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Characterization of polyphenol oxidase from blueberry (*Vaccinium corymbosum* L.)

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ABSTRACT

Polyphenol oxidase (PPO) was extracted and characterized from high-bush blueberries. PPO showed an optimum activity at pH 6.1–6.3 and 35 °C, with the enzyme showing significant activity over a wide temperature range (25–60 °C). Catechol was the most readily oxidized substrate followed by 4-methylcatechol, DL-DOPA, and dopamine. Blueberry PPO showed a K_m of 15 mM and V_{max} of 2.57 ΔA_{420} nm/min $\times 10^{-1}$, determined with catechol. PPO was completely inactivated in 20 min at 85 °C, however, after 30 min at 75 °C it showed about 10% residual activity. Thermal treatment at 55 and 65 °C for 30 min resulted in the partial inactivation of PPO. Ascorbic acid, sodium diethyldithiocarbamic acid, L-cysteine, and sodium metabisulfite were effective inhibitors of PPO at 1.0 mM. Benzoic acid and cinnamic acid series inhibitors showed relatively weak inhibition of PPO (21.8–27.6%), even at as high as 2.0 mM concentration.

1. Introduction

Blueberry (genus *Vaccinium* L.) is a native American species, with its fruit having high contents of anthocyanins and polyphenolic compounds (Lee & Wrolstad, 2004; Skrede, Wrolstad, & Durst, 2000). The U.S. is the leading blueberry producer in the world, with about three-fifth of the current world production, which has increased three-fold since 1990 (FAO, 2015). Approximately 60% of the crop is sold as fresh and the remainder goes for processing, mainly frozen. Other processed forms are dehydrated blueberries and drum-dried powder (USDA, 2015). Interest in the role of antioxidants in human health has prompted research interventions to best retain fruit antioxidants, such as polyphenols, during postharvest storage and value-added processing. Enzymatic browning reactions use phenolic compounds that subsequently result in color degradation of processed products from pigment-rich fruits, and are recognized as serious quality issues (Mayer, 2006). This warrants a need to investigate the properties of blueberry polyphenol oxidase (PPO) and methods to inactivate or inhibit this enzyme during processing.

The PPO (monophenol, dihydroxyphenylalanine: oxygen oxidoreductase; E.C. 1.14.18.1.), naturally distributed in plants, is a major contributor of enzymatic browning or color degradation in fruits and vegetables during postharvest processing operations (Fang, Zhang, Sun, & Sun, 2007; Mayer, 2006; Mishra, Gautam, & Sharma 2013). In the presence of oxygen, PPO catalyzes two types of reactions: hydroxylation of monophenols to *o*-diphenols and oxidation of *o*-diphenols to *o*-quinones representing monophenolase (cresolase) and diphenolase (catecholase) activity, respectively (Mayer, 2006; Pilar-Cano, de Ancos, & Lobo, 1995; Yoruk & Marshall, 2003). The PPO-induced browning reaction, which is triggered by the disruption of intact tissue during postharvest handling and processing, occurs due to the conversion of phenolics in plant tissue to highly reactive quinones. After undergoing polymerization and condensation reactions between proteins and polyphenols, the quinones thus formed lead to the formation of brown pigments to impart discoloration (Mayer, 2006; Gonzalez, de Ancos, & Pilar-Cano, 2000). These reactions lead to changes in physical and chemical characteristics of proteins, which, in turn, negatively impacts the quality of processed food. Vamos-Vigyazo (1981) reported that this enzyme besides negatively impacting the sensory attributes, also affects the marketability of a product by often lowering the nutrient content, too.

Given its implications in postharvest and processed fruit quality, PPO has been studied in a wide variety of fruits: apples (Chow, Louarme, Bonazzi, Nicolas, & Billaud, 2011), banana (Unal, 2007), bayberry (Fang et al., 2007), blackberry (Gonzalez et al., 2000), gooseberry (Bravo & Osorio, 2016), mulberry (Arslan,

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Erzengin, Sinan, & Ozensoy, 2004), raspberry (Gonzalez, de Ancos, & Pilar-Cano, 1999), litchi (Ruenroengklin, Sun, Shi, Xue, & Jiang, 2009), papaya (Pilar-Cano et al., 1995), pears (Siddiq & Cash, 2000), and plums (Siddiq, Sinha, & Cash, 1992). Though PPO also has been isolated and partially characterized from blueberries previously (Kader, Haluk, Nicolas, & Metche, 1998; Kader, Rovel, Girardin, & Metche, 1997; Terefe, Delon, Buckow, & Versteeg, 2015), none of these studies investigated chemical inhibition, optimum temperature range of activity, and detailed thermal inactivation at multiple incubation times. These PPO properties have significance during processing of anthocyanin-rich blueberries. Thus, our objectives were to: (a) determine the blueberry PPO kinetics, substrate specificity, and pH and temperature optima, and (b) investigate the inactivation or inhibition of blueberry PPO activity by heat treatment and chemical inhibitors. This information will be useful in devising effective methods for inhibiting discoloration while at the same time minimizing anthocyanin degradation in blueberry juice and other processed products.

