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Study and Characterization of Polyphenol Oxidase from Eggplant (*Solanum melongena* L.)

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ABSTRACT: In this study the catecholase and cresolase activities of eggplant polyphenol oxidase (PPO) were investigated. Enzyme activity was determined by measuring the increase in absorbance using catechol as substrate and 3-methyl-2-benzothiazolinone hydrazone (MBTH) as coupled reagent. The effects of substrate specificity, heat inactivation, temperature, pH, and inhibitors were investigated to understand the enzymatic alteration of ready-to-eat preparations. Browning of vegetables was determined through a colorimeter. Decrease of lightness (L^*) and increase of color difference values (ΔE^*) were correlated with tissue browning. Antibrowning agents were tested on PPO under the same conditions. The enzyme activity was strongly inhibited by 0.4 M citric acid. Under natural pH conditions, the enzyme was also inhibited by tartaric acid and acetic acid. All of the results were used to understand the best conditions for food transformation (ready-to-eat and grilled eggplant slices).

KEYWORDS: polyphenol oxidase, antibrowning, food technologies, ready-to-eat

INTRODUCTION

Browning of raw fruits, vegetables, and beverages is a major problem in the food industry and is believed to be one of the main causes of quality loss during postharvest handling and processing.[1] The mechanism of browning in foods is well characterized and can be enzymatic or nonenzymatic in origin. Enzymatic browning is catalyzed by the enzyme polyphenol oxidase (PPO; 1,2-benzenediol; oxygen oxidoreductase, EC 1.10.3.1), which is also referred to as phenol oxidase, phenolase, monophenol oxidase, diphenol oxidase, and tyrosinase. Enzyme-catalyzed browning reactions[2−4] involve the oxidation of phenolic compounds by the enzyme PPO to quinones, followed by transformation of the quinones to dark pigments. These result in deterioration of flavor, color, and nutritional quality and continue during postharvest handling and marketing of vegetables. Control of browning in fruits and vegetables hinges upon an understanding of the mechanism responsible for browning in fruits and vegetables, the properties of polyphenol oxidase enzymes, their substrates, and inhibitors, and the chemical, biological, and physical factors that affect each of these parameters. Once understood, these mechanisms may be applied in either preventing the browning reaction, or slowing its rate, thus extending the shelf life of the produce. Polyphenol oxidases were first discovered in mushrooms and are widely distributed in nature.[5,6] They appear to reside in the plastids and chloroplasts of plants, although freely existing in the cytoplasm of senescing or ripening plants.[7,8] Cloning and sequencing studies of the copper A binding region of these enzymes shows high conservation between polyphenol oxidases from plants, microorganisms, and animals. Polyphenol oxidase is thought to play an important role in the resistance of plants to microbial and viral infections and to adverse climatic conditions.[9] Phenolics, such as chlorogenic acid, caffeic acid, etc., which are substrates of this enzyme, have been shown to exhibit fungicidal properties. Plants, which exhibit comparably high resistance to climatic stress, have been shown to possess relatively higher polyphenol oxidase levels than susceptible varieties. Other enzyme systems in plants, such as chitinase, peroxidase, lipoxygenase, phenylalanine ammonia-lyase, and β-1,3-glucanase, also show increased activity when subjected to stress. It should be pointed out that the responses of enzymes to stress and infection are dependent on a number of factors, one of which is the host plant itself.[10]

The aim of this work was to identify the better cultivar eggplants suitable for the food industry using an innovative approach on PPO study and characterization to further increase the knowledge and integrate results of previous works.[11−13]

MATERIALS AND METHODS

Plant Material. The fruits analyzed were harvested from an ad hoc experimental field established at the Agricultural Experimental Farm of the University of Catania selecting from the eggplant DOFATA collection the most traditional landraces utilized in the eastern Sicily production areas, different for fruit shape and size. In particular, we focused our study on three landraces (Rotonda Violetta, Vetriola Catania, and Vetriola Adrano) actively cultivated in the Plain of Milazzo, the former characterized by its round shape and dark purple color, and in the villages around Mount Etna and in the city of Catania, the latter two landraces, respectively, characterized by oblong, light violet colored fruits. The three commercial cultivars compared with the landraces were Slim Jim, Talina F1, and Black Bell F1. The first differed from the others by its elongated purple fruit of small size, the second by its elongated fruit type and dark violet color, and finally the third by its large oval dark purple colored fruit, largely growing in greenhouses along the southeastern coast of Sicily.

