Observations on the Peroxidases System in the Locust, Schistocerca Gregaria (Forskal)

takwa E. Ellakwa1 and Tarek R. Amin2
Physical Chemistry Dept., Egyptian Russian Univ., Egypt.
2. Pest Physiology Dept., Plant Protection Research Institute,Agricultural Research Center, Giza, Egypt.

Word count: 2000

Corresponding Author: Takwa E.Ellakwa, physical chemistry Dept., Egyptian Russian Univ., Egypt

Abstract -Effects of physical and chemical treatments to peroxidases from the haemolymph of the 4th nymphal instar of the desert locust, Schistocerca gregaria were studied to detect some properties of this enzyme system. The results showed that peroxidases oxidation of O-phenylenediamine was significantly inhibited by ethylenediamine tetra acetic acid disodium salt (EDTA) and Na azide, but activated by the reducing agent (ascorbic acid) and CUSO4. The inhibition of peroxidases activity by EDTA as a chelating agent suggest that locust peroxidases are metaloenzymes. Peroxidases were more stable in acidic than alkaline media, and had optimum activity at pH 6. Peroxidases, as shown by the study, were less thermostable than some other enzyme systems. The relatively high temperature degrees (>50°C) affected thermostability of the enzyme. Locust haemolymph peroxidases activity increased gradually from 15 to 25°C and reaching to its optimal activity at 35°C. It is likely to take into consideration, during peroxidases studying, that they are sensitive to high temperature, alkalinity of the medium, and some salts and compounds, the situation that might affect locust peroxidases catalysis.

Key words: Peroxidases, temperature, pH, activators, inhibitors, locust, haemolymph.

1. INTRODUCTION

Peroxidases (EC 1.11.1.) are enzymes catalyze oxidation-reduction reactions using H_2O_2 as oxidant [1] . The functions of insect peroxidases include detoxification, stabilization of extracellular matrices, and possible involvement in insect immunity **[2].**

Properties and significance of some oxidoreductases such as phenoloxidases, specially as defensive mechanism against pathogens, have been well studied in insects **[3]; [4]; [5]; [6]; [7].** However other oxidoreductases such as peroxidases have been received, little attraction. Some studies were concerned with the structure and catalytic mechanisms such that performed on *Aedes aegypti* peroxidase **[2].** Ascorbate

peroxidase (APOX) activity, which catalyzes the oxidation of ascorbic acid was found to be resistant to many of the known inhibitors of plant Apox in larvae of *Helicoverpa zea* **[8].** A long these lines, this study was conducted to detect compounds able to inhibit peroxidases from the fourth instar nymphs of the desert locust, *Schistocerca gregaria* as well as some characteristic properties involved in its activation, inhibition and some physicochemical effects on enzyme.

2. MATERIALS AND METHODS

2.1 Insects:

Fourth nymphal instars of the desert locust was collected from Giza province cultivars, and brought to laboratory to perform the experiments**.**

2.2 Enzyme haemolymph preparation:

Nymphs were bled by cutting one of their thoracic legs and the haemolymph was collected in a chilled centrifuge tube coated with ice jacket. The collected haemolymph was centrifuged for 10 min at 3000 r.p.m. to remove the blood cells. The blood plasma proteins were precipitated with ammonium sulphate (70%), dialyzed after suspended in d H_2O_2, and used as semi-purified enzyme source which kept in a freezer (-20°C) till use for peroxidase activity assay.

Total protein in the plasma was determined as described by **[9]** using protein-dye binding method using bovine serum albumin as standard.

