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Partial purification and characterization of polyphenoloxidase from peppermint (*Mentha piperita*)

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Abstract

Polyphenoloxidase (PPO) of peppermint leaves (*Mentha piperita*) was isolated by $(NH_4)_2SO_4$ precipitation and dialysis. Its pH and temperature optima were 7.0 and 30°C, respectively. On heat-inactivation, half of the activity was lost after 6.5 and 1.5 min of treatment at 70 and 80°C, respectively. Sucrose, $(NH_4)_2SO_4$, NaCl and KCl appeared to be protective agents of peppermint PPO against thermal denaturation. Km of this enzyme ranged from 6.25×10^{-3} M with catechol to 9.00×10^{-3} M with L-dopa. The I_{50} values of inhibitors studied on PPO were determined by means of activity percentage (I) diagrams. Values were 1.4×10^{-4} M, 1.7×10^{-4} M, 9.7×10^{-5} M, 2.45×10^{-4} M, 2.16×10^{-1} M, 1.83×10^{-5} M, 6.5×10^{-5} M, 1.4×10^{-2} M, 7.5×10^{-5} M, for potassium cyanide, glutathione, ascorbic acid, thiourea, sodium azide, sodium metabisulfite, dithioerythritol, β-mercaptoethanol and sodium diethyl dithiocarbamate respectively. Therefore, sodium metabisulfite was the most effective inhibitor.

Keywords: Polyphenoloxidase; Peppermint (*Mentha piperita*); Characterization; Inhibitors

1. Introduction

Polyphenol oxidase (PPO; monophenol, dihydroxy-L-phenylalanine oxygen oxidoreductase, E.C. 1.14.18.1) is a copper protein widely distributed on the phylogenic scale and responsible for the undesired browning reactions during handling, storage and processing of damaged tissues of fresh fruits and vegetables, as well as some animal products (Mayer 1987; Mayer & Harel, 1991). PPO catalyzes two types of oxidative reactions: hydroxylation of monophenols to *o*-diphenols (cresolase activity) and oxidation to *o*-quinones (catecholase activity).

PPO characteristics have been widely studied in various plants such as grapes (Nakamura, Amano, & Kagami 1983; Yokotsuka, Makino, & Singleton, 1988), yam tubers (Anosike & Ayaebene, 1981), banana (Galeazzi, Sgabieri, & Constantinides 1981), plums (Siddiq, Sinha, & Cash, 1992), potato (Batistuti & Lourenço, 1985), tea (Coggon, Moss, & Sanderson, 1973), papaya (Cano, Lobo, Ancos, & Galeazzi, 1996), chickpea (Singh & Jambunathan, 1981) and peaches (Wong, Luh, & Whitaker, 1971). However, little research has been reported on the isolation and characterization of peppermint PPO.

Peppermint has a number of medicinal uses, due to its soothing effect on the stomach and colon. It is also a refreshing flavouring agent for food, candy, gum, and dental products.

In the present work, PPO was isolated from peppermint leaves (*Mentha piperita*) and this paper describes some properties of the enzyme. In addition, we have investigated the inhibition by thiourea, ascorbic acid, sodium metabisulfite, glutathione, sodium azide, β-mercaptoethanol, dithioerythritol, sodium diethyl dithiocarbamate and potassium cyanide.

2. Material and methods

2.1. Plant material

The peppermint leaves (*M. piperita*) were harvested fresh from the region of Menderes, in Turkey, (December).

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2.2. Reagents

Catechol, L-dopa, DL-dopa, ascorbic acid, sodium metabisulfite, glutathione, thiourea, dithioerythritol, β-mercaptoethanol and sucrose were obtained from Sigma Chemical Co. (St. Louis, USA). Sodium azide, potassium cyanide, sodium diethyl dithiocarbamate, ammonium sulfate and L-tyrosine were purchased from Merck, Germany. All other chemicals used were of analytical grade.

2.3. Partial purification of peppermint PPO

Fresh peppermint leaves (20 g) were homogenised in 100 ml 0.05 M of phosphate buffer (pH 7.0) containing 10 mM ascorbic acid and 0.5% polyethylene glycol. The homogenate was filtered through two layers of cheesecloth and then the filtered material was centrifuged at 14,000 ***g*** for 15 min at 4°C. Solid $(NH_4)_2SO_4$ was added to the supernatant to obtain 70% saturation and then centrifuged at 14,000 ***g*** for 60 min at 4°C. The precipitate was dissolved in 0.05 M phosphate buffer (pH 7.0). The enzyme extract was extensively dialyzed against the same buffer at 4°C overnight. The dialyzed sample was used as the PPO enzyme source in the following experiments.