The experimental field was sown in the middle of July by placing plants in single rows at a plant density of 0.5 plant m^{-2}. The crop was supported by conventional agro-techniques to satisfy the ordinary water

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Table 1. K_m and V_{max} Calculated with Mono- and Diphenol Substrates

substrate	PPO activity (μmol g^{-1} min^{-1})	V_{max}	K_m	V_{max}/K_m	selectivity
4-methylcatechol	303.474	5.596	2.122	2.637	3.194
catechol	195.504	2.455	2.973	0.826	1.000
chlorogenic acid	171.719	2.317	1.351	1.715	2.077
3,4-dihydroxyhydrocinnamic acid	141.888	5.452	2.122	2.569	3.111
caffeic acid	137.593	2.826	6.149	0.460	0.557
3,4-dyhydroxyphenylacetic acid	125.700				
3,4-dihydroxybenzoic acid	4.057				
4-propylphenol	1.425				
4-hydroxybenzoic acid	0.898				
4-hydroxyphenylacetic acid	0.051				
4-methylphenol	0.023				
p-coumaric acid	0.010				
3,4-hydroxyphenylpropionic acid	0.001				

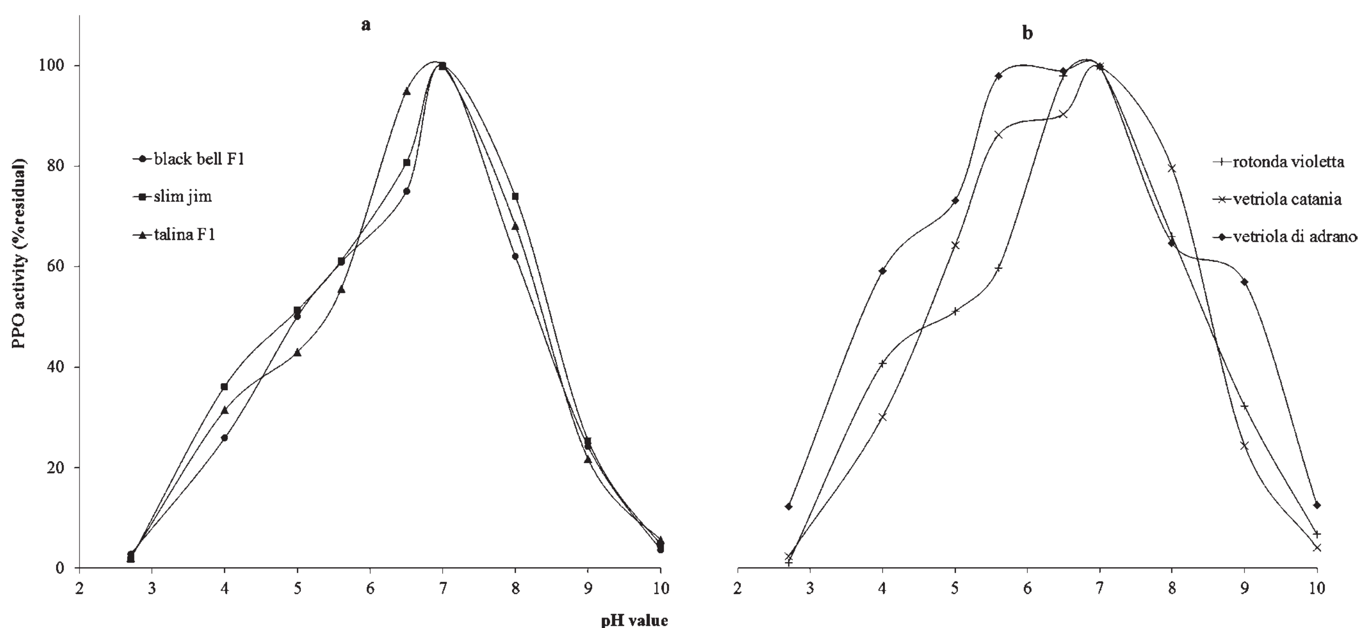

Figure 1. Optimum pH value of eggplant PPO activity for each cultivar with catechol as substrate. The assay temperature was 20 °C, and the reaction time was 10 min.

