# Potential Purification and Some Properties of Monroe Apple Peel Polyphenol Oxidase

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PVP, AG2-X8, and PVP + AG2-X8 were used as adsorbents of phenolic compounds for Monroe apple peel polyphenol oxidase (PPO) extraction with Triton X-100. Excess Triton X-100 interference in fractionation with ammonium sulfate was minimized by butanol treatment. Two PPO active peaks were separated using phenyl-Sepharose CL-4B chromatography. A 161-fold purification of PPO was obtained from the major active peak. Apparent pH optima were 4.6 with 4-methylcatechol and 5.0 with catechol. The enzyme was most stable at pH 8.0 and least stable at acidic pH. Observed optimum temperature for the PPO–catechol reaction was 30 °C. The enzyme was heat stable up to 40 °C but rapidly inactivated above 50 °C. In substrate specificity, a maximum activity was shown with 4-methylcatechol, followed by chlorogenic acid and catechol. Thiourea proved to be the most effective inhibitor of the apple PPO.

## INTRODUCTION

Most browning in fruits is caused by enzymatic oxidation of natural phenolic compounds. Polyphenol oxidase (EC 1.14.18.1; PPO) is the main enzyme which catalyzes the oxidation of phenolic compounds to quinones, which then are polymerized to brown pigments (Lee, 1991). Biochemical characteristics of polyphenol oxidases from some apple cultivars have been investigated by several researchers. Harel et al. (1964, 1965) found four isoenzymes of polyphenol oxidases present in Grand Alexander applies tissues, three of them in the chloroplasts and at least one in the mitochondria. Stelzig et al. (1972) reported that PPO of Red Delicious apple peel partially purified by calcium phosphate gel adsorption exhibited two pH optima, 4.2 and 7.0. Janovitz-Klapp et al. (1989) from Delicious apples obtained 120-fold purified PPO by ammonium sulfate precipitation and phenyl-Sepharose CL-4B chromatography.

This paper describes an improved method of PPO extraction from Monroe apple peels using anion-exchange resin, dialysis, and butanol treatment. The enzyme was further purified by the use of ammonium sulfate fractionation and hydrophobic chromatography, and the semipurified enzyme was subjected to the kinetic study.

## MATERIALS AND METHODS

**Plant Materials.** Monroe apples used for this study were grown at the New York State Agricultural Experiment Station during the 1991 season. Apples were harvested at commercial maturity. Immediately after harvest the applies were peeled by hand. The peels were put directly into a Dewar flask containing liquid nitrogen and stored at −40 °C until extraction.

**Enzyme Extraction.** The extraction solution with suspended resin consisted of 240 mL of 0.1 M sodium phosphate buffer, pH 6.0, containing 2% (v/v) Triton X-100, 5 mM phenylmethanesulfonyl fluoride (PMFS), and 67.2 g of hydrated (45% moisture) AG2-X8 resin (200–400 mesh, Bio-Rad Laboratories, 1.0 g of dry resin/g of sample) as described by Wesche-Ebeling and Montgomery (1990). Other adsorbents investigated were poly(vinylpyrrolidone) (PVP, MW 40 000, Sigma) and a combination of AG2-X8 and PVP. The anion-exchange resin was pretreated as recommended by Cooper (1977) and equilibrated overnight in the sodium phosphate buffer containing 2% Triton and 5 mM PMFS. The frozen peels were ground with liquid nitrogen in a stainless steel Waring blender. Thirty grams of the powder was extracted with the extraction solution with suspended resin for 60 min at 4 °C while stirring gently with a magnetic stirrer. The extract was filtered through glass wool. The filtrate was centrifuged at 40000*g* for 15 min at 4 °C, and the supernatant was collected. After addition of 0.33 mL of aprotinin (Sigma)/100 mL of supernatant, the crude enzyme was kept at 4 °C. The activity remained stable over a period of several days.

