reported a direct inhibitory action on PPO from several plant species (Baldry et al., 1970; Walker, 1975).

3.3.1.4. Molecular weight, electrophoretic characteristics and multimeric nature of PPO

Molecular weights of the artichoke PPO were determined by SDS-PAGE and gel filtration chromatography. The SDS-PAGE yielded four distinct PPO forms (Fig. 3.4, lanes 2,4) with apparent MW values of approximately 32, 40, 86 and 120K. Substrate staining after electrophoresis with a number of phenolic compounds revealed some interesting features of this enzymatic system. While the high MW forms exhibited activity towards monohydroxyphenols (Fig. 3.4, lane 6), o-dihydroxyphenols (Fig. 3.4, lane 7) and polyhydroxyphenols (Fig. 3.4, lane 8), the low MW subunits were specific only for o-dihydroxyphenols (Fig. 3.4, lane 7). Similar observa-

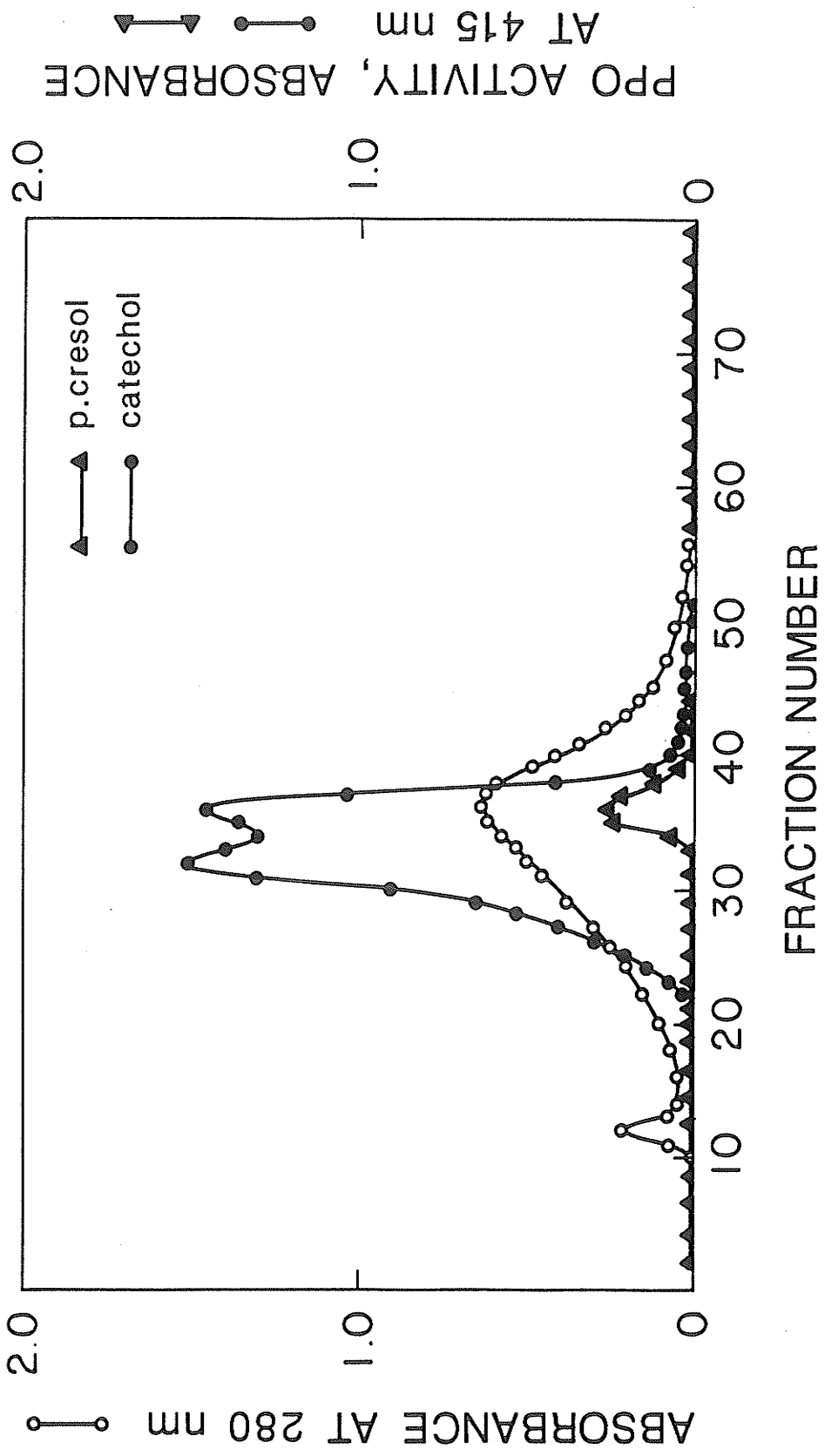
Figure 3.4 Gradient (6-12%) sodium dodecyl sulfate-polyacrylamide gel electrophoretic patterns of PPO.
1) Crude PPO extracted from acetone powder stained with catechol (3 units of PPO, protein was not determined). 2) P_1 fraction after ICAC chromatography stained with catechol (3 units of PPO). 3) P_1 fraction stained with PAS for glycoproteins (40 µg of protein). 4) P_1 fraction stained with Coomassie Brilliant Blue R-250 (CBB) for protein (40 µg of protein). 5) Low SDS-MW standards, Bio-Rad (4 µg of protein). 6-8) P_1 fraction stained for monophenol oxidase activity (lane 6; p-cresol; the same pattern also given by phenol, DL-tyrosine, ferrulic and coumaric acids), o-dihydroxyphenolase (lane 7; catechol, as well as 4-methylcatechol, DL-DOPA, caffeic, chlorogenic and procatechuic acids), and polyhydroxyphenol oxidase (lane 8; rutin, as well as gallic acid and quercetin), respectively. Unless otherwise indicated, 3 units of PPO activity (25 µg protein) were applied in each well. Unlike with the CBB stained gels, the SDS-treated PPO samples were not boiled to electrophoresis if substrate staining was used for enzyme detection in gels.


1
2
3
4
5
MW
6
7
8
92.5K
66.2K
45.0K
31.0K
21.5K
14.4K

tions regarding monophenol oxidase activity have been reported by Matheis and Belitz (1977), who observed that only the high MW forms of the potato tuber enzyme are capable of oxidizing monophenols. The MW estimates from SDS-PAGE gels of the fast moving components (32 and 40K) were in agreement with those obtained by gel filtration. Chromatography on Ultrogel AcA-44 revealed the presence of two eluting species (Fig. 3.5) with apparent MW of 42 K (catecholase activity) and 34 K (catecholase and cresolase activity). Approximately 90% of the initial PPO activity applied to the column was recovered with negligible increase in its specific activity. Moreover, an attempt to further fractionate the enzyme by gel filtration was not successful due to substantial peak overlap. A wide range of MW for PPO has been reported, thus indicating the multiplicity of this enzyme. The values obtained for the artichoke PPO (MW 32-34; 40-42K) are in good agreement with the values of 30-40K most commonly cited in the literature for plant PPO (Mayer and Harel, 1979; Vámos-Vigyázó, 1981).

To provide further insights into the nature of artichoke PPO multiplicity (i.e., aggregation vs. true subunit heterogeneity), several covalent and noncovalent bond breaking agents (urea, SDS, 2-mercaptoethanol) and an acylating reagent (succinic anhydride) were used for treating the enzyme prior to electrophoresis. All treatments brought about partial loss of PPO activity. Treatment with SDS and

Figure 3.5 Ultrogel AcA 44 column chromatography of PPO.
Purified enzyme (2 ml, 150 PPO units, 1.2 mg protein) was applied to a column (1.6 x 90 cm). The column was eluted with 20 mM sodium phosphate buffer containing 0.15 M NaCl, pH 6.5 at a flow rate of 15 ml/h. Fractions of 4 ml each were collected.