and mineral requirements during the growth cycle. The plants were transplanted in a randomized block experimental design; we used five plants per cultivar replicated four times. The fruits were harvested at the fully ripe phase, between the 65th and 75th days after the first transplanting and after the 85th and 95th days the second time, which permitted harvest of the second and fourth fruits or clusters.

Sample Preparation. The fruits were washed with tap water, dried, and stored at 4 °C until processing.

Reagents. Catechol, 4-methylcatechol, 3,4-dihydroxybenzoic acid, 3,4-dihydroxyphenylacetic acid, 3,4-dihydroxyhydrocinnamic acid, caffeic acid, 4-methylphenol, 4-hydroxybenzoic acid, *p*-hydroxyphenylacetic acid, 4-propylphenol, *p*-hydroxyphenylpropionic acid, *p*-coumaric acid, and chlorogenic acid were obtained from Sigma Chemical Co. (Milan, Italy). All other chemicals used were of analytical grade.

PPO Extraction and Assay. PPO extraction was carried out according to the methods of Pifferi[14] and Espin.[15] Pulp eggplant (150.0 g) was homogenized in 100 mL of 0.1 M phosphate buffer (pH 7.0); 20.0 g of homogenate and 140.0 mL of 0.1 M phosphate buffer (pH 7.0) were stored overnight at 4.0 ± 0.5 °C. The homogenate was filtered by two layers of cheesecloth, and then the filtered material was centrifuged at 14000*g* for 15 min at 4.0 °C. The supernatant was ultrafiltered (Millipore, 50 kDa cutoff) and its volume determined for enzymatic activity assay.

Enzyme activity was determined spectrophotometrically by measuring the increase in absorbance at 505 nm. One unit of PPO activity was defined as the amount of enzyme that causes an increase in absorbance of 0.001 min^{-1} mL^{-1}. PPO activity was assayed at 20.0 ± 0.5 °C in triplicate measurements.

Protein concentration was determined according to the dye-binding method of Bradford,[16] with bovine serum albumin as standard.

Substrate Specificity and Enzyme Kinetics. The Michaelis–Menten constant (K_m) and maximum reaction velocity (V_{max}) were determined using six substrates (3,4-dihydroxyphenylacetic acid, 3,4-dihydroxyhydrocinnamic acid, 4-methylcatechol, caffeic acid, catechol, chlorogenic acid) 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.[17]

