Original Article

CHARACTERIZATION OF POLYPHENOL OXIDASE FROM *Phyllanthus amarus* (POKOK DUKUNG ANAK) LEAVES

Nuruddin MN. and S. Al-Jasabi

Kulliyyah of Medicine and Health Sciences, INSANIAH University College, Kedah, Malaysia.

ABSTRACT

Recently much attention has been paid to biologically active components of herbs, in this study the polyphenol oxidase (PPO) was extracted, purified and characterized from herb Phyllanthus amarus, the most popular medicinal herb in South East Asia. It was extracted and purified by ammonium sulphate $(NH_4)_2SO_4$ precipitation, isolated by ion-exchange chromatography and purified by gel filtration chromatography and HPLC. The biochemical analysis revealed that the PPO extracted from Phyllanthus amarus leaves has higher affinity towards catechol (K_M = 17.4mM and V_{max} = 22,077 U/ml min^{-1}) at an optimum pH of 7.1. The enzyme had an optimum temperature of 39^0C and was relatively stable up to 56^0C for a period of 90 minutes with almost 80% activity remaining. Among the various PPO inhibitors tested, ascorbic acid was found that it is the most effective inhibitor for the enzyme with 10mM catechol as substrate.

***Key Words*:** *Phyllanthus amarus,* Catechol, Vit.C, Herbs, polyphenols

Introduction

Medicinal plants have been used all over the world for the treatment and prevention of various ailments, particularly in tropical and subtropical areas[1]. *Phyllanthus amarus* belong to the family of Euphorbiaceae and it is well known in South East Asia as indigenous medicinal herb which has a folk reputation in Ayurvedic medicine[2]. Polyphenol oxidase (E.C. 1.41.18.1) is a common copper containing enzyme responsible for melanization in animals and browning in plants also known and reported under various names (tyrosinase, phenolase, catechol oxidase, monophenol oxidase, *O*-diphenol oxidase and ortho-phenolase) based on substrate specificity[3,4]. These are widely distributed in different plant species as well as algae and fungi[5].

In all plants the enzyme has been reported that it is localized to the thylakoid membranes of chloroplasts and other plastid organelles[6]. The role of polyphenol oxidase (PPO) in plants is not yet clear, but it has been proposed that it may be involved in necrosis development around damaged leaf surfaces and in defence mechanisms against insects and plant pathogen attack[6]. It was reported that it may be involved in immunity reactions and in biosynthesis of some secondary metabolites in plant cell, and it also may play role in scavenging free radicals in photosynthesizing tissues[7,8].

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[9]. The browning is principally initiated by the activity of Polyphenol oxidase catalyses two distinct reactions: the *O*-hydroxylation of monophenols to O-diphenols and the oxidation of *O*-diphenols to *O*-quinones[10,11]. Polyphenol oxidase is frequently reported as a latent enzyme, which can be activated *in vitro* by a number of different factors such as detergents [12-15], and chemicals[13].

Biochemical characters of PPO activity were determined in a number of fruits and vegetables such as, apples (*Malus* sp.), pears, peppermint (*Mentha piperita* L.), coffee (*Coffea arabica* L.). However, no research has been reported on herb *Phyllanthus amarus* leaves PPO which is mainly grown in the tropical and sub-tropical areas of the world and used intensively in Malaysia, India and Indonesia as traditional medicine.

In the present study, PPO was extracted from leaves of *Phyllanthus amarus*, from Malaysia, partially purified and the characteristics of the enzyme were investigated.

2. Materials and Methods

2.1 Materials

Healthy undamaged leaves of herb *Phyllanthus amarus* were obtained from Ipoh area in Malaysia. Catechol, DE-AE-Sephadex A-50, Sephadex G100, caffeic acid, chlorogenic acid, phloroglucinol, Polyvinylpyrrolidine (PVP 40), ascorbic acid, tyrosine, thiourea, sodium metabisulphate and glutathione were obtained from local suppliers. and all chemicals were of analytical grade.

2.2 Enzyme extraction and purification

Hundred grams of healthy leaves were cleaned by tap water 3 times, then cleaned by phosphate buffer (pH 7.4), dried using reflected dry air then homogenized in 240 ml of 0.1M sodium phosphate buffer (pH = 7.1) containing 10mM ascorbic acid and 0.5% polyvinylpyrrolidine using Ultra homogenizer and extracted with the aid of magnetic stirrer for one hour. The crude extract was centrifuged at 40,000g for 20 min at 4^0C. Solid $(NH_4)_2SO_4$ was added to the supernatant to obtain 82% saturation. After an hour, the precipitated proteins were separated by centrifugation at 32,000g for 30 min. The precipitate was dissolved in a small amount of 5mM phosphate buffer (pH 7.1) and dialyzed in a cellulose bag (MW cut off >12,000) at 4^0C in the same buffer for 24 hours with four changes of the buffer during the analysis. In order to conduct further purification, the dialysate was transferred to a column with DEAE-Sephadex A-50 gel, balanced with 5mM phosphate buffer (pH 7.1). The column was eluted with the same buffer at the flow rate of 25 ml/h keeping linear gradient of NaCl concentration from 0 to 1.0M. Three milliliter 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, concentrated and then dissolved in 3 ml of phosphate buffer (pH 7.1). The combined fractions were transferred to a glass column filled with Sephadex G100 gel. The column was then eluted with the same buffer solution. Three milli-liter fractions were collected and the protein content and the Polyphenol oxidase (PPO) activity towards catechol were monitored spectrophotometrically. The fractions showing PPO activity were combined and concentrated.