2.3 Determination of peroxidases activity:

Peroxidases activity was determined with modifications of the method described by **[10]**. The reaction mixture consisted of 0.8 ml 0.2 M phosphate - Citrate buffer, 100 ul of 10^{-3} M O-phenylenediamine as the substrate (dissolved in 95% ethanol), 100 ul of 0.3% H_2O_2 and 25 ul of the locust plasma. The pH and temperature of the

reaction were at the optima found experimentally after detecting effect of such physical factors on the peroxidases activity. Enzyme reaction was initiated by adding the substrate solution to the reaction mixture. Prior to the inhibition of the reaction, the substrate and other ingredients of the reaction were separately incubated at the optimum temperature of the reaction. The absorbance of peroxidases activity was recorded for 10 min at 1 min interval against sample blank as the zero adjustment at 430 nm using spectronic 1201 spectrophotometer. Peroxidases activity was expressed as Δ O.D units $x10^3$ min^{-1} mg $protein^{-1}$.

2.4 Activation and inhibition of enzymatic activity:

Peroxidases activators and inhibitors were used for determining some properties, or detecting potent and new inhibitors for this system. Effect of organic compounds such as ascorbic acid as a reducing agent, and inorganic compounds such as ethylenediamineteracetic acid disodium salt (EDTA), cupper sulphate (CuSo4) and sodium azide (NaN3), on peroxidases activity was determined. Two hundreds microliters of various compounds were added to 25 ul of the plasma and incubated for 15 min at room temperature. Then peroxidases activity was determined as described above. The results were compared to reaction mixture containing 200 ul of Δ H2O instead of activators or inhibitors solutions.

2.5 Thermal and pH effect on peroxidases activity:

Locust plasma peroxidases, prior to the initiation of reaction, were incubated for 30 min at 25°C at variable pH values (4-9), and temperature (20-80°C) to determine their stability. Buffers added to the reaction were prepared with 0.1 M citric acid-sodium citrate (pH 4.5 and 5.5), 0.1 M sodium phosphate (pH 6-8), and 0.1 M Tris-HCl (pH 9). The assay of peroxidases activity was followed as described above.

2.6 Data analysis:

All obtained values were pooled from triplicate (at least n =5). Colorimetric determinations were repeated 3 times. Using Costat statistical software (Cohort Software, Brekely), means and standard deviations were obtained. The statistical significant between means were tested by Duncan's multiple range test at 1% level.

3. RESULTS AND DISCUSSION

3.1- Chemical effects:

Peroxidases activators and inhibitors were used for determining some properties of this enzyme or detecting new inhibitors of this system in *S. gregaria* fourth-nymphal instar. Incubation of haemolymph peroxidases for 15 min at 25°C, before determining the enzyme activity, with some inorganic compounds, reducing and chelating agents was studied (Table 1). The results show that peroxidase oxidation of O-phenlenediamine was significantly inhibited by all of the tested concentrations of the chelating agent; EDTA. The activity was 19.9, 11.8, 11.71 and 42.15 Δ_{430}ODx10^3/min/mg protein for haemolymph enzyme incubated with 10^{-4}, 10^{-3} and 10^{-2} M EDTA, and none treated enzyme, respectively. On the contrary, concentrations of ascorbic acid as a reducing agent activate the enzyme catalysis, and the activation was proportional to the tested concentration. After enzyme incubation with 5x10^{-3} and 2.5x10^{-2} M of ascorbic acid, the activity was 55.02 and 97.17 Δ_{430}ODx10^3/min/mg protein, respectively. The same condition was observed for $CuSo_4$, while NaN_3 inhibit *S. gregaria* haemolymph peroxidases activity when used at concentration of 10^{-1} M.

3.2- Physical effects:

The ability of peroxidases to withstand pH and temperature extremes, i.e. their stability, and optimal conditions for enzyme activity were studied. Incubation of locust plasma peroxidases for 30 min at 25°C (room temperature) in variable degrees of pH (4-9) before measuring the ability of peroxidases to oxidize O-phenylenediamine was studied (Fig. 1). The results showed that peroxidases were more stable in acidic than alkaline media. The percentage of peroxidases activity as compared to

Table (1): Effect of incubation of peroxidases with some chemical compounds on peroxidases activity of the haemolymph, of 4th nymphal instar of the locust, *S. gregaria*.