ABSORBANCE AT 280 nm
PPO ACTIVITY, ABSORBANCE AT 415 nm
FRACTION NUMBER
p.cresol
catechol
0
1.0
2.0
10
20
30
40
50
60
70

urea require more attention, since in many reports on multiplicity of plant PPO, such treatments were found to activate the enzyme (Robb et al. 1965; Swain et al., 1966; Kahn, 1977; Hutcheson et al. 1980). However, contrary to these studies and in agreement with the findings of Jolley et al. (1969), both these agents caused inhibition of the artichoke PPO. For example, enzyme incubation with various concentrations of either SDS or urea for 300 minutes caused inactivation ranging between 25 to 50%, and 25 to 35%, respectively (Fig. 3.6). Moreover, incubation with 8M urea for 5 hr led to changes in the relative electrophoretic mobility of the various enzyme forms (Fig. 3.7). The apparent multimeric nature of the 120 and 86 K species can be deduced from the fact that 8 M urea treatment (5 hr) prior to electrophoresis caused disappearance of these bands as assessed after staining the gels for mono-, di-, or polyphenol oxidase activity. In this regard, the possibility that urea treatment inhibited only the high MW species was ruled out, since both substrate and protein staining showed similar electrophoretic trends i.e. disappearance of low mobility bands upon urea treatment (Fig. 3.8). In addition, chemical modification of the enzyme with succinic anhydride (Fig. 3.9, lanes 2-4), on the contrary to the treatment with 2-mercaptoethanol (Fig. 3.9, lanes 5-7), led to similar electrophoretic results (i.e. disappearance of the high MW species). Although no further attempt was made to eluci-

Figure 3.6 Effect of SDS and urea on PPO activity.
a. effect of SDS at concentration of: 0.1% (△); 2% (○); 5% (●).
b. effect of urea at concentration of: 2 M (△); 4 M (○); 8 M (●).

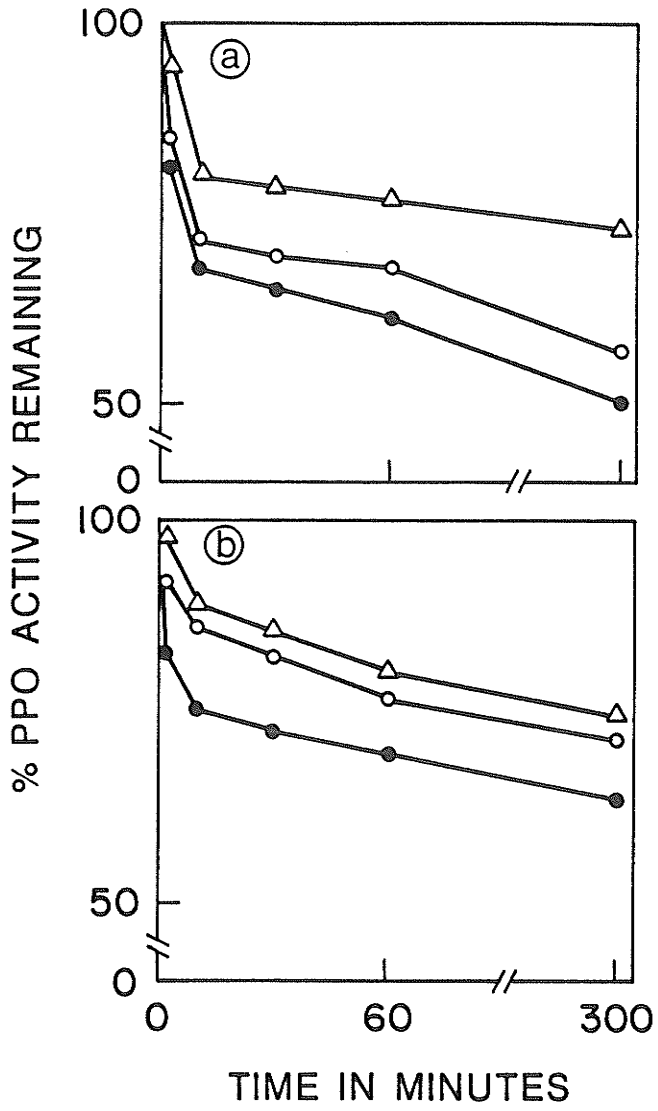

100
a
50
0
100
b
50
0
0
60
300
% PPO ACTIVITY REMAINING
TIME IN MINUTES

Figure 3.7 Gradient (6-12%) sodium dodecyl sulfate-polyacrylamide gel electrophoretic patterns of PPO following urea treatment.
1 and 2) PPO (P_1) stained with catechol before, and after urea treatment. 3 and 4) PPO stained with p-cresol before, and after urea treatment. 5 and 6) PPO stained with rutin before and after urea treatment. 7) Low SDS-MW standards, BIO-RAD (4 μg protein). Three units of PPO activity were applied in each well. The enzyme was treated with 8 M urea for 5 hr prior to electrophoresis (lanes 2, 4, 6).

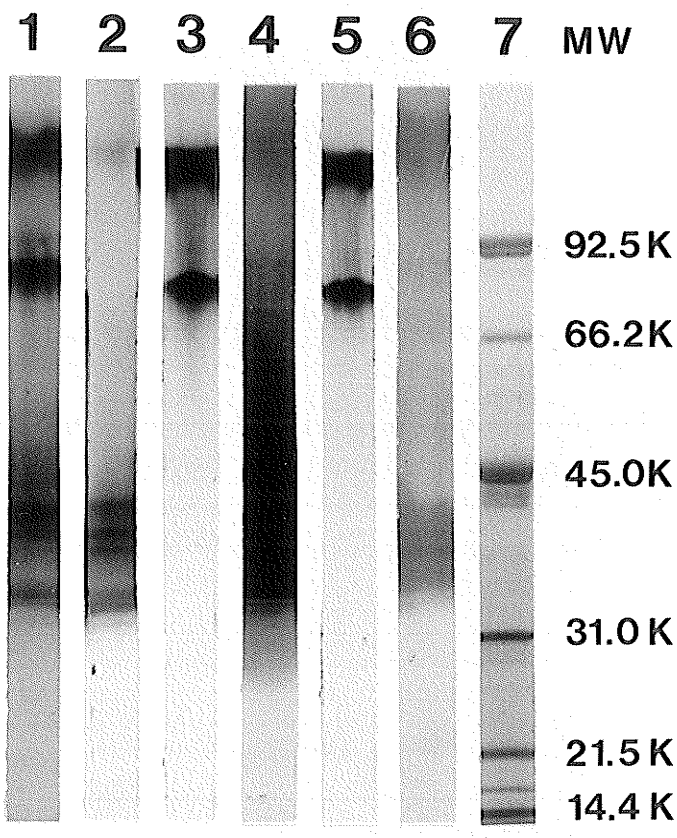

1
2
3
4
5
6
7
MW
92.5K
66.2K
45.0K
31.0K
21.5K
14.4K

Figure 3.8 Gradient (6-12%) polyacrylamide gel electrophoretic patterns of PPO following urea treatment.
a. P_1 fraction stained with p-cresol (3 units of catecholase activity were applied in each well).
b. P_1 fraction stained with CBB for protein (40 µg of protein).
1) Enzyme before urea treatment. 2) Enzyme after urea treatment.

