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RESEARCH ARTICLE

VARIATIONS INDUCED IN ELECTROPHORETIC PATTERN OF HAEMOLYMPH PROTEINS OF FLESH FLY, SARCOPHAGA ARGYROSTOMA (DIPTERA: SARCOPHAGIDAE) LARVAE CHALLENGED WITH HYDROGEN PEROXIDE.

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Abstract

Insects like all aerobic organisms are continuously exposed to oxidative stress due to products produced during oxygen metabolism and via the metabolism of the encountered toxins including allelochemicals and pesticides**.** This study was conducted to determine the variation induced in electrophoretic pattern of haemolymph proteins of third instar larvae of flesh fly, *Sarcophaga argyrostoma* challenged with hydrogen peroxide. Haemolymph was collected every four hours interval for seventy two hours post-injection with hydrogen peroxide. Total soluble protein of the haemolymph was extracted and separated using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Hydrogen peroxide challenge induced differences in number of electrophoretic protein bands with different molecular weights as compared to control. These results clearly showed that larval challenge with hydrogen peroxide could evoke the haemolymph to synthesize new proteins to overcome such stress. The quantitative analysis also clearly indicated variations in the number as well as intensity of the protein bands.

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Introduction:-

Sarcophagid flies are an important group of dipteran flies because of its medical and veterinary impact (**Singh *et al.*, 2012**). Flesh flies have been used as models to study various aspects of insects, such as physiology, biochemistry, development, reproduction and molecular/genetic identification (**Wells *et al.*, 2001; Zehner *et al.*, 2004**). The flesh flies are also an important family of insects in forensic entomology (**Byrd, 1998 & 2001**). Larvae of these flies are closely associated with human and are known to cause animal tissue myiasis. Such severe myiasis caused by this fly, is a grave problem in terms of both the animal welfare and economic loss (**Sotiraki *et al.*, 2010**). It is known that various chemicals, physical and physiological stressors can result in a stress situation that may upset functional homeostasis which is termed oxidative stress (OS), and is characterized by enhanced production of reactive oxygen species (ROS) with the simultaneous impairment of their scavenging systems. Increased concentrations of ROS result in oxidative damage to proteins, lipids, and nucleic acids, and thus the functions of cells, organs, or the whole organism may be seriously disrupted, leading to death (**Kodrik *et al.*, 2015**). Hydrogen peroxide, H_2O_2 mostly produces ROS as a steady intermediate among all kinds of stressors (**Rosa *et al.*, 2008**). As an oxidant, H_2O_2 can

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regulate the expression of many genes, for instance superoxide dismutase [SODs] (**Rhee *et al.*, 2011**). High concentrations of H_2O_2 can cause severe damage on proteins or DNA, as well initiate lipid peroxidation (**Hassan, 1997**). Hydrogen peroxide is better known for its cytotoxic effects and it has also become established as an important regulator of eukaryotic signal transduction. Insects are subjected to various environmental stressors that lead to the generation of deleterious reactive oxygen species (**Felton, 1995**). Oxidative stress reflects the disruption of an intricate balance between the formation and clearance of highly-reactive free radicals in living organisms. The increased production of reactive oxygen species that exceeds the capacities of cellular defense systems leads to oxidative stress in the cell and to the oxidation of proteins, lipids and nucleic acids (**Imlay, 2003; Stadtman & Levine, 2003**). Polyacrylamide gel electrophoresis (PAGE) has been extensively used as an excellent tool for the separation of proteins from all living organisms (**Zacharius *et al.,* 1969**). The vast majority of recent studies on insect proteins have used electrophoretic techniques. Polyacrylamide gel, with the advantages of high sensitivity and resolving power, is generally the most efficient medium to achieve separations from haemolymph samples from single insects (**Wyatt & Pan, 1978**). The proteins in insect haemolymph have been separated and in some cases characterized by various workers such as **Laufer (1960 a&b)**, **Loughton & West (1965)**, **Chen & Levenbook (1966 a&b)**, **Adiyodi (1967)** and **Marty & Zalta (1967)**. In the present study, changes in protein profile of haemolymph of *S. argyrostoma* larvae challenged with hydrogen peroxide by SDS- PAGE at different time intervals were investigated.