Compounds	Conc. (M)	Peroxidases activity (Δ_{430}ODx10^3/min/mg protein)
None	-	42.15±1.9[a]
EDTA	10^{-2}	11.71±0.9[c]
	10^{-3}	11.8±07[c]
	10^{-4}	19.9±0.5[b]
Ascorbic acid	$2.5x10^{-2}$	97.17±4.2[b]
	$5x10^{-3}$	55.02±1.8[c]
$CuSO_4$	$5x10^{-3}$	87.8±4.1[b]
	10^{-3}	91.3±3.7[b]
	10^{-4}	50.3±2.6[c]
NaN_3	10^{-1}	4.12±0.3[b]
	10^{-2}	44.5±2.4[a]

- Data presented as the means±S.D.

- Means of each compound and none treated enzyme, bearing different subscripts are significantly different (P< 1%).

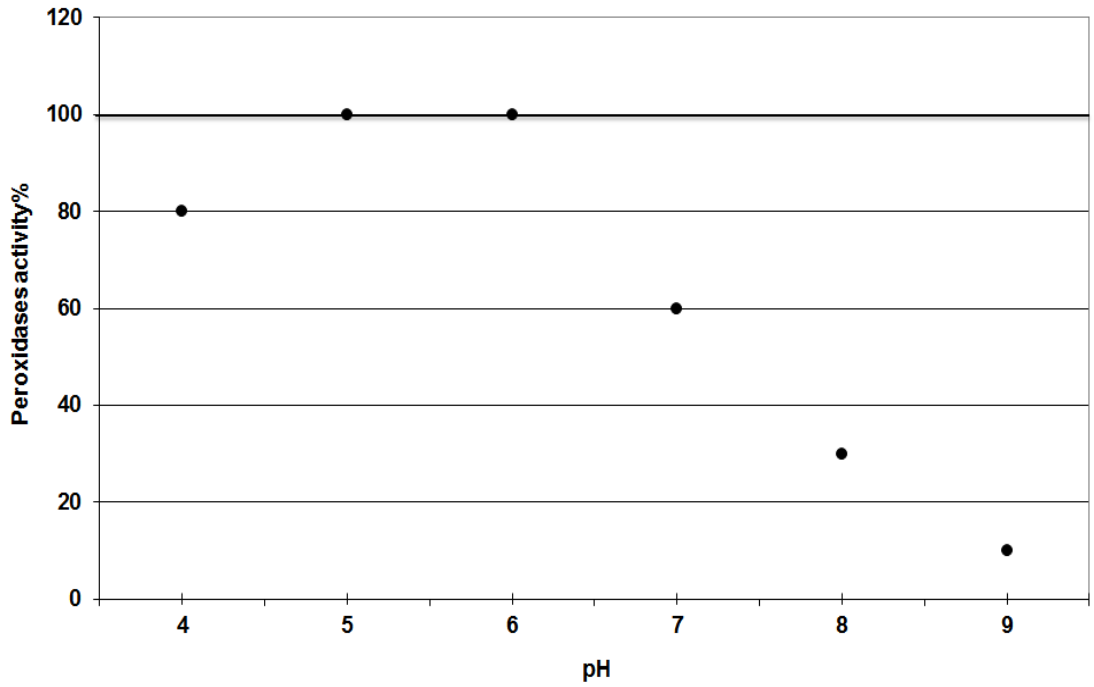

Fig. (1): **pH stability of the haemolymph peroxidases of the 4th nymphal instar of the locust, *S. gregaria* after incubation in the indicated pH for 30 min at 25°C.**

enzyme incubated at optimum pH 80, 100, 100, 60, 30 and 10% for enzyme activity incubated at pH 4, 5, 6, 7, 8 and 9, respectively.

The optimal pH of locust plasma peroxidases activity was detected (Fig. 2). The enzyme activity increased gradually when pH of the reaction mixture was 5-5.5, reaching its optimal activity at pH 6, and began to decline at pH 6.5. The decrease was sharp when pH was >6.5. Peroxidases activity at pH 6 (optimum) was 40.2 Δ_{430}ODx10^3/min/mg protein, while it was 3.4 Δ_{430}ODx10^3/min/mg protein at pH 8.