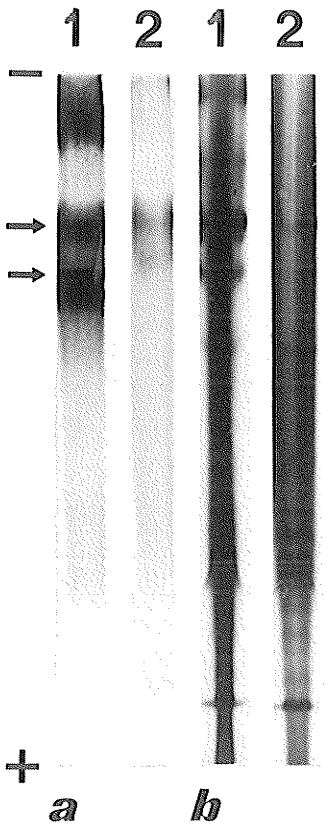
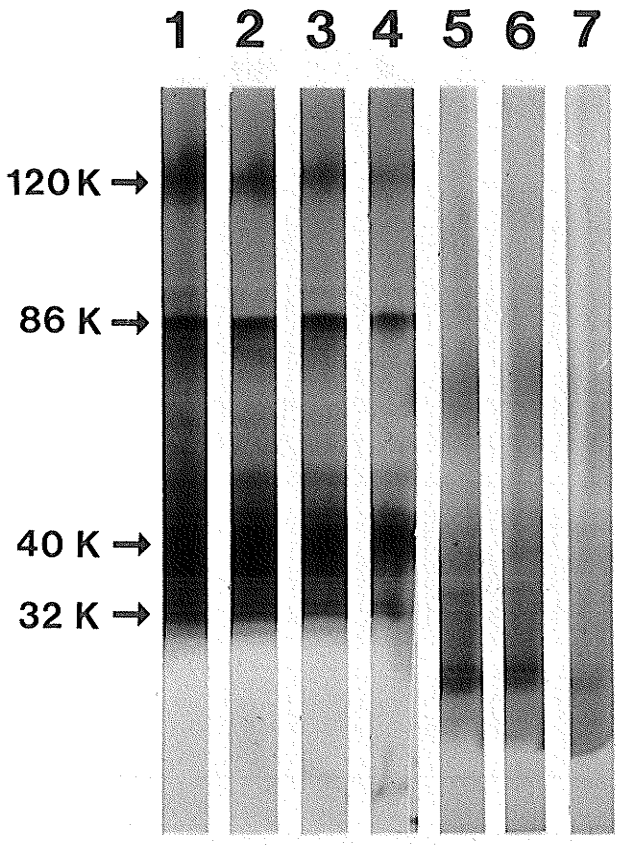

1
2
1
2
−
+
a
b

Figure 3.9 Gradient (6-12%) sodium dodecyl-sulfate polyacrylamide gel electrophoretic patterns of PPO following 2-mercaptoethanol, and succinic anhydride treatment.
1) PPO before treatment (control sample). 2, 3 and 4) PPO after treatment with 2-mercaptoethanol (2-ME) prior to electrophoresis (3 units of PPO were treated with 0.5, 1.0, and 2.0% of 2-ME, and applied in each well, respectively). 5, 6 and 7) PPO after succinylation (3 units of PPO were treated with 10, 20, and 30 mg of succinic anhydride per ml of enzyme, and applied in each well, respectively). Gels were stained with catechol.

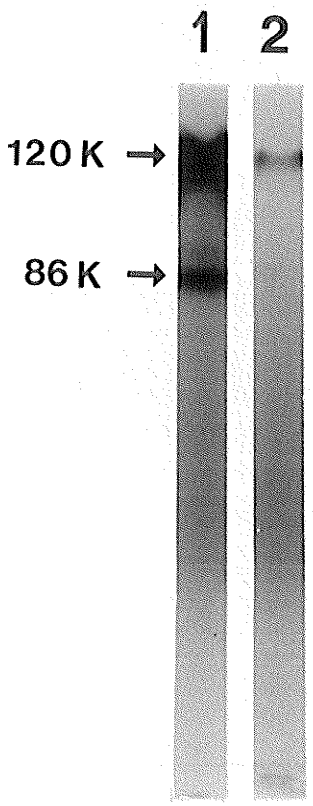

1 2 3 4 5 6 7
120 K
86 K
40 K
32 K

date the above aggregation - disaggregation mechanism, an interesting approach for this task would be that of intermolecular cross-linking with a bifunctional reagent that is labile under certain conditions. If after cross-linking and exposure to urea the high MW species remained intact, this would favor the aggregation theory. Furthermore, regeneration of the low MW species after removal of the cross-linkages would be the ultimate proof.

Interconversion between the PPO forms, presumably due to association-dissociation phenomena, would be certainly concentration dependent. This could explain the elution of only two species through the gel filtration column where the system is more dilute. Furthermore, when both 34 and 42 K species were collected after gel filtration, concentrated and analyzed by SDS-PAGE for cresolase and catecholase activities two additional bands corresponding to 86 and 120 K were found. This phenomenon was more pronounced with the 34 K fraction (Fig. 3.10). Overall, these data suggest that the 86 and 120 K species are most probably multimeric aggregates resulting from association of the low MW species. It is also noteworthy to point out that such interconversion processes between single and multiple forms of PPO enzymes have been frequently reported in the literature (Vámos-Vigyázó, 1981).

To further characterize the artichoke PPO system, analytical isoelectrofucusing of the P_1 fraction was per-

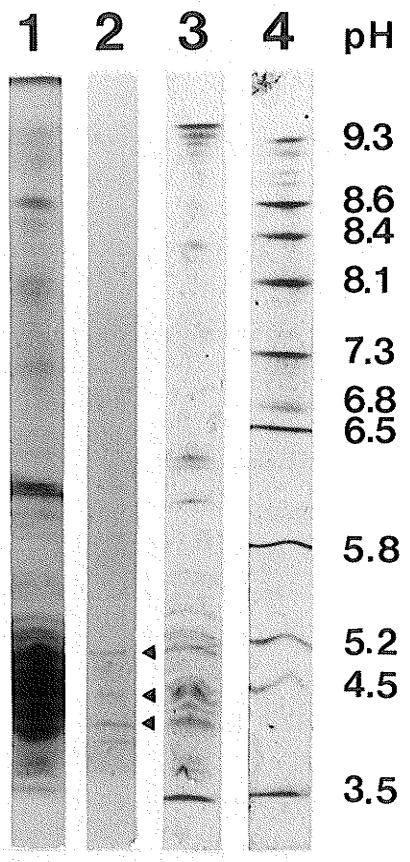
Figure 3.10 Gradient (6-12%) sodium dodecyl sulfate-polyacrylamide gel electrophoretic patterns of 34 K (1) and 42 K (2) Ultrogel AcA-44 chromatographic fractions.
Three units of catecholase activity were applied in each well, and stained with p-cresol.


1
2
120 K
86 K

formed. The isoelectric points of the enzyme when stained for catecholase activity are shown in Fig. 3.11. The isoelectric point of the predominant form was in the region of pH 4.5. Four minor species with isoelectric points (pIs) of pH 8.3, 7.7, 7.0 and 6.2 were also detected. The pI values of various plant PPO systems vary within the range of 4.0 to 10.0 (Vámos-Vigyázó, 1981). Values of 4.0 to 4.7 have been assigned for grape chloroplast (Dubernet and Ribéreau-Gayón, 1974), potato (Matheis and Belitz, 1975; Thomas et al., 1978) and mushroom PPO (Robb and Gutteridge, 1981).

Carbohydrate staining on SDS-PAGE and IEF gels of artichoke PPO was carried out to assess whether this enzyme is a glycoprotein. Both 32 and 40 K subunits (SDS-PAGE gels; Fig. 3.4, lane 3) as well as the pI 4.5 material (Fig. 3.8, lane 2) were stained with PAS reagent, as indicated by the arrows. Furthermore, carbohydrate analysis of the isolated subunits after gel filtration, using the orcinol-sulfuric acid method indicated that the 34 and 42 K subunits contained 18% and 19% carbohydrates, respectively, on a protein basis. Several authors have reported on the presence of carbohydrates in PPO preparations from various plant sources (Stelzing et al., 1972; Flurkey, 1985). Robb et al. (1965) have shown that Vicia faba PPO contained 3 to 4% carbohydrate, while Akazawa and Hara-Nishimura (1985) reported 45% of carbohydrate for sycamore PPO.

Figure 3.11 Analytical isoelectric focusing patterns of PPO.
1) PPO stained with catechol (0.6 units, 5 µg of protein/well). 2) PPO stained with PAS for glycoproteins (10 µg protein). 3) PPO stained with Coomassie Brilliant Blue R-250 for protein (10 µg protein). 4) Broad pI calibration kit, Pharmacia: trypsinogen (pI-9.3), lentil lectin-basic band (8.6), lentil lectin-middle band (8.4), lentil lectin-acidic band (8.1), myoglobin-basic band (7.3), myoglobin-acidic band (6.8), human carbonic anhydrase B (6.5), bovine carbonic anhydrase B (5.8), β-lactoglobulin A (5.2), soybean trypsin inhibitor (4.5), amylogluco-sidase (3.5).


