2011 2nd International Conference on Biotechnology and Food Science
IPCBEE vol.7 (2011) © (2011) IACSIT Press, Singapore

Partial Purification and Characterisation of Polyphenol Oxidase from *Hibiscus rosa–sinensis* L

Ikram Madani
Faculty of Science
University of Khartoum
Khartoum, Sudan
Ikramahmed3@yahoo.com

Pat M. Lee
Materials Technology Program
Universiti Teknologi MARA
Shah Alam, Selangor, Malaysia
patmlee.1@gmail.com

Lee Kong Hung
Department of Bitechnology,
Malaysia University of Science and Technology
Petaling Jaya, Selangor, Malaysia.
konghunglee@hotmail.com

***Abstract*— Polyphenol oxidase (PPO) from petals of *Hibiscus rosa–sinensis* L. (Chinese Rosa) was extracted and partially purified by ammonium sulphate precipitation. Its physical and chemical properties such as optimal pH and temperature, substrate specificity, and effects of inhibitors and activators on the enzymatic activity were investigated. The total protein contents of *Hibiscus rosa–sinensis* petals crude extract and partially purified extract were found to be 425 µg and 122 µg /100 g fresh petals respectively. The enzyme activity and its kinetic parameters, (K_m and V_{max}) were determined using 4-methylcatechol and catechol as substrates. The optimum pH and temperature for *Hibiscus* PPO activities were 6.0 and 45 °C respectively. Ascorbic acid and ethylenediaminetetraacetic acid sodium salt were found to be potent inhibitors and copper sulphate was found to be activator for *Hibiscus* PPO.**

Keywords **- *Polyphenol oxidase, Hibiscus rosa–sinensis; catechol, 4-methyl catechol , K_m and V_{max}***

I. INTRODUCTION

Polyphenol oxidase (PPO, EC 1.14.18.1) is a common copper containing enzyme which is widely distributed in nature. In the presence of oxygen it catalyses two reactions: the hydroxylation of monophenols to o–diphenols and the oxidation of o–diphenols to o–quinones. This enzyme plays an important role in many plant metabolic processes. The oxidation of phenol compounds by PPO are polymerized to form dark–coloured compounds responsible for browning in fruits and vegetables. This is regarded as an undesirable sensory attribute of the produce and decreases its commercial and nutritive values. For this reason PPO has received increasing attention [1]. PPO enzyme has been isolated and characterized from produce of many commercially important plants and fruits such as lettuce [2], aubergine [3], *Ferula* sp. [4], strawberry [5], and cocoa beans [6].

The present investigation was designed to extract PPO from petals of *Hibiscus rosa–sinensis* (Chinese Rose) which is the national flower of Malaysia. The enzyme was partially purified from the aqueous extract of flower petals, and its substrate specificity, kinetic parameters, optimum conditions of pH and temperature, and effects of inhibitor and metallic compounds on the enzymatic activity were evaluated. The results of this study would provide an understanding of the browning of the hibiscus flower and means of prolonging the shelf flower.

II. MATERIALS AND METHODS

All chemicals used were of analytical grade. Polyvinylpyrrolidone from Acros Organics; ascorbic acid and copper sulphate from Fisher Scientific; monosodium phosphate from Fluka Biochemical; disodium phosphate and iron III chloride from Sigma Chemical Co; 4-methylcatechol and catechol from Merk–Schuchardt CHG; EDTA from Progma.

A. Plant Materials

Two hundred grams of fresh red flowers of *Hibiscus rosa–sinensis,* grown in Kuala Lumpur, Malaysia, were hand-picked from the plant and left to dry at room temperature.

B. Enzyme Extraction and Partial Purification

Ten grams of dried petals were homogenized in 80 mL of 0.1M phosphate buffer (pH 6.8) containing 10 mM ascorbic acid and 0.5% polyvinylpyrrolidone (PVP40) with the aid of a magnetic stirrer for 1h. The crude extract samples were centrifuged at 32000 g for 20 min at 4ºC. Solid ammonium sulphate $(NH_4)_2SO_4$ was added to the supernatant to obtain 80% $(NH_4)_2SO_4$ saturation. After 1 h, the precipitated proteins were separated by centrifugation at 32000 g for 30 min. The precipitate was re–dissolved in a small volume of distilled water and dialyzed at 4ºC against distilled water for 24 h with 4 changes of the water during dialysis. The dialyzed sample was lyophilized and this constitutes the partial purified PPO extract and was used as the *Hibiscus* PPO enzyme source.

