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Separation of Polyphenol Oxidase and its Inhibitor from Onion Leaves on Lignin Column

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Tyrosine is a key compound of pigmentation/browning reaction through the action of polyphenol oxidase. Discrimination of this pigmentation reaction is responsible for various types of diseases and disorders. Core part of enzyme, polyphenol oxidase is same; therefore to regulate such discriminations of pigmentations/browning, searching for nontoxic endogenous inhibitor of this activity is important in developing the rational chemotherapy of pigmentation. Polyphenol oxidase and endogenous inhibitor was purified on natural matrix. Endogenous inhibitor eluted in two fractions which contains 2.15 ± 0.04 % of flavanoid and 6.1 mg/12.20 % of vitamin C. Fraction I (UV 245 nm, m.w. 66KDa) inhibits 100 % polyphenol oxidase at 14 min while fraction II (UV 224.5 nm, m.w. 98 KDa) inhibits polyphenol oxidase at 18 min. It was also confirmed by TLC.

Key Words: PPO, Onion leaves, Inhibitor, Monophenol monooxygenase, *o*-Diphenol oxidase.

INTRODUCTION

Polyphenol oxidase (PPO) is copper containing, mixed function oxidase, ubiquitous in nature. Tyrosine is a key compound for melanin formation/pigmentation, which is visible in skin, hair and eyes of mammals. Some of the pleiotropic function of melanin includes (1) sexual interaction (2) absorption of radiation over a wide range of wavelength which provides protection to the cell from damages caused by ultraviolet light (3), the scavenging of toxic free radicals generated within cell[1] and other possible neurotransmitter functions among others[2].

In India, Maharashtra is the major place where fruits and vegetables are cultivated on large scale. In the peak season, if transport facility becomes unavailable, fruits and vegetables are perishable, resulting in great economic loss to farmers. Because of year round growing onion and onion leaves provide continuous source of income to farmers. Further onion leaves have importance for vegetarian and non-vegetarian diet due to medicinal and nutritional value. However, when onion leaves are cut they turn into black due to pigmentation reaction. This undesirable reaction is responsible for unpleasant sensory qualities and losses in nutritional quality. The prevention of browning reaction has always been a challenge to food scientists[3]. Until now a number of synthetic inhibitors have been reported including arbutin[4], kojic acid[5] and linolenic acid[6]. However, they are toxic to human beings. Dopa and its analogues are available in tissues of human body[7]. Therefore searching for non-toxic inhibitor of this activity is important in developing a rational chemotherapy of pigmentation.

EXPERIMENTAL

L-Dopa, L-tyrosine, sodium phosphate (mono and dibasic), ammonium sulfate, comassie brilliant blue R 250, acrylamide, bisacrylamide, TEMED, -was obtained from SD Fine. Citron X-100, EDTA, ascorbic acid from E-Merck (India). Sephadex G-25 from pharmacia. Silica gel, ammonium hydroxide, diethylether, chloroform, acetone from Loba chemicals, butanol from SRL, ethanol was obtained locally and distilled prior to use.

Locally available variety of onion leaves having maximum activity monophenol monooxygenase and *o*-diphenol oxidase was used. Fresh onion leaves were clean and exposed to atmospheric oxygen round the clock.

Separation of endogenous regulator was carried out by salt and solvent method. Monophenol monooxygenase assay was carried out by Kahn and Andrawis[8], while *o*-diphenol oxidase assay by Shimodo *et al.*[9], protein content was measured as per Lowry method[10].

Isolation of monophenol monooxygenase, *o*-diphenol oxidase and endogenous inhibitor of polyphenol oxidase on natural affiant: Fresh onion leaves of 100 g were homogenized with ice cold acetone, water with Citron X-100 (80:19:1 v/v) for *ca.* 24 h (-18 ºC). The resulting acetone powder was dried for *ca.* 0.5 h at room temperature. The acetone powder was suspended in 124 mL of 100 mM phosphate buffer pH

6.8 containing 100 mM EDTA sonicated for 1 h, followed by centrifugation at 5600 g for 15 min. Precipitation was carried out by using solid ammonium sulphate. Resulting pellets was homogenized in 50 mM phosphate buffer pH 7.0, dialyzed for against same buffer for 24 h. Monophenol monooxygenase, *o*-diphenol oxidase and protein were measured at each stage. After partial purification, a sample containing 68 mg of protein with 7700 IU activity for monophenol monooxygenase and 1900 IU activity *o*-diphenol oxidase (Fig. 1A Table-1) was loaded on natural affiant lignin which was prepared by method of Blende[11].