1
2
3
4
pH
9.3
8.6
8.4
8.1
7.3
6.8
6.5
5.8
5.2
4.5
3.5

3.3.2 Characteristics of the PPO acidic fraction

3.3.2.1 pH optima

The pH optima for the activity of the acidic PPO fraction occurred at pH 6.0 for all three substrates (4-methylcatechol, catechol, and chlorogenic acid) examined (Fig. 3.12). This value coincides with that of the crude PPO extract from artichoke tubers, as well as with that of P_1 fraction. Similar pH optima for different phenolic substrates being oxidized by a particular PPO preparation have been reported in the literature (Abukharma and Woolhouse, 1965; Sharma and Ali, 1980).

3.3.2.2 Amino acid composition

The amino acid analysis data of the acidic PPO fraction are given in Table 3.2, while a chromatographic elution profile is depicted in Appendix I. The fraction contained high levels of glycine followed by alanine. The content of sulphur amino acids was about 3.0%. A comparative analysis of our data with those reported for potato (Balasingam and Ferdinand, 1970) and wheat (Interesse et al., 1983) PPO, indicated that there is a similarity in composition for all three enzymes as related to their contents in hydrophobic and sulphur amino acids. On the other hand, the artichoke PPO is distinguished from the other two enzymes by the greater residue content of glycine and alanine and the lower content of basic (lysine, argi-

Figure 3.12 Effect of pH on the activity of PPO using 4-methylcatechol (△), catechol (●), and chlorogenic acid (○) as substrates.

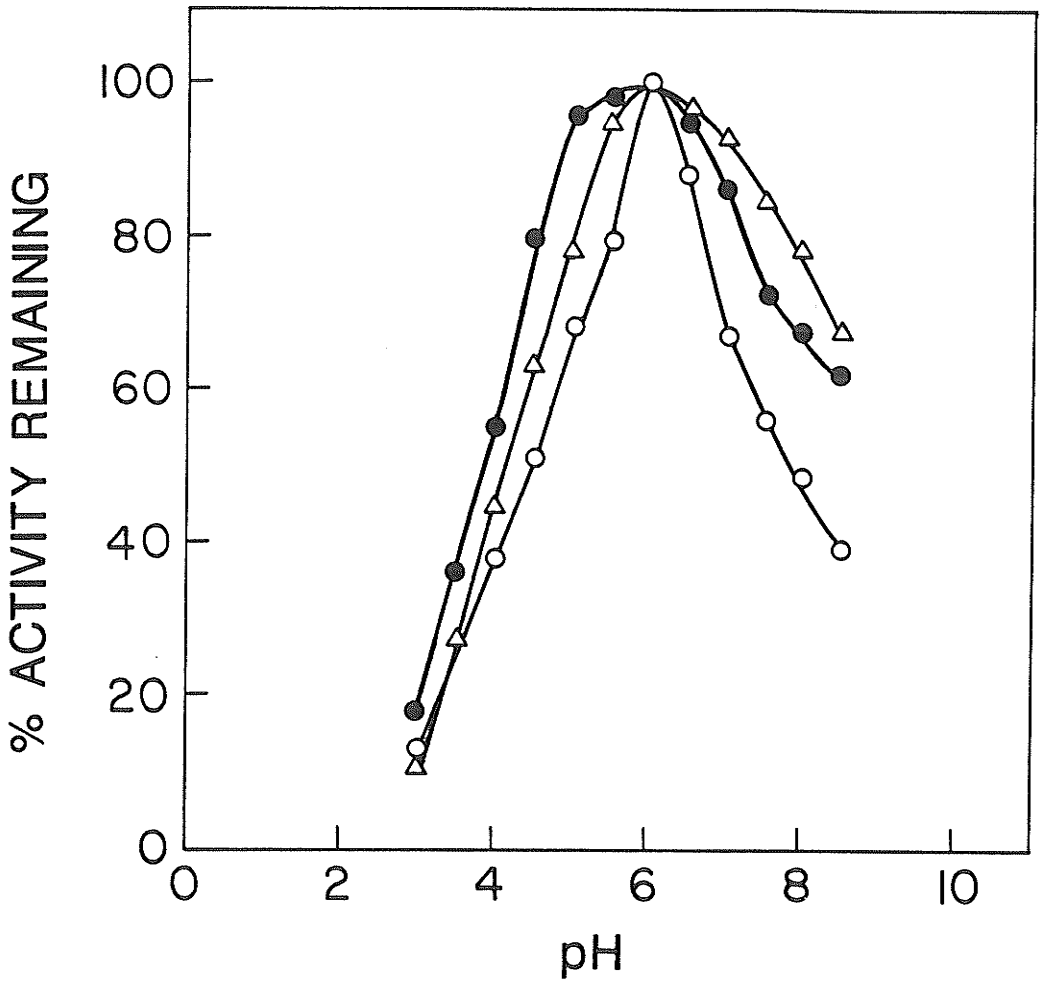

% ACTIVITY REMAINING
100
80
60
40
20
0
0
2
4
6
8
10
pH

Table 3.2 Amino acid composition of the PPO acidic fraction[a].

Amino Acid	Average value[b] (mole %)
Alanine	11.0 (±0.2)
Arginine	2.2 (±0.0)
Aspartic acid[c]	7.1 (±0.0)
Cysteine	1.7 (±0.1)
Glutamic acid[d]	10.2 (±0.1)
Glycine	12.6 (±0.2)
Histidine	0.5 (±0.0)
Isoleucine	4.3 (±0.0)
Leucine	8.4 (±0.0)
Lysine	4.8 (±1.3)
Methionine	1.2 (±0.0)
Phenylalanine	4.4 (±0.1)
Proline	6.0 (±0.1)
Serine	8.4 (±0.1)
Threonine	7.5 (±0.1)
Tyrosine	2.5 (±0.1)
Valine	7.2 (±0.1)

a- mole percent of the total amino acids, tryptophan not determined; b- means from two replications are given in the table, standard deviations are in parentheses; c- includes asparagine; d- includes glutamine.

nine, histidine) amino acids. These compositional differences do account for the relatively low isoelectric point (pI 4.5) of the artichoke enzyme; wheat PPO has been reported to exhibit a pI of 9.6 (Interesse et al., 1983). It is of interest further to note that the content of part of the hydrophobic residues (proline, valine, isoleucine, leucine, phenylalanine) of the artichoke PPO (30.3%) lies at the suggested crossover point (30.0%) between single chain (nonassociating) and multichain (associating) proteins (Jolley et al., 1969). These data could thus account, at least in part, for the tendency of this enzyme to form multimeric aggregates.

3.3.2.3 PPO kinetics

As was reported previously, the artichoke PPO is primarily an o-diphenol oxidase showing highest activity towards chlorogenic acid, 4-methylcatechol and catechol. In this respect, classical kinetic studies were performed on the PPO acidic fraction to examine the effect of concentration of the above o-dihydroxyphenols on the enzyme activity. Substrate concentration analyses via the Lineweaver - Burk plots exhibited apparent K_m values of 1.8, 3.6, and 4.0 mM for chlorogenic acid, 4-methylcatechol and catechol, respectively. In addition, the values of $V_{max}/2K_m$ (an expression recomended by some authors to indicate the efficiency of a given substrate for an enzyme preparation; Vámos-Vigyázó, 1981) for the above substrates were 66.7,

34.2 and 15.3 $\mu M\ O_2\ min^{-1}.mg^{-1}$ protein.mM^{-1} substrate, respectively. The K_m and substrate efficiency values are of similar order of magnitude with those reported previously for the oxidation of these substrates by the preparation obtained using IMAC chromatography (P_1 fraction).