C. Determination of Hibiscus PPO Activity

Hibiscus–PPO activity was determined by measuring the absorbance at 420 nm using a spectrophotometer (Pharmatech, Model UV–1700). To determine the best concentration of enzyme preparation corresponding to the highest enzyme activity, the activity was assayed in 3 mL of reaction mixture consisting of 0.5 mL substrate (0.02 M 4–methylcatechol and 0.02 M catechol separately) and different concentrations (0.025-0.5 mL) of the enzyme preparation (1mg/mL). This mixture was topped-up to 3.0 mL with the phosphate buffer (pH 6.8) in a 1 cm light path quartz cuvette. The blank consisted of 3.0 mL 0.1 M phosphate buffer (pH 6.8). Two controls were prepared: the cuvette of the first

control contained 2.5 mL buffer solution and 0.5 mL substrate, whereas the second control cuvette contained 2.9 mL buffer and 0.1 mL enzyme preparation. Absorbance values of these controls were subtracted from that of the sample. *Hibiscus*–PPO activity was calculated from the linear portion of the curve. The initial rate of *Hibiscus*–PPO catalyzed oxidation reaction was calculated from the slope of the absorbance–time curve. The results were expressed as absorbance increment min^{-1}. An increase in absorbance of 0.001 min^{-1} was taken as one unit of enzyme activity [7]. An enzyme preparation of 0.1 mL showed the highest activity using 4–methylcatechol as a substrate which was used in all other experiments.

D. Substrate Concentration and Specificity of Hibiscus PPO

An attempt was made to select the best substrate and its optimum concentration corresponding to the highest enzyme activity. The *Hibiscus*–PPO activity was determined using two different substrates namely catechol and 4–methylcatechol at different concentrations (0.01-0.16 M). The activity was assayed in 3 mL of reaction mixture consisting of 2.4 mL phosphate buffer (pH 6.8), 0.5 mL substrate and 0.1 mL enzyme preparation (1 mg/mL). The blank consisted of 3.0 mL of 0.1 M phosphate buffer (pH 6.8). The highest enzyme activity was obtained with 0.04 M of 4–methylcatechol. Therefore the concentration of 0.04 M of 4–methylcatechol was used as the substrate in all other experiments.

E. Protein Estimation and Determination of Molecular Weight

Protein content was estimated by the Lowry method [8]. Bovine serum albumin was used as a standard for the assay. To determine the molecular weight of protein in the extract, 15% gel electrophoresis (both native and SDS–denature) was run with protein marker (prestained SDS–PAGE Standards, Broad Rang, Catalog 161–0318, Control 310007460) which contains myosin (209.998 K Da), B–Galactosidase (117.068 K Da), Bovine serum albumin(97.834 K Da), Ovalbumin (55.145 K Da), Carbonic anhydrase (29.00 K Da), Lysozyme (19.720 K Da) and Aprotinin (6.50 K Da). The bands were visualized with Coomassie blue staining and their relative mobility values were measured and then calculated from the standard curve of the marker (relative mobility values against molecular weight).

F. Enzyme Kinetics

For determination of Michaelis constant (K_m) and maximum velocity (V_{max}) values of the enzyme, *Hibiscus*–PPO activities were measured with two substrates at various concentrations. 1/V and 1/[S] values, obtained from these activity measurements, were used for drawing Lineweaver–Burk graphs. K_m and V_{max} values of *Hibiscus*–PPO, for each substrate, were calculated from Lineweaver– Burk graphs.

