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Characterization of polyphenol oxidase from broccoli (*Brassica oleracea* var. *botrytis italica*) florets

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Abstract

Polyphenol oxidase (PPO) from broccoli florets was extracted and purified through $(NH_4)_2SO_4$ precipitation, ion-exchange and gel filtration chromatography. The molecular weight was estimated to lie between 51.3 and 57 kDa by sodium dodecyl sulphate-polyacrylamide gel electophoresis (SDS-PAGE) and gel filtration. The effects of substrate specificity, pH, and sensitivity to various inhibitors: citric acid, ascorbic acid, sodium sulphate and EDTA (sodium salt of ethylenediaminetetraacetic acid) of partially purified PPO were investigated. Polyphenol oxidase showed the best activity toward catechol ($K_M = 12.34 \pm 0.057$ mM, $V_{max} = 2000 \pm 8736$ U/ml/min) and 4-methyl catechol ($K_M = 21 \pm 0.087$ mM, $V_{max} = 28.20 \pm 0.525$ U/ml/min). The optimum pH for broccoli PPO was 5.7 with catechol and 4-methylcatechol as substrates. The most effective inhibitor was sodium sulphate.

Keywords: Polyphenol oxidase; Broccoli; Inhibitors

1. Introduction

Polyphenol oxidase (PPO) is a common copper-containing enzyme which is responsible for melanization in animals and browning in plants. The enzyme catalyzes two distinct reactions: the o-hydroxylation of monophenols to *o*-diphenols (acts like cresolase (E.C. 1.14.18.1.)) and the oxidation of *o*-diphenols to *o*-quinones (acts like catecholase (E.C. 1.10.3.2.)) (Rodríguez-López et al., 2001; van Gelder, Flurkey, & Wichers, 1997). The role of PPO in plants is not yet clear. It is suggested that it may be involved in immunity reactions and in biosynthesis of plant components, and it also may play the role of a scavenger of free radicals in photo-synthesizing tissues (Heimdal, Larsen, & Poll, 1994). Quinones are highly reactive electrophilic molecules that can polymerise, leading to the formation of brown or black pigments. The phenomenon of enzymatic browning often occurs in fruits and vegetables and is the cause of a decrease in their sensory properties and nutritional value (Prota, 1988). For this reason, the activity of PPO has been studied in apples (*Malus* sp.) (Espin, Morales, Varon, Tudela, & Garcia-Carnovas, 1995), pears (*Pyrus* sp.) (Hwang, Yoon, & Kim, 1996), potatoes (*Solanum tumerosum* L.) (Chen et al., 1992), artichokes (*Cynara scolymus* L.) (Leoni & Palmeri, 1990), broad beans (*Vicia faba* L.) (Ganesa, Fox, & Flurkey, 1992), lettuce (*Lectuca sativa* L.) (Heimdal et al., 1994), banana (*Musa cavendishii* L.) (Galleazi, Sgarbieri, & Constantinides, 1981), plums (*Prunus* sp.) (Siddiq, Sinha, & Cash, 1992), peppermint (*Mentha piperita* L.) (Kavrayan & Aydemir, 2001), coffee (*Coffea arabica* L.) (Mazzafera & Robinson, 2000) and seeds of field bean (*Dolichos lablab*) (Paul & Gowda, 2000). The literature on the subjects contains no data on purification and characterization of PPO from broccoli. The main objective of this investigation was to characterize PPO from broccoli florets and to elucidate the mechanism of its inhibition by selected chemical compounds.

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2. Materials and methods

2.1. Materials

Frozen broccoli florets/Hortex Company/were used. Catechol, 4-methylcatechol, polyvinylpyrrolidone (PVP40), DEAE-Sephadex A-50, Sephadex G50, Sephadex-G100, Bradford reagent, vaniline, ferulic acid, phloroglucinol (1,3,5-trihydroxybenzene), chlorogenic acid, caffeic acid, protein markers, ascorbic acid, citric acid, sodium sulphate, EDTA (sodium salt of ethylenediaminetetraacetic acid) were purchased from Sigma–Aldrich, USA. All others chemicals were of analytical grade.