2.4. Measurement of PPO activity

PPO activity was determined by measuring the initial rate of quinone formation as indicated by an increase in absorbance at 420 nm (Coseteng & Lee, 1987). A Jasco spectrophotometer was employed throughout the investigation. One unit of enzyme activity was defined as the amount of enzyme that caused a change in absorbance of 0.001 per min. PPO activity was assayed in triplicate measurements. The sample cuvette contained 2.95 ml of 10 mM catechol solution in 0.05 M phosphate buffer (pH 7.0) and 0.05 ml of the enzyme solution. The blank sample contained only 3 ml of substrate solution.

2.5. Protein determination

Protein concentration was determined according to the dye-binding method of Bradford (1976), with bovine serum albumin as standard.

2.6. Optimum pH and stability

Enzyme activity, as a function of pH, was determined with 10 mM catechol in 0.05 M phosphate buffer, ranging from pH 3.0 to 10.0.

pH stability was determined by incubating the enzyme in 0.05 M phosphate buffer (pH 4.0–9.0) for 30 min at 30°C. PPO activity was assayed under standard conditions with catechol as the substrate.

2.7. Optimum temperature and stability

Polyphenol oxidase activity as a function of temperature was determined at various temperatures from 10–70°C. The heat-stability of the enzyme was determined by placing the enzyme solution in a test tube set up in a water bath. After 30 min of heating at various temperatures (30–80°C), the enzyme solution was rapidly cooled in ice and the remaining activity was assayed in 0.05 M phosphate buffer (pH 7.0) at 30°C.

In addition, to study the effect of additions on heat stability PPO was incubated in the presence of either salt or sugar.

Renaturation of the enzyme was investigated by measuring the activity at certain intervals, at temperatures below to 30°C.

2.8. Inhibitor effects

The effects of several inhibitors (ascorbic acid, sodium metabisulfite, glutathione, thiourea, sodium azide, potassium cyanide, β-mercaptoethanol, dithioerythritol, sodium diethyl dithiocarbamate), on peppermint PPO activity were studied.

To determine the effects of inhibitors, reactions containing 10 mM catechol and a constant amount of enzyme in 0.05 M phosphate buffer, (pH 7.0), were run at 30°C in the presence and absence of the inhibitor. Using five different concentrations of the substrates, PPO activities were measured at three constant inhibitor concentrations with the inhibitors indicated above. Values 1/V and 1/[S] were employed to draw Lineweaver–Burk graphs. Finally K_i constant values were obtained from the graphs. To determine the inhibitor concentration that reduced the enzyme activity by 50% (I_{50}), regression analysis graphs were drawn by using percent inhibition values by a statistical package program on a computer. I_{50} values were determined from the graphs.

2.9. Substrate specificity and enzyme kinetics

Michaelis-Menten constant (K_m) and maximum reaction velocity (V_{max}) were determined using four substrates (catechol, DL-dopa, L-dopa, L-tyrosine) in five different concentrations and in standard conditions. Data were plotted as 1/V and 1/[S] concentration according to the method of Lineweaver and Burk (1934).

3. Results and discussion

3.1. Effect of pH on PPO activity and stability

pH optima for PPO activity differed for each substrate. This value was found to be 7.0 for peppermint