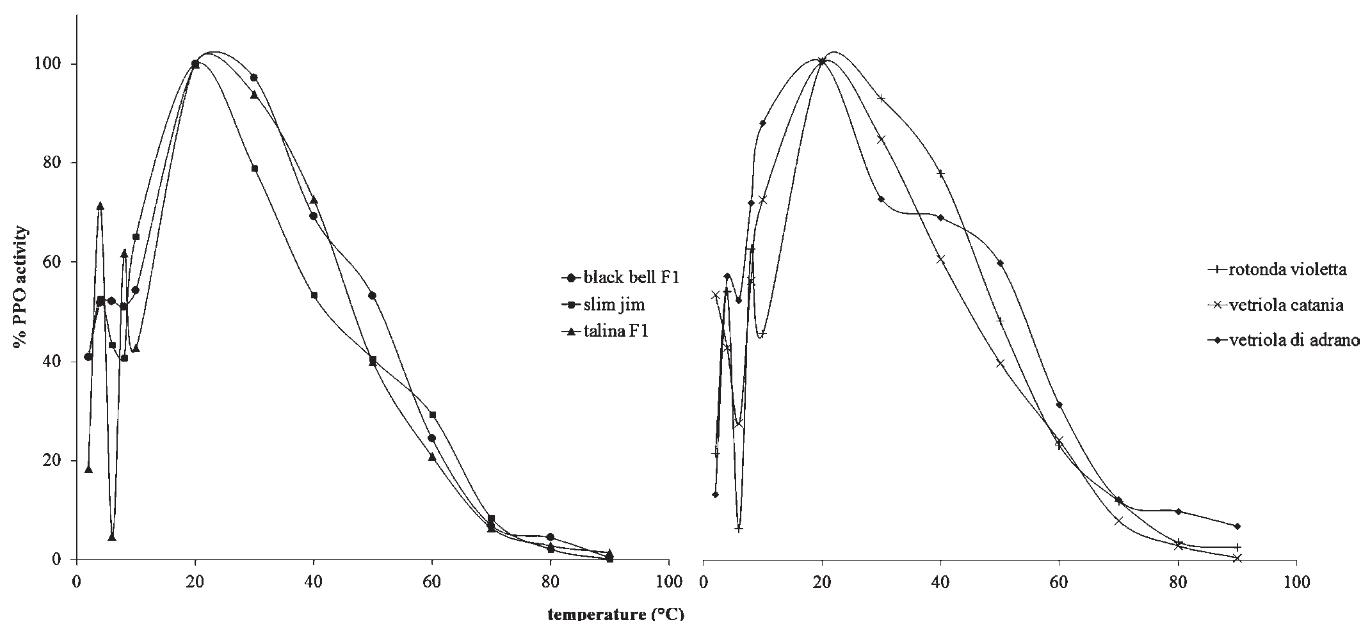

Figure 2. Optimum temperature of all cultivars at pH 5.70 and reaction time 10 min.

Optimum pH and Stability. The activity of the enzyme was determined over a pH range of 2.7−9.0 by in 0.1 M acetate (pH 2.7−6.0) and 0.1 M phosphate (pH 6.0−9.0) buffer adjusted with 0.1 M NaOH or 0.1 M HCl, using catechol as substrate. pH stability was determined by incubating the enzyme in 0.1 M acetate (pH 3.0−6.0) and 0.1 M phosphate (pH 6.0−9.0) buffer for 30, 60, and 120 min at 20.0 °C, using catechol as substrate.

Optimum Temperature and Stability. PPO activity was assayed at various reaction-controlled temperatures by a circulation water bath; the temperature was varied over the range of 4.0−70.0 ± 0.1 °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 (30.0−80.0 ± 0.1 °C), the enzyme solution was rapidly cooled in ice and the remaining activity was assayed in 0.1 M phosphate buffer (pH 5.7) at 20.0 ± 0.5 °C.

Inhibitor Tests. Acetic acid, citric acid, and tartaric acid, at five different concentrations, were dissolved in the assay medium, and PPO activity was measured at 20.0 °C to determine their inhibitor effects by using different concentrations of the inhibitor.

Physicochemical Properties of Samples. Titratable acidity (TA) was quantified by titrating 5.0 g of homogenized sample with 0.1 N NaOH to an end point of pH 8.1 (Inolab pH/Oxi Level one 600 WTW) and expressed as grams of citric acid per 100 mL. The total soluble solid (TSS) content was measured with a digital refractometer (Abbe 1S, Milan, Italy) and expressed as °Brix at 20.0 °C. The pH was measured with the same equipment used for measuring TA. Color was determined with a compact tristimulus chromameter (Minolta CR-300, Ramsey, NJ) with an 8 mm ∅ viewing aperture: white plate reference (Y), 94.3; x, 0.3142; y, 0.3211; and C illuminant (CIE, 2° observer). Readings were expressed as L^*, a^*, and b^* parameters. Chroma $[(a^{*2} + b^{*2})1/2]$ and hue angle $[\tan - 1(b^*/a^*)]$ were calculated.

Statistical Analysis. All determinations were conducted at least three times. Analysis of variance (ANOVA) of the data was performed by the Statistical Analysis System (SAS version 9.0). Duncan's multiple-range test was employed to determine the statistical significance of the differences between the means ($p < 0.05$).

Determination of Total Phenolics. The amount of total phenolics extract was determinated with the Folin−Ciocalteu reagent using the method of Singleton and Rossi.[18] Results was expressed as milligrams of chlorogenic acid per 100 g of fresh weight material.