The oxidation of chlorogenic acid by PPO requires more attention, since this substrate exhibited a marked inhibition at concentrations above 10 mM. Enzyme inhibition by excess of substrate was observed for the oxidation of chlorogenic acid (Abukharma and Woolhouse, 1966; Tremolieres and Bieth, 1984) as well as 4-methylcatechol (Interesee et. al., 1983) by PPO from different plant sources. This phenomenon is often rationalized by assuming that at high substrate concentrations, the enzyme - substrate complex formation could lead to the production of both active (ES) and inactive (ES_2) enzyme - substrate complexes (Koller and Neukom, 1969). For the latter, it has been suggested that a second substrate molecule bound ineffectivelly near the active center of the enzyme retards its overall catalytic ability (Trowbridge et al., 1963, Tremolieres and Bieth, 1984). The process is illustrated in the following scheme:

$$E \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + P$$

$$ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} ES_2$$

The reaction rate (v), as a function of substrate concentration (S), can be thus described by the following rate equation (Dixon and Webb, 1960):

$$v = \frac{Vmax}{1 + Km/(S) + 1/K'x\ (S)}$$

where V_{max} is the maximum reaction velocity, K_m is the Michaelis constant, and K' is the equilibrium constant of the reaction (dissociation constant of ES_2):

$$ES + S \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} ES_2$$

The above equation was used for the Lineweaver-Burk (Fig. 3.13a) and Dixon (Fig. 3.13b) plots. Estimates of K_m (1.8 mM) and K' (20.5 mM) constants were obtained by extrapolation of the corresponding linear portions of these plots.

The effect of sodium cyanide on the oxidation of 4-methylcatechol by PPO was examined using the Dixon plot (i.e. 1/V vs. inhibitor concentration). Kinetic analysis (Fig. 3.14) showed that the overall inhibitory action was of a noncompetitive type with an apparent K_i value of

Figure 3.13 Lineweaver-Burk (a) and Dixon (b) plots for PPO oxidation of chlorogenic acid: K_m, Michaelis constant; K', dissociation constant; S, chlorogenic acid concentration.

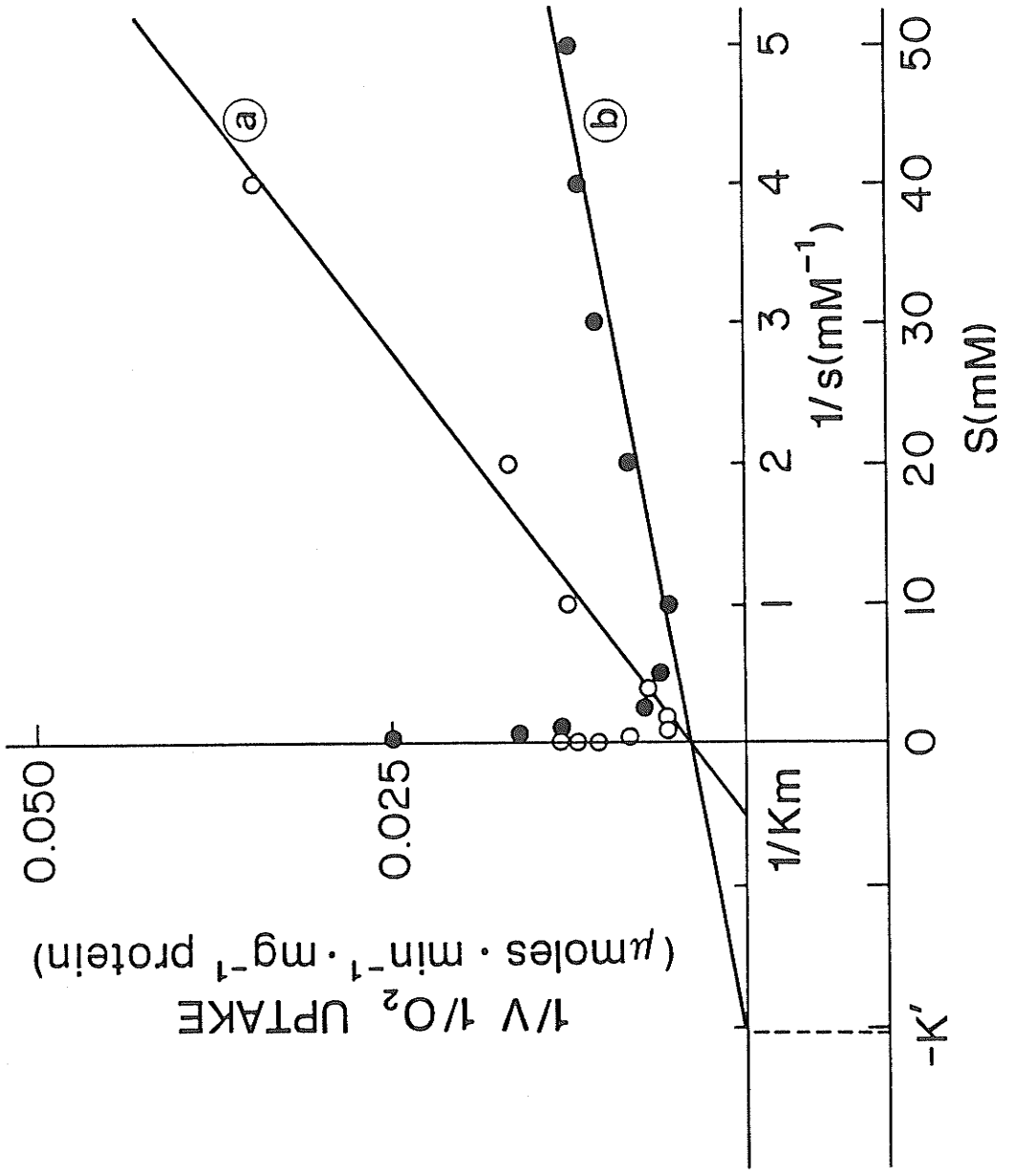
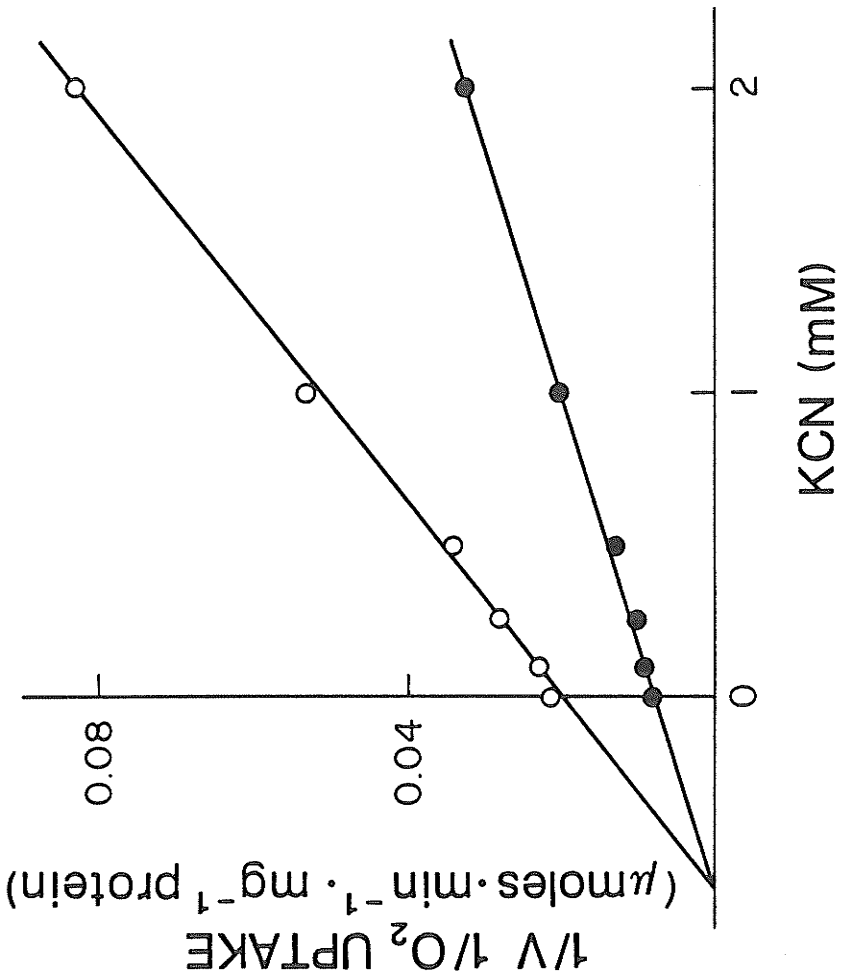

1/V 1/O2 UPTAKE
(μmoles · min⁻¹ · mg⁻¹ protein)
0.050
0.025
a
b
1/Km
-K'
0
10
20
30
40
50
1
2
3
4
5
1/s(mM⁻¹)
S(mM)

Figure 3.14 Dixon plot of potassium cyanide inhibition of PPO catalyzed oxidation of 1 mM (○), and 5 mM (●) 4-methylcatechol.


