Research article Available online www.ijsrr.org ISSN: 2279–0543

International Journal of Scientific Research and Reviews

Apoptotic and Biochemical Studies of Silkworm (*Bombyx mori L.)* During Larval-Pupal Metamorphosis

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ABSTRACT:

In the present study apoptotic and biochemical studies of *Bombyxmori L*. were investigated during larval to pupal transformation. Haemolymph was collected from silkworms during 5thinstar larvae, the total viable haemocytes count was determined by trypan blue test. The concentration of protein content was determined, acid phophatase and glutathione activity was tested by using silkworm fat body tissue during larval to pupal transformation. SDS-PAGE was carried out by using 12.5% separating gel and 5% stacking gel and morphological changes in larval midgut were observed by histological sections. The total number of viable haemocytes count and the concentration of haemolymph proteins were decreased during larval to pupal metamorphosis. Glutathione (GSH) levels were decreased during larval to pupal metamorphosis, increases cellular susceptibility to apoptosis. Acid phosphate activity was detected in the fat body tissue of silkworm during metamorphosis, indicates presence of autophagy. Disappearance of protein bands were observed through the SDS-PAGE electrophoresis indicates utilization of proteins during spinning of cocoon and degradation proteins to maintain amino acid concentration in the haemolymph. Morphological changes were observed in the midgut sections of silkworm, the midgut was degenerated during metamorphosis. The present study clearly indicated that apoptosis and autophagy responsible for larval to pupal metamorphosis.

KEYWORDS: Apoptosis, autophagy, haemolymph, SDS-PAGE, acid phosphates,glutathione.

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INTRODUCTION:

Apoptosis is the process of programmed cell death that occurs in all multicellular organisms[1]. Apoptosis accompanied by chromatin condensation, cell shrinkage, membrane blebbing, apoptotic body formation and DNA fragmentation[2]. Dysfunction of the apoptosis process results variety of cancer and autoimmune diseases. Apoptosis process present in all mammals and insects[3], nematodes[4]. Insects are the largest group of living organisms, more than one million species of insects have been reported[5]. Lepidoptera are the most widely used species for the study of apoptosis and autophagy during metamorphosis[6]. *Bombyx mori* is the domesticated, holometabolous insect it undergoes complete metamorphosis between final instar and adult stages. The silkworm life cycle consists of four stages embryo, larva, pupa and adult moth. Newly laid eggs are a creamy yellow colour, after hatching from the eggs the larvae passed through five instars. During fifth instar the larvae spin a cocoon for protection. After a final molt inside the cocoon, the larva change into the brown pupa, finally adult mouth emerges from cocoon. Silkworm has an open circulatory haemolymph system, especially at the fifth instar a lot of haemolymph proteins are synthesized in fat bodies and secreted into the haemolymph, and they are required to participate in diverse biochemical and physiological processes. During insect metamorphosis, profound biochemical changes occur in the haemolymph, in particular, the concentration of proteins[7]. During metamorphosis degradation of several larval tissue and organs takes place. Juvenile hormone (JH) and ecdysteroid titers are important factors for insect metamorphosis, 20-hydroxyecdysone (20E) as the regulator of degeneration of larval tissues and organs[8]. Apoptosis and autophagy is responsible for the degradation of midgut epithelial cells and silk glands and fat body tissue in the silkworm during larval to adult transformation. The insect fat body equivalent to the vertebrate liver in function and central organ for metabolism and synthesis of haemolymph components[9]. During metamorphosis the larval midgut epithelium degenerates and a new adult midgut epithelium formed[10].

MATERIALS AND METHODS:

Rearing of B. Mori Larvae:

In the present investigation the parentage of CSR×2 silkworm varieties were used as test insects. Various stages of the silkworms are maintained on mulberry leaves at a temperature of 27º C and relative humidity of 75 %. The life span of the silkworm under these conditions was 30-32 days.