Another physical factor which expected to affect locust peroxidases activity is temperature. Figure (3) shows that high temperature degrees affect thermostability of the enzyme. When the enzyme subjected to heat treatment before measuring its activity, the relatively high temperature degrees led peroxidases to be less

active, where peroxidases activity% was affected at 60°C. It was 63% of the activity of control enzyme that not subjected to heat treatment. The enzyme lost its major activity (90%) at 80°C.

The optimal temperature of peroxidases activity was detected as shown in Fig. (4). The figure shows that oxidation of O-phenylenediamine by locust peroxidases increased gradually from 15 to 25°C and reaching to its optimum activity at 35°C. The activity was 19, 35.29 and 42.15 Δ_{430}ODx10^3/min/mg protein at 15, 25 and 35°C, respectively. At 40 and 50°C, the activity decreased only by 7 and 25.6%, respectively as compared to that of optimum temperature. The activity showed dramatic changes when the temperature of the reaction mixture was>50°C.

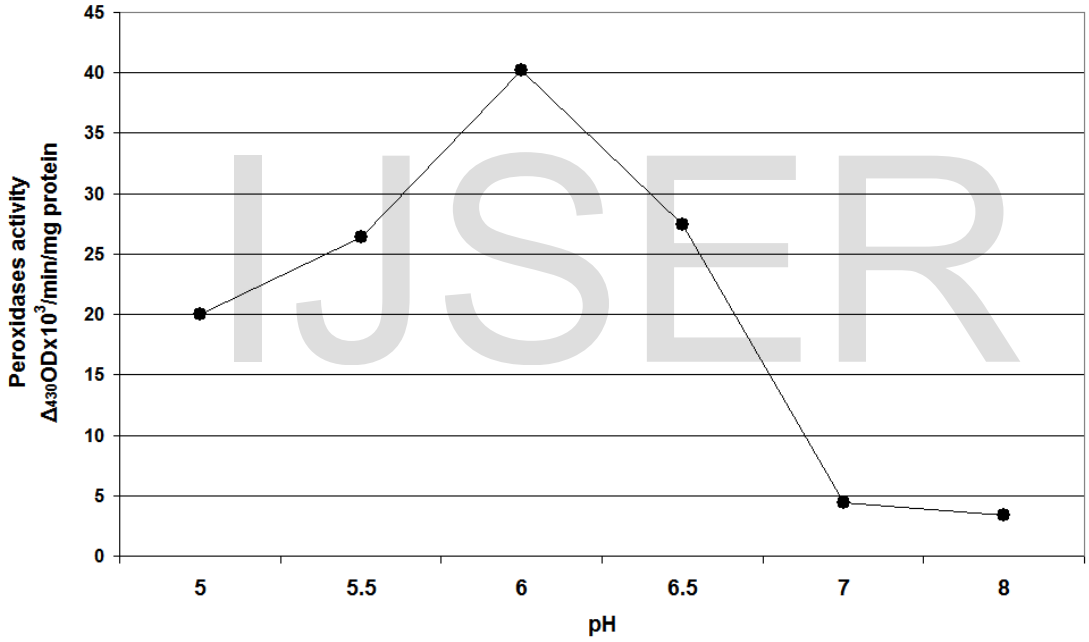

Fig. (2): **Optimum pH conditions for peroxidases activity of the locust, *S. gregaria* haemolymph. The reaction time was 10 min at 35°C.**