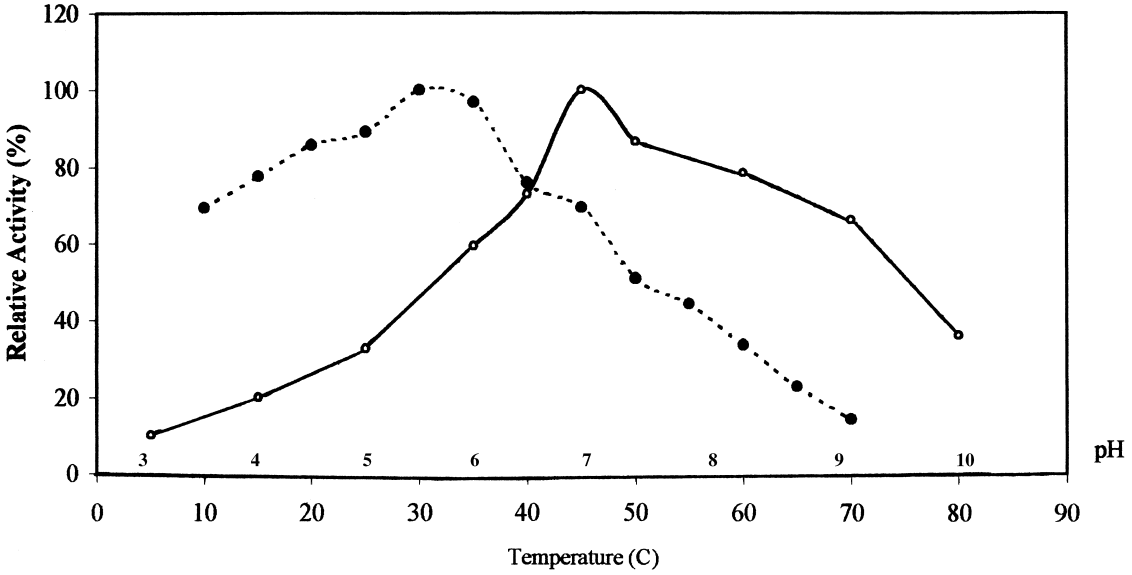

Fig. 1. The effect of pH and stability on peppermint polyphenoloxidase activity pH (— ○ —), temperature (— ● —).

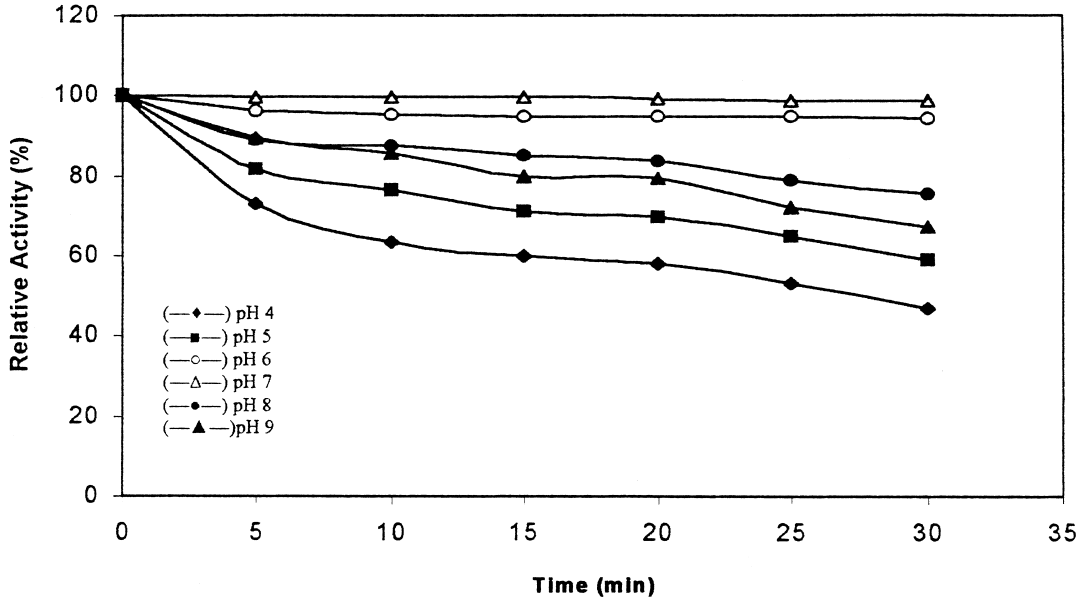

Fig. 2. The effect of pH on stability of peppermint polyphenoloxidase.

(*M. piperita*) PPO with catechol as substrate (Fig. 1). PPO activity decreased below and above pH 7.0, but the enzyme was still active at pH 4.0 with a relative activity close to 20%. At pH 10, the retained activity of enzyme was 36%. Maximum activity with DL-dopa and L-dopa were obtained at pH 7.5. Differences in pH optima with several substrates have been reported for PPO from strawberries and other sources (Mayer & Harel, 1979; Wesche-Ebeling & Montgomery, 1990). Aylward and Haisman (1969) reported that the optimum pH for maximum activity of PPO varies from about 4.0 to 7.0 depending on the extraction methods, substrates and localization of the enzyme in the cell (Aylward & Haisman, 1969).