Materials and methods:-

Insect rearing:-

The stock colony was established from flies originally collected from Faculty of Veterinary Medicine, Cairo University, Giza Governorate by placing a piece of fresh meat in an open wooden box in order to attract gravid females for laying larvae, the larvae were collected, identified and reared to the adult stage. *Sarcophaga argyrostoma* was maintained in the insectary of Zoology Department, Faculty of Science, Al-Azhar University (girls branch) under laboratory conditions of (25 ± 1 °C, 14 L: 10D and 60 ± 5% RH. Adults were fed on 10% sucrose solution while larvae were reared on bovine meat (**Bai *et al.*, 1988**).

Hydrogen peroxide challenge:-

Hydrogen peroxide challenge was performed by injecting the newly moulted third instar larvae with 2µl of 30% hydrogen peroxide using a sterile, thin-needled microsyringe (**Kim *et al.,* 2011**).

Haemolymph collection:-

Haemolymph was pooled by cutting off the anterior tip of the larvae with sterile fine scissors. Haemolymph was collected every four hours post injection for three days (5 µl each), in an ice-cold Eppendorf containing few crystals of phenylthiourea to prevent melanization. A haemolymph sample was collected from untreated larvae as control one. All haemolymph samples were preserved in liquid nitrogen till analysis.

Protein extraction and gel preparation:-

Proteins were extracted from the samples by using Tris buffer system as described by (**Dunn, 1993**), with 2% (w/v) [SDS] and 5% (w/v) 2-mercaptoethanol to cleave the disulphide bonds. The slurry was cooling centrifuged for 20 min. at 12000 rpm. The samples were heated in a boiling water bath for 15 minutes before loading to ensure dissociation. Preparation of the gels followed the sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method described by (**Laemmli, 1970**). Resolving gel (15%) was used according to (**Hames, 1981**). Bromophenol blue (0.001%) tracking dye was used for marking the buffer front during electrophoresis. The gel was electrophoresed at 25–30 mA constant current, at 200V. Staining was done using Coomassie brilliant blue, and a solution of 10% acetic acid and 45% methanol was used for destaining. Protein standards (markers) were used to estimate the molecular weights of the separated bands.

Results:-

Figures (1-3) and Tables (1-3) illustrated haemolymph proteins of control and treated larvae that electrophoretically separated by SDS-PAGE using 15% polyacrylamide gel.

The results of protein banding pattern in Fig. 1 and Table 1 showed that the treated haemolymph at 4, 8 and 12 h post injection had the same protein bands of the untreated haemolymph with more or less increased intensity of some bands. The molecular weights of separated protein bands were 205.127, 198.886, 137.462, 79.604, 64.122,

60.279, 58.336, 56.123, 54.789, 52.322, 50.125, 48.203, 46.683, 44.763, 40.113, 38.234, 33.987, 28.356, 26.115 and 11.234 kDa. Bands 1, 4, 6, 7, 8, 9, 10, 11, 12, 13 and 17 appeared in haemolymph of both control and all hydrogen peroxide-challenged larvae, while bands 2, 3, 5, 18 and 20 were observed at all treated samples and disappeared at 16 h p.i. Bands 14, 15, 16 and 19 were detected in hydrogen peroxide-challenged larvae at 4, 8 and 12 h p.i. and disappeared at 16, 20, and 24 h post injection. At 16, 20 and 24 h p.i., there was a unique band with molecular weight of 39.245 kDa and not observed in both control and other treated samples. Another unique band with molecular weight of 15.921 kDa., appeared only at 16, 20 and 24 h p.i.