Collection of Haemolymph From Silkworm:

The 5th instar larvae are chilled on crushed ice for 10 min and then swabbed with 70 % alcohol. A small cut was made on the proleg cuticle and the haemolymph was collected in sterile vials containing 0.5 g phenol thiourea, it prevents the melanization of the haemolymph .

Protein Extraction from Haemolymph:

Haemolymph from healthy silkworms was collected in to 10% cold TCA and kept at 4°C for 10 minutes. The samples were centrifuged at 2000 ×g for 5 minutes and the pellet was washed twice with cold 5 % TCA followed by a wash with alcohol ether (3:1) mixture. The pellet was dissolved in 0.1 N NaOH and used for protein estimation [11, 12].

Estimation of Cell Viability -Trypan Blue Test:

0.2 ml of the haemocyte suspension was mixed with 0.3 ml of PBS and 0.5 ml of trypan blue in a small test tube. An aliquot is then placed on hemocytometer and count number of viable cells under the microscope. The plasma membrane of the viable cells does not permit the entry of electrolyte dye substance. This phenomenon is used to distinguish dead cells from living haemocytes. Percentage of viability was calculated by the following formula.

$$\% \text{ of viable cells: } = \frac{\text{Number of viable cells}}{\text{Number of viable cells + Number of dead cells}} \times 100$$

Estimation of Protein from Cell Free Extracts:

The total protein content was determined with Folin Ciocalteau's reagent according to the Lowry et al., [13]. For the estimation of protein content 0.1 ml of protein solution was treated with 0.9 ml of alkaline copper sulphate solution. Further, 5 ml of alkaline copper sulphate solution was added and allowed to stand for 10 minutes at room temperature. Then 0.5 ml of Follin reagent was added, thoroughly mixed by vertexing with a cyclomixer and allowed to stand for 30 minutes for color development. Absorbance was measured at 750 nm in a Spectrophotometer. Bovine serum albumin was used as standard.

Glutathione Assay:

0.5ml of tissue homogenate was treated with 3.5ml of 5%TCA. The precipitate was removed by centrifugation. To 0.5ml of supernatant, 3.0ml of phosphate buffer and 0.5ml of Ellman's reagent were added. The yellow color developed was read at 412nm. A series of standard's along with a blank containing 3.5ml of buffer. Values were expressed as µg of Glutathione (GSH)/mg protein.

Acid Phosphatase Activity Assay:

Acid phosphatase activities were determined by the liberation of p-nitrophenol from the appropriate p-nitrophenyl substrate according to the method reported by De Couet H.G. et al., [14]. The assay mixture contained 10 µl of tissue homogenate and 200 µl of substrate solution (2 mg/ml p – nitrophenyl phosphate, 1 mg/ml bovine serum albumin in homogenization buffer with 0.1 mol/l TRIS-maleate at pH 5.2). The enzyme reaction was carried out at 21°C and samples arrested at 15 min intervals by addition of 50 µl 4 N NaOH over 1 h period. The reaction mixtures were then centrifuged at 1,000 g for 10 min at room temperature and remove precipitate. Blanks were prepared by addition of NaOH prior to enzyme solution. Color development was determined at 405 nm using spectrophotometer. Readings were compared to a standard curve prepared with p-nitrophenol, the activity of enzyme was represented by mg pi/gm/hr.

SDS-Polyacrylamide Gel Electrophoresis (SDS PAGE):

SDS-PAGE was carried out according to the method of Laemmli et al,.[15]. After estimation of protein concentration in haemolymph SDS-PAGE was carried out by using 12 % separation gel and 5 % stacking gel. The gels were stained with Coomassie brilliant blue. The separating gel consisted of 10 % (W/V) acrylamide, N,N-methylene bis acrylamide (Sigma, USA) 0.375 M, Tris-HCL (pH 8.8) and 0.1 % SDS. It was chemically polymerized with 0.05 % (W/V) ammonium persulphate (Sigma, USA)) and 0.05 % (W/V) TEMED (Merck, FRG). The solution was cast into slabs and overlayed with n- butanol to exclude contact with air. The st acking gel containing 4 % (W/V) acrylamide 0.12M, Tris-HCl (pH 6.8), 0.1% SDS, 0.05% (W/V) ammonium persulphate, 0.05 % (V/V) TEMED. Samples, 50-200 µgs were digested with an equal volume of sample buffer (0.0625 M Tris-HCl, pH 6.8) 10% (V/V) glycerol, 5 % ß-Mercaptoethanol, 2 % SDS and 0.02 % bromophenol blue by heating in a boiling water bath for 3 minutes. After cooling, the samples along with protein markers were loaded into the slots. The samples were stacked and run at 120 V for about 6 hr using 0.025 M Tris, 0.192M glycine buffer (pH 8.3)