The pH stability curve of PPO activity is shown in Fig. 2. The peppermint enzyme, at basic pH, was more stable than at acidic pH. The pH stability curve indicates that PPO retained more than 95% of its initial activity within the pH range 6.0–7.0. At pH 5.0 and pH 4.0, PPO activities decreased by about 59 and 47% of the maximum activities, respectively. At pH 9.0, only about 33% of its activity was lost. This result was similar to that for Monroe apple PPO, reported by Zhou, Smith, and Lee (1993).

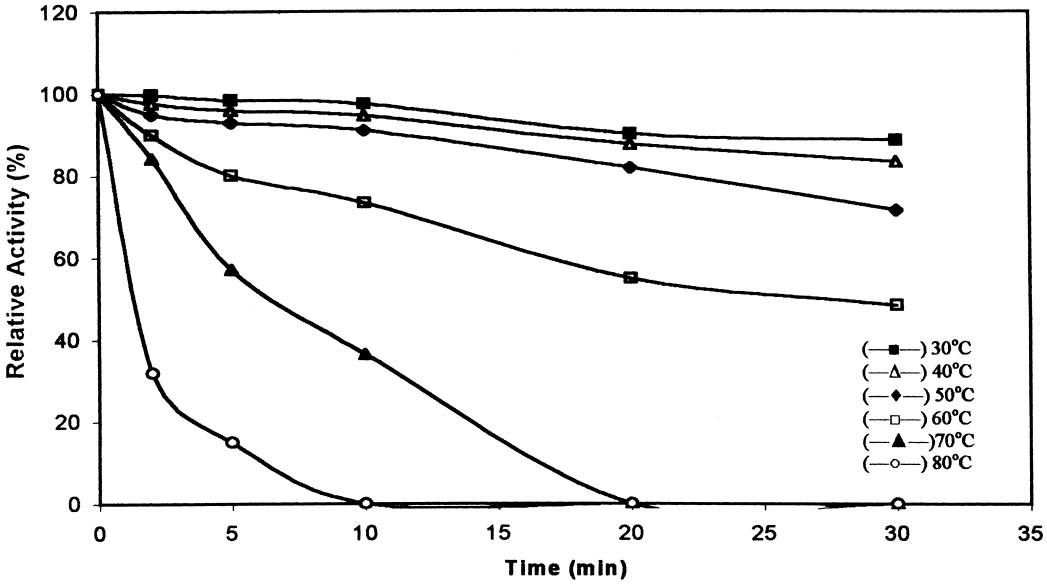

Fig. 3. Heat-inactivation of peppermint polyphenoloxidase at various temperatures.

3.2. Effect of temperature on PPO activity and stability

The activity and stability profiles for the oxidation of catechol by peppermint PPO are shown in Figs. 1 and 3. The optimum temperature for the PPO catechol reaction was 30°C.

The enzyme was stable at lower temperatures but unstable at higher temperatures. Heating for 30 min at 30°C caused no change of enzymic activity. At the higher temperature, the more rapidly the enzyme activity was lost. For instance, when the temperature was increased from 40 to 60°C, the relative activity of PPO decreased from 83 to 48. This indicated that the enzyme was rapidly denatured at higher temperatures. The times required for 50% inactivation of activity at 70 and 80°C were found to be 6.5 min and 1.5 min, respectively. In this respect, half-life of PPO activity at 70°C was similar to the PPO extracted from other sources, i.e. 8 min for avocado PPO (Kahn, 1976), 15 min for green olive PPO (Ben-Shalom, Kahn, Harel, & Mayer, 1977) and 11.7 min for d'Anjou pear (Halim & Montgomery, 1978). Siddiq, Cash, and Akhter (1994) showed that kiwi fruit PPO was completely inactivated at 65°C when heated for 5 min.

3.3. The effect of salts and sugars on the thermal stability of PPO enzyme

Salts, sugars and polyhydric alcohols have been used for many years as stabilizing agents for the maintenance of the biological activity of macromolecules (Frigon & Lee, 1972; Neucere & Angelo, 1972). The basic observations are that these additives prevent the loss of enzymic activities (Bradbury & Jakoby, 1972), inhibit irreversible aggregation (Frigon & Lee, 1972) or increase the thermal transition temperature of macromolecules (Gerisma, 1968; Neucere & Angelo, 1972). Lee and Timasheff (1981) found a significant contribution to protein stabilization by sugars in aqueous solution, the preferential interaction being a strong function of the increase in the surface tension of water by the addition of sucrose.

