Polyphenol Oxidase from *Allium* sp.

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Polyphenol oxidase (PPO) was isolated from *Allium* sp. PPO showed activity to catechol and DL-dopa (K_m values were 25 mM for cathecol and 33 mM for DL-dopa; V_{max} values were 666 EU/mL·min for cathecol and 166 EU/mL·min for DL-dopa). Catechol was the most suitable substrate for *Allium* sp. PPO (lowest K_m value). The optimum pH for the PPO was 7.5 on substrates catechol and DL-dopa. Heat inactivation studies showed temperature >40 °C resulted in loss of enzyme activity. Heating for 30 min at 40 °C did not cause a significant loss of enzymatic activity. *Allium* sp. PPO was significantly inhibited in the presence of ascorbic acid, 2-mercaptoethanol, and sodium metabisulfide.

Keywords: *Polyphenol oxidase; Allium sp.; inhibitors; herb cheese*

INTRODUCTION

Enzymatic browning catalyzed by polyphenol oxidase (PPO) occurs when plant tissues are damaged and is an economic problem for processors and consumers. The main step in enzymatic browning is the oxidation of phenolic compounds to corresponding quinones by PPO in the presence of oxygen. The quinones then condense to form darkened pigments (Matheis, 1983). PPO is a copper-containing enzyme and widely distributed in plants (Mayer, 1987). It has been related to enzymatic browning in several plant tissues (Zhow and Feng, 1991; Vamos and Gaszago, 1978; Sharon and Khan, 1979; Tan, 1984; Galeazzi and Sparbieri, 1981; Roudsari et al., 1981; Augustin et al., 1985).

An important food product in the east Anatolian part of Turkey is herb cheese. A number of herbs such as *Allium* sp., *Thymus* sp., and *Ferule* sp. are used in making herb cheese. However, the most widely used one is *Allium* sp. These herbs are added into the vat to get the desired flavor for the cheese. The herbs are collected from plateaus in spring season. Producers can used either single or mixed herbs to prepare pickle. After washing well, they are cut into slices and placed into a plastic container. Brine with concentration of 16% salt is poured into the container. For a period of 15 or 20 days, pickled herbs are stored in a cool place. Producers mostly use whey brine instead of tap water brine. After that, the pickled herbs are ready to be added into the cheese. Approximately 2% of herbs depending on the producer is used for the vat-cheese milk (Akyüz et al., 1996). The pickled herb is also sold in markets, so they can be found throughout the year.

PPO from different plant tissues shows different substrate specificities and degrees of inhibition (Vamos-Vigyazo, 1981). Therefore, characterization of the enzyme could help to develop more effective methods for controlling browning of plants and products.

Our objective was to isolate PPO from *Allium* sp. and to characterize the enzyme at different pH values and temperatures. Substrate and inhibitor effects were also studied.

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EXPERIMENTAL PROCEDURES

Plant Material. *Allium* sp. used in this study was obtayned from Van, Turkey, and was kept for 2 day in deep freezer (−15 °C) before it was used for extraction of PPO.

Isolation of PPO. *Allium* sp. plants were harvested fresh (in Van, Turkey) and stored in a deep freezer (−15 °C). For preparing extract, 3 g of *Allium* sp. plants was placed in a dewar flask under liquid nitrogen, which decomposes cell membrane, transferred to a stainless steel Waring blender, and ground to a powder under liquid nitrogen. Before use, the powder was transferred to a small beaker. The frozen plant powder was added to the extraction solution (0.1 M sodium citrate/0.2 M potassium phosphate buffer adjusted to pH 5.8 with 0.1 M NaOH or 0.1 N HCl) and mixed with a magnetic stirrer for 4 min at 4 °C. The suspension was centrifuged at 11000*g* for 10 min at 0 °C, and the supernatants were filtered through Whatman No. 1 filter paper. The final crude extract was dialyzed against 0.2 M phosphate buffer (pH 6.8) for 2 days with three changes of buffer (Wesche and Montgomery, 1990).