containing 0.1 % SDS as electrode buffer. After electrophoresis, gels were fixed in PBS buffer for 1hr, and then the gels were treated with 50 % ethanol for 10 minutes. Gels were washed with distilled water and soaked in Coomassie brilliant blue solution for ½ hr. Further gels were washed with distilled water.

HISTOCHEMISTY:

Tissue Preparation for Histology:

The midgut of the final instars larvae were excised and immediately fix in 10% fomalin for 24hrs, then washed under tap water for 15 min. After fixation they are dehydrated in 80 %, 90 %, and 100 % solutions of alcohol and acetone for 2 hrs each and cleared in xylene for three times for 30 min each. The tissues are impregnated in paraffin wax at 50º-60 ºC over night and sections of 3-4 microns are made using microtome. The prepared sections are dried in oven for 1hr and later transferred to xylene solution.

Sections are deparaffinised by repeated transferring of the sections in to xylene for three times for every 10 min each. Then the sections are hydrated in absolute alcohol followed by 90 % 80 %, 70 % and 50 % alcohol respectively for 5 min each. They were then washed under running tap water for 5 min, processed in hematoxylline for 15min, washed under tap water for 5min, dipped in 1% acid alcohol for 2-3 times, washed in tap water for 2 min, dipped in 0.5 % ammomia solution for 2-3 times, washed in tap water for 5-10 min. The sections were dipped in 95 % alcohol for 10 times, stained in 1 % eosin solution for 5 min, excess stain was removed by dipping in 70 % alcohol for about 5 times. The stained sections were mounted on slide by using DPX mounting fluid which provides a high refractive index for microscopy. The slides thus prepared were photographed by the photo micrographic technique, the magnification was 450X.

RESULTS:

Estimation of Cell Viability -Trypan Blue Test:

The total haemocytes counts indicate that variations in the number of haemocytes during developmental stages of silkworm. The total viable haemocytes counts increase from day one to day 6 of 5^{th} instar larva, and reached maximum peak at 6^{th} day of 5^{th} instar larva, and decreased during spinning day 1, day 2 and pre-pupal stage of silkworm (Fig.1). Number of viable haemocytes increased continuously throughout the development and decreased during metamorphosis.