The kinetics of the heat-inactivation of the enzyme at 30–80°C are shown in Fig. 3. Fig. 4 shows the effect of sucrose at 20 and 40% (w/w) on thermal inactivation of PPO at 80°C. The data shown in Fig. 4 reveal that activity was more stable when PPO was heated in the presence of sucrose. In this case, PPO retained 68% of the original activity after 2 min of heating at 80°C compared to a 33% value obtained in the absence of sugar. In contrast, the enzyme heated in 20% sucrose was not markedly protected by sucrose.

According to our results, the heat treatment of the enzyme at 70°C in 20 and 40% sucrose solutions did not protect the enzyme against heat-inactivations, as compared to a control heated under similar conditions in the absence of sugar. After heating for 40 min, the control enzyme had no activity, but PPO enzyme in the presence of sugar showed about 10–20% activity. The mechanism of heat-stabilization of proteins by sugars has been reported by many authors. Gerlsma (1968, 1970) and Gerlsma and Sturr (1972) have shown that polyhydric alcohols and sugars increased the transition temperatures of some proteins in aqueous solution and they ascribed the stabilizing actions of these substances to their induction, in water, of a decrease in hydrogen-bond rupturing potency. Back, Oakenfull, and Smith (1979) have shown that glucose, as well as other sugars, had a stabilizing action against the thermal denaturation of proteins. Lee and Timasheff (1981) have suggested that

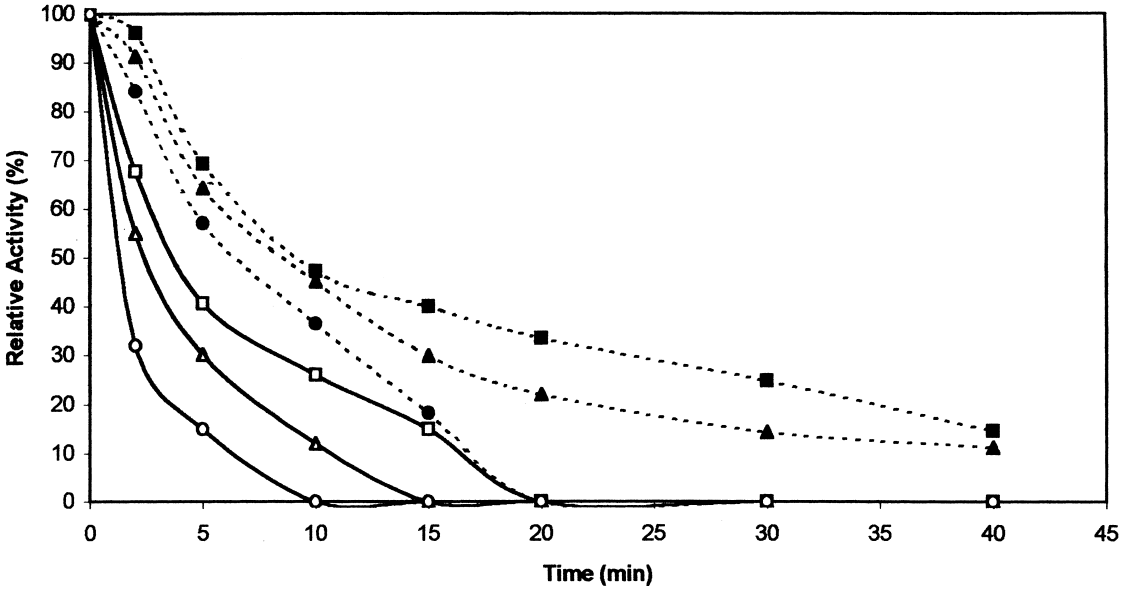

Fig. 4. Heat-inactivation of peppermint polyphenoloxidase in the presence of sucrose at 70 and 80°C heating at different temperatures; the enzyme solution was rapidly cooled in ice and remaining activity was assayed with catechol as subsrate at 30°C. 70°C, Control (— ● —), sucrose concentrations:20% (— ▲ —), 40% (— ■ —), 80°C, Control (— ○ —), sucrose concentrations:20% (— Δ —), 40% (— □ —).

