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Purificiation and Some Properties of Polyphenol Oxidase from Van Apple (Golden Delicious)

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Polyphenol oxidase was isolated from van apple, with a 4.0-fold purification of polyphenol oxidase by ammonium sulfate and DEAE cellulose being achieved. The optimum pH was 4 and for this enzyme, the optimum temperature was 50°C. V_{max} and K_m values were found of Lineweaver-Burk graphs. Potassium cyanide, benzoic acid, citric acid, $MnCl_2$, NaCl, $NaNO_2$ and $CuSO_4$ were used as inhibitors. The enzyme activity was inhibited by benzoic acid, $CuSO_4$ and NaCl, polyphenol oxidase activity at 10 mM, whereas $NaNO_2$, citric acid, KCN and $MnCl_2$ enhanced by polyphenol oxidase activity at 10 mM.

Key Words: Polyphenol oxidase, Characterization, Purification, Inhibitors.

INTRODUCTION

Enzymatic browning occurs in many vegetables and fruits after brushing or cutting or during storage. This results from oxidation of phenolic compounds to quinones by polyphenol oxidase in the presence of oxygen[1]. Polyphenol oxidase (PPO) is a ubiquitous plastic associated plant enzyme[2]. In the plant itself, browning, *i.e.*, phenolic oxidation is thought to have a positive role in disease resistance[3]. Polyphenol oxidase has been widely studied in many fruits and vegetables to determine how to prevent browning which results in the loss of their marketability. Undesirable browning of damaged tissue is known to be catalyzed by polyphenol oxidase during storage and processing of fresh fruits and vegetables. Therefore, enzymatic browning is an economic problem for processors and consumers. Browning has been attributed to oxidation of phenolics by polyphenoloxidase, brown coloured by products[4–7]. In future, PPO may have further industrial and environmental applications, such as in the *in-vitro* production of black teas, the production of flavonid-derived colorants and antioxidants and the removal of oestrogenic substances from aquatic environments[2]. However, purification and characterization of some properties of van apple have not yet been done and there is no knowledge about its enzyme properties. Therefore, van apple is preferred for this study.

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The objective of this study was to characterize the PPO from van apple. This information will be useful in devising effective methods for inhibiting browning during storage.

EXPERIMENTAL

Van apple fruits used in this study were obtained from local Van region. Catechol, polyvinylpyrolidone, DEAE cellulose, $(NH_4)_2SO_4$ and other chemicals were obtained from Sigma Chemical Co., St. Louis, MO.

Extraction and purification: The van apple was homogenized with 0.1 M sodium phosphate buffer (pH 6.8) with insoluble high molecular weight grade of polyvirylpyrolidone, 1% of fruit apple in weight, using an ultra-All. After the filtrate had been centrifuged at 20 g for 30 min, the supernatant was collected. Then the samples were fractionated with solid ammonium sulfate, the precipitate from 20–80% saturation was dissolved in a minimal volume of the same buffer column with phosphate buffer and washed. The active fractions were applied to DEAE-cellulose column (1 × 5 cm) previously equilibrated with sodium phosphate buffer (pH 6.3). The fractions containing PPO activity were appropriately combined, dialyzed against extraction buffer and distilled water. After overnight dialyzing, the solution was collected as PPO[1, 4–7].

Determination of protein: Protein concentration was determined according to the dye binding method of Bradford [8] with bovine serum albumin as standard.

Enzyme assay: The assay mixture consisted of 2.8 mL of 0.001 M sodium phosphate buffer (NaPi), pH 7.3, 0.2 mL of 0.1 M catechol and 0.2 mL of enzyme extract. The increase in absorbance at 420 nm was measured. One unit of enzyme activity is defined as the amount of the enzyme that causes an increase in absorbance of 0.001 min at 25°C[6].

Effect of pH: The effect of pH on PPO activity was obtained at different pH values (pH 4.0–10.0).

Effect of temperature: The effect of temperature on PPO activity was obtained at different temperature values (20–80°C)

Kinetic determinations and effect of inhibitors: V_{max} and K_m values were determined by Lineweaver-Burk graphs. Effects of inhibitors potassium cyanide, citric acid, benzoic acid, $MnCl_2$, NaCl, $NaNO_2$ and $CuSO_4$ were investigated.

RESULTS AND DISCUSSION

pH optimum and stability

The activity of PPO was determined at different pH values during incubations at 25°C for 5 min in NaPi buffer. The optimal pH for PPO activity was found as 4 in catechol substrate (Fig. 3). The optimum pH for PPO from other studies has been reported[5–7]. This value was different from those of raspberry (8.0 and 5.5)[9], *Allium* sp (7.5)[10], Amasya apple (7.0)[11], Ercis apple (7.1)[5] using catechol as substrate; on the other hand, the optimum pH of Van apple PPO was similar to those of graphe and Chinese cabbage[1, 12].