G. Effect of pH on Hibiscus–PPO Activity

The effect of pH on *Hibiscus*–PPO activity was determined under standard laboratory conditions using 0.1 mL enzyme preparation, 1 mL of 0.04 M 4–methylcatechol and topped-up to 3 mL of 0.1 M sodium acetate buffer (pH 3.5-5.5) or 0.1 M sodium phosphate buffer (pH 4.5-7.4). The blank contains 3.0 mL buffer only. The optimum pH that corresponded to the highest *Hibiscus*–PPO activity was used for the study of the effect of inhibitors and temperature on enzyme activity.

H. Effect of Inhibitors and Metallic Ions on PPO Activity

The effects of two inhibitors (ascorbic acid and ethylenediaminetetraacetic acid sodium salt (EDTA) and two activators (copper sulphate and ferric chloride) on *Hibiscus*–PPO activity were evaluated using 0.1 mL enzyme preparation, 1 mL of 0.04 M 4–methylcatechol as substrate, 1 mL inhibitor or activator and topped-up to 3mL with 0.1 M sodium phosphate buffer pH 6.0 at room temperature. The change in absorbance was measured spectrophotometrically at 420 nm. Control tests for inhibitors plus substrate plus buffer were also run at the same time. In addition, comparative study of the effect of copper sulphate inhibitor on enzyme activity was done at different concentrations of 0.25-0.4 mM copper sulphate using 0.04 M 4–methylcatechol at pH 6.0 and room temperature.

I. Effect of Temperature on PPO Activity

To determine the optimum temperature for *Hibiscus*–PPO, the activity of the enzyme was measured at different temperatures (25-70°C) using 0.1 mL enzyme, 1.0 mL of 0.04 M 4–methylcatechol as substrate and completed to 3 mL with 0.1 M sodium phosphate buffer (pH 6.0). The blank consisted of 3.0 mL of 0.1 M phosphate buffer. Controls were run under the same tested temperature. The reaction mixture (buffer and substrate) was heated to the appropriate temperatures by using a temperature controller attached to the cell-holder of the UV-Vis spectrophotometer. Once temperature equilibrium was reached, enzyme was added and the reaction was followed spectrophotometrically at constant temperature at given time intervals.

III. Results and Discussion

A. Extraction and partial purification of PPO

The total protein concentrations of the crude extract and ammonium sulphate extract estimated using Lowry method [8] were found to be 10.113 µg mL^{-1} and 3.042 µg mL^{-1} respectively. On a fresh weight basis the total protein content of *Hibiscus* petals crude extract and ammonium sulfate extract were 425 µg and 122 µg /100 g fresh petals.

B. Molecular Weight Estimation

The analysis of SDS-PAGE gel revealed some bands in native conditions both on crude and purified extracts. As shown in Fig.1, the crude extract showed several bands with a more distinct band corresponding to a molecular weight of 70.795 K Da while the partial purified extract showed less number of bands and with a more distinct band

corresponding to a molecular weight of 39.810 K Da. The molecular weight of PPO from other species has been reported as follows: cabbage 39 K Da [9], banana 62 K Da [10], field bean seed 120 K Da [11], and Chinese cabbage 65 K Da [12]. Our results indicate that the molecular weight of *Hibiscus*-PPO is similar to that of cabbage but lower than those of banana, field bean seed and Chinese cabbage

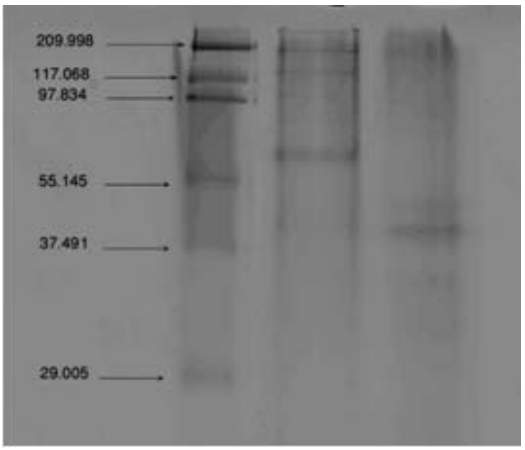

Figure 1. Electrophoretogram of PPO from extracts of petals of *Hibiscus rosa–sinensis*. Lane 1 protein marker; lane 2 crude extract; lane 3 partial purified extract