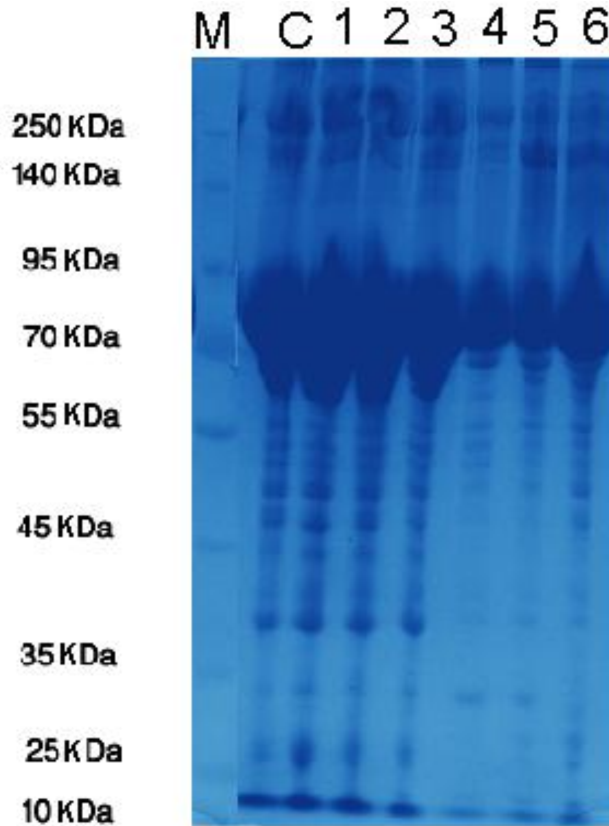

Fig. 1:- Changes in haemolymph protein banding patterns of third larval instar of *S. argyrostoma* challenged by hydrogen peroxide using SDS-PAGE. Lane M: Marker, C: control and lanes 1-6: treated larvae at 4, 8, 12, 16, 20 and 24 h post injection.

Table 1:- The molecular weight analysis of haemolymph proteins of third larval instar of *S. argyrostoma* challenged by hydrogen peroxide using SDS-PAGE. LaneM: Marker, C :control and L1-L6 treated larvae at 4, 8, 12, 16, 20 and 24 h post injection.

M	Number of band	C	L 1	L 2	L 3	L 4	L 5	L 6
250								
	1	205.127	205.127	205.127	205.127	205.127	205.127	205.127
	2	198.886	198.886	198.886	198.886	-	198.886	198.886
140								
	3	137.462	137.462	137.462	137.462	-	137.462	137.462
95								
	4	79.604	79.604	79.604	79.604	79.604	79.604	79.604
70								
	5	64.122	64.122	64.122	64.122	-	64.122	64.122
	6	60.279	60.279	60.279	60.279	60.279	60.279	60.279
	7	58.336	58.336	58.336	58.336	58.336	58.336	58.336
	8	56.123	56.123	56.123	56.123	56.123	56.123	56.123
55								
	9	54.789	54.789	54.789	54.789	54.789	54.789	54.789
	10	52.322	52.322	52.322	52.322	52.322	52.322	52.322
	11	50.125	50.125	50.125	50.125	50.125	50.125	50.125
	12	48.203	48.203	48.203	48.203	48.203	48.203	48.203
	13	46.683	46.683	46.683	46.683	46.683	46.683	46.683
45								
	14	44.763	44.763	44.763	44.763	-	-	-
	15	40.113	40.113	40.113	40.113	-	-	-
						39.245	39.245	39.245
	16	38.234	38.234	38.234	38.234	-	-	-
35								
	17	33.987	33.987	33.987	33.987	33.987	33.987	33.987
	18	28.356	28.356	28.356	28.356	-	28.356	28.356
	19	26.115	26.115	26.115	26.115	-	-	-
25								
						15.921	15.921	15.921
	20	11.234	11.234	11.234	11.234	-	11.234	11.234
10								

Figure 2 and Table 2 illustrated the protein banding pattern of the haemolymph at 28, 32, 36, 40, 44 and 48 h post injection compared to marker and the untreated haemolymph. It was obvious that the treated samples had somewhat different numbers of bands ranging from 20 to 12 bands. The same twenty bands were present in control larvae, and 32 h post injection. The bands 4, 12, 13, 15, 16, 19 and 20 were not affected with treatment and appeared in all lanes, while drastic changes happened at 40, 44 and 48 h post injection that several protein bands of untreated haemolymph disappeared after these periods of injections. On the other hand a unique protein band with molecular weight 129.214 kDa was observed in the haemolymph at 40 and 44 h post injection.