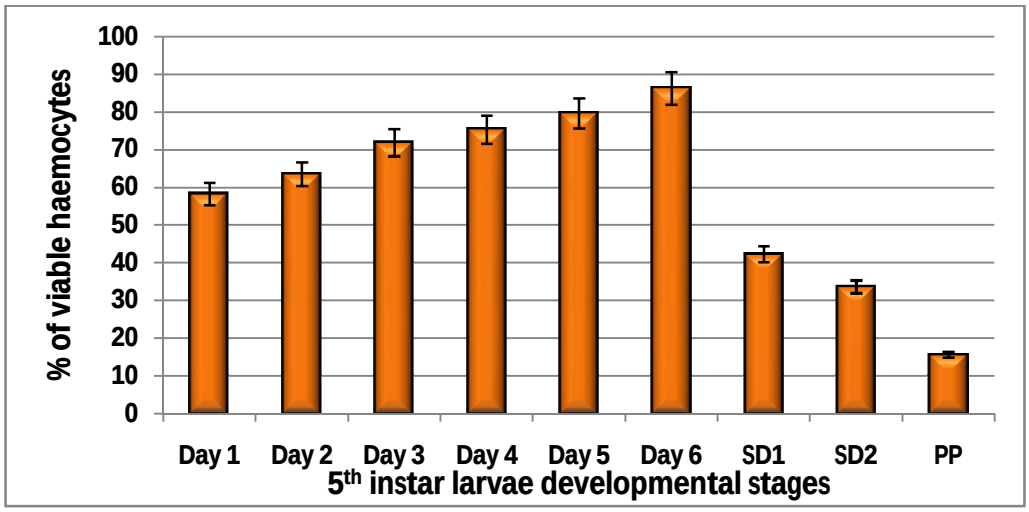

Fig.1: Total viable haemocyte count of 5th instar silkworm larvae. Data are represented as mean ± S.E.

Estimation of Protein from Cell Free Extracts:

Changes in the total protein concentration of haemolymph was observed during day 1 to day 6 of 5th instar larvae and spinning day 1,day 2 larvae. Total protein concentration increased up to day 6thof 5th instar larvae after that protein concentration was decreased from spinning day 1 to pre pupal stage of silkworm (Fig.2). Silkworm haemolymph contains different types of storage proteins, involved during metamorphosis of silkworm.

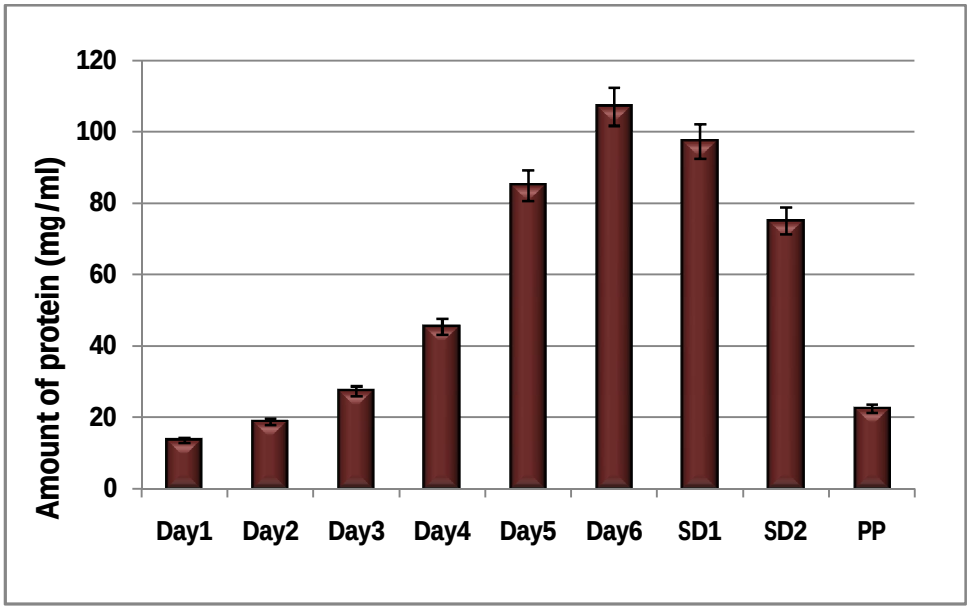

Fig.2: Protein levels in the larval haemolymph during developmental stages of silkworm. Data are represented as mean ± S.E.

Glutathione (GSH) Assay:

Changes in the Glutathione (GSH) were observed in the fat body of the silkworm during larval to pupal transformation. Glutathione (GSH) levels increased during 1st and 6th day of 5th instar, and decreased gradually during the spinning period and a subsequent decrease at prepupal stage (Fig.3).

