Bull. Fac .Agric. Cairo Univ., 60: 408-414 (2009)

SOME PROPERTIES OF THE POLYPHENOL OXIDASE FROM *Cyclamen persicum*

(Received: 17.8.2009)

By
O.M. Atrooz

Department of Biological Sciences, Mutah University, Jordan

ABSTRACT

A study of polyphenol oxidase (PPO) from *Cyclamen persicum* Mill. (*C. persicum*) tubers was carried out to investigate its characteristics in terms of pH and temperature optima, kinetic parameters and effects of some inhibitors on its activity. Optimum pH for the PPO activities in the presence of catechol as a substrate were 4.0 and 7.0 and the optimum temperature was 40 ^{0}C. It was found that the best substrate for the PPO was catechol, by considering the K_m, V_{max}, and V_{max}/K_m ratio (5.34) compared to catechin (1.67) and L-tyrosine (1.66). Six inhibitors of PPO activity were tested. The most potent inhibitor (at 10 mM) was thiourea with relative activity 4.7% followed by sodium thiosulphate (9.3%), L-ascorbic acid (11%) and L-cysteine (12%), while benzoic acid and citric acid showed poor potent activities with relative activities of 50% and 74.6%, respectively.

***Key words**: browning, Cyclamen persicum Mil., polyphenol oxidase.*

1. INTRODUCTION

Cyclamen is a genus of 23 species of flowering plants, traditionally classified in the family Primulaceae. The genus is most widely known by its scientific name *Cyclamen*. They are perennial herbaceous aestivating plants, with a surface or underground tuber, which produces leaves in late winter, and flowers in the autumn (Yesson & Culham, 2006 and Yesson *et al*., 2009).

According to folk medicine some species belonging to the genus *Cyclamen* were used for their biological activities. Early investigation of the different species of the *Cyclamen* resulted in the isolation of triterpenic saponins from cyclamen tubers, which have anti-inflammatory and antinociceptive activities in rats and mice (Speroni *et al*., 2007). *Cyclamen* is a houseplant that has a toxic saponin in the tuberous rhizomes (Spoerk *et al*., 1987), and its pollen causes an allergy (Ariano and Panzani,).

Polyphenol oxidase (PPO) is a copper-containing enzyme, which is also known as catechol oxidase, catecholase, diphenol oxidase, O-diphenolase, phenolase and tyrosinase (Victoria and Whitaker, 1995). It is present in some bacteria, fungi, most plants, some arthropods and all mammals. In all cases, it is responsible for melanization in animals (Mercedes and Francisco, 1996) and browning in plants (Tan and Harris, 1995) due to the oxidation of phenolic compounds which have a role in disease resistance and in photosynthetic regulation (Ridgway and Tucker, 1999). It catalyzes two distinct reactions using oxygen as a second substrate (Carine *et al*., for 2004) the hydroxylation of monophenols and the oxidation of o-diphenols to o-quinones (Mercedes and Francisco, 1996) and then react following different pathways according to their electrophilic and/ or oxidant character to produce secondary products (Mayer, 1987; Mayer and Harel, 1979,1991 and Eskin, 1990).

A lot of previous works deal with characterization of the PPO enzymes of different plants, determination of their activity, kinetics parameters, and their inhibition (Carine *et al*., 2004), but to our knowledge non on PPO enzyme of *Cyclamen*.

Several chemical compounds have already been proposed to inhibit PPO. Among these chemicals, some act on the enzyme such as halide salts, organic acids and chelating agents, while others may act upon the reaction products like reducing agents (Catherine *et al*., 2003).

The objective of this work was to study the activity, kinetics, pH and temperature optima, and some properties of the enzyme polyphenol oxidase from *Cyclamen persicum* tubers. Since, it is the first study on PPO of C. *persicum* , some illustrations on its characteristics, and its usage and purifications are considered true.

2.MATERIALS AND METHODS

2.1. Plant material

Cyclamen persicum tubers were collected from Perguish region in the north of Jordan in April, 2009.