Effect of temperature

The activity of PPO was measured at different temperatures at 20–80°C. The substrate and pure enzyme were incubated for 15 min at various temperatures from 20 to 80°C and then spectrophotometric measurement for 5 min was carried out at 25°C[7]. The enzyme showed the highest activity at 50°C (Fig. 2). This value was different from those of Amasya apple (18°C)[11] and grape (25°C)[12]. On the other hand, the optimum temperature of PPO in apple was similar to that of Ercis apple and Chinese cabbage[5].

Effect of inhibitors

Enzymatic browning of vegetables may be delayed or eliminated by removing the reactants such as oxygen and phenolic compounds or by using PPO inhibitors[1]. Complete elimination of oxygen from vegetables during processing is difficult because oxygen is ubiquitous. However, it is noted that benzoic acid and NaCl completely inhibit the PPO activity[7]. In addition, ascorbic acid, citric acid, $FeSO_4$ and acetic acid have been used as inhibitors[5]. Similar effects of compounds were found in the PPOs of banana peel[4]. Benzoic acid, $CuSO_4$ and NaCl inhibited the PPO activity at 10 mM, whereas $NaNO_2$, KCN and $MnCl_2$ enhanced PPO activity (Table-2). Similar inhibitory effects of these chemicals on PPOs were also reported in banana peel, head lettuce and banana pulp[4, 13, 14]. In addition, in polyphenoloxidase from apple, partial purification and same properties have been shown. PPO characteristrics have been investigated in apple[15]. Studies on the polyphenoloxidase of *Malus pumila* (apple) have been done[16].

These results suggest that benzoic acid, $CuSO_4$ and NaCl are able to be used as good inhibitors of enzymatic browning in van apple.

TABLE-1
PARTIAL PURIFICATION OF PPO FROM VAN APPLE

Purification step	Protein (μg/mL)	Activity (units/mL)	Specific activity (units/mg)	Total activity (units)	Purification (fold)	Yield (%)
Crude extract	74.6	129	1.729	64500	1.0	100
$(NH_4)_2SO_4$	77.0	205	2.662	41000	1.5	63.6
DEAE-cellulose	30.0	209	6.972	10450	4.0	16.2

Effect of time on enzyme activity

Enzyme purified solution was stored at +4°C for 10 days and the residual activity was measured. The result showed that activity was retained for 1 day but suddenly decreased after 2 days (Fig. 1). PPO activity reported that 15% of the enzyme activity was retained during storage at +4°C for 15 days[14]. On the other hand, Kowalski *et al.*[17] reported that wild potato PPO was stable at –70°C for several weeks, but it was stable at +4°C for only 2 days.

TABLE-2
EFFECT OF VARIOUS COMPOUNDS ON PPO

Inhibitor	Concn (mM)	Relative activity (%)
$CuSO_4$	1	98.2
	5	96.4
	10	95.2
Benzoic acid	1	97.0
	5	91.7
	10	94.7
NaCl	10	37.0
$MnCl_2$	1	97.4
	5	96.0
	10	99.6
$NaNO_2$	1	80.3
	5	97.4
	10	99.8
KCN	1	98.3
	5	99.1
	10	117.0
Citric acid	1	73.0
	5	71.7
	10	78.3

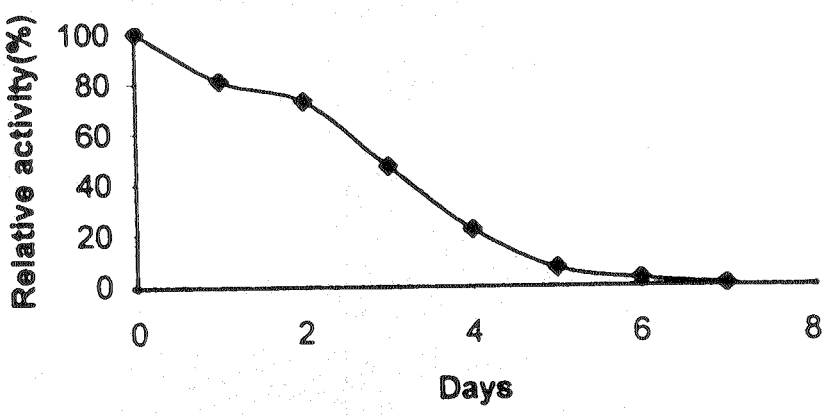

Fig. 1. Effects of time on the activity of van apple PPO

Substrate specificity

The substrate specificity of the enzyme was determined by using catechol as substrate. Michaelis constants (K_m) and maximum reaction velocities (V_{max}) were determined using these substrates under various concentrations 37.3, 34.67, 33.33, 32 and 31.67 mM. Lineweaver-Burk graph analysis of this enzyme preparation showed K_m value of 14.28 mM for catechol (Fig. 4). K_m is similar to that reported