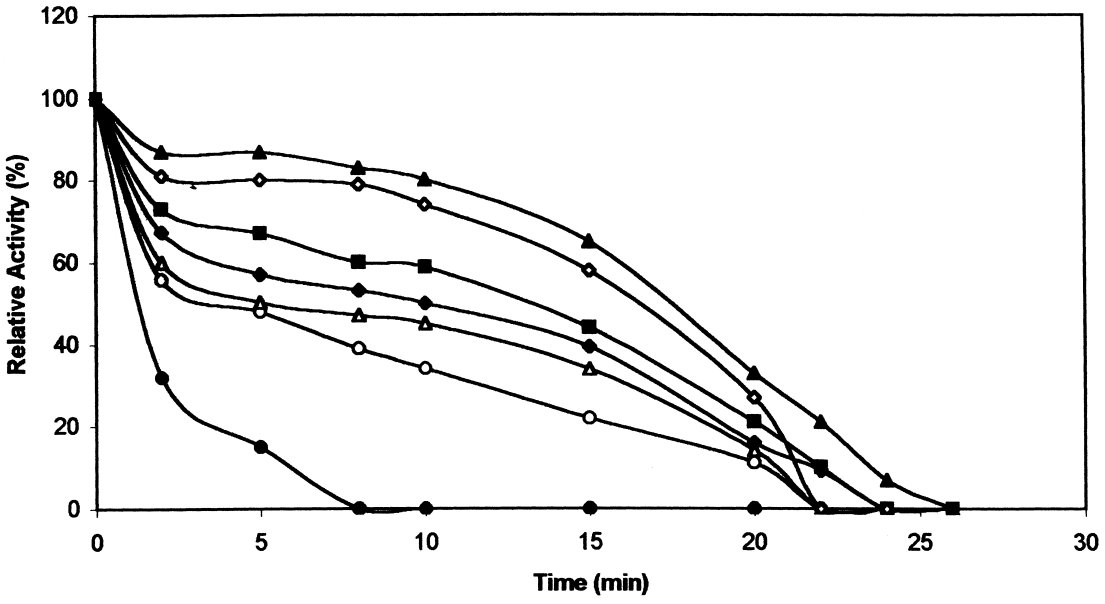

Fig. 5. Heat-inactivation of peppermint polyphenoloxidase in the presence of salts and heating at different temperatures. The enzyme solution was rapidly cooled in ice and remaining activity was assayed with catechol as subsrate at 30°C. Control (— ● —); salt concentrations: 1 M KCI (— ○ —), 1 M NaCl (— Δ —); 1 M $(NH_4)_2S0_4$ (— ▲ —); 2 M NaCI (— ■ —), 2 M KCl (— ◆ —), 1 M Na_2SO_4 (— ◇ —).

the cohesive force of sugars responsible for the increase in the surface tension of water is a very important factor governing the preferential interaction of proteins with solvent components in aqueous sugar systems, and hence, the stabilization of proteins.

The protective effect on the heat-stabilization of protein increases in the order $(NH_4)_2SO_4 > Na_2SO_4 >$ NaCl > KCl, as shown in Fig. 5. The heat-inactivation, in the presence of salts, seemed to induce some heat-stabilization of the PPO structure, as measured by loss of initial activation. The results show that the protective effect on PPO activity depends on the concentration and nature of the salts. It was also observed that, at the same concentrations, Na_2SO_4 was more effective than NaCl in protecting the enzyme activity. Both at identical anion concentration (1 M) and at identical Na^+ concentrations (1 M), and at pHs where the protein is quite stable, the exclusion of the salt from the protein is stronger for SO_4^{-2} as is evident from Fig. 5. The effect of salts on protein stability depends on the concentration