2.2. Chemicals

Sodium fluoride (CODEX LTD Carloerba, Milan, Italy); Catechol, L-tyrosine and Thiourea (BDH Laboratory Supplies, Poole, England); Di-sodium hydrogen phosphate anhydrous (Fluka–Chemieka AGCH.9470 Buchy); Sodium-dihydrogen phosphate (Panreac, Montplet & Esteban SA, Barcelona-Espana); Sodium acetate: (Riedel-Dehaen.Sigma-Aldrich Laborchemilealien GmbH Seelze); Catechin (Sigma-Aldrich CO., USA); Sodium thiosulphate (Pharmacos LTD. Southend-On-Sea, Essex, England); L-Ascorbic acid (Surechem Products LTD, Needham Market, Suffolk, England); L-cysteine (GRG, Avondale Laboratories- Supplies and Services limited, Banbury, Oxon, England); Benzoic acid (CBH Lab Chemicals, Nottingham, UK).

2.3. Crude enzyme preparation

Fresh *C. persicum* tubers (50 grams) were homogenized with the addition of sodium fluoride solution (1:5 w/v) in a blender for 3 min. The homogenate was filtered through several filter papers, and then was centrifuged for 10 min at low speed. The supernatant was collected as crude enzyme solution and was kept at 4 ^{0}C.

2.4. Browning intensities measurement

A sample, (1.5 ml) of freshly prepared crude enzyme solution in a cuvette was monitored for its optical densities (absorbance) at wavelength 410 nm for 60 min by spectrophotometer (Pharmacia. Novaspec II, LKB Biochrom, England). The absorbance was taken as the browning intensity.

2.5. Assay of PPO activity

Polyphenol oxidase activity was determined by measuring the increase in absorbance at 410 nm at 37 ^{0}C with a spectrophotometer. The sample tube contained 2.0 mL of 20 mM catechol (prepared in 0.2 M sodium phosphate buffer, pH 7.0), 0.9 ml of 0.2 M sodium phosphate buffer (pH 7.0) and 0.1 ml of enzyme solution. The reference tube (blank) contained 2.0 ml of the same substrate solution and 1.0 ml of 0.2 M sodium phosphate buffer. The initial velocity was calculated from the slope of the absorbance-time curve. One unit of PPO activity was defined as the change in absorbance of 0.001 min^{-1} (Mustafa *et al*., 2007).

2.5.1. Effect of pH on PPO activity

Two kinds of buffer solutions were used for this study: 0.2 M sodium acetate buffer for the pH range of 3.6–) 5.6, and 0.2 M sodium phosphate buffer for pH range 6.0–8.0. Catechol (20 mM) was used as a substrate to determine the effect of pH on PPO activity.

To determine the effect of pH on PPO activity, 0.1 ml of enzyme solution was incubated in 0.9 ml of various buffer solutions (pH 3.6–8.0) for 10 min at 40 ^{0}C, and the residual activity was measured at 0 and 60 min, The enzyme activity was measured according to the method described above. Residual PPO activity was determined in the form of percentage residual PPO activity at the optimum pH.

2.5.2. Effect of temperature on PPO activity

The PPO activity was determined at various temperatures: 20 ^{0}C - 80 ^{0}C using water bath (Gesellschaft Fur Labortechnik mbH D 3006 Burgwedel (GFL), Germany). The mixtures of 0.9 ml of 0.2 M sodium phosphate buffer (pH 7.0) and 2.0 mL of catechol solution (20 mM) were incubated for 5 min at various temperatures over the range of 20 ^{0}C - 80 ^{0}C, prior to the addition of 0.1 ml of enzyme solution. The relative activity of PPO at a specific temperature was determined spectrophotometrically by addition of enzyme to the mixture as rapidly as possible. Residual PPO activity was determined in the form of percentage residual PPO activity at the optimum temperature. (Segel, 1976).

2.5.3. Substrate specificity

Michaelis-Menten constant (K_m) and maximum reaction velocity (V_{max}) of the enzyme PPO were determined using three substrates (catechol, catechin, and L-tyrosine).

