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Partially Purification and Characterization of Polyphenol Oxidase of Quince

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Polyphenol oxidase (PPO, EC 1.14.18.1) was extracted from quince (*Cydonia oblonga*) by using 0.1 M phosphate buffer, pH 6.8. The polyphenol oxidase of quince was partially purified by $(NH_4)_2SO_4$ and dialysis. Substrate specificity experiments were carried out with catechol, pyrogallol, L-DOPA, p-cresole and tyrosine. Catechol was the most suitable substrate compound for quince PPO. The Michaelis constants were 4.54 mM, 7.35mM and 17.8 mM for catechol, pyrogallol and L-DOPA, respectively at 25°C. The optimum pH and temperature were determined with the specific substrate catechol as 8.0 and 40°C, respectively. Of eight inhibitors tested L-cysteine, ascorbic acid and potassium cyanide were the most effective against quince PPO.

Key Words: Quince, polyphenoloxidase, purification, characterization

Introduction

In fruits and vegetables, browning occurs due to the oxidation of polyphenols. Oxidative browning is catalysed by polyphenol oxidase (PPO; monophenol, dihydroxyphenyl alanine:oxygen oxidoreductase; EC.1.14.18.1). When fresh products are damaged oxidative browning occurs, and this is an economic problem for producers and consumers. Polyphenoloxidase, which is a copper-containing enzyme, catalyses the aerobic regioselective oxidation of monophenols to o-diphenols followed by dehydrogenation to o-quinons[1]. PPO is widely distributed in plants and microorganisms[2,3] and many researchers are interested in the PPO isolated from various sources such as the banana[4], Amasya apple[5], kiwi fruit[6], edible burdock[7], head lettuce[8], pear[9,10], palmito[11], cocoa bean[12] and oil bean[13]. However, there are only a few articles about quince PPO[1].

The purpose of this study was to isolate PPO from quince and determine some of its enzymatic properties. In the study, the characteristics of the enzyme were investigated at different pH levels and temperatures. Substrate and inhibitor effects were also studied. The assays of PPO activity were carried out in the presence of air oxygen.

Experimental

Isolation of Polyphenoloxidase

Quinces (Cydonia oblonga) were obtained from the local market of Edirne, Turkey and stored at 4°C. For the preparation of the crude extract, 200 g of quince was cut quickly into thin slices and homogenised with 200 ml of 0.1 M phosphate buffer, pH 6.8, containing 10 mM ascorbic acid, 0.1% polyvinylpyrrolidone and 0.5% Triton X-100 in a Waring blender at 5000 rpm for 3 min. The homogenate was filtered through glass wool and the filtrate was centrifuged at 160000xg for 30 min at 4°C by a Sanyo MS 60 Ultracentrifuge. The supernatant was brought to 30-90% $(NH_4)_2SO_4$ saturation with solid $(NH_4)_2SO_4$, pH 6.8. The precipitated PPO was separated by centrifugation at 160000xg for 30 min. The precipitate was dissolved in a small amount of 0.1 M phosphate buffer, pH 6.8, and dialysed at 4°C in the same buffer for 6 h with three changes of buffer during the dialysis.

Assay of PPO Activity

PPO activity was determined by measuring the increase in absorbance at 420 nm by a Shimadzu UV-160 A spectrophotometer in the presence of air oxygen. The reaction mixture contained 0.2 ml of enzyme solution (0.680 mg protein/ml) and 2.8 ml of 0.02 M substrate solution in 0.05 M phosphate buffer, pH 6.8, at 25°C. The blank sample contained only 3.0 ml of substrate solution. Enzyme activity was calculated from the linear portion of the curve[14]. One unit of PPO activity was defined as the amount of enzyme that caused an increase in absorbance of 0.001 per min. The protein concentration was determined by the method of Lowry, with bovine serum albumin as the standard[15].

Substrate Specificity

Substrate specificity was determined by using five different substrates (catechol, pyrogallol, L-DOPA, p-cresole and tyrosine). All substrate solutions were prepared at 0.02 M in 0.05 M phosphate buffer, pH 6.8.