1/V 1/O2 UPTAKE
(μmoles·min⁻¹· mg⁻¹ protein)
0.08
0.04
0
1
2
KCN (mM)

approximately 0.6 mM. The potent inhibition by KCN has been also reported for PPO oxidation of chlorogenic acid (Alberghina, 1964; Hasegawa and Maier, 1980) and catechol (Halim and Montgomery, 1978; Anosike and Ayaebene, 1981; Roudsari et al., 1981). Noncompetitive inhibition toward phenolic substrates by cyanide is considered to originate from interaction of this inhibitor with the PPO copper (Mayer and Harel, 1979).

3.3.2.4 Heat inactivation

The results of thermal inactivation of artichoke PPO (pH 6.0) at several temperatures are presented in Fig. 3.15. The enzyme showed a relatively high heat stability with half - lives of 48, 26, 18, 12, and 6 minutes at 60, 65, 70, 75 and 80^{o} C, respectively. The rate of heat inactivation was greater with increasing temperature and followed first order kinetics. The same kinetic order has been also shown for PPO from several plant sources (Chan and Yang, 1971; Halim and Montgomery, 1978; McCord and Kilara, 1983).

The corresponding reaction rate constants at various temperatures were obtained by regression analysis of the activity data (Fig. 3.15). An apparent activation energy (Ea) of 26.2 kcal/mol for the heat inactivation of PPO was estimated from the Arrhenius plot of Fig. 3.16. Although, this value was about half that reported for the crude enzyme, the artichoke PPO should be still considered as a

Figure 3.15 Heat inactivation of PPO.

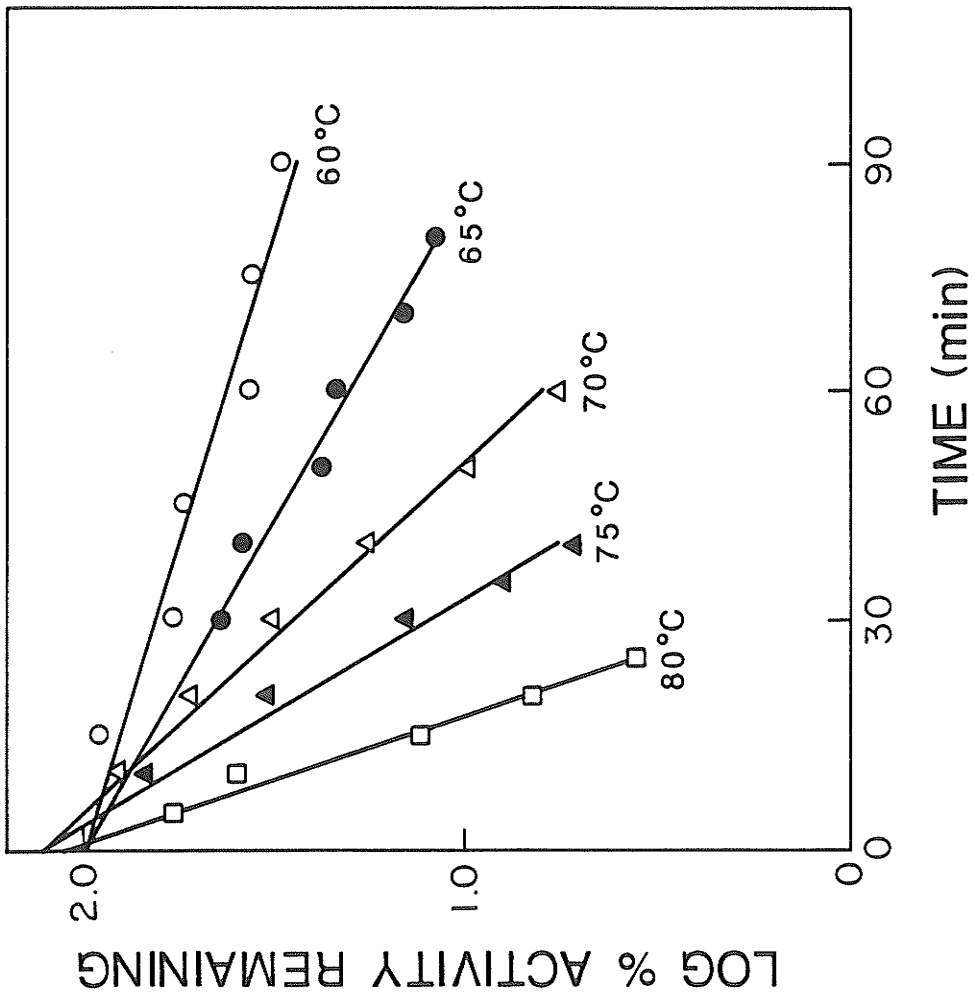
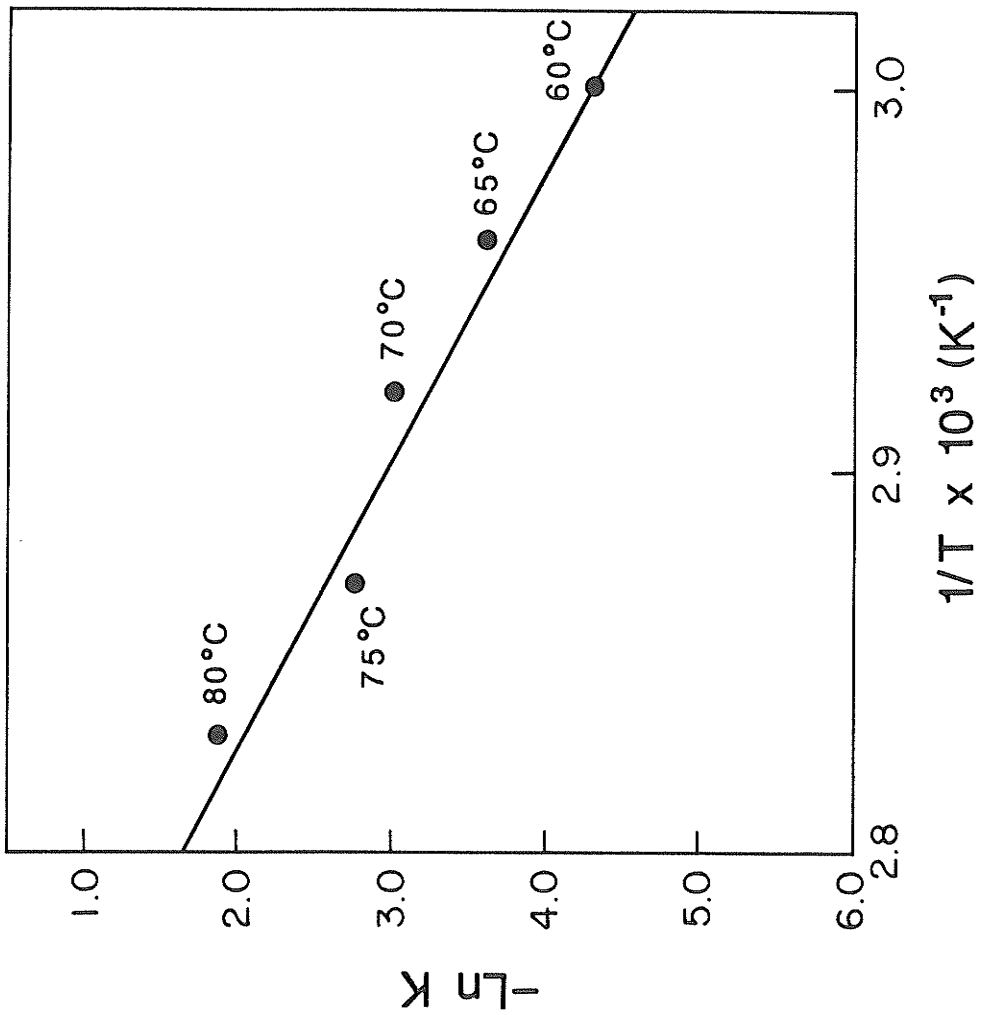

LOG % ACTIVITY REMAINING
2.0
1.0
0
0
30
60
90
TIME (min)
60°C
65°C
70°C
75°C
80°C

Figure 3.16 Arrhenius plot for the thermal inactivation of PPO.


