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Characterization of Polyphenol Oxidase from Jerusalem Artichoke (Helianthus tuberosus)

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Polyphenol oxidases (PPO) in Jerusalem arthichoke (*Helianthus tuberosus*) skin and flesh were extracted and purified through $(NH_4)_2SO_4$ precipitation, dialysis and gel filtration chromatography. The samples obtained from ammonium sulfate precipitation and dialysis were used for the characterization of crude skin and flesh PPO. Optimum pH values were 7.5 for skin PPO and 8.0 for flesh PPO with 50 mM catechol. The optimum temperatures for skin and flesh PPO were 25 °C and 30 °C respectively with catechol. Six inhibitors were tested in the study and the most effective inhibitors were found to be sodium azide and thiourea for both flesh and skin PPO. Km and V_{max} values were 5.09 mM and 363.6 unit/min.ml for skin PPO and 4.03 mM and 714.2 unit/min.ml for flesh PPO respectively with 50 ml catechol substrate. Thermal inactivation data indicated that apparent activation energies with catechol substrate were 29.34 kcal/mol for skin PPO and 42.56 kcal/mol for flesh PPO.

Introduction

Polyphenoloxidase (monophenol, dihidroxyphenylalanine, oxygen oxidoreductase, E.C. 1.14.18.1) catalyzes two distinct reactions involving molecular oxygen, namely 1) the o-hydroxylation of monophenols to o-diphenols, or cresolase activity; and 2) the subsequent oxidation of o-diphenols to o-quinones, or catecholase activity. The catalytic action of PPO is connected to undesirable browning and off-flavor generation in stored and processed foods of plant origin[1]. In this respect, the extensive discoloration encountered during the processing of artichoke tubers for the production of inulin hydrolyzates has been linked to the presence of a highly active PPO system[2].

Jerusalem artichoke (*Helianthus tuberosus* L.), which is recognized as a good source of stored inulin (fructan), has been reported to exhibit discoloration reactions during processing[3]. Jerusalem artichoke tubers contain 75-85% (dry weight) inulin and related fructans (2→1 β- D- fructofranans) as storage polysaccharides, which are recognized as a potential source of fructose. Inulin is a dietary fiber, the consumption of which confers a number of health advantages[4]. It is not digested by humans and as a consequence has tremendous utility as a bulking agent in low calorie formulated foods. The Jerusalem artichoke is an excellent crop for inulin production and the United States, Russia and some European countries use it in their food and pharmaceutical industries as a raw material because of its valuable properties.

The enzyme PPO in the Jerusalem artichoke has been studied by Zawistowski et al.[5,6], McLaurin et al.[7], Gunnarson et al.[8], and Somda et al.[9]. In order to control the action of PPO, it is important to know the characteristics of the enzyme. The purpose of this study was to isolate, purify and characterize the PPO enzyme from a Jerusalem artichoke's skin and flesh. The characterization of the PPO enzyme could help in the developmment of more effective methods of controlling browning in the Jerusalem artichoke.

Experimental

Materials The Jerusalem artichoke used as research material was harvested at maturation in Ankara, Turkey. It was stored at 4 °C for 2 weeks after harvest. Most chemicals were obtained from Sigma Co., 4-methyl catechol was purchased from Fluka, while catechol, pyrogallol and gallic acid were obtained from Merck. Ammonium sulfate (especially low in heavy metals, for enzyme work) was also purchased from Sigma.