Effects of pH and Temperature on the Activity

PPO activity was determined with 0.02 M catechol at 25°C using appropriate buffers (0.1 M citrate/0.2 M phosphate and 0.1 M phosphate) between pH 4 and 9. To determine the optimum temperature of PPO, the enzyme activity was measured in the temperature range from 5°C to 90°C by using catechol solution.

The thermal stability of quince PPO was determined by putting 2.0 ml of the enzyme solution in a test tube in a water bath at the appropriate temperatures; 25-80°C. Then 0.2 ml of enzyme solution was withdrawn at appropriate time intervals and rapidly cooled in an ice bath. Then the PPO activity was assayed as described above, pH 6.8, at 25°C.

Kinetic Study

Michaelis constant (K_m) and maximum rates (V_{max}) were determined by using catechol solution in the range of concentrations 0.001-0.05 M at pH 6.8 and 25°C. The reaction was followed in a spectrophotometer and data were plotted according to Lineweaver-Burk.

Effect of inhibitors

PPO activity was measured by using seven different inhibitors (citric acid, EDTA, ascorbic acid, cysteine, urea, potassium cyanide, thioacetamid and sodium bisulphite) at two concentrations with catechol as the substrate. Each inhibitor solution was prepared at concentrations of 0.2 and 0.02 M in 0.05 M phosphate buffer, pH 6.8. The substrate was 0.02 M catechol in the same buffer. The enzyme and inhibitor solutions used were 0.2 ml and 0.1 ml, respectively. The PPO activity was measured under previous reaction conditions (pH 6.8, 25°C). The results were reported as percentage catechol inhibition.

Results, Discussion

Quince PPO was partially purified by ammonium sulphate fractionation and dialysis. The results are summarised in Table 1. The partial purification resulted in about a six-fold increase in enzyme activity.

Table 1. Partial purification of quince PPO

		Protein		Enzyme			
Procedures	Total Volume (ml)	mg/ml	Total mg	Unit/ml	Total Activity (unit)	Specific Activity (U/mg protein)	Purification Factor (fold)
Crude extract	418	1.35	564	110.5	46 189	81.8	1
Ammonium sulphate fractionation	70	0.69	48	328.5	22 995	479.0	5.85
Dialysate	70	0.68	47.6	325	22 750	477.9	5.84

Substrate specificity

In Table 2, K_m and V_{max} data for quince PPO reacting with various mono-phenols, o-diphenols and pyrogallol are summarised.

As shown in Table 2, partially purified enzyme oxidised catechol, L-DOPA and pyrogallol but did not oxidise with tyrosine and p-cresole. No reaction was observed for up to 1 h after the enzyme was mixed tyrosine and p-cresole. This indicated that quince PPO showed activity toward ortho-diphenols but not monophenols. It has been reported that the cocoa bean[12]and pear[9]have similar activities.

Table 2. Substrate specificity of quince PPO

Substrate	Specific activity (unit/mg protein)	K_m (M)	V_{max} (unit/mg protein)
Catechol	2445	4.54×10^{-3}	3125
Pyrogallol	720	7.35×10^{-3}	1674
L-DOPA	460	17.8×10^{-3}	1110
Tyrosine	-	-	-
p-cresole	-	-	-

-no activity

The kinetic properties of quince PPO are shown in Table 2. Lineweaver-Burk plots of catechol, L-DOPA, and pyrogallol were determined at pH 6.8, 25°C. As shown in Figure 1, the Michaelis constants (K_m) for quince PPO were 4.5, 7.3 and 17.8 mM with catechol, pyrogallol and L-DOPA, respectively. The value for catechol approximated that of oil bean seeds PPO (5.71 mM)[13]. Our findings showed that the enzyme has the greatest reactivity towards catechol among the substrates used. Catechol was the best substrate, having the lowest K_m and the highest V_{max}. The K_m of the enzyme for the pyrogallol was 7.3 mM as calculated

from the Lineweaver-Burk plots, while that of pyrogallol oxidase from mandarin orange and edible burdock was 7.1 and 1.8 mM, respectively[7].