2.3 PPO activity assay

PPO activity assay was determined by measuring the initial rate of quinine formation as indicated by an increase in absorbance at 420 nm[14]. One unit of enzyme activity was defined as the amount of enzyme that caused a change in absorbance of 0.001 min^{-1} [15]. The PPO activity was assayed in triplicate measurements. The sample cuvette contained 2.95 ml of 10mM catechol solution in 0.1 ml phosphate buffer (pH 6.8) and 0.05 ml of the enzyme solution. The blank sample contained only 3ml of the substrate solution.

2.4 Protein determination

Protein content of the enzyme extract was determined using Bovine Serum Albumin (BSA) as standard according to Bradford[16].

2.5 Biochemical characterization of PPO

The PPO activity as a function of pH was determined under standard conditions using various buffers in the pH buffer range 2.0 – 12.0 using 0.02 M catechol as the substrate. The buffer solution was prepared according to Britton – Robinson. PPO activity was assayed as described before with catechol as the substrate. The pH value corresponding to the highest enzyme activity was taken as the optimal pH.

The specificity of PPO extracted from *Phyllanthus amarus* leaves was investigated in five different substrates like catechol, caffeic acid, chlorogenic acid, phloroglucinol and tyrosine at a concentration of 10mM as reported before as in our previous reported method. The activity of PPO extract as function of the concentration of catechol was investigated. Michaelis constant (k_M) of the PPO was determined by Lineweaver – Burk's method.

PPO activity as a function of temperature was determined under standard assay conditions using temperatures from 5 to 80^0C. Thermal stability of PPO was determined by heating the enzyme solution at various temperatures between 20 to 80^0C for 60 minutes at pH 7.1. Residual PPO activity was measured under standard assay conditions as we have reported before[17].

The inhibitory effects of ascorbic acid, thiourea, sodium metabisulphate and glutathione on PPO activity were determined. 5mM concentration of each of these compound and were tested using 10mM of catechol as substrate. The corresponding control contained the same concentration of enzyme in the absence of the inhibitor.

Results and Discussion

The elution profile of the PPO with DEAE Sephadex A-50 and Sephadex G100, and purification of fold of 1.87 relative to a protein yield of 24.5% was achieved (Table 1). The technique of gel filtration is widely used in enzyme separation. The activity of PPO was measured at different pH using catechol. The optimum pH of the enzyme was found to be 7.1 using catechol as the substrate. It is seen that in general, most plants show PPO activity at or near neutral pH values. The result shown in this study corresponds well with the results obtained by others[5], for broccoli (Brassica oleracea var. botrytis italic) florets. It also corresponds with the results obtained before[18].

Though there are several compounds that are used as substrates for polyphenol oxidase, in this study we selected the most commonly used substrates such as catechol, caffeic acid chlorogenic acid phloroglucinol and tyrosine. As shown in Table 2, Polyphenol oxidase showed the highest activity towards catechol (dihydroxy phenols) and the lowest activity towards phloroglucinol (trihydroxy phenol), whereas, no activity was shown towards tyrosine (monophenols). Cho and Ahn[19] used catechol in the studies of kinetic properties of PPO from potatoes, whereas, Janovitz-Klapp[20] compared the activity of PPO in apples against several substrates.

The Lineweaver – Burk plot analysis of polyphenols oxidase from *Phyllanthus amarus* leaves showed that the Michaelis Menten constant (K_m) and the maximum reaction velocity (V_{max}) were 11.2mM and 16,400 $U/mlmin^{-1}$ respectively for catechol. This value for catechol was similar to that of Tea leaf (13.6 mM) and also with field bean seed (10.5 mM)[21]. The effect of temperatures between 5 and 80^0C on PPO activity has shown that the optimum temperature for the PPO enzyme from *Phyllanthus amarus* leaves to be 37^0C. This value was similar to that obtained by others[22] from plum (37^0C). The value is also similar to

Table 1: The results of the yield % and the purification fold

Purification Steps	Protein conc. mg/ml	Activity U/ml	Specific activity U/mg	Purification fold	Yield (%)
Crude extract	3.44	39.90	12.47	1.00	100.00
$(NH_4)_2SO_4$	2.55	35.70	15.47	1.24	89.70
DEAE- Sephadex A-50	1.81	18.30	17.52	1.40	50.00
Sephadex G-100	0.40	9.59	23.27	1.87	24.50

Table 2: The results show the relative activity of *Phyllanthus amarus* PPO.