**Purification Procedures.** All procedures were done at 4 °C, unless otherwise noted. The crude enzyme extract was dialyzed overnight against 1.0 mM phosphate buffer, pH 6.0. The dialysate was transferred to a separatory funnel and washed with cold (−20 °C) 1-butanol (1:1 v/v), and the layers were allowed to develop. The aqueous phase was redialyzed against the phosphate buffer for 24 h with three changes of buffer to remove residual butanol. The dialyzed enzyme solution was fractionated with solid ammonium sulfate (30–80% saturation), and the precipitate was collected by centrifugation at 12500*g* for 30 min. The precipitate was redissolved in 6.0 mL of 0.05 M sodium phosphate buffer pH 6.5 containing 1.0 M ammonium sulfate and dialyzed overnight against that buffer. After centrifugation at 40000*g* for 30 min, the total volume of supernatant was applied onto a phenyl-Sepharose CL-4B column (0.9 × 20 cm) previously equilibrated at 18 °C with an elution buffer (EB, 0.05 M sodium phosphate buffer, pH 6.5, containing 1.0 M ammonium sulfate and 1.0 M KCl). PPO was eluted from the column by 60 mL of EB at different concentrations (1.00, 0.80, 0.40, 0.20, and 0.05 EB) in a stepwise manner, followed by 50% ethylene glycol and water. The flow rate was 0.8 mL/min, and 4-mL fractions were collected. Fractions containing PPO activity were combined.

**Enzyme Assay and Protein Determination.** Polyphenol oxidase activity was assayed with catechol as the substrate according to a spectrophotometric procedure (Coseteng and Lee, 1987). The assay was performed using 0.2 mL of enzyme solution and 2.8 mL of 0.05 M catechol prepared in 0.1 M citrate–0.2 M sodium phosphate buffer, pH 5.0. The increase in absorbance at 420 nm at 24 °C was measured on a spectrophotometer (Hewlett-Packard, 8452A). One unit of enzyme activity was defined as the amount of the enzyme which caused a change in absorbance of 0.001/min.

Protein concentration was determined according to the dye binding method of Bradford (1976) with bovine serum albumin as a standard. To prevent interference from Triton X-100 during the protein assay, the samples were treated with Bio-Beads SM-2 (Bio-Rad Laboratories) according to the procedure of Holloway (1973). Moist Bio-Beads were added to the samples (0.6 g/mL) and were then stirred gently for 2 h at 4 °C.

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Table I. Summary of the Extraction and Purification of Monroe Apple Peel Polyphenol Oxidase

| purification step | vol, mL | act., units/mL | total act., units | protein, mg/mL | sp act., units/mg | yield, % |
|---|---|---|---|---|---|---|
| crude extract (AG2-X8 treated) | 230 | 540 | 124 200 | 0.154 | 3506 | 100 |
| dialyzed extract | 266 | 358 | 95 228 | 0.072 | 4972 | 77 |
| butanol extract | 245 | 439 | 107 555 | 0.073 | 6014 | 87 |
| butanol extract (dialyzed) | 360 | 297 | 106 920 | 0.045 | 6600 | 86 |
| 30–80% $(NH_4)_2SO_4$ | 6 | 10285 | 61 710 | 0.256 | 40176 | 50 |
| phenyl-Sepharose | | | | | | |
| major peak | 20 | 1128 | 22 560 | 0.002 | 564000 | 18 |
| small peak | 15 | 155 | 2 320 | <0.001 | | 2 |

**Effect of pH on PPO Activity and Stability.** Three kinds of buffer solutions used for this study were as follows: 0.1 M citric acid–0.2 M disodium phosphate, for pH 3–8; tris(hydroxymethyl)aminomethane, pH 8.5; and 0.2 M glycine–0.2 N NaOH, pH 9–10.