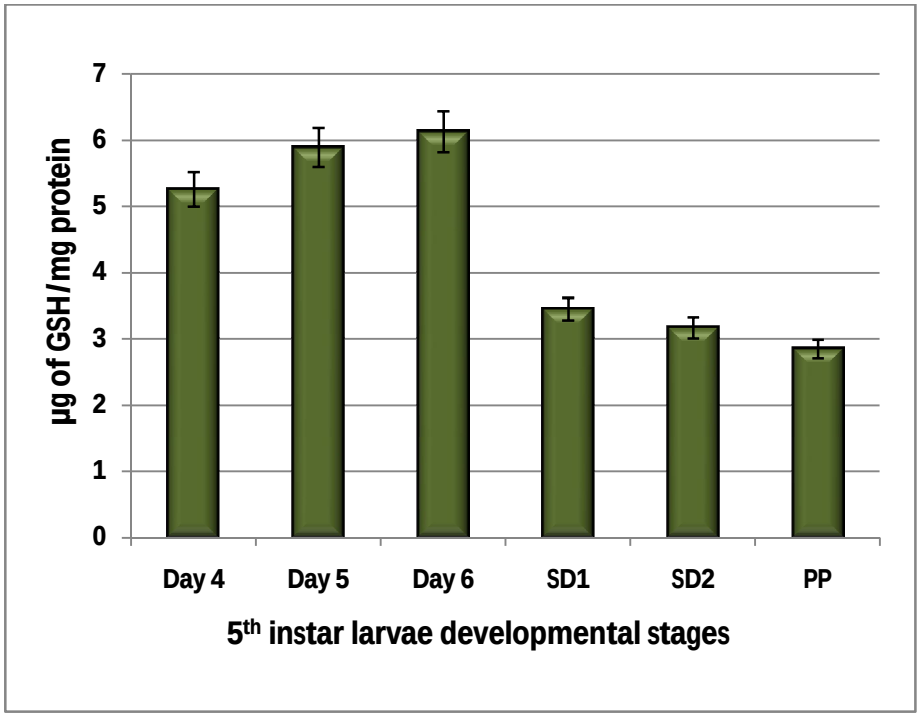

Fig.3: Analysis of glutathione activity in silkworm fat body during larval to pupal transformation. Data are represented as mean ± S.E.

ACID PHOSPHATASE ACTIVITY ASSAY:

Changes in the acid phosphatases were observed in the fat body of the silkworm during larval to pupal transformation. Acid phosphatase activity was low during 5th instar larvae. The enzyme activity increased gradually during spinning day 1 and spinning day 2 larvae and pupal stages (Fig 4).

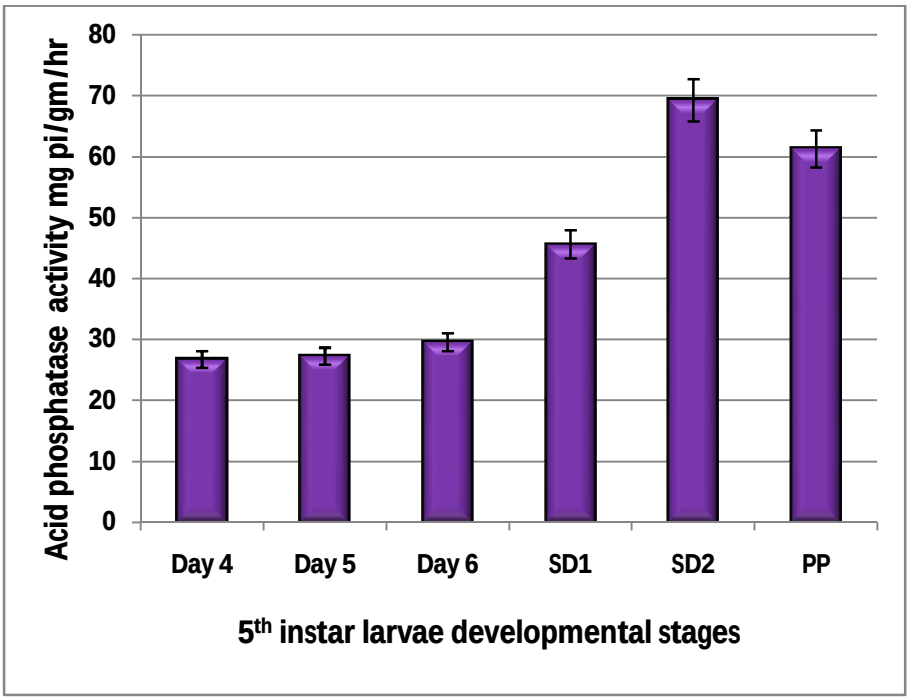

Fig.4: Analysis of acid phosphatase activity in silkworm fat body during larval to pupal transformation. Data are represented as mean ± S.E.