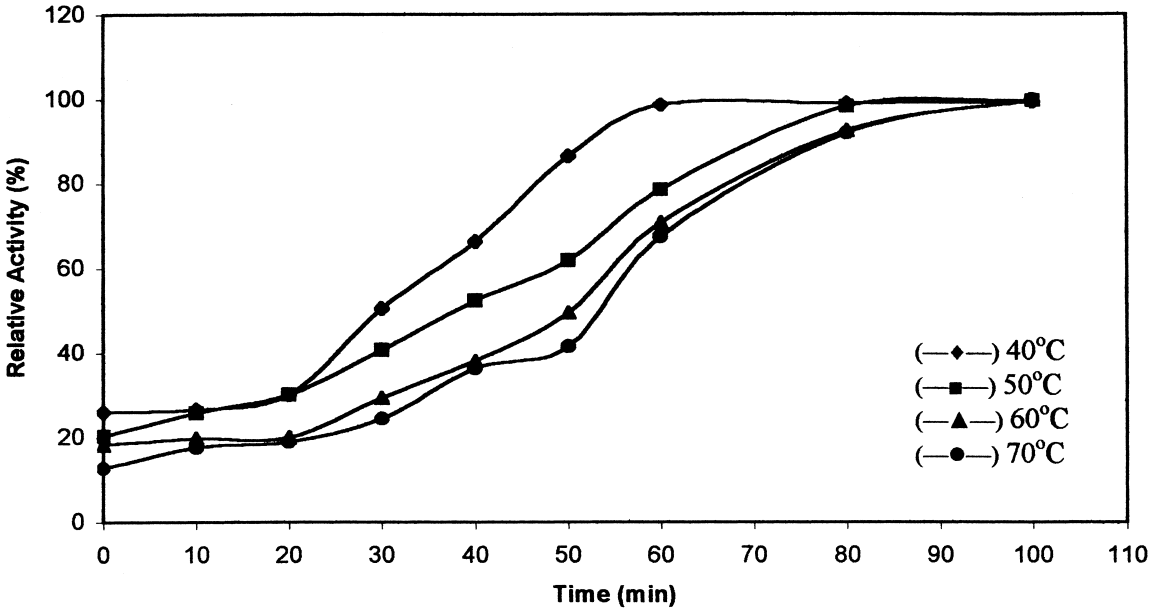

Fig. 6. Renaturation property of the peppermint polyphenoloxidase denaturedat different temperatures.

Table 1
Optimum pH, temperature, K_m and V_{max} values of the polyphenoloxidase for different substrate

Substrate	Optimum pH	Optimum temperture (°C)	K_m(M)	V_{max}(IU/mg.min)
Catechol	7	30	6.25×10^{-3}	15047
D-L dopa	7.5	50	7.93×10^{-3}	7526
L-dopa	7.5	55	9.00×10^{-3}	5709

and ionic strength of the salts. At low salt concentration, the stabilizing effect of electrolytes on protein conformation has been attributed to an electrostatic response. At high salt concentration, the ability of salts to stabilize protein structure has been related to the preferential hydration of the protein molecule, as a result of a salt-induced alteration of the water structure in the vicinity of the protein, and can be referred to as a lyotropic effect. Recently, Melander and Horvarth (1977) applied the cavity formation theory to aqueous salt solutions and proposed that protein stability in aqueous salt solution can be explained in terms of the surface tension of the solvent. As there is a good correlation between the molal surface tension increments of salts and the anionic lyotropic series. Kuntz and Kauzmann (1974) have reviewed the available data on the effect of salt on protein hydration, but the information is insufficient to interpret the widely different salt effects in terms of preferential interactions.

Renaturation of the peppermint PPO was also studied by lowering the temperature to 30°C. When the denatured enzyme was incubated at 30°C for a 10–100-min duration, it recovered activity again (Fig. 6). Peppermint PPO therefore shows this renaturation property. This is undesirable from the point of food processing. Therefore, we do not recommend the heat-inactivation method for preventing enzymatic browning of peppermint.

3.4. Substrate specificity and enzyme kinetics

Peppermint *(Mentha piperita)* PPO showed activity with all the *o*-diphenolic substrates in this study. Maximum activity was detected toward catechol, followed by DL-dopa and L-dopa. It is generaly known that the main phenolic compounds contained in the plant used as enzyme source serve as good substrates to the enzyme (Kimberly & Lee, 1980). L-tyrosine was also tested but was not oxidized by peppermint PPO. These findings are similar to those for Yali pear, Bartlett pears and Koshu grapes PPO (Nakamura et al., 1983; Rivas & Whitaker, 1973; Zhou & Feng, 1991).

The apparent K_m and V_{max} for the three substrates are shown in Table 1. The enzyme has a relatively high affinity towards catechol, which was the best substrate of those tested (lowest K_m value). The K_m for catechol, as calculated by the Lineweaver–Burk plot was 6.25 mM. The peppermint K_m value is lower than the 20 mM for plum and compares with a K_m value of 67 mM for

Table 2
Ki values and inhibition modes of the polyphenoloxidase with different inhibitors