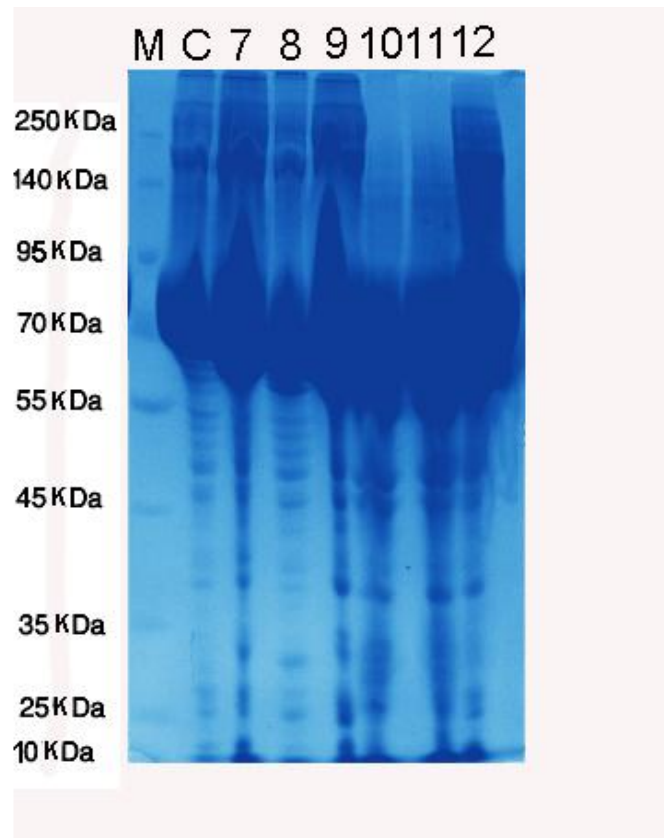

Fig. 2:- Changes in haemolymph protein banding patterns of third larval instar of *S. argyrostoma* challenged by hydrogen peroxide using SDS-PAGE. LaneM: Marker, C :control and lanes 7-12 treated larvae at 28, 32, 36, 40, 44 and 48 h post injection.

Table 2:- The molecular weight analysis of haemolymph proteins of third larval instar of *S. argyrostoma* challenged by hydrogen peroxide using SDS-PAGE. LaneM: Marker, C :control and lanes L7- L12 treated larvae at 28, 32, 36, 40, 44 and 48 h post injection.

M	Number of band	C	L 7	L 8	L 9	L 10	L 11	L 12
250								
	1	205.127	205.127	205.127	205.127	-	-	205.127
	2	198.886	198.886	198.886	198.886	-	-	198.886
140								
	3	137.462	137.462	137.462	137.462	137.462	137.462	137.462
		-	-	-	-	129.214	129.214	129.214
90								
	4	79.604	79.604	79.604	79.604	79.604	79.604	79.604
70								
	5	64.122	-	64.122	-	-	-	-
	6	60.279	-	60.279	-	-	-	-
	7	58.336	-	58.336	-	-	-	-
	8	56.123	-	56.123	-	-	-	-
55								
	9	54.789	54.789	54.789	54.789	-	-	-
	10	52.322	52.322	52.322	52.322	-	-	-
	11	50.125	50.125	50.125	50.125	-	-	-
	12	48.203	48.203	48.203	48.203	48.203	48.203	48.203
	13	46.683	46.683	46.683	46.683	46.683	46.683	46.683
45								
	14	44.763	44.763	44.763	44.763	-	-	44.763
	15	40.113	40.113	40.113	40.113	40.113	40.113	40.113
	16	38.234	38.234	38.234	38.234	38.234	38.234	38.234
35								
	17	33.987	33.987	33.987	33.987	33.987	33.987	-
	18	28.356	28.356	28.356	28.356	-	-	28.356
	19	26.115	26.115	26.115	26.115	26.115	26.115	26.115
25								
		-	24.127		24.127	24.127	24.127	24.127
		-	15.921		15.921	15.921	15.921	-
	20	11.234	11.234	11.234	11.234	11.234	11.234	11.234
10								

The variations that were observed at 40, 44 and 48 h post injection changed on the third day of treatment (Fig. 3 and Table 3). Most of disappeared protein bands were observed again at 52, 56, 60 and 64 h post injection, while some of these bands disappeared again at 68 and 72 h post injection. Another unique protein band with molecular weight 39.154 kDa was observed at 72 h post injection. It was clear from the overall results of SDS-PAGE that larvae challenged with hydrogen peroxide could evoke the haemolymph to synthesise new proteins.