Enzyme Extraction Enzyme extraction was carried out at 4 °C and the reagents and equipment were cooled to that temperature before use. One hundred gram batches of the Jerusalem artichoke were washed with deionized water and then peeled with a stainles steel knife. The skin and flesh were separated. The flesh was cut into 1 cm cubes. The skin was studied separately from the flesh. Each part was blended with 300 ml phosphate buffer (pH 7.2) in the presence of 10% polyethylene glycol (PEG) and 50 mM ascorbic acid by using a Waring blender for 2 min. The homogenate was filtered through cheese cloth and the filtrate was centrifuged at 30,000 x g for 35 min at 4 °C. It was named crude extract. The supernatant was brought to 80% saturation with solid $(NH_4)_2SO_4$. The precipitated PPO was separated by centrifugation at 50,000 x g for 40 min. The precipitate was dissolved in small portions of phosphate buffer pH 7.2 and dialyzed at 4 °C in cellulose dialysis tubing (Sigma Chemical Co. mol. wt. cut of 12000-14000 daltons) for 18 h with three changes of the buffer. The dialyzed samples were kept in stoppered test tubes at -20 °C and were used as the PPO enzyme source in the following experiments. The dialyzed aliquots were fractioned by gel filtration. The protein concentration of the samples at different stages of purification was determined by the method of Bradford[10].

Gel Filtration In the gel filtration chromatography, a column (1.0 x 60.0 cm) was prepared using Sephadex G-100 (Sigma, dry bead diameter 20-50 μm) with 0.2 mM phosphate buffer containing 10% glycerin. The dialyzed skin and flesh PPO solutions were passed through the column separately. The elution rates were adjusted to 0.5 ml/min. The eluates were collected in 4 ml volumes in tubes by a fraction collector. Every 4 ml fraction was examined at 280 nm for protein content and assayed for enzyme activity under standard conditions. The obtained values were graphed against the tube number. The fractions having PPO activity were pooled and dialyzed against 0.2 mM phosphate buffer at 4 °C for 8 h. To determine specific activity, PPO activities and quantitative protein measurements were carried out before and after purification.

PPO Activity Measurement PPO activity was determined by measuring the increase in absorbance at 420 nm for 2 min by means of a spectrophotometer (Jenway 6105 UV/vis.). Dialyzed stock enzyme solution was diluted 1/10 (v/v) with phosphate buffer. The sample cuvette contained 2.8 ml substrate in phosphate buffer and 0.2 ml enzyme solution. The blank sample contained only 3 ml substrate solution in phosphate buffer. Enzyme activity was calculated from the linear portion of the curve, which was DA_{420} nm/min due to the oxidation of substrates[11]. One unit of PPO activity was defined as the amount of enzyme that caused

an increase in absorbance of 0.001/min at room temperature. The mean values are of three determinations and the relative standard deviations are less than ±1%.

Characterization of Jerusalem Artichoke PPO

The crude enzyme samples obtained from ammonium sulfate precipitation and dialysis were used in the characterization of PPO.

Substrate Specificity Nine different commercial grade substrates (catechol, 4-methyl catechol, D-tyrosine, L-dopa, pyrogallol, caffeic acid, chlorogenic acid, p-cresol, and gallic acid) were used to study their specificity at concentrations of 50 mM except D-tyrosine and caffeic acid (2 mM) due to their limited solubilities.

Effect of Enzyme Concentration The effect of enzyme concentration on browning was determined with 50 mM catechol as substrate. The enzyme concentration ranged from 1 to 22 ml/50 ml reaction mixture. For smaller concentrations of the enzyme, the stock enzyme solution was diluted with 0.2 mM phosphate buffer. The activities were plotted against enzyme concentration.

Effect of Hydrogen Ion Concentration The rate of catechol oxidation by polyphenol oxidase was studied in the pH range 4.0 - 9.2 and the concentration of catechol was 50 mM. Appropriate buffers (0.1 mM citrate for pH 4.0 - 5.5, 0.2 mM phosphate 5.5 - 7.0 and 0.05 mM tris-HCl for pH 7.0-9.2) were used for determining the optimum pH of PPO. The optimum pH value obtained from this assay was used in all other experiments.

Effect of Temperature For determining optimum temperature values of the enzyme, PPO activity was measured at different temperatures in the range of 10 °C-80 °C using 50 mM catechol as substrate.

Heat Stability For the heat treatment studies 10 ml enzyme solution was pipetted into a test tube in a water bath. After each heating interval, 0.5 ml of the sample was withdrawn immediately in crushed ice and then assayed for the remaining activity of PPO. The denaturation property of the enzyme was determined by measuring PPO activity at 75 °C for 60 min at (2, 10, 15, 30, 45, and 60 min) intervals using 50 mM catechol as substrate.