To determine the effect of pH on PPO activity, 0.2 mL of 0.5 M catechol or 0.5 M 4-methylcatechol solution (prepared in 0.1 M citrate–0.2 M sodium phosphate buffer, pH 5.0) was added to 2.7 mL of various buffer solutions followed by the addition of 0.1 mL of partially purified enzyme solution. Enzyme activity was measured at 420 nm using the spectrophotometric procedure as previously described.

To determine the effect of pH on PPO stability, 0.2 mL of the enzyme solution was incubated in 0.4 mL of various buffer solutions ranging from pH 2.5 to 10 for 30 min at 24 °C. Residual PPO activity was assayed by mixing 2.7 mL of 0.05 M catechol in 0.1 M citrate–0.2 M sodium phosphate buffer, pH 5.0, with 0.3 mL of the incubated PPO solution.

**Effect of Temperature on PPO Activity and Stability.** The optimum temperature of the PPO–catechol reaction was determined by adding 0.1 mL of the partially purified enzyme solution to 2.9 mL of 0.05 M catechol solution, pH 5.0, preequilibrated for 5 min at various temperatures ranging from 20 to 70 °C prior to the addition of the enzyme and measuring the rate of the reaction. In the thermal stability studies, the enzyme solution was incubated at various temperatures for 30 min and rapidly cooled in an ice bath for 5 min. The enzyme solution (0.1 mL) was then added to 2.9 mL of 0.05 M catechol solution, pH 5.0, and the residual enzyme activity was assayed.

**Substrate Specificity.** In the studies of the substrate specificity, the reaction system consisted of 0.1 mL of the enzyme solution and 2.9 mL of various substrate solutions prepared in 0.1 M citrate–0.2 M sodium phosphate buffer, pH 5.0. The increase in absorbance at the optimum wavelength for each substrate was measured.

For the determination of $K_m$, the concentrations of 4-methylcatechol were varied from 2 to 25 mM at pH 4.6. The reaction system was the same as above. Initial rates were measured as the increase in absorbance at 420 nm and at 24 °C. Relative $K_m$ value was calculated from the Lineweaver and Burk (1934) plot.

**Effect of Inhibitors.** To determine the effect of inhibitors on enzyme activity, 2.7 mL of 10 mM catechol in 0.1 M citrate–0.2 M sodium phosphate, pH 5.0, and 0.2 mL of inhibitor solution with different concentrations were mixed immediately before the addition of 0.1 mL of enzyme solution. Relative enzyme activity was determined from the slope of the reaction curve following any delay in change in absorbance at 420 nm due to the inhibitors.

## RESULTS AND DISCUSSION

**Extraction and Purification of PPO.** A summary of Monroe apple peel PPO extraction and purification is shown in Table I. A 161-fold purification of the sample PPO was achieved.

During the extraction of PPO, the main problem lies in the interference of endogenous phenolic compounds that react with the enzyme and cause its inactivation and molecular modification. In this experiment PVP, anion-exchange resin AG2-X8, and a combination of PVP and AG2-X8 (1:1) were used as adsorbents of phenolic compounds during the extraction of PPO as shown in Table II. When only PVP was used, the PPO extract exhibited a peak at 324 nm and became slightly brown overnight at 4 °C. This indicated that residual phenolic compounds remained in the extract. The 324-nm peak may suggest the presence of hydroxycinnamic acids in the extract, most likely chlorogenic acid (Sioud and Luh, 1966). Chlorogenic acid is the main substrate for the catalytic action of polyphenolase which leads to the browning of tissue when apples are cut (Hulme, 1958; Walker, 1962). Anderson and Sowers (1968) also reported that PVP is unable to bond chlorogenic acid efficiently. The absorbances at 324 nm of the extracts prepared with AG2-X8 or with the combination of PVP and AG2-X8 were much lower than that of PVP extract. The AG2-8X extract and the AG2-X8/PVP (1:1) extract showed no visible browning.