They were assayed at different concentrations for each substrate: 1.0 mM, 3.0 mM, and 5.0 mM at pH 6.0, temperature 40 ^{0}C and at wavelength 410 nm. Data were plotted as 1/velocity and 1/substrate concentration according to the method of Lineweaver & Burk (1934). Substrate specificity (V_{max}/K_m) was calculated by using the data obtained on a Lineweaver-Burk plot.

2.5.4. Effect of inhibitors

The reaction mixture contained 2.0 ml of 20 mM catechol, 0.4 ml of 0.2 M sodium phosphate buffer (pH 7.0), and 0.5 ml of inhibitor solution in 0.2 M sodium phosphate buffer (pH 7.0) and 0.1 ml of enzyme solution. Each of the inhibitors was assayed at various concentrations (0.1 mM, 1.0 mM and 10 mM). Relative enzyme activity was calculated as a percentage of the activity without any inhibitor.

2.6. Statistical analysis

Data were expressed as means ± standard deviation of three measurements made on one extract. A significant difference was considered at the level of $p<0.05$.

3. RESULTS AND DISCUSSION

3.1. Effects on browning and enzyme activity

Fig. (1) shows the browning intensities of *Cyclamen* PPO for 60 minutes. It was observed that the browning intensities were significantly increased with time using the acetate buffer (pH

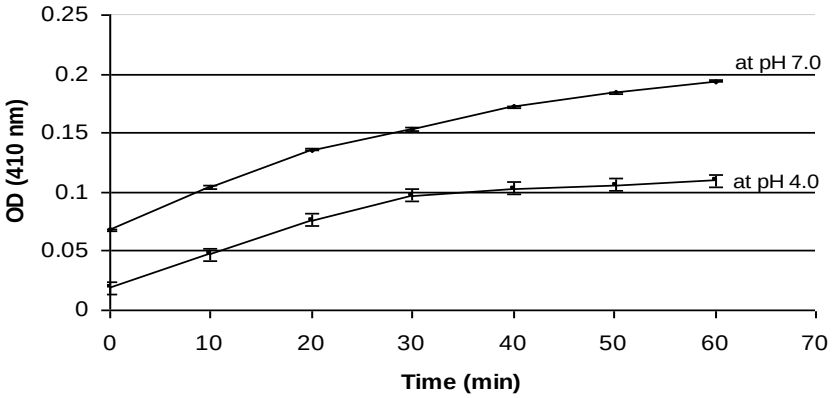

Fig. (1) The browning intensities of *Cyclamen persicum* **PPO with time at (410 nm) at pH 4.0 and 7.0. Each value is the mean for the three replicates, and vertical bars indicate the standard deviations**.

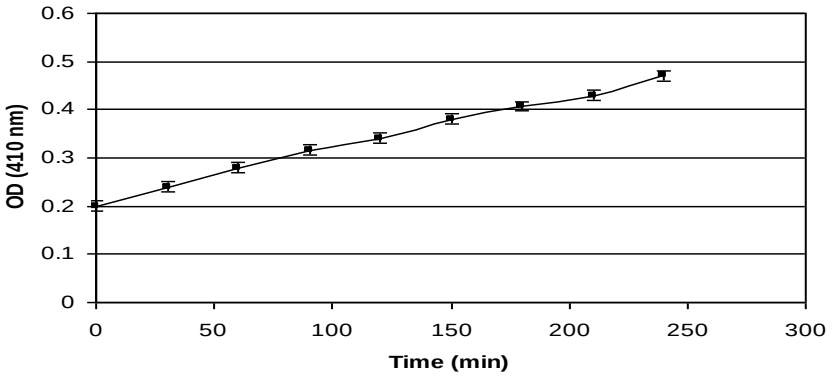

Fig. (2) Activity of *Cyclamen persicum* **PPO with time (at 410 nm) at pH 7.0. Each value is the mean for three replicates, and vertical bars indicate the standard deviations.**