Activation Energy Activation energy studies of PPO enzyme action on 50 mM catechol were run at 30, 50, 60 and 80 °C. When the reaction rate log values were plotted versus the reciprocal of absolute temperature, the activation energy was calculated from the slope. The Arrhenius equation was used for the determination of activation energy.

Enzyme Kinetics To determine the Michaelis constant (Km) and maximum velocity (V_{max}) values of the enzyme, PPO activites were measured with catechol at varying concentrations (0.8, 1.0, 1.25, 1.5, 2.4 and 10 mM) in optimum conditions of pH and temperature. Km and V_{max} values of PPO were calculated from a plot of 1/V vs. 1/[S] by the method of Lineweaver and Burk[12].

Effect of Inhibitors The inhibitor effects on PPO activity were determined by using L-cysteine, L-ascorbic acid, and sodium azide, sodium diethyl dithiocarbamate (DETC), citric acid, and thiourea. Each inhibitor was prepared in 0.2 M phosphate buffer pH 7.2. Inhibition of PPO activity was tested in a reaction mixture (3 ml) consisting of 50 mM (2.5 ml) catechol, 0.3 ml inhibition solution and 0.2 ml enzyme solution in the same buffer.

Results and Discussion

Extraction and Purification The extraction of PPO was carried out in 0.2 mM phosphate buffer (pH 7.2) containing 10% PEG and 50 mM ascorbic acid, and then precipitated by the $(NH_4)_2SO_4$ method. Several precipitations with solid ammonium sulfate between 20 and 80% were tested to find the suitable saturation point. As a result, the PPO activity of the precipitate of 80% $(NH_4)_2SO_4$ saturation was found to be the highest and this saturation point was used for all extraction processes. Polyethylene glycol was used to bind the phenolic compounds that may inactivate the PPO during extraction. It is well known that the oxidation of plant phenolics in the presence of PPO produces quinones that can inhibit many enzymes[13], including polyphenol oxidase[14]. Badran and Jones[15] and Arakji and Yang[16] reported that PEG was more effective as a phenolic binding agent than polyvinylpyrrolidone for extracting PPO from green banana and cranberry. Ascorbic acid was also used to reduce quinones to phenolic substrates during extraction. After ammonium sulfate precipitation the dialyzed enzyme extract was used for the purification and characterization of the PPO. The results for the purification of the PPO are shown in Table 1. The elution profile of PPO from Sephadex G-100 revealed two protein fractions for flesh and three protein fractions for skin (Figure 1). The purification degrees of skin PPO and flesh PPO were 18.0-12.1 fold after gel filtration, respectively.

Table 1. Purification of PPO from Jerusalem artichoke.

Purification	Volume (ml)	Activity (unit/ml)	Total activity (unit)	Protein (mg/ml)	Total Protein (mg)	Specific Activity (unit/mg)	Recovery	Purification (n-fold)
Skin (47 g)								
Crude extract	180	112.8	20304.0	0.678	122.04	166.37	100.0	0
$(NH_4)_2SO_4$ precipitation and dialysis	72	280.7	20210.4	0.498	35.85	563.65	99.5	3.4
Results of Sephadex G-100	30	588.4	17652.0	0.197	5.91	2986.80	86.9	18.0
Flesh (92 g)								
Crude extract	300	197.2	59160.0	0.818	245.4	241.07	100	0
$(NH_4)_2SO_4$ precipication and dialysis	102	424.6	43309.2	0.589	60.08	720.88	73.2	2.3
Results of Sephadex G-100	45	820.7	36931.5	0.281	12.64	2920.64	62.4	12.1