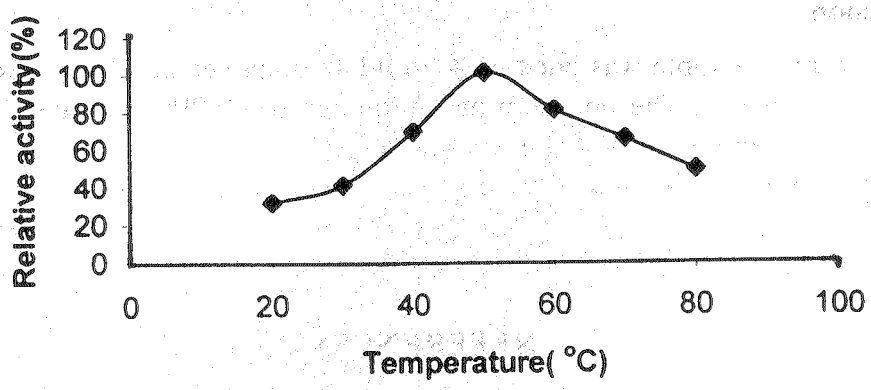

Fig. 2. Effect of temperature on the activity of van apple PPO

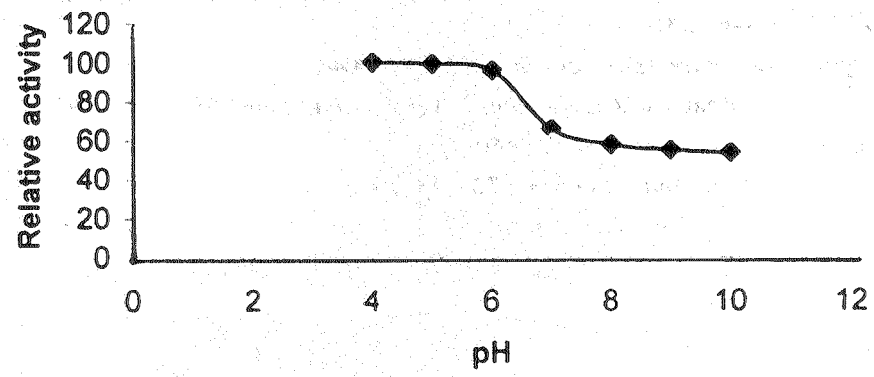

Fig. 3. Effect of pH on the activity of van apple PPO

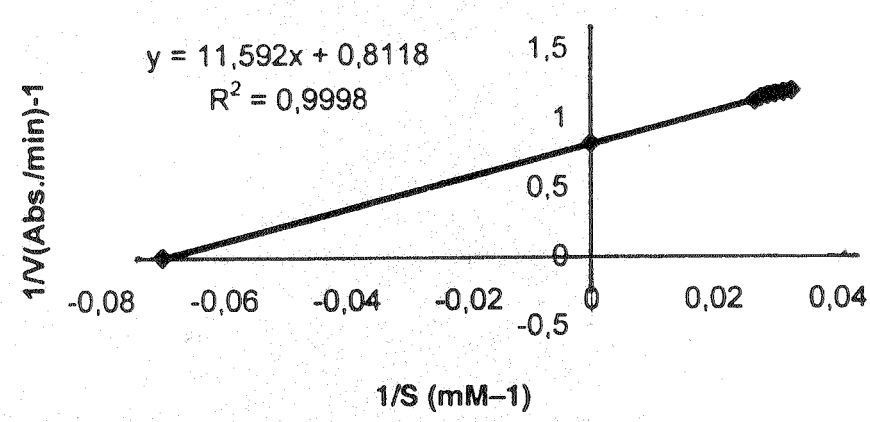

Fig. 4. Lineweaver-Burk graph

for apple peel[18]. This value for catechol was different from that of tea leaf 12.52 mM[19], amasya apple 34 mM[11], Stanley plum 20 mM[20] and herb 25 mM[10]. It has been reported that K_m values for pyrogallol are as follows: apple 27 mM[11], peach 0.2 mM[21], spinach 15.7 mM[22] and tea leaf 17.8 mM[23]. In addition, K_m values for chlorogenic acid were different from those of apple: 0.122 mM[24], pear 5.9 mM[25], spinach 11.6 mM[22]. V_{max} value was found as 1,232 Abs/min for PPO in catechol.

Conclusion

PPO from van apple was purified 4.0-fold, to homogenate, 3-step purification procedure (Table-1). The optimum pH of the van apple PPO for catechol was 4. The optimum temperature of the enzyme was found as 50°C. The K_m and V_{max} values for catechol calculated from a Lineweaver-Burk graph were 14.28 mM and 1,232 Abs/min, respectively (Fig. 4). Some properties of the enzyme van apple were determined.

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