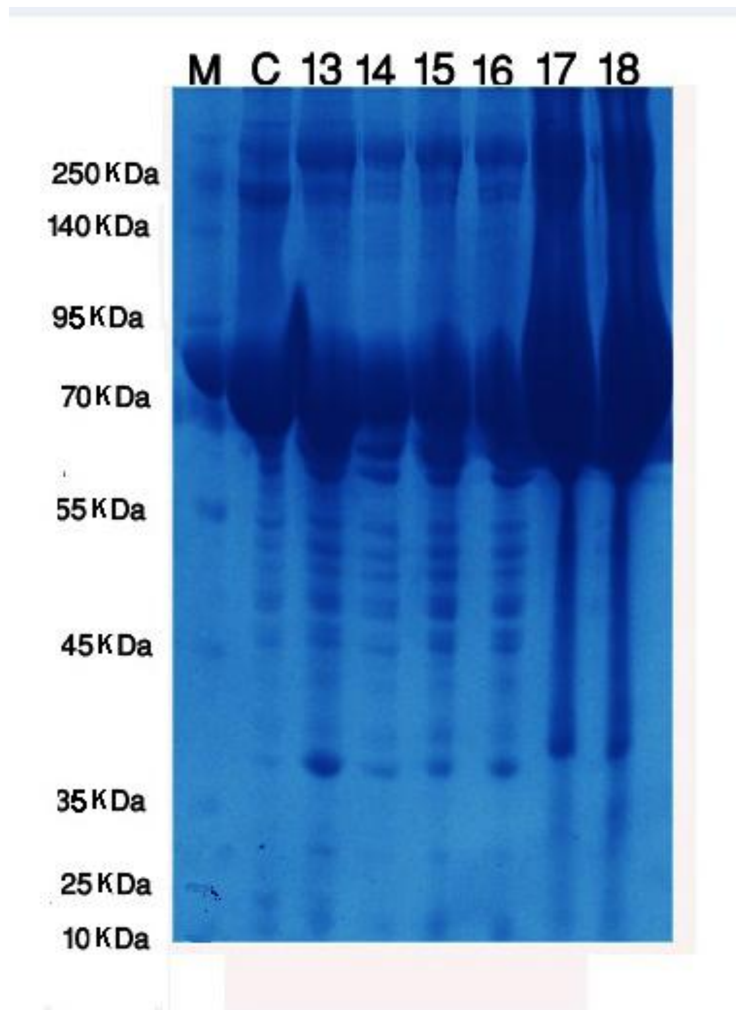

Fig . 3:- Changes in haemolymph protein banding patterns of third larval instar of S. argyrostoma challenged by hydrogen peroxide using SDS-PAGE. LaneM: Marker,C :control and lanes 13-18 treated larvae at 52,56, 60, 64, 68 and 72 h post injection.

Table 3:- The molecular weight analysis of haemolymph proteins of third larval instar of *S. argyrostoma* challenged by hydrogen peroxide using SDS-PAGE. LaneM: Marker, C :control and lanes L13- L18 treated larvae at 52,56, 60, 64, 68 and 72 h post injection.

M	Number of band	C	L 13	L 14	L 15	L 16	L 17	L 18
250								
	1	205.127	205.127	205.127	205.127	205.127	205.127	205.127
	2	198.886	198.886	198.886	198.886	198.886	198.886	198.886
140								
95	3	137.462	137.462	137.462	137.462	137.462	137.462	137.462
	4	79.604	79.604	79.604	79.604	79.604	79.604	79.604
70								
	5	64.122	64.122	64.122	64.122	64.122		
	6	60.279	60.279	60.279	60.279	60.279	60.279	60.279
	7	58.336						
	8	56.123	-					
55								
	9	54.789	54.789	54.789	54.789	54.789		
	10	52.322	52.322	52.322	52.322	52.322		
	11	50.125	50.125	50.125	50.125	50.125		
			49.211	49.211	49.211	49.211		
	12	48.203	48.203	48.203	48.203	48.203		
			47.120	-	47.120	47.120		
	13	46.683	46.683	-	46.683	46.683		
45								
	14	44.763	44.763	44.763	44.763	44.763		
	15	40.113	40.113	40.113	40.113	40.113		
							39.154	39.154
	16	38.234	38.234	38.234	38.234	38.234		
35								
	17	33.987	33.987	33.987	33.987	33.987	33.987	33.987
	18	28.356	28.356	28.356	28.356	28.356	28.356	28.356
	19	26.115	26.115	26.115	26.115	26.115	26.115	26.115
25								
			24.127	24.127	24.127	24.127	24.127	24.127
	20	11.234	11.234	11.234	11.234	11.234	11.234	11.234
10								