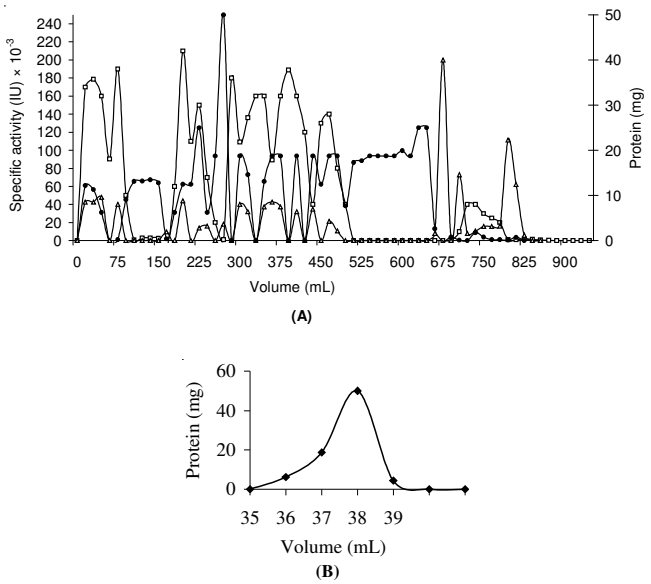


Fig. 1. Purification of PPO and proteinous fraction/regulator on natural affiant. (A) (□-□) represents monophenol monooxygenase, (Δ-Δ) represents *o*-diphenol oxidase and (•-•) represents (B) proteinous fraction separated and loaded on silica gel appeared as a single peak

TABLE-1
SPECIFIC ACTIVITY OF MONOPHENOL MONOOXYGENASE AND *o*-DIPHENOL OXIDASE

Peaks	Specific activity of	
	Monophenol monooxygenase (IU)	*o*-Diphenol oxidase (IU)
Peak 1	30,000	4000
Peak 2	55,000	1452
Peak 3	10,000	2270
Peak 4	90,000	–
Peak 5	75,000	–
Peak 6	80,000	–
Peak 7	60,000	–
Peak 8	20,000	–
Peak 9	50,000	–

Characterization of protein peak: The pure protein fraction I (60 mL) (volume 105-165 mL) and fraction II (150 mL) (volume from 540-690 mL) from affinity column (Fig. 1A) was concentrated and passed through sephadex G-25 (Fig. 1B). Fractions monitored by all above methods were freeze-dried and stored at ambient temperature for further study. The fractions were subjected to SDS-PAGE (Fig. 2), PAGE, UV and TLC. Total flavanoid content of the fraction was measured by using aluminium chloride, colorimetrically and spectrophotometrically[12]. Estimation of vitamin C was carried out by iodometric method.

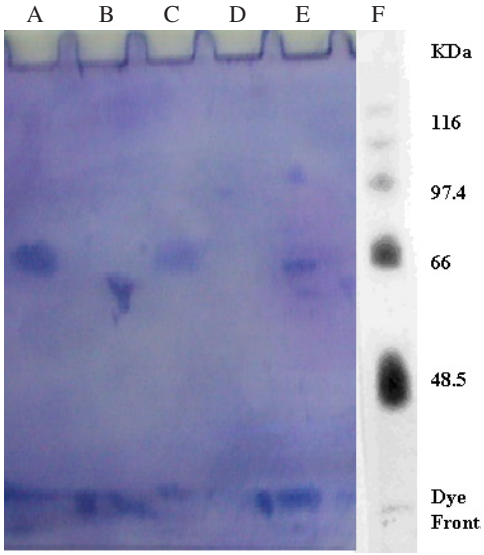


Fig. 2. SDS-PAGE- (A) endogenous inhibitor/proteinous fraction I (B) *o*-diphenol oxidase activity, (C) monophenol monoxygenase activity from onion leaves and (D) and (E) endogenous inhibitor/proteinous fraction II, (F) MW marker

The UV/vis spectra of fraction I and fraction II were recorded on a UV-1800 spectrophotometer in 10-mm quartz cuvettes (Shimadzu corp.). The sample of onion leaves proteinous fraction was prepared at concentrations of 0.50 mg/mL pH 6.8, sodium phosphate buffer. The change of the absorption was recorded at room temperature.

Effect of proteinous fraction on monophenol monooxygenase and *o*-diphenol oxidase activity: Monophenol monooxygenase and *o*-diphenol oxidase was isolated from onion leaves on natural column. Various concentrations of isolated proteinous fractions I and II were incubated separately with 10 µg of respective enzyme. Inhibition of monophenol monooxygenase and *o*-diphenol oxidase was measured spectrophotometrically, colorimetrically and TLC.

RESULTS AND DISCUSSION

Partial purification of enzyme was carried out by 60 % ammonium sulphate saturation. After dialysis, the fraction was loaded on lignin column. Separation of protein and enzyme was eluted with 100 mM sodium phosphate buffer pH 6.8 at 4 ºC. The elution profile pattern shows nine peaks for monophenol monooxygenase and three peaks of *o*-diphenol oxidase (Fig. 1). Protein fraction I (15 mL) was eluted from 271-285 mL and protein fraction II (150 mL) from 540-690 mL. Both protein fractions were passed through sephadex G-25 for the confirmation of the purity of the protein. These proteinous fractions act endogenous inhibitor/regulator of PPO (Fig. 1B). The protein fraction I and II were concentrated and subjected to SDS-PAGE, UV and TLC.