Table II. Comparison of Monroe Apple Peel PPO Extracts Prepared Using Different Adsorbents[a]

| adsorbent used[b] | PPO act., units/mL | $A_{324nm}$ | yield, % |
|---|---|---|---|
| PVP | 523 | >3 | 68 |
| AG2-X8 resin | 766 | 0.432 | 100 |
| PVP + AG2-X8 | 558 | 0.621 | 73 |

[a] All extracts were prepared by adding 2.5 g of sample, powdered under liquid nitrogen, to 20 mL of 0.1 M sodium phosphate buffer, pH 6.0, containing 2% (v/v) Triton X-100, 5 mM phenylmethanesulfonyl fluoride, and different adsorbents. See Materials and Methods for extraction procedure and activity assay of PPO. [b] All adsorbents were added at a level of 1 g of dry wt/g of sample.

When the PPO activities of the extracts are compared, the PVP extract showed 32% less enzyme activity than the AG2-X8 extract. However, the extract with a combination of AG2-X8 and PVP had almost the same level of activity as the PVP extract. These results indicated that AG2-X8 protected apple PPO from inactivation better than PVP or PVP combined with AG2-X8 during enzyme extraction. The anion-exchange resin (AG2-8X) was known to remove apple endogenous phenolic compounds efficiently during extraction due to its ability to complex with phenolic compounds through hydrophobic, ionic, and hydrogen-bonding mechanisms (Smith and Montgomery, 1985; Loomis, 1974). Hulme et al. (1964) found that mitochondrial preparations from apple peel in the absence of PVP contained highly active PPO, while in the presence of PVP the PPO activity was considerably reduced.

The nonionic detergent Triton X-100 has been successfully used for apple PPO extraction due to its disruption of chloroplast membranes, thus facilitating the release of PPO. An increase of enzyme activity by this detergent treatment has been reported (Harel et al., 1965; Stelzig et al., 1972). However, Triton X-100 also extracted the green pigments from the apple peels; thus, the enzyme extract had a light green color. The original attempt to purify PPO, released by the Triton X-100 treatment, with an ammonium sulfate fractionation was unsuccessful due to the interference of chlorophyll and excess Triton X-100, which could not be adequately removed by dialysis. When the Triton X-100 extract was dialyzed and then treated with 1-butanol, the green color moved into the butanol

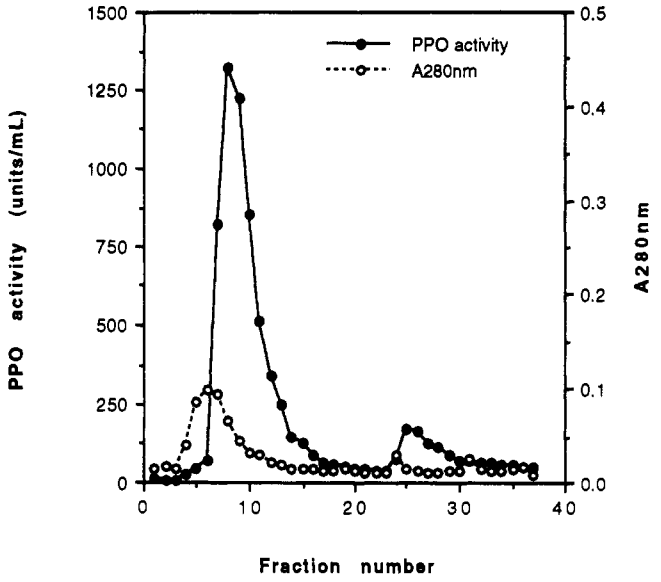


**Figure 1.** Phenyl-Sepharose CL-4B chromatography of Monroe apple peel PPO.

phase and the PPO remained in the aqueous phase. A 113% recovery of PPO activity was obtained in the 1-butanol extraction step. The resulting PPO was probably well solubilized (Stelzig et al., 1972). Perhaps solubilization of the enzyme and removal of phenols contributed to an increase in the total activity. To increase the recovery of enzyme precipitation by ammonium sulfate, the dialysate was concentrated by ultrafiltration and then fractionated by adding solid ammonium sulfate. A 6-fold purification with a recovery of 58% was obtained over the applied enzyme activity.