Enzyme Assay. Enzyme activity was determined by measuring the increase in absorbance at 420 nm with a spectrophotometer (LKB-Biochrom). The sample cuvette contained 2.8 mL of substrate in various concentrations prepared in the homogenization buffer and 0.2 mL of the enzyme extract. The blank sample contained only 3 mL of substrate solution. Enzyme activity was calculated from the linear portion of the curve. One unit of PPO activity was defined as the amount of enzyme that caused an increase in absorbance of 0.001/min (Oktay et al., 1995).

Effect of pH. The activity of the enzyme was determined in the pH range 5.5−8.5 by using 0.1 M phosphate buffer adjusted with 0.1 M NaOH or 0.1 M HCl. The optimum pH for the PPO was obtained using two different substrates (catechol and DL-dopa).

Heat Inactivation of PPO. The thermal denaturation of PPO was studied at 10, 40, 60, and 80 °C. For the study, 0.5 mL of enzyme in a test tube was incubated at the required temperature for fixed time intervals. At the end of the required time interval, the test tube was cooled by immersion in ice. The activity of the enzyme was then determined.

Enzyme Kinetics and Substrate Specificity. Michaelis constant (K_m) and maximum velocity (V_{max}) were determined using two substrates (catechol and DL-dopa) in various concentrations. The reaction was followed in a spectrophotometer, and data were plotted according to the method of Lineweaver and Burk (1934). For substrate specificity, three compounds at various concentrations were tested as substrates for PPO: catechol, DL-dopa and L-tyrosine. The absorbance of the products was scanned from 398 to 420 nm by means of a spectrophotometer.

S0021-8561(96)00929-6 CCC: $14.00

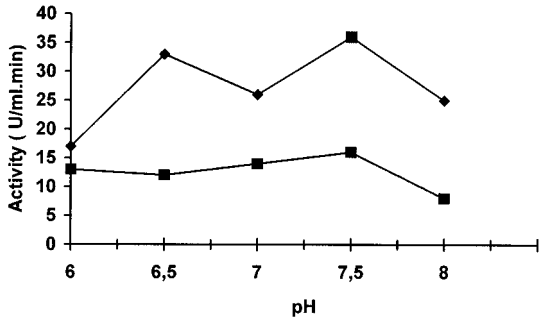

Figure 1. Effect of pH on PPO activity: (◆) DL-dopa; (■) catechol.

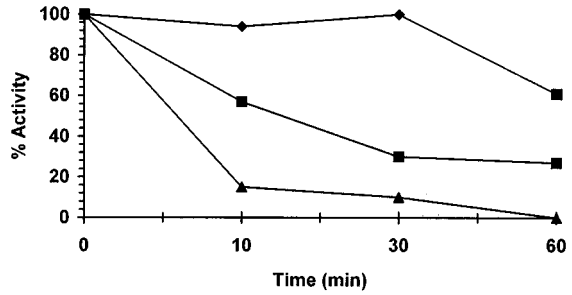

Figure 2. Heat inactivation of PPO at various temperatures (for DL-dopa): (◆) 40 °C; (■) 60 °C; (▲) 80 °C.

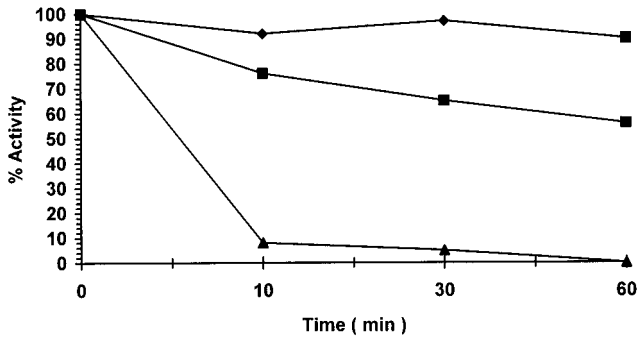

Figure 3. Heat inactivation of PPO at various temperatures (for catechol): (◆) 40 °C; (■) 60 °C; (▲) 80 °C.

Effect of Inhibitors. To determine effects of inhibitors, the reaction assay contained 0.3 mL of 0.1 M catechol, 0.1 mL of enzyme solution, 2.6 mL of 0.1 M phosphate buffer, and inhibitor solution at various concentrations. Studied inhibitors were ascorbic acid, sodium metabisulfide, and 2-mercaptoethanol. Results were expressed as percent catechol inhibition.