2. Materials and methods

2.1. Materials

Market-ripe blueberries were purchased from a local source and were kept frozen at −20 °C until the extraction of PPO enzyme. All of the PPO substrates, inhibitors, and extractions chemicals and buffers were of reagent grade, and purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Extraction of PPO

Frozen blueberries were thawed overnight in a refrigerator. In order to minimize PPO activity, extraction of the enzyme was carried out in a cold room (4 ± 1 °C), and all extraction buffers and glassware were maintained at 4 ± 1 °C as well. The PPO extraction was carried out using a modification of the method of Siddiq and Cash (2000). Briefly, 50 g thawed blueberries were blended in 100 mL of 0.1 M, pH 9.5 TRIZMA buffer and 0.5% Triton X-100 for 3 min using a Waring blender. The blended macerate was filtered through a fine-mesh nylon cloth, and 200 mL of −20 °C acetone added to precipitate the filtrate. The contents were filtered through a fine-mesh nylon cloth and the precipitate was suspended in 25 mL of cold 0.1 M pH 6.0 sodium phosphate buffer and 7.5 mL of 0.3 M calcium chloride added to precipitate pectic substances. The contents were stirred at low speed using a magnetic stirrer. After all the precipitate was dissolved (~10 min), the viscous slurry was then centrifuged at 4,500*g* for 20 min in a refrigerated centrifuge. The supernatant was filtered through Whatman No. 1 filter paper and kept in tightly capped glass vials at −20 °C, until used for further characterization of the enzyme. All the PPO extraction and subsequent experiments on the characterization of the enzyme were performed using three replicates.

2.3. Assay of PPO activity

The enzyme activity was assayed according to the method of Siddiq & Cash (2000). The reaction mixture consisted of 3.8 mL of 0.3 M catechol in 0.1 M citrate phosphate buffer (pH 6.1) and 0.2 mL of PPO extract, containing equivalent of 0.237 mg/mL protein (Bradford, 1976). The change in absorbance at 420 nm was observed for 3 min at 25 °C, using a Lambda Perkin Elmer spectrophotomer, equipped with enzyme kinetics software. The reaction velocity (V) was calculated from the linear part of the plot of absorbance (A) against time (t). The unit of PPO activity was defined as the change in the absorbance of 0.001/min (ΔA_{420}nm/min) due to the oxidation of substrate.

2.4. pH and temperature optima

The blueberry PPO activity as function of pH was determined with 0.03 M catechol as substrate in 0.1 M citrate phosphate buffer (pH 3.0–7.2) and 0.1 M sodium phosphate buffer (pH 5.5–8.0). The optimum pH, for the maximum activity of the enzyme with 0.1 M citrate phosphate buffer, was used for subsequent characterization of the blueberry PPO. The temperature optima was determined at a temperature range of 25–85 °C, using a shaking water bath. The 0.1 M citrate phosphate buffer (pH 6.3) and PPO extract was heated in 10 mL capped test tubes. The temperature was monitored using stainless steel stem digital thermometer, inserted in the test tube through a hole in the cap. One min after reaching a predetermined temperature equilibrium, 0.03 M catechol was added and the enzyme activity assayed as above.

2.5. Substrate specificity and enzyme kinetics

A range of reagent-grade substrates at 0.03 M conc. were used to determine their affinity towards PPO in citrate phosphate buffer. Substrates that had poor water solubility were dissolved in 2x diluted ethanol. The results are expressed relative to catechol, with which the PPO showed maximum activity.

Blueberry PPO kinetics, Michaelis-Menten constant (K_m) and maximum velocity (V_{max}) were determined with 0.01 M−0.5 M catechol in 0.1 M citrate phosphate buffer (pH 6.3). The K_m and V_{max} values were determined by plotting 1/activity ($1/V$) versus 1/-substrate (1/[S]), according to the direct linear plot method of Lineweaver and Burk (1934).

2.6. PPO inhibition and heat inactivation

2.6.1. Chemical inhibition

A wide range of inhibitors (benzoic acid and cinnamic acid series, and others) were studied to assess their inhibitory effect on blueberry PPO. Each inhibitor (0.l mL) was added to the reaction mixture (citrate phosphate buffer and 0.03 M catechol) at 0.2, 1.0, or 2.0 mM concentration, and PPO activity assayed as above. The results are reported as percent catechol inhibition of blueberry PPO.

2.6.2. Heat inactivation kinetics

The blueberry PPO in the assay buffer in 10-mL tightly capped glass test tubes was heated in a shaking water bath, maintained at 55, 65, 75, or 85 °C. The temperature of the mixture was monitored using stainless steel stem digital thermometer, inserted in one of the test tubes through a hole in test tube cap. After heating for a predetermined time (2, 5, 10, 20 or 30 min) the test tubes were taken out of the water bath and rapidly cooled down to <30 °C in an ice bath, and the enzyme activity assayed. The result are reported relative to the initial PPO activity (% A_0).

2.7. Storage stability of PPO

In order to assess the retention of PPO activity, the storage stability of blueberry PPO was determined over 20 weeks at temperatures of −20, 4, and 22 °C, corresponding to frozen, refrigerated, and room temperatures storage, respectively. The activity was assayed weekly for the first 4 weeks, and then after 6, 8, 12, 16, and 20 weeks. The PPO extract stored at −20 °C were kept in separate vials to avoid freeze-thaw effect on PPO at each observation interval. The result are reported relative to the initial PPO activity (% A_0)