Phenyl-Sepharose CL-4B chromatography has been used for the purification of PPO from various fruits, such as peaches (Flurkey and Jen, 1980), pears (Wissemann and Montgomery, 1985), grapes (Wissemann and Lee, 1980), strawberries (Wesche-Ebeling and Montgomery, 1990), and apples (Janovitz-Klapp et al., 1989). The elution profile of Monroe apple PPO from the phenyl-Sepharose CL-4B column is presented in Figure 1. Two peaks with PPO activity were separated by the column chromatography. The major peak was eluted using full-strength elution buffer (EB) and the small peak at 0.8 EB. The recovery of PPO activity by chromatography was 40% over the applied PPO activity. The major PPO fractions were pooled and used for kinetic study.

**pH Optima and Stability.** The pH–activity profile for the oxidation of catechol and 4-methylcatechol by purified PPO from Monroe apple peel is shown in Figure 2. PPO activity has an observed optimum pH at 5.0 with catechol, followed by a small shoulder at pH 7.0. The pH–activity curve is characterized by a rapid decrease in activity between pH 5.0 and 5.5. The observed activity at pH 5.5 was only 20% of the activity at pH 5.0. With 4-methylcatechol as a substrate, the pH–activity exhibited a bell-shaped curve between pH 3.5 and 5.5, with an apparent optimum pH at 4.6, followed by a small shoulder at pH 6.5. Differences in pH optima with different substrates have been reported for PPO from strawberries (Wesche-Ebeling and Montgomery, 1990) and tea leaves (Gregory and Bendall, 1966). Aylward and Haisman (1969) reported that the optimum pH for maximum activity of PPO varies from about 4.0 to 7.0 depending upon the originating material, extraction methods, and substrates. Harel et al. (1965) reported that apple PPO has two pH optima, 5.1 from chloroplast PPO and 7.3 from mitochondria PPO, and also found that Triton X-100 is preferential for extracting PPO from the chloroplasts rather than from mitochondria. According to the pH optimum, the Monroe apple PPO purified in this study appeared to be, mainly, a chloroplast enzyme and contained a very small amount of mitochondria enzyme.

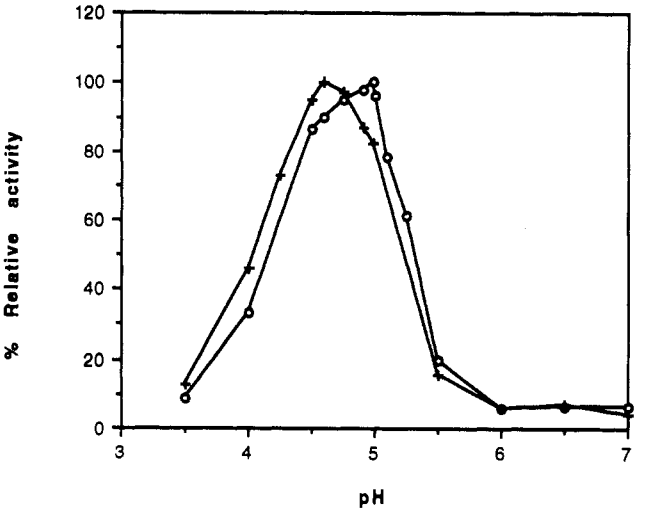


**Figure 2.** Observed pH optima for activity of Monroe apple peel PPO. Maximum activity with catechol (O) was 908 units/mL and with 4-methylcatechol (+) 1175 units/mL.