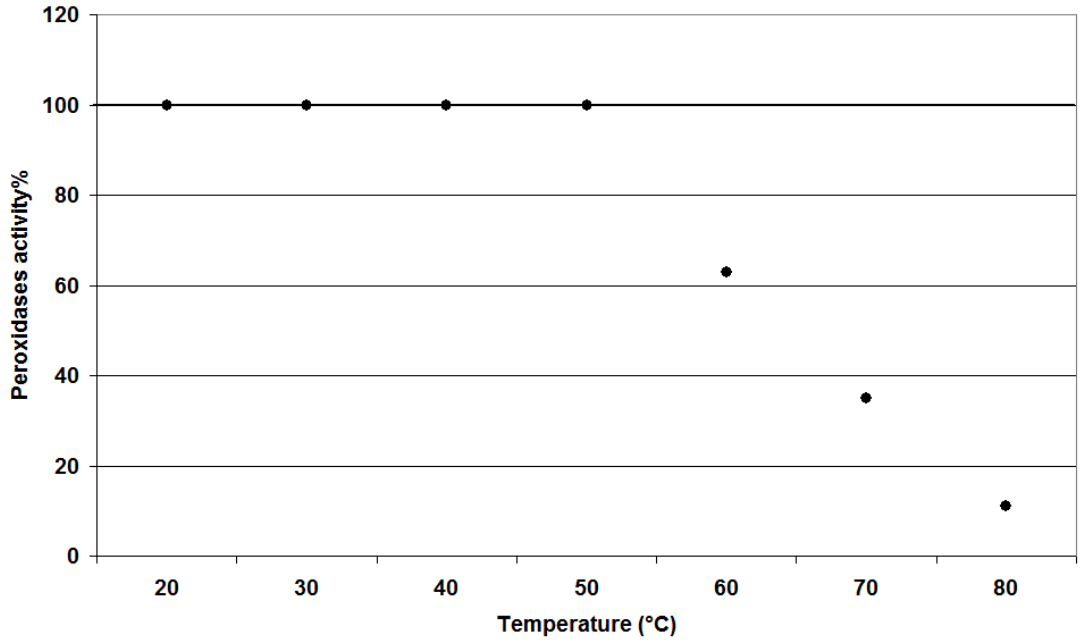

Fig. (3): **Thermostability of the haemolymph peroxidases of the 4th nymphal instar of the locust, *S. gregaria* after incubation at the indicated temperature for 30 min at pH 6.**

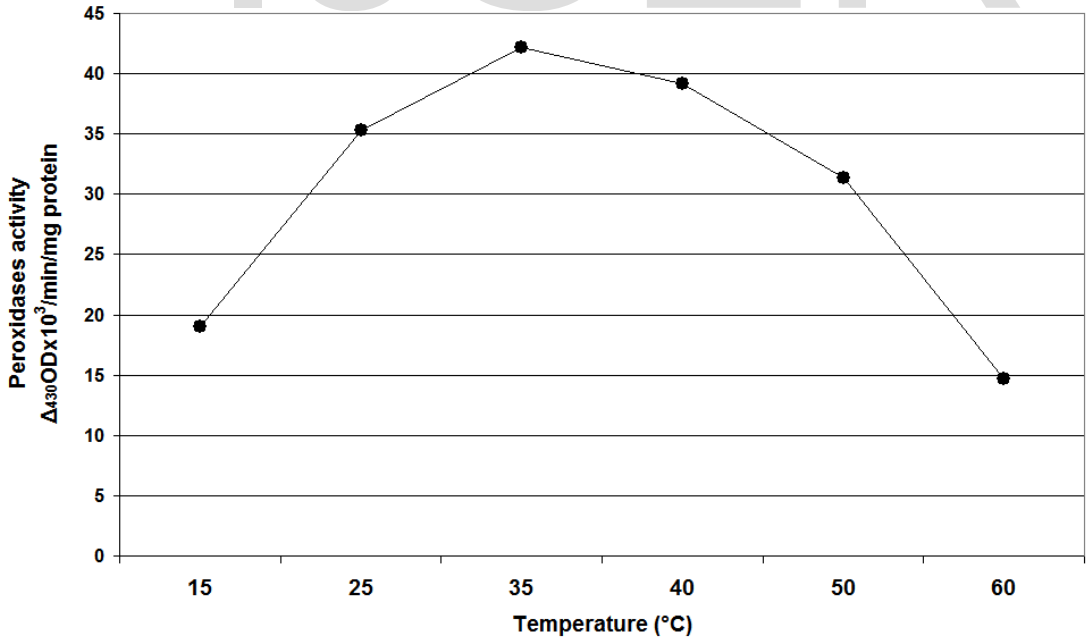

Fig. (4): Optimum thermal conditions for peroxidases activity of the locust, *S. gregaria* haemolymph. The reaction time was 10 min at pH 6.