2.2. Enzyme extraction and purification

Ten grams of material were homogenized in 80 ml of 0.1 M sodium phosphate buffer pH 6.8 containing 10 mM ascorbic acid and 0.5% polyvinylpyrrolidone (PVP40) and extracted with the aid of a magnetic stirrer for 1 h. The crude extract samples were centrifuged at 32,000*g* for 20 min. The process was conducted at the temperature of 4 °C. Solid $(NH_4)_2SO_4$ was added to the supernatant to obtain 80% saturation. After an hour, the precipitated proteins were separated by centrifugation at 32,000*g* for 30 min. The precipitate was dissolved in a small amount of 5 mM phosphate buffer (pH 6.8) and dialyzed at 4 °C in the same buffer for 24 h (MW cut-off >12,000) with four changes of the buffer during dialysis. In order to conduct further purification, the dialysate was transferred to a column (2.5 × 100 cm) filled with DEAE-Sephadex A-50 gel, balanced with 5 mM phosphate buffer, pH 6.8. The column was eluted with the same eluent at the flow rate of 30 ml/h and linear gradient of NaCl concentration from 0 to 1.0 M. Five millilitre fractions were collected in which the protein level and PPO activity towards catechol as substrate were monitored. The fractions which showed PPO activity were collected and concentrated and then dissolved in 3 ml of phosphate buffer, pH 6.8. The combined fractions were transferred to a glass column (2.5 × 100 cm) filled with Sephadex G50 gel. The column was eluted with the same buffer solution. 3-ml fractions were collected and the protein content and PPO towards catechol was monitored in them spectrophotometrically. The fractions showing PPO activity were combined, concentrated and transferred to a column (2.5 × 100 cm) filled with Sephadex-G100 gel. The column was eluted as described above.

2.3. Enzyme assay

PPO activity was determined by measuring the initial rate of quinone formation, as indicated by an increase in absorbance at 420 nm (Perkin–Elmer Lambda 40 UV–Vis spectrophotometer was used) (Wisserman & Lee, 1980). An increase in absorbance of 0.001 min^{-1} was taken as one unit of enzyme activity (Ho, 1999). The increase in absorbance was linear with time for the first 120 s. The sample cuvette contained 2.95 ml of substrate solution (10 mM catechol or 4-methylcatechol) in 50 mM phosphate buffer (pH 6.8) and 0.05 ml of the enzyme solution. The blank sample contained 2.95 ml of substrate solution and 0.05 ml of phosphate buffer.

2.4. Protein estimation

Protein content was determined according to the dye-binding method of Bradford (1976) using bovine serum albumin as standard.

2.5. Determination of molecular weight

The molecular weight of the purified enzyme was estimated by SDS-PAGE and gel permeation. SDS-PAGE was performed according to the method of Laemmli (1970). Proteins were dissolved in a 12.5% polyacrylamide gel and visualized with colloidal Coomassie staining.

The molecular weight of PPO was also estimated on a Sephadex G 100 with protein markers of cytochrome C (12.40 kDa), albumin (66.00 kDa), carbonic anhydrase (29.00 kDa), aprotinin (6.50 kDa) and aldolase (158.00 k kDa). Blue dextran 2000 was used to determine the void volume (V_0). The protein markers were chromatographed and the elution volume (V_e) was measured. The distribution coefficient (K_{av}) was given by equation:

$$K_{av} = (V_e - V_0)(V_t - V_0)^{-1}$$

where V_t stands for the total bed volume. The calibration curve was obtained by plotting log MW versus K_{av}.

2.6. Characterization of PPO

2.6.1. Effect of pH on enzyme activity

PPO activity, as a function of pH, was determined under standard conditions using various buffers in a pH range 2.0–12.0. The buffer systems were prepared according to Britton–Robinson (Kłyszejko-Stefanowicz, 2003). The optimum pH for the PPO was obtained using two substrates: 10 mM catechol and 10 mM 4-methyl catechol. The pH value corresponding to the highest enzyme activity was taken as the optimal pH.

2.6.2. Kinetic data analysis and substrate specificity

The specificity of broccoli PPO extract was investigated for seven commercial grade substrates: vaniline, ferulic acid, phloroglucinol (1,3,5-trihydroxybenzene), chlorogenic acid, caffeic acid, catechol and (4-methylcatechol) at concentrations 10 mM. PPO activity was assayed in triplicate. The activity of PPO extract as a function of the concentration of catechol and 4-methyl catechol was investigated. Michaelis constant (K_m) and V_{max} of the PPO was determined by Lineweaver–Burk's method.