SDS-Polyacrylamide Gel Electrophoresis (SDS PAGE):

Variations in protein bands were observed in SDS-PAGE of silkworm haemolymph during larval to pupa metamorphosis. The bands corresponding to molecular weight of 37 kDa, 25 kDa and 18 kDa protein bands are completely disappear in spinning stage and prepual stage, whereas bands corresponding to 45 kDa, 31 kDa and 21 kDa were found in all stages (Fig 5). Disappearance of protein bands indicates utilization of proteins during spinning of cocoon and degradation proteins to maintain amino acid concentration in the haemolymph.

Histological Studies of MIDGUT:

Morphological changes were observed in the midgut of the silkworm larva during larval to pupal metamorphosis. No significant changes were observed during 6^{th} day of 5^{th} instar larvae. Where as extensive modifications were identified during spinning day 1and spinning day 2 and prepupal stages.

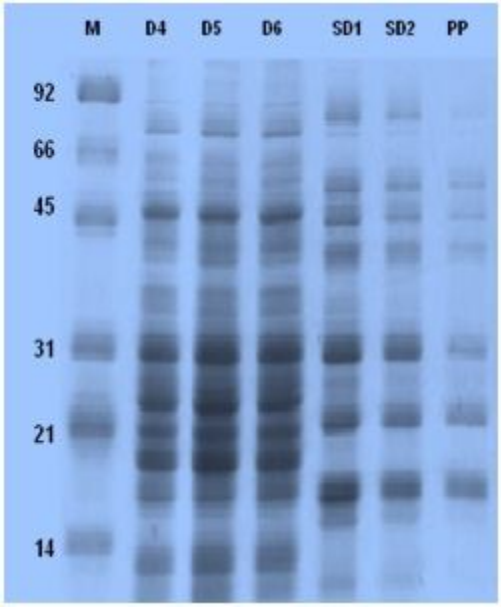

Fig: 5.Haemolymph protein profiles of fifth instar silkworm larvae.

Majority of the dead cells were observed in the midgut of silkworm larvae during larval to pupal metamorphosis, which indicates apoptosis and autophagy degradation of midgut cells of silkworm larva (Fig 6).

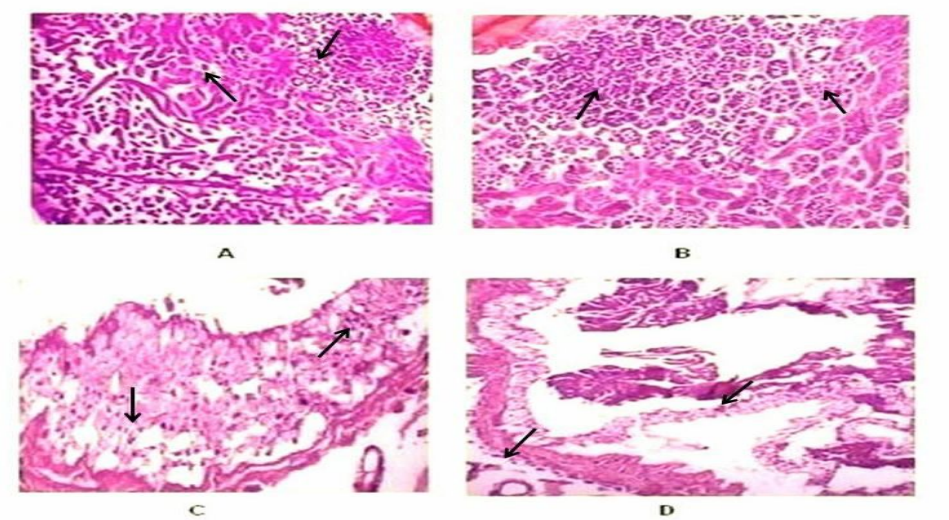

Fig.6: Midgut sections of silkworm during larval to pupal transformation.(A,B). Midgut sections of 5th instar day 6 larva and spinning day 1 larva. (C,D) Midgut sections of spinning day 2 larva and prepupal stage of silkworm.