It is known that any salts or detergents that may be introduced during extraction or purification could be potentially inhibit or activate the enzyme. The results in the present work revealed that the response of peroxidases to be inhibited or activated was varied and depend on the compound that incubated with the enzyme prior to the initiation of the reaction. The activation effect of ascorbic acid and $CuSO_4$ was observed for peroxidases from *Malva neglecta* when incubated with copper compounds such as $CuCl_3$ **[11].** The activation was probably due to the ability of these ions to form symmetrical compounds known as octahedral **[12].**

Ascorbic acid found to activate locust peroxidases. Peroxidases reduce H_2O_2 to water while oxidizing a variety of substrates, thus peroxidases are oxidoreductases which use H_2O_2 as an electron receptor for catalyzing different oxidative reactions. A variety of election donors are used, including phenols and enediols like ascorbic acid.

The results showed that EDTA as a chelating agent significantly inhibited *S. gregaria* peroxidases. This confirms that peroxidases system is metalloenzyme in which iron constitute an important part for the enzyme activity. EDTA works by drawing Fe^{+2} exist in the active site and form complexes lead to inhibition of enzyme activity **[13].** NaN_3 inhibit peroxidases only at conc. 10^{-1} M. NaN_3 also inhibits horseradish peroxidases. This might due to the ability of NaN_3 to denature the enzyme. The results agreed with many studies of effects of reduction and denaturation materials on enzymes **[14]; [15].**

The oxidation of O-phenylenediamine by locust 4th nymphal instar peroxidases as show in the present study, was affected by temperature or pH values with respect to peroxidases stability or optimum conditions. Effect of hydrogen ion concentration on peroxidases catalytic activity was more pronounced in alkaline media. Alkalinity gets peroxidases to lose their activity than do acidic media. On the other hand, the optimal pH of locust peroxidases was slight acidic (pH=6). Locust peroxidases resemble plant peroxidases in the effect of pH on activity and stability of the enzyme. **[14]** showed that the optimum pH of peroxidases purified from

Helimthus tuberosus ranging between 5 and 6. The effect of pH on enzyme stability is due to the effect of pH on enzyme structure which leads to denaturation of enzyme molecules or change in the ionic state of the active sites, also pH affects secondary or tertiary structure of the enzyme that lead to losing activity in buffers that for away from optimum pH **[16].**

Peroxidases are considered to be less thermostable as compared to some of other systems. The enzyme, in the present paper lost its major activity when incubated at temperatures above 50°C. Also, locust peroxidases had a relatively moderate optimal temperature (35°C). **[17]** found that phenoloxidases from the tomato leafminer, *Tuta absoluta* had optimal temperature of 35°C. Peroxidases purified from *Litchi pericarp* had the highest activity when incubated 40°C for 10 min. above this temperature, peroxidases activity decreased by 58.5 and 76.6% at 60 and 70°C, respectively, as compared to optimum conditions **[18].** There are some enzyme systems such as esterases that withstand temperatures up to 100°C for 30 min without losing esteriolytic activity in *Triatoma infestans* **[19]**. The difference in thermostability of enzymes depend on many factors such as the type of substrate, molecular weight of the enzyme, enzyme source and the type of isozymes because acidic isozymes resist high degrees of temperature as compared to alkaline ones **[20].**

It is likely, to take into consideration during studying of peroxidases that this system is sensitive to high temperature, alkalinity of the medium, and the presence of some salts, the situation that might effects locust peroxidases catalysis.

References

[1] Hall, J.L.; T.J. Flowers and R.M. Roberts : The molecules of cells. In Plant cell structure and metabolism. Ed. Longman House, Burnt Mill, England,1984, Page 102.