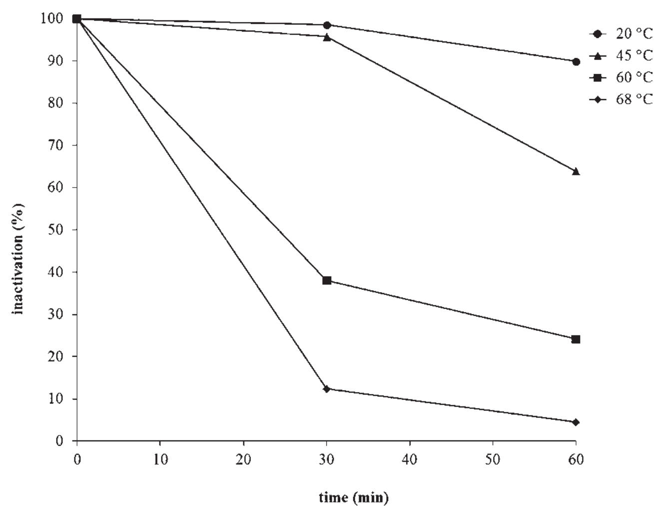

Figure 3. Heat inactivation of Black Bell F1 eggplant PPO at pH 5.70.

RESULTS AND DISCUSSION

Substrate Specificity and Enzyme Kinetics. PPO activity in partially purified extracts was examined with regard to its monophenolase and diphenol oxidase activities. The substrate specificity of the enzyme was investigated by using six chemicals (3,4-dihydroxyphenylacetic acid, 3,4-dihydroxyhydrocinnamic acid, 4-methylcatechol, caffeic acid, catechol, chlorogenic acid) as substrates. Eggplant PPO showed very low activity toward some diphenolic substrates (3,4-dihydroxybenzoic acid, 3,4-hydroxyphenylpropionic acid) and no activity versus monophenols such as 4-hydroxybenzoic acid, 4-hydroxyphenylacetic acid, 4-methylphenol, 4-propylphenol, and p-coumaric acid. Therefore, in this study catechol was used as substrate. The enzyme

Table 2. *D* and *Z* Parameters Calculated at 100 °C at Different pH Values, 4.23 and 5.66, in Different Cultivars

	heat treatment at 100 °C											
	pH 5.66						pH 4.23					
	Black Bell F1	Slim Jim	Talina F1	Rotonda Violetta	Vetriola Catania	Vetriola Adrano	Black Bell F1	Slim Jim	Talina F1	Rotonda Violetta	Vetriola Catania	Vetriola Adrano
PPO activity	15.5	28.4	5.3	3.2	10.7	1.5	5.09	11.81	1.71	1.38	4.17	1.04
D	8.3	9.5	22.7	26.3	5.2	19.9	0.8		0.9	1.1	10.7	1.9
Z	39.7	44.8	116.6	49.1	36.1	46.6	25.5		56.8	29.8	59.3	71.3
D value corrected[a]	83.2	174.7	77.4	55	36.1	19.9	3.8		1.5	1.5	42.9	1.9

[a] All relative values were calculated using cultivar BB as reference.

Table 3. Inhibitor Effect on PPO Activity with Organic Acids at Different Concentrations

		PPO activity decrease					
	M	Black Bell F1	Slim Jim	Talina F1	Rotonda Violetta	Vetriola Catania	Vetriola Adrano
DL-tartaric acid	0.00	100.00	100.00	100.00	100.00	100.00	100.00
	0.04	99.00	99.06	99.25	99.89	98.25	99.14
	0.11	45.01	48.62	49.85	48.11	48.84	47.62
	0.16	46.00	45.22	46.15	47.20	47.13	46.71
	0.21	48.93	46.23	47.00	49.85	48.31	48.53
	0.42	54.07	50.39	50.22	48.78	48.79	47.96
citric acid	0.00	100.00	100.00	100.00	100.00	100.00	100.00
	0.04	63.72	68.52	66.8	64.51	69.32	67.59
	0.11	32.82	31.89	30.99	33.00	32.25	31.35
	0.16	26.00	29.31	28.54	26.32	29.64	28.89
	0.21	22.87	27.70	27.00	23.15	28.01	27.31
	0.42	3.80	5.64	5.32	3.82	5.69	5.36
acetic acid	0.00	100.00	100.00	100.00	100.00	100.00	100.00
	0.04	85.91	84.23	82.16	83.74	84.65	83.81
	0.11	80.36	81.22	78.96	78.36	81.63	80.54
	0.16	78.00	77.23	75.23	76.00	77.21	76.76
	0.21	76.34	74.86	72.98	74.43	75.96	74.89
	0.42	30.84	28.13	27.45	30.07	28.36	28.01