RESULTS AND DISCUSSION

Effect of pH. The influence of pH on the activity of PPO is shown in Figure 1. Maximum PPO activities were at pH 7.5 for catechol and DL-dopa substrates. In general, most plants showed maximum activity at or near neutral pH vulues (Oktay et al., 1995). In our study, we found similar results for catechol and DL-dopa. Optimum pH values for the enzyme from different sources have been reported. The optimum pH for PPO activity has been found to vary with the source of the enzyme and substrate in a relatively wide range of pH values (Oktay et al., 1995).

Heat Inactivation of PPO. Figures 2 and 3 show that temperatures >40 °C resulted in loss of enzyme activity. Heating for 30 min at 40 °C did not cause a significant loss of enzymatic activity. At the higher temperature, the enzyme activity was lost most rapidly. It has been noted that heat stability of the enzyme may be related to the ripeness of the plant, and in some cases it is also dependent on pH. In addition, different molecular forms from the same source may have different thermostabilities (Zhow and Feng, 1991).

Table 1. Effects of Some Inhibitors on the Activity of PPO from *Allium* sp.

inhibitors	$M \times 10^{-5}$	% inhibition
sodium metabisulfite	1.60	47
	2.50	61
	3.30	73
ascorbic acid	0.83	45
	1.66	75
	2.50	90
2-mercaptoethanol	0.33	20
	3.30	40
	8.30	80

Enzyme Kinetics and Substrate Specificity. Values calculated from Lineweaver−Burk graphs showed K_m values were 25 mM for catechol and 33 mM for DL-dopa. V_{max} values were 666 EU/mL·min for catechol and 166 EU/mL·min for DL-dopa. To determine substrate specifitiy, *o*-dihydroxyphenols and monohydroxyphenolic compounds were tested. Catechol and DL-dopa were oxidized by *Allium* sp. PPO. L-Tyrosine (monohydroxy) was also tested, but no reaction took place. According to these results, the *Allium* sp. PPO could be a diphenol oxidase. This is similar to the findings for Yali PPO (Roudsari et al., 1981) and Bartlett pears (Rivas and Whitaker, 1973) and kiwi fruit (Park and Luh, 1985). The enzyme has a relatively high affinity toward catechol, which was the best substrate (lowest K_m value). The K_m value for *Allium* sp. (using catechol as substrate for PPO) is lower than obtained values for olive enzyme (Ben-Sholom et al., 1977) and Yali PPO enzyme (Roudsari et al., 1981). A K_m value of 25 mM was obtained with catechol as substrate. This is close to the value reported for PPO from Yali (Zhow and Feng, 1991) with chlorogenic acid as the substrate. It was found that the K_m for PPO varies with source of the enzyme (Park and Luh, 1985).

Effect of Inhibitors. Enzymatic browning of plants may be delayed or eliminated by removing the reactants such as oxygen and phenolic compounds or by using PPO inhibitors. Complete elimination of oxygen from fruits during processing is difficult because oxygen is ubiquitous (Roudsari et al., 1981). Several compounds were tested for their inhibitory action on *Allium* sp. PPO. The effects of these inhibitors on PPO are shown in Table 1. There was a significant decrease in PPO activity by ascorbic acid and 2-mercaptoethanol.

The prevention of enzymatic browning by a specific inhibitor may involve a single mechanism or be the result of an interplay of two or more mechanisms of inhibitor action (Roudsari et al., 1981). Ascorbic acid and 2-mercaptoethanol act by reducing the quinones formed by PPO to polyphenols while they are themselves oxidized. These inhibitors at a concentration of 10^{-5} M caused inhibition effects.

Ascorbic acid can be used safely in making herb cheese. Ascorbic acid will reduce quinones to phenols and will prevent enzymatic browning only as long as it is present in the reduced form.

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Received for review November 25, 1996. Revised manuscript received April 14, 1997. Accepted May 9, 1997.[⊗]

JF960929W

[⊗] Abstract published in *Advance ACS Abstracts,* July 1, 1997.