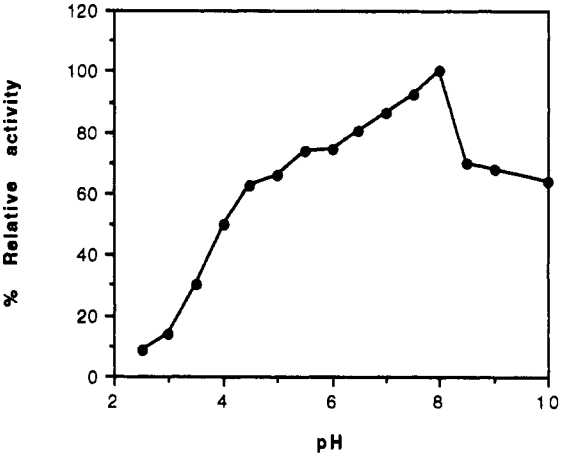


**Figure 3.** Apparent pH stability of Monroe apple peel PPO after incubation for 30 min at 24 °C with catechol as substrate.

The pH stability curve of PPO activity is shown in Figure 3. The apple enzyme was most stable at pH 8.0 and less stable at acidic pH. The pH stability of PPO increased from pH 2.5 to 8.0 and decreased above pH 8.0. This result was similar to that of grape PPO as reported by Yokotsuka et al. (1988).

**Optimum Temperature and Stability.** Profiles of variation in the activity and stability of apple PPO with temperature are shown in Figures 4 and 5. The optimum temperature for the PPO–catechol reaction was 30 °C. The enzyme was stable at lower temperatures but unstable at higher temperatures. For instance, when the temperature was increased from 20 to 40 °C, the relative activity of PPO decreased from 100 to 96%. The relative activity, however, decreased from 87 to 6% when the temperature was raised from 50 to 70 °C. It indicated that the enzyme was rapidly denatured at higher temperatures. In this respect, Monroe apple PPO was similar to the PPO extracted from grapes (Wissemann and Lee, 1980).