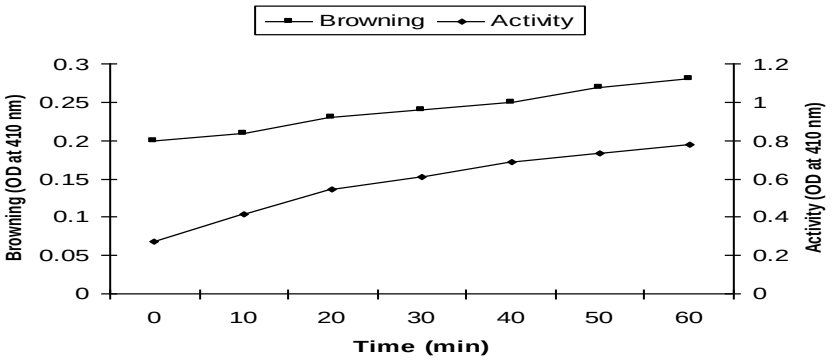

Fig. (3) The relationship between the browning intensities and PPO activity of *Cyclamen persicum* **with time (at pH 7.0).**

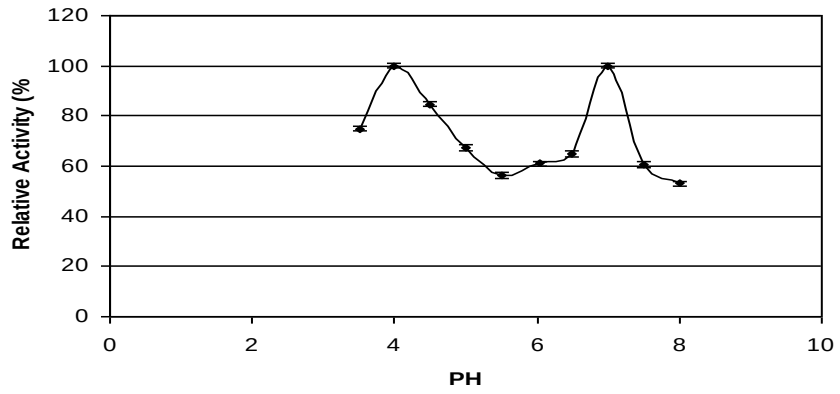

Fig. (4) The activity of *Cyclamen persicum* **PPO as a function of pH with catechol as a substrate. The maximum activity was considered as 100%. Each value is the mean of the three replicates, and vertical bars indicate the standard deviations**.

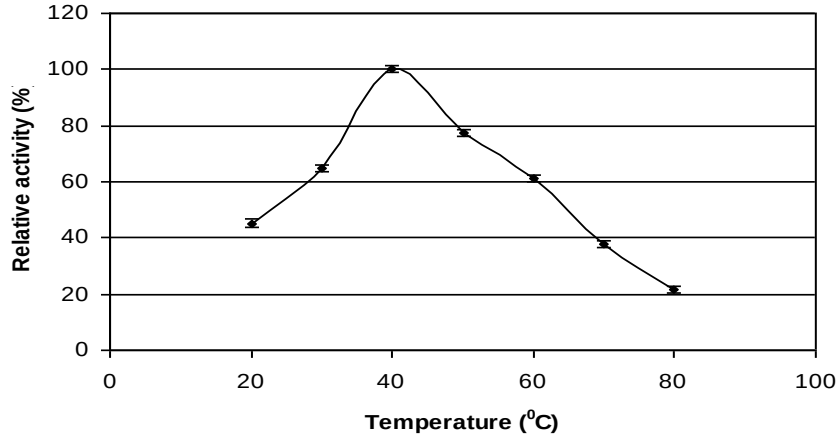

Fig. (5) The optimum temperature of *Cyclamen. persicum* **PPO with catechol as a substrate. The maximum activity was considered as 100%. Each value is the mean for the three replicates, and vertical bars indicate the standard deviations.**

4.0) and phosphate buffer (pH 7.0). The browning intensities in terms of optical density (OD) were higher for the phosphate buffer (0.196) than the acetate buffer (0.111). Fig. (2) shows the increment in the enzyme activity. The increase in the browning was correlated with the increase in the enzyme activity (Fig. 3). This indicates that PPO activity is one of the principal factors affecting the browning of *Cyclamen* tubers.