Discussion:-

Protein is necessary for various biological activities during development, metamorphosis and maintenance of various physiological functions in different tissues **(Kumar *et al.,* 2011)**. The various aspects of protein metabolism including quantitative changes in haemolymph protein synthesis and metabolic activity of specific enzymes have attracted the interest of many insect biochemists. The proteins play an important role in the haemolymph of insects not only in specific transport functions, but also in their enzyme action. The synthesis and utilisation of haemolymph proteins are controlled by genetic and hormonal factors **(Hurlimann *et al.*, 1974)**. Proteins could be antibacterial agents **(Riddiford *et al.*, 1983)**, detoxifying agents **(Furukawa *et al*., 1999)**, hormone carriers **(Fengwu *et al.*, 1997)**, morphogenesis proteins **(Kiheung *et al.*, 1998)** or even similar to some human proteins. As the haemolymph composition of the insects reflects the nature and degree of metabolism of the tissue suffused in this fluid, changes in the proteins of the haemolymph may show the level of modification in the organism. The qualitative variations in the protein bands of different treatments and in different days during the larval life indicate both utilities of the specific proteins as well as the synthesis of new proteins by the insect **(Lokesh *et al.,* 2006)**. Results obtained from the SDS-PAGE of haemolymph of H_2O_2-challenged larvae at different time intervals demonstrated that there were

changes in the protein pattern of the challenged larvae as compared to control. Thus, H_2O_2 was capable of changing the profiles of haemolymph proteins qualitatively. The appearance of different bands in challenged larvae may be attributed to the induction of new proteins. The synthesis of new proteins may be a result of simultaneous induction of challenging with hydrogen peroxide. Such change in protein profile between normal and challenged larvae may be attributed to the increased production of antioxidants and repair proteins that allow adaptation to these oxidative conditions **(Storz & Tartaglia, 1992; Jamieson, 1998)** and may reflect specialization and adaptation in the organisms based on subtle metabolic alterations **(Witmore & Gilbert, 1974)**. Total protein changes and the electrophoretic protein bands studies in insects after various treatments were recorded by many authors **(Latha *et al.,* 1996; Zidan *et al.,* 1996; Chau faux *et al.*, 1997; Kawaski, 1998; Salama *et al.*, 1999)**. Till now there have not been any published reports regarding variations induced in protein profiles of *Sarcophaga sp* following hydrogen peroxide challenge. However, some studies are available with other chemical and physical stressors.The present results agreed with **Amin (2010)** who determined the variation induced in electrophoretic protein pattern of first and third instar larvae of flesh fly, *S. bullata* irradiated with Gamma rays (physical stressor) comparing with unirradiated larvae and found that the challenging with radiation resulted in appearance of unique protein bands in the treated sample by using SDS-PAGE and disappearance of protein bands as compared with control. Also, **Bedenarova *et al.* (2013)** investigated injection of hydrogen peroxide into the body of adult female *Pyrrhocoris apterus* (Heteroptera: Pyrrhocoridae) and noticed a highly significant increase in protein carbonyl levels and AKH titres: 2.8-fold in CNS and 3.8-fold in haemolymph compared with controls. Changes in protein profiles of insect larvae subjected to different chemical and physical stressors were investigated in *Heliothis zea* **(Vinson & Lewis, 1969)** and *Pieris brassicae* **(Bai & Degheele, 1988)**. **Omar *et al.* (2005)** reported that (SDS-PAGE) analysis of greater wax moth (*Galleria mellonella*) infected with *B. thuringinsis* showed four peptide groups in the range of 11- 120 kDa. **El-Shiekh *et al*. (2010)** found that the protein profiles of *S. littoralis* treated with five profenofos formulations, after stored at cold and hot conditions, were differently changed and distinguished into several separated protein bands in the range of 3.6 to 195.5 kDa. It can be concluded that the proteins play an important role in the haemolymph of insects not only in specific transport functions, but also in their enzyme and hormone action as antibacterial agents or detoxifying agents. This conclusion was reinforced by **Ahmad (1992)** who reported that oxidative stress may lead to membrane or the whole cell damage by lipid peroxidation and protein oxidation resulting in uncontrolled apoptosis. Eukaryotes, including insects, possess a suite of antioxidant enzymes that protects their cells from oxidative radicals.

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