C. *Enzyme Kinetics and Substrate Specificity:*

A number of compounds was used as substrates for PPO in the literature. In this study, catechol and 4–methylcatechol were used as substrates since they are considered as the most common compounds used as substrates for PPO enzyme [2]. *Hibiscus*–PPO was determined by measuring the initial rate of quinone formation, as indicated by an increase in absorbance at 420 nm [13]. An increase in absorbance of 0.001 min^{-1} was taken as one unit of enzyme activity [7]. In the present study the highest activity was detected with 0.08 M, 4–methylcatechol and 0.02 M catechol. Fig. 2 shows the variation of enzyme activity with substrate concentration. At lower concentrations (0.01 M and 0.02 M) catechol showed greater activity compared to 4–methylcatechol while at higher concentrations (0.04 - 0.16 M) 4–methylcatechol showed higher activities. The enzyme activity decreased with catechol at higher concentrations (Fig. 1b).

Michaelis constants (K_m) and maximum reaction velocities (V_{max}) and specificity (V_{max}/K_m) of the *Hibiscus*–PPO were determined at optimum pH 6.0 and 25°C using catechol and 4–methylcatechol at various concentrations. Analysis of the double reciprocal Lineweaver–Burk plots of *Hibiscus*–PPO gave K_m values of 19.23 mM for catechol and 21.98 mM for 4–methylcatechol (Table 1).

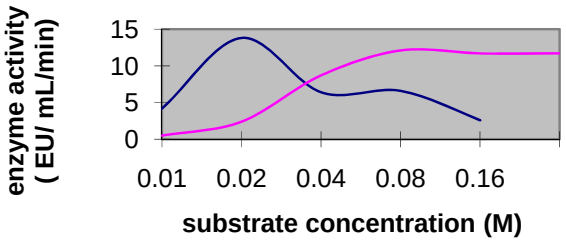

Figure 2. Variation of *Hibiscus*–PPO activity with substrate concentrations. (a): 4-methyl -catechol and (b) catechol.

The K_m values were close for both substrates. The lower activities for *Hibiscus*–PPO were observed with lower concentrations of 4-methylcatechol as substrate (Fig. 1). K_m values obtained for catechol in the literature are in the range of 19 to 21 mM for lettuce [14] whereas K_m values obtained for 4–methylcatechol by some workers were as high as 94.3 mM observed for aubergine [7].

TABLE 1 K_M, V_{MAX}, VALUES FOR *HIBISCUS*-PPO

Substrate	V_{max}(EU/mL/min)	K_m(mM)
Catechol	2137	19.23
4-methylcatechol	1616	21.98

D. *Optimum pH*

Since pH exerts a strong effect on enzymatic activity, it was of interest to study the effect of pH on *Hibiscus*–PPO activity using 4-methylcatechol as a substrate. As shown in Fig.3, it was found that the optimum pH value for *Hibiscus*-PPO was 6.0 which was similar to that reported for aubergine [3] and for *Ferula* sp. [4], In general, most plants show maximum PPO activity at or near neutral pH values [15]. However, different optimum pHs for PPO obtained from various sources are reported in the literature. For example, it is reported that optimum pH values are 5.5 for strawberry [5], 7.0 for Amasya apple [16], 6.8 for cocoa beans [6] using 4–methylcatechol as a substrate

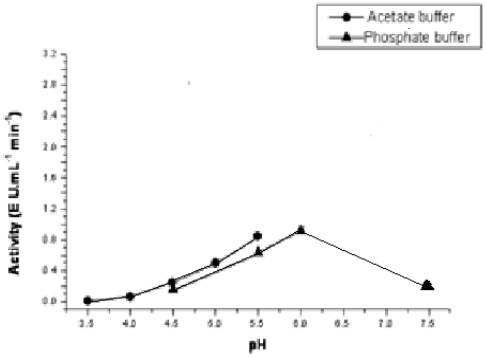

Figure 3. Effect of pH on *Hibiscus rosa–sinensis* PPO activity using sodium acetate buffer and phosphate buffer

E. *Effect of Temperature*

Effect of temperatures were assayed using 4–methylcatechol as a substrate.over a temperature range of 25-70 °C at the optimum pH. The results are shown in Fig. 4. *Hibiscus*-PPO activity was found to increase with increasing temperature with the maximum activity being attained at 45 °C and dropped to a sub-minimum at 70 °C. In the literature the reported optimum temperature values were 20 °C for dog rose [17], 30 °C for aubergine [3] and 56 °C for amasya apple [18], using 4–methylcatechol as a substrate.