3.2 pH optima

The *Cyclamen* PPO activity as a function of pH was determined with two kinds of buffer solutions: sodium acetate for the pH range 3.5-5.5 and phosphate buffer for pt range 6.0-8.0. Two pH optima were observed with catechol as a substrate (Fig.4) the first peak was recorded at pH 4.0 and the other at p^H 7.0.

The enzyme showed very high relative activity (%) at pH 4.0 and 7.0. A sharp drop in enzyme activity was observed at PH between 4.0-5.5 and between 7.0-8.0, while an increase in the activity was seen between pH 3.5 to 4.0 and 5.5 to 7.0.

In most plants, PPO enzyme from different parts has only one pH optimum, such as in potato it was 5.0 (Balasingam and Ferdinand, 1970), medlar fruits 6.5 (Barbaros *et al.,* 2002), longan fruit 7.0 (Jiang, 1999), but some have two pH optima, such as in apple (Shannon and Pratt, 1967) and sweet cherry (Pifferi and Cultrera, 1974). In all, the optimum pH for maximum PPO activity in plants lies between 4.0 to 7.0, dependsing on extraction methods, substrate used and the localization of the enzyme in the plant cell (Aylward and Haisman, 1969).

3.3. Temperature optima

The effect of temperature on PPO is shown in Fig. (5). The optimum temperature of *Cyclamen* PPO activity was 40 ^{0}C. The relative activity of PPO was 45% at 20 ^{0}C and continues increasing to reach a maximum (100%) at 40 C^0. Increasing temperature. above 40 ^{0}C results in a drastic decline in the activity, which reached 20% at 80 ^{0}C, and this may be due to the effect of denaturation. This optimum temprature for *Cyclamen* PPO was higher than the optimaum temperature of PPO from Stanly plum (25 ^{0}C) (Siddig *et al.,* 1992), Medler friuts (35 ^{0}C) (Yang *et al.,* 2000) and grape (25-30 ^{0}C) (Mustafa *et al*., 2007).

3.4. Kinetic parameters:

Cyclamen PPO showed different activities towards different substrates. K_m value is a measure of affinity of the enzyme for the substrate, the smaller the value of K_m, the greater the affinity of the enzyme for that substrate. The maximum velocity (V_{max}) of an enzyme-catalyzed reaction shows the catalytic efficiency. Table (1) indicated that *Cyclamen* PPO had different K_m and V_{max} values for catechol, catechin, and L-tyrosine substrates.

Table (1): Kinetic parameters for the oxidation of various substrates by *Cyclamen persicum* **PPO at optimum pH and temperature.**

Substrate	Km (mM)	Vmax (µmol/	V_{max}/K
Catechin	20.00	33.33	1.67
Catechol	20.08	11.11	5.34
L-tyrosine	6.25	10.00	1.66

The best substrate for any enzyme depends on the strong binding of the substrate to the enzyme (low K_m) and high catalytic activity (high V_{max}). The ratio of V_{max}/K_m will evaluate the best substrate (Palmer, 1995). The stronger substrate binding was recorded with chatechol (K_m 2.08) while catechin was the least binding (K_m 20). By considering the ratio (V_{max}/K_m), it was observed that catechol was the best substrate for *Cyclamen* PPO (ratio 5.30) compared to catechin (1.67) and L-tyrosine (1.66).

3.5. Effect of inhibitors

The effects of inhibitors of various concentrations on *Cyclamen* PPO are shown in Table (2). The results were reported as relative activity (%). The effect of inhibitors was in the following order (at concentration 10 mM): 4.7>9.3>11>12>50>74.6 for thiourea, sodium thiosulphate, L-ascorbic acid, L-cysteine, benzoic acid and citric acid, respectively.

The mechanism of action for these inhibitors was different. Thiourea and L-cystein react with copper (cofactor) of the enzyme to inhibit its activity or form a complex with o-quinones.