Substrate (10 mM)	Relative activity (%)
Catechol	100.00±3.110
Caffeic acid	14.11±0.870
Chlorogenic acid	2.88±0.660
Phloroglucinol	0.75±0.007
Tyrosine	0.00

Table 3 : The results show inhibition % using different substrates

Inhibitor	K_i(M)	Type of inhibition	Inhibition (%)
Ascorbic acid	$13.7x10^{-5}$	Competitive	69
Thiourea	$2.8x10^{-5}$	Non competitive	59
Sodium meta bisulphate	$7.1x10^{-5}$	Non competitive	58
Glutathione	$6.6x10^{-5}$	Non competitive	60

that obtained before[5] in investigations of PPO from butter lettuce.

The thermal stability profile of *Phyllanthus amarus* leaves PPO, showed as residual activity after pre-incubation at the specified temperature. This PPO showed thermal stability up to 50^0C for a period of 60 minutes with almost 80% activity remaining. The enzyme from meddler fruits was stable for 30 min at 60^0C [23,24]. It has been reported that *Allium* sp. PPO was stable at 40^0C for 30 min[25]. PPO from latex of *Hevea brasiliensis* was stable up to 60^0C for 60 min [26,27].

The effect of various inhibitors like ascorbic acid, thiourea, sodium metabisulphate and glutathione on *Phyllanthus amarus* leaves PPO with catechol as the substrate is shown in Table 3. From the K_i constants, it is concluded that the inhibition modes for thiourea, sodium metabisulphate and glutathione are non competitive and ascorbic acid competitive [28-33]. Enzymatic browning of plants and fruits may be delayed or eliminated by removing the reactants such as oxygen and phenolic compounds or by using PPO inhibitors. There are a number of inhibitors used by researchers to prevent enzymatic browning[27]. The inhibitory reaction mechanism differs and depends on the reducing agent that is employed. Ascorbic acid has also been found to show competitive activity towards PPO isolated from peppermint[15] and potato[23].

References

1. Mayer, A. and E. Harel, *Polyphenol oxidases in plants.* Phytochemistry, 1979. **18**(2): p. 193-215.
2. Paithankar V., S. Raut, R.Charde and V.Vyas, 2011. *Phyllanthus Niruri*: a magic herb. Research in Pharmacy, 1(4): 1-9.
3. Divya K., R. Ankur, R. Seema and U. Khan, 2011. *Phyllanthus amarus* An ample therapeutic potential herb. Inter. J. of Research Ayureveda& Pharmacy, 2 (4): 1096-1099.
4. Mayer, A.M., *Polyphenol oxidase in plants recent progress.* Phytochemistry, 1987. **26**: p. 10.
5. Sherman, T., K. Vaughn, and S. Duke, *A limited survey of the phylogenetic distribution of polyphenol oxidase.* Phytochemistry, 1991. **30**(8): p. 2499-2506.