[2] Zhao, X.; C.T. Smart; J. Li and B.M. Christensen : *Aedes aegypti* peroxidase gene characterization and developmental expression. Insect Biochem. Mol. Biol.,volume 31,2001,page 481-90.

[3] Ishaaya, I. : Studies of the haemolymp and cuticular phenoxidase in *Spodoptera littoralis* larvae. Insect Biochem.,volume 2,1972,page 409-419.

[4] Duvic, B. and M. Brehèlin : Two major proteins from locust plasma are involved in coagulation and are specifically precipitated by laminarin, a B-1,3-glucan. Insect Biochem. And Molec. Biol., volume 28,1998, page 959-967.

[5] Mullen, L.M.; M.E. Lightfoot and G.J. Goldswerthy : Induced hyperlipaemia and immune challenge in locusts. J. Insect Physiol., volume 50, 2004, page 409-417.

[6] Shelby, K.S. and H.J.R. Poham : Plasma phenoloxidase of the larval tobacco budworm, *Heliothis virancens*, is virucidal. J. Insect Sci., volume 6, 2006, page 220-232

[7] Amin, T.R. and A.M. Azazy : Suppression of humoral immune reaction of the red palm weevil, *Rhynchophorus ferruigineus* to entomopathogenic nematodes. Egypt J. Agric. Res.,volume 86, 2008, page 531-541.

[8] Mathews, M.C.; C.B. Summers and G.W. Felton : Ascorbic peroxidase : A novel antioxidant enzyme in insects. Archs. Insect Biochem. Physiol., volume34,1998, page 57-68.

[9] Bradford, M.M. : A rapid and sensitive method for quantitation of protein utilization principle of protein-dye binding. Analyt Biochem., volume72,1976, page 248-254.

[10] Vetter, J.L.; M.P. Steinberg and A.I. Nelson : Quantative determination of peroxidase in sweet corn. J. Agric. Food Chem.,volume 6,1958,page 39-41.

[11] Al-badri, R.H. (2012): Extraction, partial purification and characterization of peroxidase from *Malva neglecta*. M.Sc. Thesis, Collge of Sci., Baghdad Univ.

[12] Wang, L.W.; A.M. Showalter and I.A. Ungar : Effect of salinity on growth, ion content and cell wall chemistry in *Atriplex prosturate* (*Chenopodia ceae*). American J. Botany, volume84,1997, page 1247 (Abstract).

[13] North, M.J. : Comparative biochemistry of proteinases of Eucaryotic microorganisms. Micro. Rev., volume49,1982, page 308-340.

[14] Yoon, E.S.; S-J. Kang; B-S. Noh and E-H. Choi : Isolation and characterization of peroxidase from Jerusalem artichoke tubers. J. Food Sci. Techno., volume 25, 1993, page 565-570.

[15] Silva and Valdir : Soluble and bound peroxidase from papaya fruit. Phytochemistry, volume 29,1990, page 1051-1056.

[16] Segel, J.J. (1976): Biochemical calculation. Jhon Wiley and Sons.

[17] Amin, T.R.; T.E. Ellakwa and D.E. Ellakwa : Properties of phenoloxidases from the tomato leafminer, *Tuta absoluta* (Myrick). J. Appl. Sci.,volume 13,2013page 929-933.n and developmental expression. Insect Biochem. Mol. Biol., 31: 481-90.

[18] Gisele, P.M.; L.F. Fernando; A.R. Rolf; L. Lumila and F.M. Wanger : Effect of pH and temperature on peroxidase and phenoloxidae activities of *Litchi pericarp*. Sci. Agric.,volume 67,2010 page 213-217.

[19] de Malkenson, N.C.; E.J. Wood and E.N. Zebra : Isolation and characterization of an esterase of *Triatoma infestans* with a critical role in the degradation of organophosphorus esters. Insect Biochem., volume14,1984 page 481-486.

[20] Nessel, A. and M. Mader : Physiological significance of the isoenzyme groups of peroxidase from tobacco demonstrated by biochemical properties. Micro. Rev., volume 39,1977, page 3008-3012.