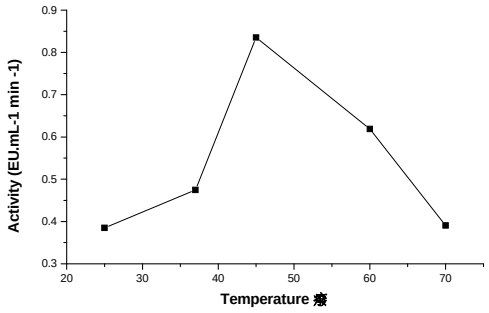

Figure 4. Effect of temperature on *Hibiscus* PPO activity

F. *Effect of Inhibitors and Metallic compounds*

There are several inhibitors, such as ascorbic acid used by researchers to prevent enzymatic browning of plants. In this work, ascorbic acid and EDTA-were used to study their inhibitory effects on the activity of PPO. Fig. 5 shows that ascorbic acid is a stronger inhibitor than EDTA while iron chloride did not show any effect on the *Hibiscus* PPO activity.

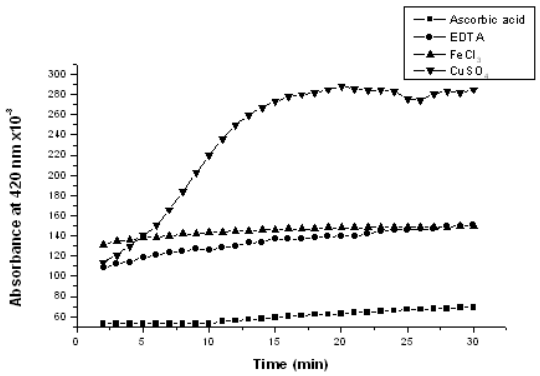

Figure 5. Variation of Absorbance with time for different inhibitors and activators.

However copper sulphate was found to have an activating effect on the activity of the enzyme. Therefore an experiment was conducted to study the *Hibiscus* PPO activity as a function of copper sulfate concentrations. From the results shown in Fig. 6, it is noted that the activity increases with increase concentration of copper sulphate and reaches a plateau at concentration range of 2.5-4 mM. This indicates that *Hibiscus* PPO is a copper enzyme and copper sulphate serves as an activator for its activity.

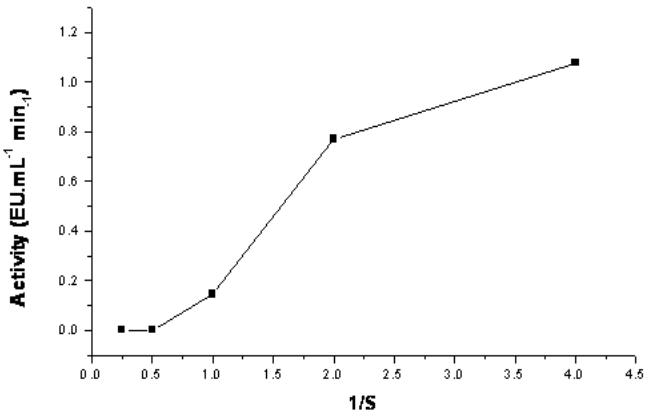

Figure 6. Effect of copper sulphate on *Hibiscus* PPO activity

IV. CONCLUSION

This is the first report on the studies of PPO from the extract of a flower, in particular *Hibiscus rosa-sinensis*. Ammonium sulphate precipitated fraction of the aqueous extract of *Hibiscus rosa-sinensis* flower demonstrated polyphenol oxidase activity and its characteristic physico-chemical properties. This warrants further studies on the isolation and purification of PPO from the extract of this flower.