is an *o*-diphenol oxidase as no cresolase activity was present. Michaelis constants (K_m) and maximum reaction velocities (V_{max}) were determined using these substrates at various concentrations (Table 1).

The V_{max}/K_m ratio is called the "catalytic power", and it is a better parameter to find the most effective substrate. On the basis of the ratio V_{max}/K_m, it can be said that 3,4-dihydroxyhydrocinnamic acid and methylcatechol are the most suitable substrates for eggplant PPO activity.

Optimum pH. PPO showed a clear pH optimum around 7.0 (Figure 1). It was possible to note a significant drop in activity around pH 4.0 for cultivar 'Black Bell F1' between commercial cultivars and 'Vetriola Catania' between landraces, which may be suitable for the food industry.

Optimum Temperature. The optimum temperature of activity for eggplant PPO was 20.0 °C, whereas at cold-storage temperatures (2.0−8.0 ± 0.5 °C) fluctuations in activity were seen (Figure 2). This common trend has been noted in another paper.[19]

Heat Inactivation. The thermal stability profile for eggplant PPO, presented in the form of the residual percentage activity, is shown in Figure 3. The enzyme activity decreased due to heat denaturation of the enzyme with increasing temperature and inactivation time. The drop in percentage residual activity at high temperatures is probably due to the unfolding of the tertiary structure of the enzyme. For instance, when the temperature was increased from 45 °C for 30 min to 68 °C for 30 min, the activity of PPO decreased from 95 to 12% with catechol as substrate; for 60 min of heating the change was from 64 to 5%, respectively. This indicated that the enzyme was rapidly denatured at higher temperatures.[20−24] To better understand the thermal technological properties of cultivars, *D* and *z* were calculated at 100 °C at two different pH values (Table 2). Because different cultivars showed different PPO levels, it was necessary to include the

contribution of PPO activity on *D* definition. 'Black Bell F1' showed the lowest absolute *D* value, so it was selected as reference to calculate all relative values according to the following formula:

$$D_{relative} = (D_{absolute}/PPO_{cultivar}) \times PPO_{BB}$$

Inhibition of PPO. In this study, inhibition of eggplant PPO by several organic acids[25] was investigated with catechol as substrate. Table 3 shows the decrease of PPO activity when the concentration of different acids tested increased; PPO activity decreased from 100% to ca. 40%. The prevention of enzymatic browning by a specific inhibitor may involve a single mechanism or may be the result of interplay of two or more mechanisms of inhibitor action.

Citric, tartaric, and acetic acid treatments had similar inhibitory effects on different cultivars; in particular, citric acid was the most successful in reducing the activity at high concentrations (about 95% reduction), whereas tartaric and acetic acid resulted in 50 and 20% activity reductions, respectively, at the same concentration.

The inhibitory action of citric acid on all cultivars was noted at low concentrations (0.11 M), whereas this effect on different cultivars was similar.

With regard to eggplants as raw material for minimal processing, it could be concluded that all cultivars seem to be suitable for minimal processing due to the low PPO activity. The characterization of the PPO extracted showed optimal conditions of catalysis and inhibition by organic acids. This information could be useful in the production of minimally processed eggplant slices. Citric acid was found to be the most suitable organic acid to increase the quality and shelf life of eggplant because it was effective at low concentration and because it is unlikely to affect sensory parameters and, at the same time, may increase the produce's nutritional value with low additional cost.

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