-Ln K
1.0
2.0
3.0
4.0
5.0
6.0
2.8
2.9
3.0
1/T x 10³ (K⁻¹)
80°C
75°C
70°C
65°C
60°C

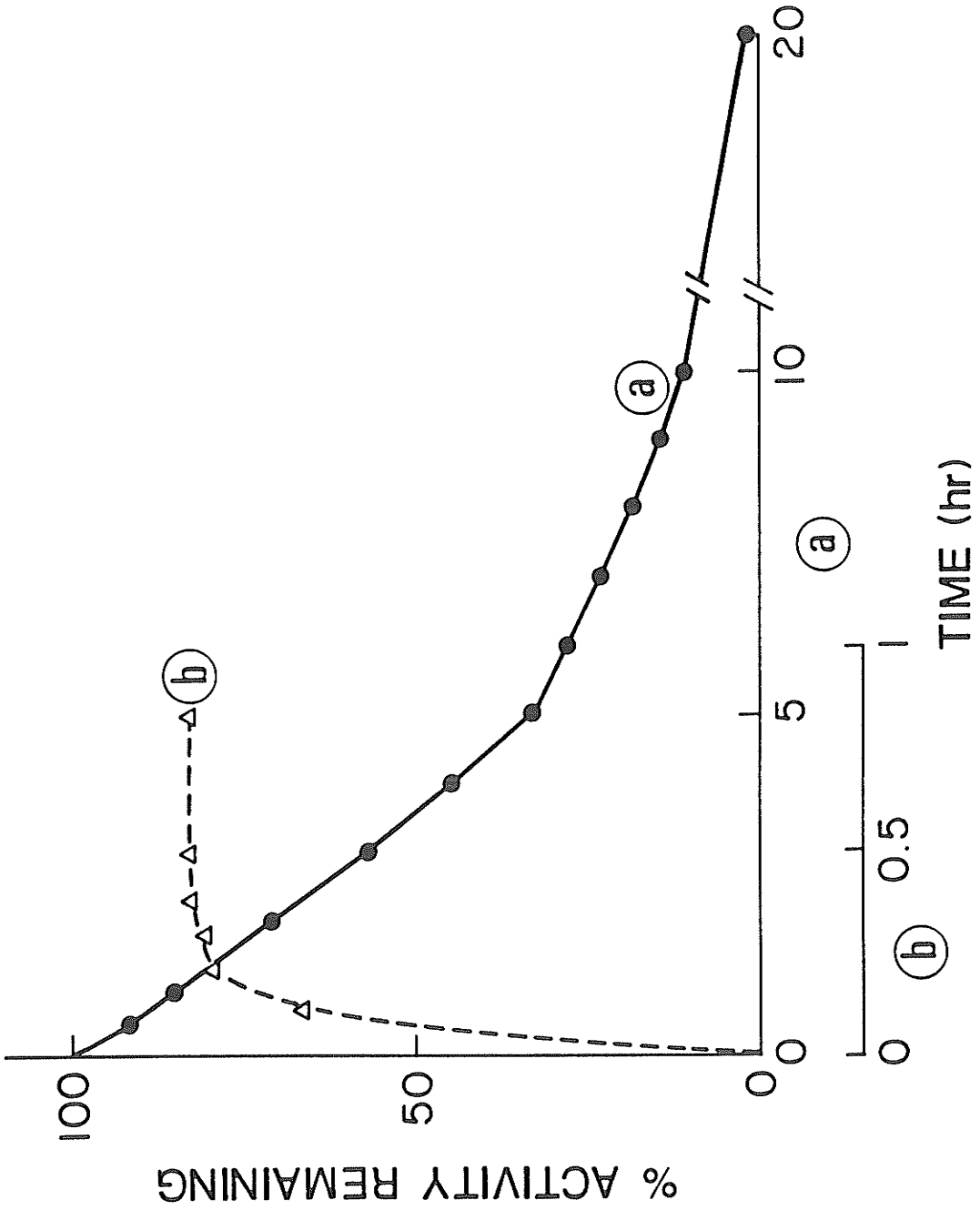
relatively thermostable enzyme. In this respect, Park and Luh (1985) determined that the Ea for two PPO isoenzymes isolated and purified from kiwi fruit were 4.0 and 7.0 kcal/mol, respectively.

3.3.2.5 Metallo-enzyme properties of PPO

When native PPO was subjected to dialysis against potassium cyanide, changes in enzymatic activity were observed over the time course of the experiment (Fig. 3.17a). During the first phase of dialysis (5 hr) the enzyme activity decreased relatively fast, up to approximately 30%. A constant decrease at a much slower rate was seen for the next 15 hr of the process and the preparation finally attained a limiting activity value close to 1%. This last value was persistent during the course of dialysis for an additional period of 8 hr, and was in good agreement with the data reported for apoforms of Neurospora sp. tyrosinase (Phiffner and Lerch, 1981; Beltramini and Lerch, 1983). Reconstitution of PPO from its apoform and cupric ions is shown in Fig. 3.17b. The reactivation process was very rapid and approximately 84% of the initial activity was restored during the first 30 minutes of dialysis.

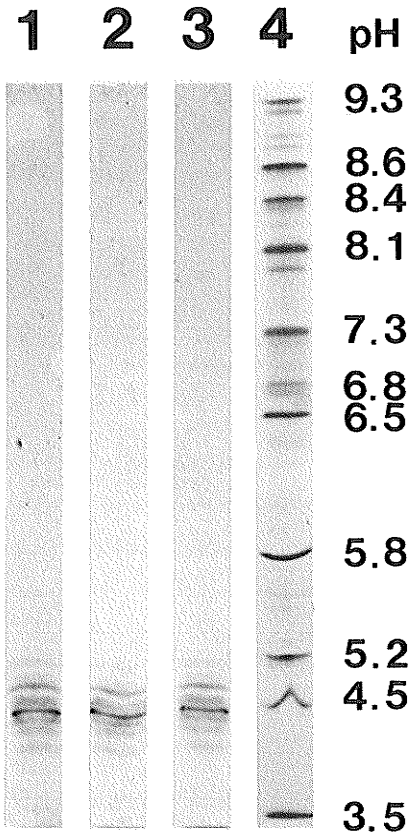
The above results, along with the data obtained from KCN and DIECA inhibition imply that the artichoke PPO is a copper - enzyme, i.e. a true polyphenol oxidase, as has been also shown for PPO preparations from several plant sources (Vámos-Vigyázó, 1981).

Figure 3.17 Time course of deactivation of PPO (a), and restoration of its enzymatic activity (b).


% ACTIVITY REMAINING
100
50
0
0
5
10
20
TIME (hr)
0
0.5
1
a
b
a
b

To assess whether removal and re-addition of copper ions from PPO, by KCN treatment, affected the pI value of the enzyme, holo, apo, and reconstituted forms were analyzed by isoelectric focusing (Fig. 3.18). In all three cases the enzymatic proteins displayed essentially the same electrophoretic pattern, which suggests that the surface charge properties of PPO are not significantly affected by the bound metal ions.

Figure 3.18 Analytical isoelectric focusing of (1) holoform, (2) apoform, and (3) reconstituted PPO (10 μg of protein were applied in each well), (4) broad pI calibration kit (see Fig.3.11); all gels were stained with CBB.