ACKNOWLEDGMENTS

Ikram Madani would like to acknowledge the help of University of Khartoum for sponsoring the Post-doctorate leave to spend six months in Malaysia University of Science and Technology. K.H. Lee and Pat M. Lee would like to thank the Ministry of Science, Technology and Innovation for the Science Fund (Project nos. 02-02-05-SF0030 and 02-01-01-SF0214).

REFERENCES

[1] Yoruk, R., and Marshall, M. R. (2003). Physicochemical properties and function of plant polyphenol oxidase: A review. Journal of Food Biochemistry, 27, 361–422.

[2] Gawlik–Dziki, U., Zlotek, U., and Swieca, M. (2008). Characterization of polyphenol oxidase from butter lettuce (Lactuca sativa var. capitata L.). Food Chemistry, 107, 128–135.

[3] Dogan, M., Arslan, O., and Dogan, S. (2002). Substrate specificity, heating activation and inhibition of polyphenol oxidase from different aubergine cultivars. International Journal of Food Science and Technology, 37, 415–423.

[4] Erat M, Sakiroglu H, Kufrevioglu IO (2006) Purification and characterization of polyphenol oxidase from Ferula sp. Food Chem. 95:503–508.

[5] Wesche-Ebeling P, Montgomery MW. (1990) Strawberry polyphenol oxidase: extraction and partial characterization. J Food Sci 55:1320–1325.

[6] Lee, PM., Lee, KH and Karim, M.IA (1991) Biochemical studies of cocoa beans polyphenoloxidase Journal of Food Science and Agri. 55, 251-260.

[7] Ho, K. (1999). Chaeacterisation of poly phenol oxidase from aeril root of an orchid Aran ,csaline 130. Plant Psiology et Biochemistry, 37 (11), 841-848.

[8] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951). Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193 (1): 265–75.

[9] Fujita, S., Saari, NB, Maegawa, M, Tetsuka, T, Hayashi, N, and Tono, T (1995). Purification and properties of polyphenol oxidase from cabbage. Journal of Agricultural and Food Chemistry, 43, 1138.

[10] Galeazzi, MAM, Sgarbieri, VC and Constantinides, SM (1981). Purification and physicochemical characterization of polyphenol oxidase from a dwarf variety of banana. Journal of Food Science, 46, 150.

[11] Beena, P and Gowda, LR (2000). Purification and characterization of polyphenol oxidase from the seeds of field bean. Journal of Agricultural and Food Chemistry, 48, 3839.

[12] Nagai, T, and Suzuki, N (2001). Partial purification of polyphenol oxidase from chinese cabbage. Journal of Agricultural and Food Chemistry, 49, 3922.

[13] Wisserman, K. W., and Lee, C. Y. (1980). Purification of grape polyphenoloxidase with hydrophobic chromatography. Journal of Chromatography, 192, 232–235.

[14] Dogan S and Salman U (2007) Partial characterisation of lettuce (Lactuca sativa L.) polyphenol oxidase. Eur Food Res Technol 226:93–103.

[15] Betrosian, K., Steinburg, MP., and Nelson, AI. (1960). Effect of borates and other inhibitors on enzymic browning in apple tissue,2. Mechanisms in Food Technology, 14, 480.

[16] Siddiq, M., Sinha, NK., and Cash, JN. (1992). Characterization of polyphenol oxidase from Stanley plums. Journal of Food Science, 57, 1177–1179.

[17] Sakiroglu, H., Kufrevioglu, IO., Kocacaliskan, I., Oktay, M., and Onganer, Y. (1996). Purification and characterization of Dog-rose (Rosa dumalis Rechst.) polyphenol oxidase. Journal of Agricultural and Food Chemistry, 44, 2982–2986.

[18] Oktay, M., Keufrevioglu, I., Kocacaliskan, I., and Sakiroglu, H. (1995). Polyphenol oxidase from Amasya apple. Journal of Food Science, 60, 495–499.