Characterization of PPO

Substrate specificity A number of monohydroxy, dihydroxy and trihydroxy phenols were used to test substrate specificity (Table 2). The skin and flesh enzymes showed activity toward the ortho-diphenols (catecholase activity). A low activity for trihydroxy phenols like pyrogallol and gallic acid was detected. The enzyme from the flesh showed higher activity on ortho-diphenols than the skin PPO. In addition, both skin and flesh enzymes showed high activity on catechol. Since catechol resulted in the highest level of enzyme activity, it was used as the standard for comparison. The number of hydroxyl groups and their position in the benzene ring of the substrate affected oxidase activity. Mason[17] suggested that cresolase and catecholase

differed in substrate specificity, while Oktay et al.[18] noted that catechol and 4-methylcatechol exibited a very high affinity for Amasya PPO. Tate et al.[19] also reported very high activity toward o-diphenolic substrates for Bartlet pear PPO. Cash et al.[20], found only catecholase activity with Concord grape PPO and no activity when monohydroxy phenols were used as substrates. Zawistowsky et al.[2], demonstrated that the PPO enzyme in the Jerusalem artichoke functions primarily as an o-diphenol oxidase, but also has low ability to hydroxylate monophenols. The evidence indicates that the PPO enzyme systems of all plants are primarily specific for ortho-diphenolic substrates.

Table 2. Substrate specificity of Jerusalem artichoke PPO

Substrate	Concentration (mM)	Type of Configuration	Relative Activity	
			Skin	Flesh
Catechol	50	Ortho-dihydroxy	100.0	100.0
4-methylcatechol	50	Ortho-dihydroxy	78.7	87.7
Chlorogenic acid	50	Ortho-dihydroxy	59.1	89.5
Caffeic acid	2	Ortho-dihydroxy	65.8	78.6
Gallic acid	50	Trihydroxy	6.8	11.9
Pyrogallol	50	Trihydroxy	18.5	10.1
p-cresol	50	Monohydroxy	1.4	22.4
p-tyrosine	2	Monohydroxy	1.7	21.1

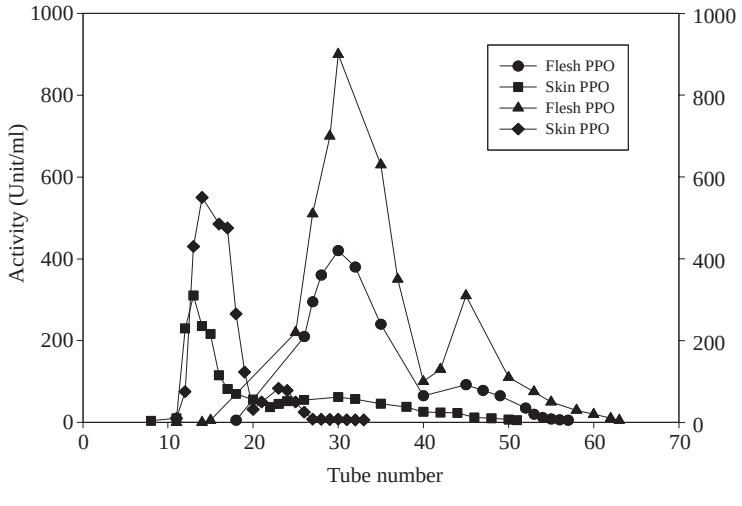

Figure 1. Elution profile of flesh and skin PPO: ▲ Flesh PPO, ♦ Skin PPO. PPO Activity of flesh and skin PPO: ● Flesh PPO, ■ Skin PPO.

Effect of Enzyme Concentration The rate of the PPO catalyzed reaction was proportional to the enzyme concentration. If a higher concentration of the enzyme was used, the activation of the enzyme might be decreased because of the inhibiting effect of ortho-quinones[21].

Effect of Hydrogen Ion Concentration PPO activities were measured in the pH range 4.0–9.2 with 50 mM catechol as substrate. Optimum pH values were 7.5 for skin PPO and 8.0 for flesh PPO. In general, most plant PPO showed maximum activity at about neutral pH values[22–24] (Figure 2). Şakiroğlu et al.[25] found the optimum pH of the dog-rose fruit to be 8.5 for catechol. The optimum pH of fruits may vary depending on maturity, variety and horticultural factors.