6. Gawlik-Dziki, U., U. Szymanowska, and B. Baraniak, *Characterization of polyphenol oxidase from broccoli (Brassica oleracea var. botrytis italica) florets.* Food chemistry, 2007. **105**(3): p. 1047-1053.
7. Heimdal, H., L. Larsen, and L. Poll, *Characterization of polyphenol oxidase from photosynthetic and vascular lettuce tissues (Lactuca sativa).* Journal of Agricultural and Food Chemistry, 1994. **42**(7): p. 1428-1433.
8. Prota, G., *ENZYMIC AND LIGHT-INDUCED CONVERSION OF 5, 6-DIHYDR0XYIND0LE (S) TO MELANIN (S).* Light in biology and medicine, 1988: p. 329.
9. Moore, B. and W. Flurkey, *Sodium dodecyl sulfate activation of a plant polyphenoloxidase. Effect of sodium dodecyl sulfate on enzymatic and physical characteristics of purified broad bean polyphenoloxidase.* Journal of Biological Chemistry, 1990. **265**(9): p. 4982.
10. Golbeck, J. and K. Cammarata, *Spinach thylakoid polyphenol oxidase: isolation, activation, and properties of the native chloroplast enzyme.* Plant Physiology, 1981. **67**(5): p. 977.
11. Espı´n, J.C.M., M.; Varo´n, R.; Tudela, J.; Garcı´a- and F. Ca´novas, *Monophenolase activity of polyphenol oxidase from Verdoncella apple.* J. Agric. Food Chem, 1995. **b** (43): p. 6.
12. Hwang, I., W. Kim, and K. Yoon, *Rapid measurement of the enzymatic browning of pear juice by the addition of L-DOPA.* Foods and Biotechnology (Korea Republic), 1996.
13. Kavrayan, D. and T. Aydemir, *Partial purification and characterization of polyphenoloxidase from peppermint (Mentha piperita).* Food chemistry, 2001. **74**(2): p. 147-154.
14. Mazzafera, P. and S. Robinson, *Characterization of polyphenol oxidase in coffee.* Phytochemistry, 2000. **55**(4): p. 285-296.
15. Ho, K., *Characterization of polyphenol oxidase from aerial roots of an orchid, Aranda [] Christine 130'.* Plant Physiology and Biochemistry, 1999. **37**(11): p. 841-848.
16. Bradford, M., *A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding.* Analytical biochemistry, 1976. **72**(1-2): p. 248-254.
17. Dincer, B., et al., *Characterization of polyphenoloxidase from medlar fruits (Mespilus germanica L., Rosaceae).* Food chemistry, 2002. **77**(1): p. 1-7
18. Dogan, S. and M. Dogan, *Determination of kinetic properties of polyphenol oxidase from Thymus (Thymus longicaulis subsp. chaubardii var. chaubardii).* Food chemistry, 2004. **88**(1): p. 69-77.
19. CHO, Y. and H. AHN, *Purification and Characterization of Polyphenol Oxidase from Potato: II. Inhibition and Catalytic Mechanism.* Journal of Food Biochemistry, 1999. **23**(6): p. 593-605.
20. Janovitz-Klapp, A., F. Richard, and J. Nicolas, *Polyphenoloxidase from apple, partial purification and some properties.* Phytochemistry, 1989. **28**(11): p. 2903-2907.
21. Halder, J., P. Tamuli, and A. Bhaduri, *Isolation and characterization of polyphenol oxidase from Indian tea leaf (Camellia sinensis)* 1.* The Journal of Nutritional Biochemistry, 1998. **9**(2): p. 75-80.
22. Paul, B. and L. Gowda, *Purification and characterization of a polyphenol oxidase from the seeds of field bean (Dolichos lablab).* J. Agric. Food Chem, 2000. **48** (9): p. 3839-3846.
23. Arslan, O., A. Temur, and Tozlu, *Polyphenol oxidase from Allium sp.* J. Agric. Food Chem, 1997. **45**(8): p. 2861-2863.
24. King, R. and W. Flurkey, *Effects of limited proteolysis on broad bean polyphenoloxidase.* Journal of the Science of Food and Agriculture, 1987. **41**(3): p. 231-240.
25. Kłyszejko-Stefanowicz, L., *Anatomia i histogeneza ro lin naczyniowych. Organy wegetatywne. Wydawnictwo Naukowe PWN, Warszawa*. 2003, Polish.
26. Xu, J., et al., *Purification and characterization of polyphenol oxidase from Henry chestnuts (Castanea henryi).* Journal of Wood Science, 2004. **50**(3): p. 260-265.
27. Sellés-Marchart, S., J. Casado-Vela, and R. Bru-Martínez, *Isolation of a latent polyphenol oxidase from loquat fruit (Eriobotrya japonica Lindl.): Kinetic characterization and comparison with the active form.* Archives of biochemistry and biophysics, 2006. **446** (2): p. 175-185.
28. Wititsuwannakul, D., et al., *Polyphenol oxidases from latex of Hevea brasiliensis: purification and characterization.* Phytochemistry, 2002. **61**(2): p. 115-121.
29. Dogan, M., O. Arslan, and S. Dogan, *Substrate specificity, heat inactivation and inhibition of polyphenol oxidase from different aubergine cultivars.* International Journal of Food Science & Technology, 2002. **37**(4): p. 415-423.
30. Sakiro lu, H., et al., *Purification and characterization of Dog-rose (Rosa dumalis Rechst.) polyphenol oxidase.* J. Agric. Food Chem, 1996. **44**(10): p. 2982-2986.
31. Yang, C., et al., *Partial purification and characterization of polyphenol oxidase from banana (Musa sapientum L.) peel.* J. Agric. Food Chem, 2001. **49**(3): p. 1446-1449.
32. Aydemir, T., *Partial purification and characterization of polyphenol oxidase from artichoke (Cynara scolymus L.) heads.* Food chemistry, 2004. **87**(1): p. 59-67.
33. Rapeanu, G., et al., *Biochemical characterization and process stability of polyphenoloxidase extracted from Victoria grape (Vitis vinifera ssp. Sativa).* Food chemistry, 2006. **94**(2): p. 253-261.

Corresponding author: Prof. Dr. Saad Al-Jasabi
Email address: smohd84@gmail.com
Tel: +60-173067907

Received: May 2016
Accepted for publication: May 2016