	I (M)	Ki	Type of inhibition	% Inhibition
Potassium cyanide	9×10^{-5}	5.40×10^{-5} M		48.3
	2×10^{-4}	6.40×10^{-5} M	Competitive	64.9
	3×10^{-4}	3.70×10^{-5} M		69.5
Glutathione	3×10^{-5}	1.60×10^{-4} M		15.3
	5×10^{-5}	1.50×10^{-4} M	Competitive	20.3
	9×10^{-5}	1.40×10^{-4} M		59.0
Ascorbic acid	3×10^{-5}	6.25×10^{-5} M		22.3
	6×10^{-5}	7.3×10^{-5} M	Competitive	34.9
	9×10^{-5}	6.1×10^{-5} M		72.0
Thiourea	8×10^{-5}	1.88×10^{-4} M		21.6
	1.5×10^{-4}	1.37×10^{-4} M	Competitive	46.7
	3.3×10^{-4}	1.11×10^{-4} M		63.6
Sodium azide	5×10^{-2}	6.03×10^{-2} M		12.7
	1×10^{-1}	3.17×10^{-2} M	Competitive	50.0
	3×10^{-1}	4.69×10^{-2} M		67.3
Sodium metabisulfite	1×10^{-5}	9.41×10^{-6} M		43.5
	4×10^{-5}	9.20×10^{-6} M	Competitive	68.9
	6×10^{-5}	7.60×10^{-6} M		79.4
Dithioerythritol	$3\times10^{-}$	1.35×10^{-5} M		38.0
	5×10^{-5}	2.93×10^{-5} M	Competitive	44.9
	7×10^{-5}	5.27×10^{-5} M		51.4
β-Mercaptoethanol	1.4×10^{-3}	2.05×10^{-2} M		15.4
	6×10^{-3}	2.63×10^{-2} M	Noncompetitive	27.7
	9×10^{-3}	2.40×10^{-3} M		67.0
Sodium diethyl dithiocarbamate	3×10^{-5}	1.76×10^{-4} M		14.3
	5×10^{-5}	1.94×10^{-4} M	Uncompetitive	29.9
	7×10^{-5}	2.04×10^{-4} M		62.0

Concord grape PPO, with catechol as substrate. This is close to the value of 5.0 mM reported for polyphenol oxidase from litchi (Tan & Li, 1984), 6.2 mM from cocoyam (Anosike & Ojimelukwe, 1982), 8.3 mM from green olive (Ben-Shalom et al., 1977), and 9.1 mM from *Diascorea bulbifera* (Anosike & Ayaebene, 1981), all using catechol as the substrate.

3.5. Storage stability

Effects of different storage temperatures on partially purified peppermint PPO were studied over a 21-day period at pH 7.0. At 25°C, the enzyme completely lost its activity after 4 days. At 4°C, 20% loss in PPO activity was observed during the first 5 days; however, at the end of 12 days, more than 80% of its activity was lost. The enzyme was more stable at −15°C; it lost only 10% enzyme activity on storage for 6 days and was completely inactivated after 21 days. The loss of PPO activity could be attributed to the partially purified enzyme.

3.6. Effect of inhibitors

Ki values and inhibition modes for nine inhibitors used in the study are shown in Table 2. From the Ki constants, it has been concluded that inhibition modes of the inhibitors are as follows: sodium diethyl dithiocarbamate, uncompetitive; β-mercaptoethanol, noncompetitive, and the others, competitive. I_{50} values were also obtained with these inhibitors, using catechol as the substrate. The values were 1.4×10^{-4} M, 1.7×10^{-4} M, 9.7×10^{-5} M, 2.45×10^{-4} M, 2.16×10^{-1} M, 1.83×10^{-5} M, 6.5×10^{-5} M, 1.4×10^{-2} M and 7.5×10^{-5} M for potassium cyanide, glutathione, ascorbic acid, thiourea, sodium azide, sodium metabisulfite, dithioerythritol, β-mercaptoethanol and sodium diethyl dithiocarbamate, respectively. The strongest inhibitor was found to be sodium metabisulfite. That sodium metabisulfite can act as a reducing agent for *o*-benzoquinones, as has been shown by Wong et al. (1971). Sodium diethyl dithiocarbamate, potassium cyanide (KCN) and dithioerythritol are

effective PPO inhibitors because of their capacity to bind to copper at the active site, while KCN and sodium diethyl dithiocarbamate are reported to react with quinones (Mayer & Harel, 1979). Ascorbate reduces the initial quinone formed by the enzyme to the original diphenol, before it undergoes secondary reactions which lead to browning (Matheis & Whitaker, 1984). Golan-Goldhirsh and Whitaker (1984) have reported that ascorbic acid causes the irreversible inhibition of mushroom PPO.

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