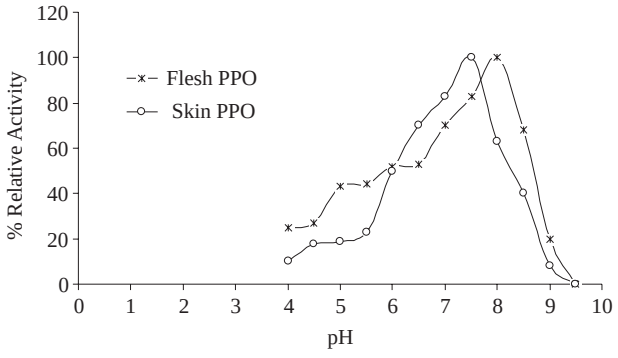

Figure 2. pH optima of skin and flesh PPO.

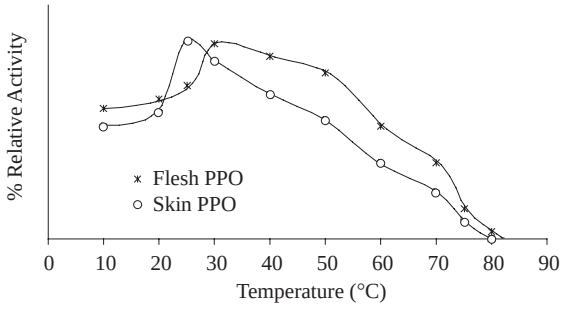

Figure 3. Temperature optima of skin and flesh PPO.

Effect of Temperature Optimum temperatures were 25 °C and 30 °C for skin and flesh PPO respectively, using catechol as substrate (Figure 3). Above 40 °C the activity declined as the temperature increased, but the enzyme was not completely inactivated even at 75 °C for flesh and skin PPO. Cash et al.[20], and Nakamura et al.[26], reported optimum temperatures of 25 °C and 30 °C respectively for grape PPO.

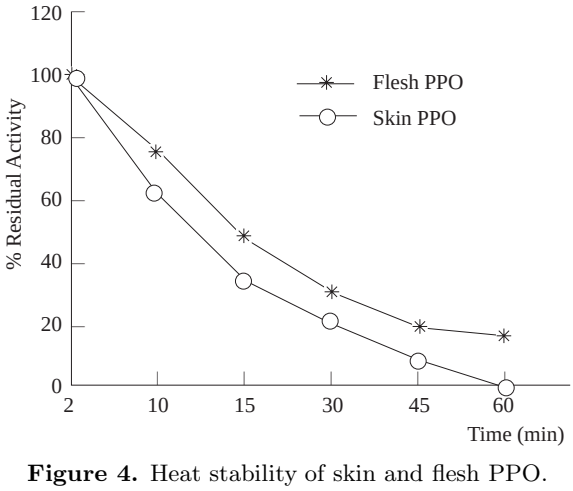

Figure 4. Heat stability of skin and flesh PPO.

Heat Stability As seen in Figure 4, skin PPO was less heat stable than flesh PPO. An 80% decrease in flesh PPO activity was found after heating at 75 °C for 60 min. However, the skin PPO enzyme was completely inactivated after 60 min. The PPOs of Clingstone peaches[11], Royal Ann cherries[27] and Bartlett pears[28] are also characterized by a relatively high stability to heat.

Activation Energy The activation energy for denaturation of the enzyme was determined by an Arrhenius plot of the log reaction rate (ln k) vs. the reciprocal of the absolute temperature. The activation energy of flesh PPO was 42.56 kcal/mol and that of skin PPO 29.34 kcal/mol. Zawistowski et al.[2] determined that the (E_a) for the thermal inactivation of Jerusalem arthichoke PPO was 54.0 kcal/mol. Artichoke PPO should be considered a relatively thermostable enzyme.