Table (2): Effects of inhibitors on *Cyclamen. persicum* **PPO activity. The concentrations of each inhibitor were 0.1, 1.0 and 10 mM.**

Inhibitor	Concentration (mM)	Relative activity (%)
Thiourea	0.1	67.2
	1.0	38.7
	10.0	4.7
Sodium sulphete	0.1	35.3
	1.00	18.4
	10.0	9.3
Ascorbic acid	0.1	56.8
	1.00	33.0
	10.0	11.0
L-cystein	0.1	55.0
	1.0	25.0
	10.0	12.0
Benzoic acid	0.1	7 0.1
	1.0	59.7
	10.0	50.0
Citric acid	0.1	100.0
	1.0	85.0
	10.0	74.6

Sodium thiosulphate is a reducing agent and reacts with o-quinones to form a colourless complex by reacting with disulfide bonds of PPO structure, leadsing to a change in the tertiary structure and thus inhibits its activity (Lerch, 1987). L-Ascorbic acid also reacts with copper of the enzyme and acts as a reducing agent preventing the formation of coloured complex compounds (Wang, 1991).

Conclusion:

Recently, more attention was paid to Cyclamen tubers for the usage of its content in medicine (*e.g*. saponin and terpenes). However, the problem of browning hinders its usage. Data on pH, temperature, inhibitors and substrate specificity (kinetics) of *Cyclamen* PPO can give us information about some properties and conditions.

4. REFERENCES

Ariano R. M. and, Panzani R.C. (2006). Occupational respiratory allergy to cyclamen pollen: a case report. Allergy Immunol: (Paris).38(3): 90-3.

Aylward, F. and Haisman D.R. (1969). Oxidation systems in fruits and vegetables-Their relation to the quality of preserved products. Advances in Food Research, 17: 71-76.

Balasingam K. and Ferdinand W. (1970). The purification and properties of a ribonucloenzyme o-diphenol oxidase from potatoes. Biochemistry Journal, 118: 15–23.

Barbaros D., Ahmet C., Nese A., Asim K. and Saadettin G. (2002) Characterization of polyphenoloxidase from *medlar fruits (Mespilus germanica* L., Rosaceae). Food Chemistry, 77: 1–7.

Carine Le Bourvellec, Jean-Michel Le Q., Philippe Sanoner, Jean-Francois D. and Sylvain Guyot (2004) Inhibition of apple Polyphenol oxidase activity by procyanidins and polyphenol oxidation products. Journal of Agricultural and Food Chemistry 52:122-130.

Catherine B., Emeline R., Sophie B., Christelle M, and Jacques N. (2003). Inhibitory effect of unheated and heated D-glucose, D-fructose and L-cysteine solutions and maillard reaction product model systems on polyphenoloxidase from apple. Food Chemistry, 81: 35-50.

Eskin N.A.M. (1990). Biochemistry of Food Spoilage: Enzymatic Browning. In Biochemistry of Foods (2nd Ed.). Academic Press: New York. p. 401-432.

Jiang Y.M. (1999). Purification and some properties of polyphenol oxidase of longan fruit. Food Chemistry, 66: 75–79.

Lerch K. (1987): Molecular and active site structure of tyrosinase. Life Chemistry Report, 5: 221- 234.

Lineweaver H. and Burk D. (1934). The determination of enzyme dissociation constant. Journal of American Chemistry Society, 56: 658–661.

Mayer A.M. (1987) Polyphenol oxidases in plants-Recent progress phytochemistry. 26:11-20.

Mayer A.M. and Harel E. (1979) Polyphenol oxidases in plants. Phytochemistry, 18:193-215.

Mayer A.M. and Harel E. (1991) Polyphenol oxidases and their significance in fruits and vegetables. In P.F. Fox (ed), Food Enzymology. p. 373-399. London: Elsevier Applied Sciences.

Mercedes J. and Francisco G.C. (1996). Kinetics of the slow pH-mediated transition of polyphenol oxidase. Archives of Biochemistry and Biophysics, 331(1): 15-22.

Mustafa U.U., Aysun S. and Kemal S. (2007) Characterization of Sultaniye grape (*Vitis vinifera* L. cv. Sultana) polyphenol oxidase. International Journal of Food Science and Technology, 1:1-5.