Spectral studies have given new dimension for the detection of flavanoid. Flavanoids are polyphenolic antioxidant naturally present in fruits and vegetables. In the Fig. 3, TLC plate 1 under UV chamber fluorescence spot was observed of (i) proteinous fraction I, (ii) proteinous fraction II, (iii) disappearance of indicates 100 % inhibition of onion leaves

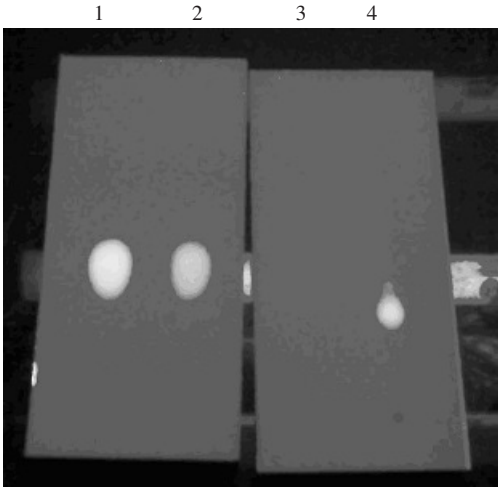
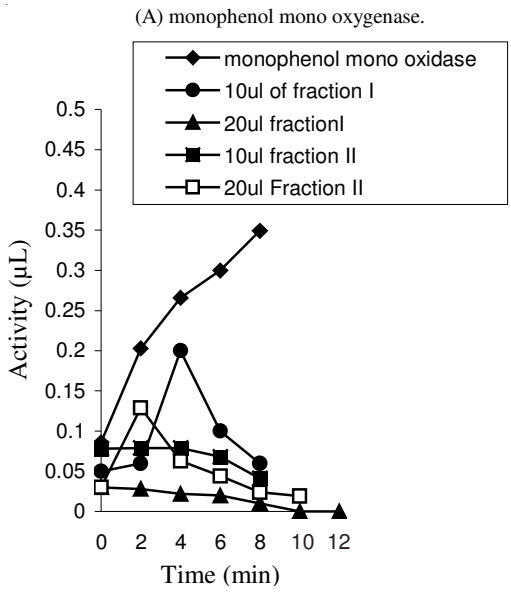


Fig. 3. TLC plate 1 under UV chamber fluorescence spot (1) Proteinous fraction I, (2) Proteinous fraction II, (3) Disappearance of indicates 100 % inhibition of onion leaves *o*-diphenol oxidase, (4) Reduce the intensity of spot is inhibition of onion leaves monophenol monoxygenase

o-diphenol oxidase, (iv) reduced the intensity of spot. It indicates that the inhibition of onion leaves monophenol monoxygenase. Hundred percent inhibition of monophenol monooxygenase and *o*-diphenol oxidase was observed within 14-18 min while oxidation of PPO was observed at 20-30 min. (Fig. 4 A and B). Therefore, this proteinous fractions leads to develop new drugs for the treatment of monophenol monooxygenase/tyrosinase and *o*-diphenol. Onion leaves contain 2.15 ± 0.04 % of flavanoid and 6.1 mg/12.20 % of vitamin C per 100 mg. Vitamin C acts as enzymatic co-factor and responsible for dark coloration. Its physiological function in human body includes the synthesis of tyrosine which is a key compound of melanin formation/pigmentation. Absorbance of fraction I is observed 1.702 and 1.070 indicates presence of monomer and dimmer while 0.770 and 0.580 of fraction II indicates presence of monomer at 290 and 328 nm, respectively at room temperature. UV spectrum has maximum profile in 250-300 nm region. The UV maxima of fraction II was at 265 nm and minima at 224.5 nm.



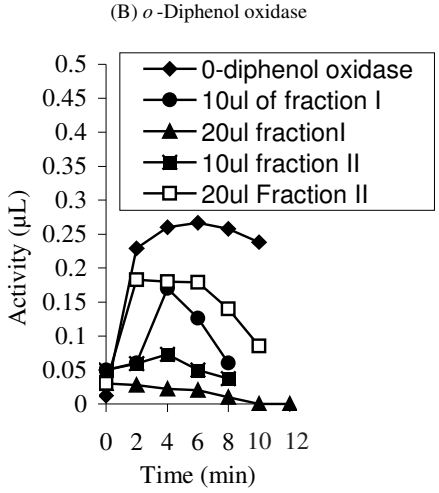


Fig. 4. Effect of proteinous fraction I and II on the activity of (A) monophenol monooxygenase (B) *o*-diphenol oxidase

Salt and solvent method is the green method to isolate endogenous inhibitor. This method is one pot two shots: such as enzyme and endogenous inhibitor purification at a time. It requires less time, cost effective, ecofriendly and nontoxic to operate. Environmentally "green" and high safety profile, mild conditions (pH 5-8, 20-40 ºC) compatible with each other, catalyze a broad spectrum of reactions, chemoselective, regioselective, diastereoselective, enantioselective, *etc*.

Conclusion

Endogenous inhibitor and PPO were isolated from onion leaves on natural column, two protein fractions were eluted. Isolated proteinous inhibitor is natural, non-toxic. It requires 14-18 min for 100 % inhibition of pigmentation reaction. This inhibitor extends the shelf-life of onion leaves with the aid of this endogenous inhibitors one can formulate drugs for the treatment of various diseases and disorders.

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