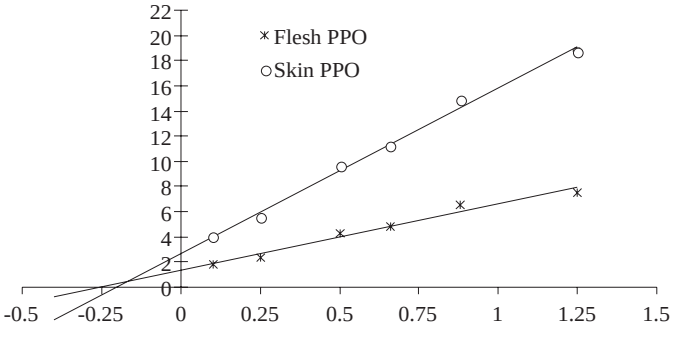

Figure 5. Lineweaver and Burk plot for skin and flesh PPO.

Enzyme Kinetics K_m and V_{max} values calculated from Lineweaver-Burk graphs are shown in Figure 5. As seen in Figure 3, K_m values were 5.09 mM for skin PPO and 4.03 mM for flesh PPO. Oktay et al.[18] have reported a K_m of 4.6 mM for the Amasya apple. Jen and Kahler[29] also found a K_m value of 29.0 mM for peach. Zawistowski et al.[6] have reported a K_m of 3.9 mM for catechol substrate in Jerusalem arthichoke tubers. The maximum reaction velocity (V_{max}) values were 714.2 unit/min.ml for flesh PPO and 363.6 unit/min.ml for skin PPO.

Table 3. Effect of inhibitor on PPO (% activity remaining after addition of inhibitor)

Inhibitor	Flesh PPO	Skin PPO
L-cysteine	100	53
L-ascorbic acid	85	44
Sodium azide	17	11
Sodium diethyl dithiocarbamate	25	19
Thiourea	9	8
Citric acid	89	50

Effect of inhibitors Inhibition study experiments were devised to determine the effect of six types of inhibitors on PPO activity, the enzyme which catalyzes the browning of Jerusalem artichoke tissue. Enzymatic browning of the plants may be retarded or eliminated by removing reactants such as oxygen and phenolic compounds or by the use of PPO inhibitors. There was a significant decrease in flesh and skin PPO activities caused by sodium azide and thiourea (Table 3). The inhibitory action of DETC implies that artichoke PPO most likely is a metalloprotein, as this inhibitor has been shown to complex the copper prosthetic group of PPO from a variety of plant sources[11,27,30]. Knapp[31] reported that DETC and 1-phenyl 2-thiourea were inhibitors of Lula avocado PPO. These inhibitors are copper chelating agents and they suppress browning activities, thus supporting theories that copper is directly involved in the oxidation of phenolic compounds[32].

Effect of storage on stability For stability testing, PPO enzymes were kept in small erlenmayer flasks at 4 °C and 20 °C for 32 days. Samples were taken each day to determine the loss of PPO activity

over time as shown in Figure 6. PPO activity decreased slowly as storage was prolonged. At 4 °C PPO activities for flesh and skin appeared to be stable over a 4-day period before activity began to decline slowly.

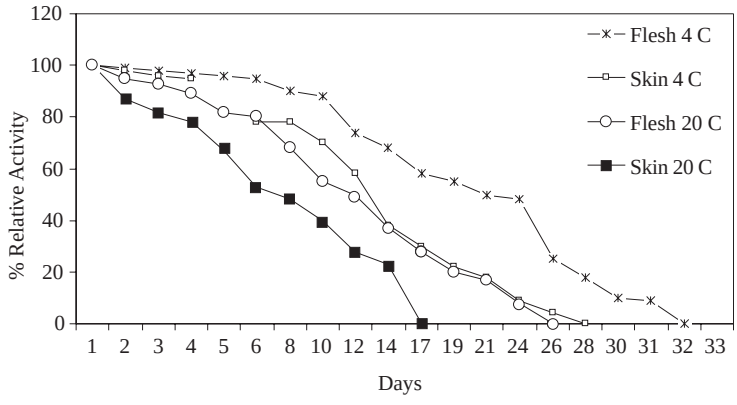

Figure 6. Stability of flesh and skin PPO during storag.

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