Palmer T. (1995). Kinetics of single-substrate enzyme catalysed reactions. In: Understanding Enzymes, 4th edn. pp: 107–127.

Patricia de Fatima P.G., Jose D.A., Marcelo, M.M., Luiz C.O. and, Laudiene E.M. (2003) Purification of Polyphenoloxidase from coffee fruits. Food Chemistry, 83: 7-11.

Pifferi P.G. and Cultrera, R. (1974) Enzymatic degradation of anthocyanins: the role of sweet cherry polyphenol oxidase. Journal of Food Science, 39: 786–791.

Ridgway T.J. and Tucker G.A. (1999) Procedure for the partial purification of apple leaf polyphenol oxidase suitable for commercial application. Enzyme and Microbial Technology, 24: 225-231.

Segel I.H. (1976). Biochemical Calculations. 273 pp. New York, NY: John Wiley Sons.

Shannon C.T. and Pratt D.E. (1967). Apple polyphenol oxidase activity in relation to various phenolic compounds. Journal of Food Science, 32: 479–483.

Siddig M., Sinha N.K. and Cash Y.N. (1992) Characterization of a polyphenol oxidase

from Stanley plums. Journal of Food Science, 57: 1177–1179.

Speroni E., Cervellati R., Costa S. , Dall'acqua S., Guerra M. C. , Panizzolo C. , Utan A. and Innocenti G. (2007) Analgesic and anti-inflammatory activity of *Cyclamen repandum* tubers in rats and mice. Phytotherapy Research 21(7): 684-689.

Spoerk D. G., Spoerke S.E., Hall A. and Rumack, B.H. (1987). Toxicity of *Cyclamen persicum* Mill. Vet. Hum. Toxicol. 29: 250-251.

Tan B.K. and Harris N.D. (1995). Maillard reaction products inhibit apple polyphenoloxidase. Food Chemistry 53: 267-273.

Tien C. l., Vachon C., Mateescu M.A., and Lacroix, M. (2001) Milk protein coatings prevent oxidative browning of Apples and potatoes. Journal of Food Science, 66(4):

Victoria M. and Whitaker J.R. (1995). The biochemistry and control of enzymatic browning. Trends in Food Science& Technology, 6: 195-200.

Wang Z. (1991). Food enzymology. 268. Beijing, China: China Light Industry Press.

Yang C.P., Fujita, S., Ashrafuzzaman M.A., Nakamura N. and Hayashi N. (2000). Purification and chracterization of polyphenol oxidase from banana (*Musa sapientum* L.) pulp. Journal of Agricultural Food Chemistry, 48: 2732–2735.

Yesson C. and Culham A. (2006). A Phyloclimatic study of Cyclamen. BMC Evolutionary Biology, 6:72.

Yesson C., Toomey N.H. and Culham A. (2009). Cyclamen: Time sea and speciation biogeography using a temporally calibrated phylogeny. Journal of Biography, 36(7): 1234-1252.

بعض خصائص إنزيم البولى فينول أوكسيديز المستخلص من نبات *Cyclamen persicum* **Mill**

عمر محمد عطروز

قسم العلوم الحياتية-جامعة مؤتة-الأردن

ملخص

تمت دراسة فعالية و بعض خصائص انزيم البولي فينول اوكسيداز في درنات نبات السيكلامن(*Cyclamen persicum*) بيرسيكم من حيث درجة الحرارة و الحموضة المثلى و بعض المعايير المتعلقة بثابت و سرعة التفاعل بالإضافة لتأثير بعض المثبطات على فعاليةالانزيم.

وقد أظهرت النتائج أنه عند استخدام الكاتيكول كوسط ، فإن درجة الحموضة المثلى هي 4,0 و 7,0 بينما درجة الحرارة المثلى هي 40.0 م^0 ، وان المادة المتفاعلة الكاتيكول هي الأنسب مقارنة مع الكاتكين و الثيروسين اعتمادا على ثابت و سرعة التفاعل. كما أثبتت نتائج المثبطات أن الثيويريا هو الأكثر فعالية بينما حامض السيتريك هو الأقل.