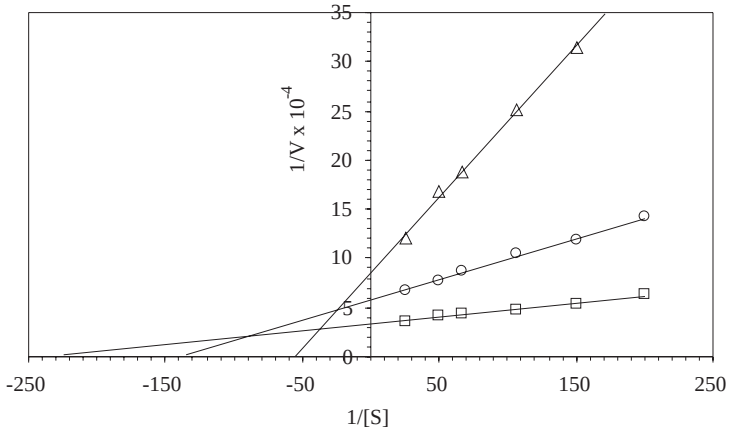

Figure 1. Lineweaver-Burk plots of quince PPO

Effect of pH and temperature

The pH optimum for enzymatic catalysed oxidation of catechol in phosphate buffer was found to occur at pH 8.0 (Figure 2). At pH >9.0, the activity decreased very rapidly. The PPO system in fruits has been shown to be most attractive at or near neutral pH values[1]. It was reported that the pH optimum of PPO from some sources also occurs in the range of pH 6.0-8.0. Maximum activity at pH 7.0 was found in d'Anjou pears[9], burdock[7], cocoa beans[12], oil bean seeds[13] and Amasya apples[5]. Laurenço *et al.* reported the pH optimum of palmito PPO to be 6.6. According to Lee *et al.* the rapid deactivation of the enzyme at pH >8.0 was attributed to the following possibilities: conformational change in the enzyme under alkaline conditions and/or the enzyme may react more rapidly with o-quinon through the Maillard reaction and/or Strecker degradation.

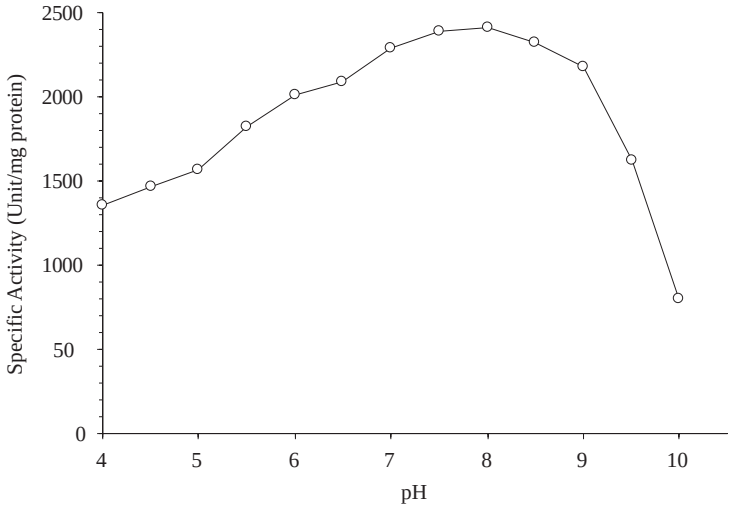

Figure 2. Effect of pH on quince PPO activity

The optimum temperature for the maximum PPO activity with catechol substrate at pH 8.0 was around 40°C (Figure 3). This parameter for cocoa bean and burdock PPO was 45°C and 60°C, respectively.

Quince PPO lost about 20% of the original activity at 60°C and about 65% of it at 70°C for 30 min. (Figure 4). These enzymatic properties were similar to those from the palmito[11], pear[9], cocoa bean[12] and burdock[7]. At temperatures >60°C, as expected, the rate of inactivation was greater with increasing temperature. However, the enzyme showed relatively high stability. It can be seen that quince PPO is relatively thermostable.