1
2
3
4
pH
9.3
8.6
8.4
8.1
7.3
6.8
6.5
5.8
5.2
4.5
3.5

CONCLUSIONS AND RECOMMENDATIONS

An active and stable PPO system has been shown to occur in Jerusalem artichoke tubers (Helianthus tuberosus L.). The presence of PPO appears to be largely responsible for color formation during processing of this crop into fructose syrups. Preliminary results revealed that the specific activity level of crude PPO is approximately 13 times the level found in potatoes and 3 times that of sugar beets. The bulk of enzyme activity is located in subcellular fractions of tubers (outer peels). In addition, it was shown that the enzyme was inactivated on heating and that this effect was dependent upon time and temperature. However, under most common experimental conditions, total inactivation was not achieved. Application of chemical inhibitors to suppress enzymatic browning is restricted only to a few compounds since most of the inhibitors impart toxicity or off-flavor and odor. Among them, metabisulfite and cysteine, were the most promising in providing effective control of enzymatic activity and greatly reducing discoloration during processing of artichoke tubers. Furthermore, metabisulfite is preferable to cysteine for control of browning in the long stored artichoke extracts, because it provides a better stabilization effect at a lower concentration. Since pH has a synergistic effect on metabisulfite inhibition of PPO activity, lowering the pH to 5.0 is desirable during

processing of tubers. However, the use of acidic pH may cause fructose decomposition and product loss during processing of tubers. Thus, an optimization study is recommended to determine the pH optimum that can be used during fructose preparation. Moreover, a combination of treatments involving heat and chemical inhibitors should be considered. This could not only reduce problems associated with each particular treatment but also reduce processing costs.

Several chromatographic methods were evaluated to establish an optimal procedure for purification of PPO from artichoke tubers; among them immobilized copper affinity chromatography was the most efficient, yielding an enzyme preparation (P_1 fraction) with a high degree of purity. During the course of the purification, artichoke PPO was separated into four fractions of activity differing in affinity for immobilized copper. This may reflect differences in the distribution of amino acid residues, capable of forming coordination complexes with Cu^{2+}, on the surface of the enzyme molecules. In addition these fractions exhibited differences in relative electrophoretic mobilities based on electrophoretic analysis. Thus, future work should be directed to further characterize the components of these fractions and thus develop a complete understanding of the artichoke PPO molecular properties.

The purified PPO catalyzes primarily the oxidation of o-dihydroxyphenols, but it is also capable of hydroxylating

monohydroxyphenols. The enzyme had the highest affinity towards chlorogenic acid, followed by 4-methylcatechol, and catechol. It is possible that these phenolic compounds also exist as endogeneous phenolics in the artichoke tubers; further work is required to clarify this point. In addition, the enzyme exhibited the same pH optimum with all substrates examined. Inhibition studies carried out on a purified enzyme preparation confirmed that metabisulfite was the most effective inhibitor of artichoke PPO. Although the purified enzyme is less thermostable than its crude extract, thermal inactivation data suggested that the enzyme is relatively stable upon heat treatment. Kinetics of deactivation, upon copper removal, and reconstitution reactions demonstrated that artichoke PPO is a copper containing enzyme. The isoelectric points of PPO were within the range of 4.0 to 10.0. The enzyme was also found to contain appreciable amounts of associated carbohydrate material. Electrophoretic studies revealed the complex nature of this enzyme. Four distinct enzymatic forms were identified; two high molecular weight, presumably aggregates with a broad specificity toward mono- and polyphenols, and two low molecular weight subunits that exhibit activity for o-dihydroxyphenols. The presence of multiple forms, which may have resulted from association-dissociatiion phenomena of low molecular weight species, was shown to be concentration dependent. Moreover, urea treat-

ment prior to electrophoresis caused disappearance of the high MW species, thus supporting the aggregation hypothesis. However, it would be worthwhile to prove this hypothesis using intermolecular cross-linking, with a bifunctional cross-linking reagent that is labile under certain conditions. Furthermore, it would be interesting to separate all four enzymatic forms (e.g. using preparative electrophoresis) and study the effects of enzyme conentration, temperature, pH, ionic strentgh on the aggregation phenomena.

Finaly, since the isolated PPO was a soluble enzyme preparation it would be of interest to establish whether particulate-bound enzymatic forms are present in artichoke tubers and whether their molecular properties are similar to those of the soluble enzyme.

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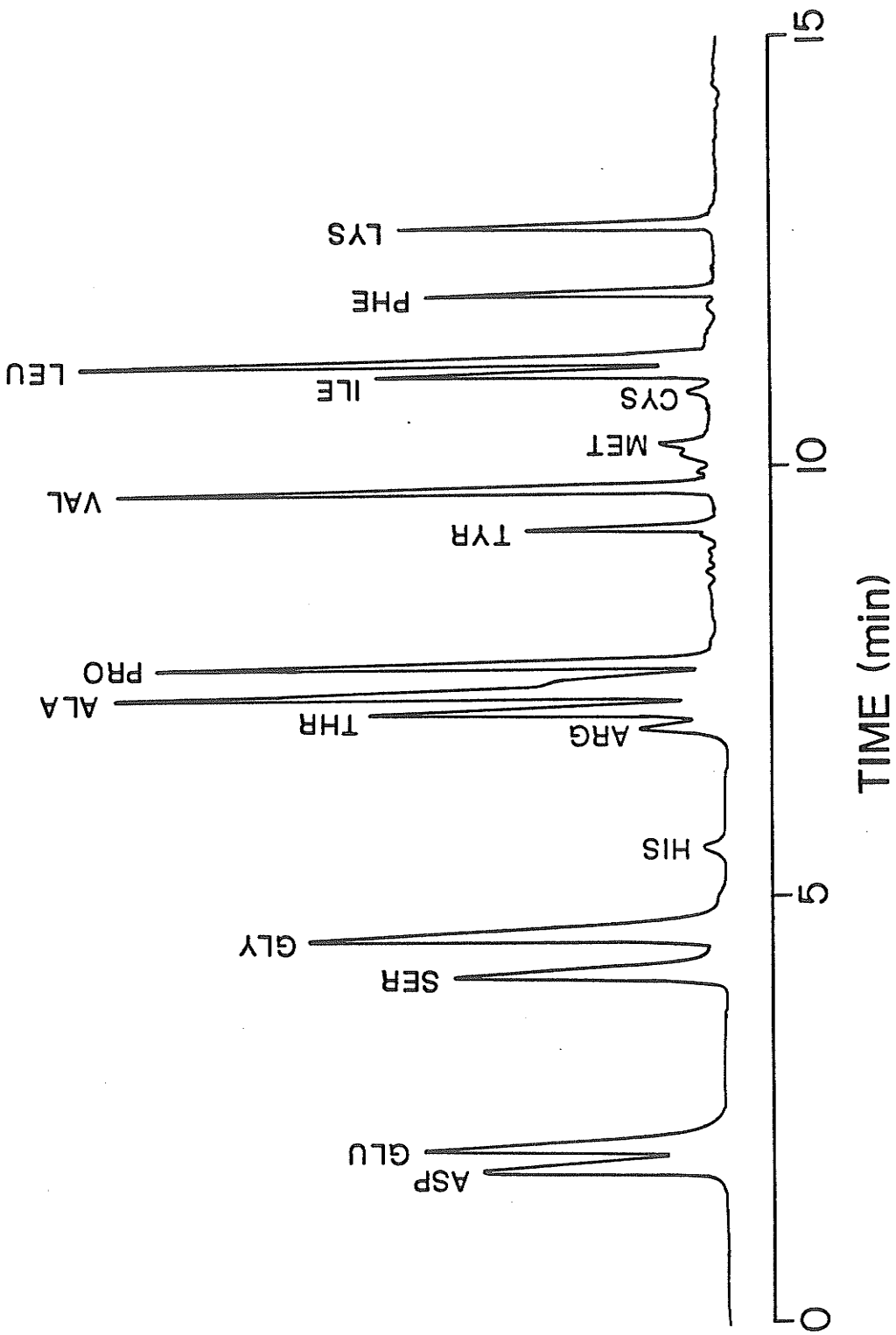
APPENDIX I HPLC elution profile of amino acids of the PPO acidc fraction.
Sample preparation and chromatographic conditions were as described in the text.


ABSORBANCE AT 254 nm
0
5
10
15
TIME (min)
ASP
GLU
SER
GLY
HIS
ARG
THR
ALA
PRO
TYR
VAL
MET
CYS
ILE
LEU
PHE
LYS