**Substrate Specificity.** PPO activities using different substrates are shown in Table III. Relative activities of

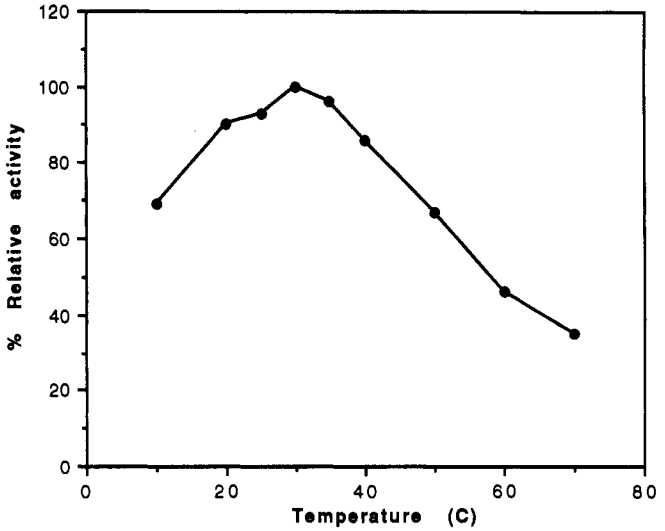


**Figure 4.** Temperature optimum of Monroe apple peel PPO.

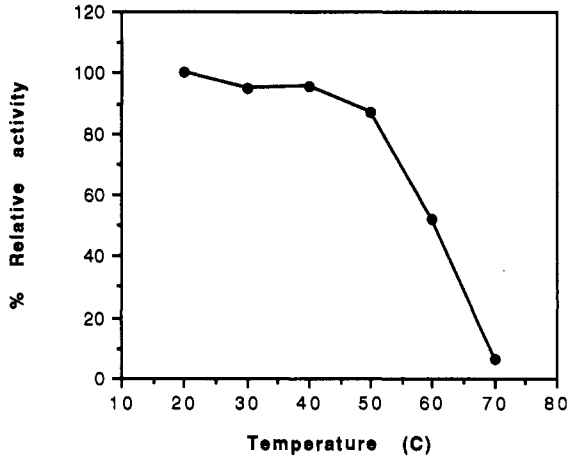


**Figure 5.** Temperature stability of Monroe apple peel PPO.

**Table III. Substrate Specificity of Monroe Apple Peel Polyphenol Oxidase**

| substrate | concn, mM | wavelength, nm | act., units/mL | rel act. compared to that of catechol, % |
|---|---|---|---|---|
| 4-methylcatechol | 10 | 400 | 665 | 181 |
| catechol | 10 | 400 | 367 | 100 |
| chlorogenic acid | 5 | 400 | 376 | 102 |
| *d*-catechin | 10 | 400 | 197 | 54 |
| pyrogallol | 10 | 334 | 138 | 38 |
| dopamine | 10 | 400 | 134 | 37 |
| DL-Dopa | 5 | 460 | 43 | 12 |
| tyrosine | 2.5 | 472 | 10 | 3 |

PPO, measured at each substrate's optimum wavelength, were calculated using catechol as the basis of comparison. The results indicated that the Monroe apple PPO was more active toward *o*-diphenols rather than monophenols. Maximum activity was detected toward 4-methylcatechol, followed by chlorogenic acid, catechol, and *d*-catechin. High activities using these substrates were reported for PPO from Delicious apple peel (Stelzig et al., 1972) and Grand Alexander apple (Harel et al., 1964).

The $K_m$ for 4-methylcatechol as calculated by the Lineweaver–Burk plot (data not shown) was 10.96 mM. This $K_m$ is almost twice as high as values reported for apples, perhaps because of source differences (Harel et al., 1964, 1965; Janovitz-Klapp et al., 1989).

**Inhibitors.** The effect of different inhibitors on the apple PPO is shown in Table IV. With catechol as the substrate a lag period was observed when the inhibitors L-cysteine, sodium diethyldithiocarbamate, potassium metabisulfite, 2-mercaptoethanol, thiourea, and ascorbic acid were used at a concentration of 1.5 mM. Residual PPO activity was determined from the slope of the reaction curve after the lag period.

**Table IV. Effect of Various Inhibitors on Monroe Apple Peel PPO Activity**

| inhibitor | concn, mM | inhibition, % |
|---|---|---|
| L-cysteine | 15 | 100 |
| | 1.5 | 46 |
| | 0.15 | 33 |
| sodium diethyldithiocarbamate | 15 | 100 |
| | 1.5 | 43 |
| | 0.15 | 23 |
| potassium metabisulfite | 15 | 100 |
| | 1.5 | 34 |
| | 0.15 | 4 |
| 2-mercaptoethanol | 15 | 100 |
| | 1.5 | 22 |
| | 0.15 | 0 |
| thiourea | 15 | 100 |
| | 1.5 | 67 |
| | 0.15 | 30 |
| ascorbic acid | 15 | 100 |
| | 1.5 | 37 |
| | 0.15 | 5 |
| sodium cyanide | 15 | 41 |
| | 1.5 | 22 |
| | 0.15 | 19 |

Of all inhibitors used in the study, thiourea was most effective for inhibition of observed apple PPO activity, followed by L-cysteine and sodium diethyldithiocarbamate. L-Cysteine can easily form complexes with quinones and has been shown to inhibit PPO by the formation of additional products formed by cysteine with *o*-quinones of 4-methylcatechol (Sanada et al., 1972; Janovitz-Klapp et al., 1990). This amino acid has been suggested to prevent enzymatic browning in processed apple products (Walker and Reddish, 1964). Janovitz-Klapp et al. (1990) reported that, using Red Delicious apple PPO, the higher the cysteine concentration, the longer the lag period and the lower the rate following the lag period. Sodium diethyldithiocarbamate and thiourea derivatives inhibit PPO activity due to their action on the copper in the enzyme (Mayer and Harel, 1978). Since the results of apple PPO activity measured by spectrophotometry for color development and polarography for oxygen uptake are varied due to the effects of the secondary reaction products formed from *o*-quiniones and inhibitors (Janovitz-Klapp et al., 1990), we plan to compare the inhibitory effects by polarography.

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