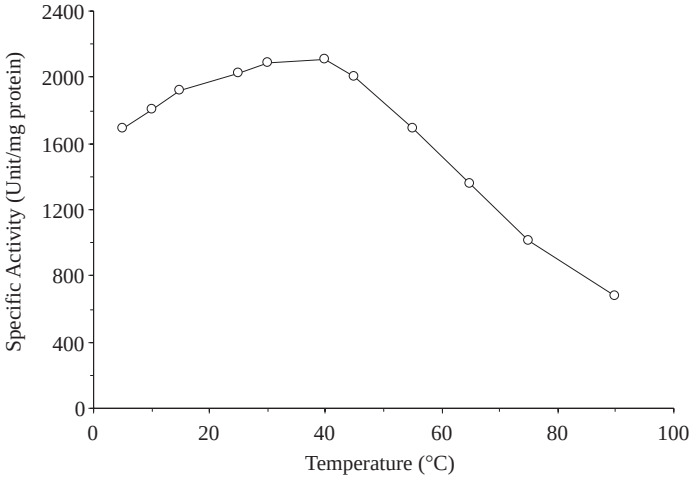

Figure 3. Effect of temperature on quince PPO activity

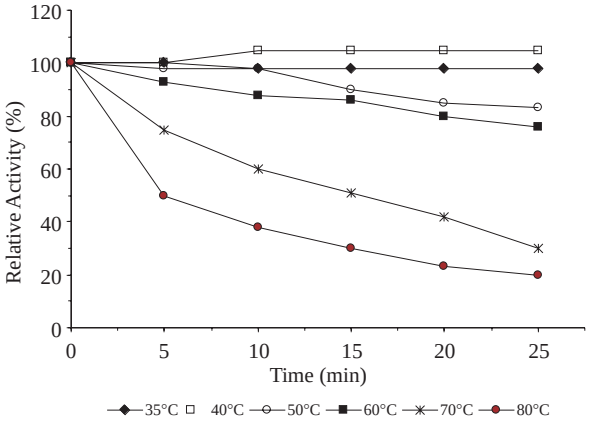

Figure 4. Thermal stability profile of quince PPO. The enzyme solution was incubated for various time intervals (5-25 min) at the specified temperature (25-80°C) and rapidly cooled. The activity measured at 25°C was taken as 100% and activities measured at 35-80°C were compared with the activity measured at 25°C.

Effect of inhibitors

The effects of various compounds on the catecholase activity of quince PPO are listed in Table 3. It was markedly inhibited by potassium cyanide, L-cysteine, ascorbic acid and sodium bisulphite. Catecholase activity was also inhibited by EDTA, thioacetamid, urea and citric acid but the inhibitory effects were less affected. Table 3 shows that potassium cyanide, L-cysteine, ascorbic acid and sodium bisulphite had very high inhibition % values and are thus strong inhibitors of the enzyme. According to Mayer *et al.* and Lee

et al. these inhibitors might inhibit the enzyme reaction by reacting with the enzyme molecule. Potassium cyanide might interact with the cofactor of the enzyme as a chelating agent. Dehydroascorbic acid, the oxidation product of ascorbic acid, can react with amino groups in close proximitiy to the active site(s) of the enzyme through Strecker degradation. Cysteine and sodium bisulphite, on the other hand, may react directly with sulfhydryl groups with the reduction of o-quinone[3,12].

Table 3. Effect of inhibitors on quince PPO activity

	% Inhibition	
Inhibitors	2 mM	20 mM
Citric acid	9	23
Cysteine	99	100
Ascorbic acid	98	100
EDTA	15	50
Sodiummetabisulphite	52	98
Urea	-	39
Potassium cyanide	100	100
Thioacetamid	35	91

L-cysteine, which is a naturally occurring aminoacid and is non-toxic, is used the food technology, like ascorbic acid. Thus these two compounds may be useful in preventing enzymatic browning of quince products.

Conclusion

Quince PPO is active towards o-diphenols only. The enzyme maximum activity had a pH optimum at 8.0 and the temperature optimum was 40°C when catechol was used as the substrate. Ascorbic acid and cysteine can be used in quince products. The Michaelis constant K_m for catechol was 4.5 mM. Enzyme activity was inhibited by ascorbic acid, cysteine and potassium cyanide.

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