DISCUSSION:

Cell death occurs during development and metamorphosis of holometabolous insects to eliminate tissues and organs that are only necessary only for embryonic or larval life[16]. Lepidoptera are the most

widely used species for the study of autophagy during metamorphosis, their size and available molecular background information on endocrine regulation of development make them ideal models to explain the molecular pathways of programmed cell death (PCD). The development of the worm is depending on metamorphosis process which is a dynamic biochemical process [17]. Metamorphosis is even more extreme in insects, in which many cells of the larvae are destroyed, while cells that will become the future adult undergo differentiation and morphogenesis[18]. Metamorphosis involves the breakdown of larval structures, though the programmed cell death (PCD) [19].

Haemocytes are circulating cells and found in the haemolymph of insects responsible for the defense mechanism against foreign body[20]. In present study significant changes in total haemocyte count was observed, the number of viable haemocytes was gradually decreased during larval to pupal transformation. Haemocytes perform various physiological functions in the insect body, changes in total haemocyte counts of particular insect directly or indirectly affect the insect [21]. Changes in the expressed proteins that are known to be involved in the process of apoptotic cell death[22]. During metamorphosis the protein concentration decreases gradually in haemolymph. In insects various biochemical changes occurs in the total concentration of haemolymph amino acids and proteins during metamorphosis[23]**.** The haemolymph is store house of various kinds of proteins and enzymes[24]. These proteins functions as the main reservoirs for the supply of amino acids during larval moults and metamorphosis[25]. In present study changes in the levels of protein in haemolymph of silkworm during larval to pupal metamorphosis was observed. During final instar the haemolymph proteins were significantly increase due to the synthesis of new proteins by fat body tissue and release into haemolymph. The proteins levels were gradually decreased during spinning stage and prepual stage of silkworm.

Glutathione (GSH) is a tripeptide, dominant intracellular thiol plays an important protective role against oxidative stress[26]. Enzymatically GSH participates in detoxification of H_2O_2, and GSH peroxidases thus GSH is an important antioxidant that functions directly in elimination of toxic peroxides. The nucleus exhibits an independent GSH pool that plays an important role in protection against oxidant and ionizing radiation induced DNA damage[27]. GSH functions as hydrogen donor in ribonucleotide reductase catalysed reduction of ribonucleotides to deoxyribonucleotides and thus play a contributory role in DNA synthesis[28]. A decrease in cellular GSH concentration has long been reported to be an early event in the apoptotic cascade induced by death receptor activation[29]. Decrease in GSH

concentration may increases in proapoptotic Bax and Bcl-xL and initiation of mitochondrial apoptotic cascade [30].

Acid phosphatase used as a marker enzyme for lysosomes and for apoptosis, involved in the degradation of insect tissues[31]. During the metamorphosis acid phosphatase activity was increased and play important role in the degradation of fat body tissue and silk gland. Lysosomal participation during the PCD of PV tissues has been confirmed by the elevated level of the marker enzyme, acid phosphatase[22]. Disappearance of protein bands indicates degradation of haemolymph proteins to maintain amino acid concentration in the haemolymph. The hydrolysis of proteins has occurred during the larval period to form amino acids, which in turn utilized to form silk proteins. The migut of 6th day of fifth instar larva shows well developed midgut with high population of epithelial cells. During larval to pupal metamorphosis fat body tissue and silk gland undergoes programmed cell death. Histological midgut sections of silkworm during larval to pupal metamorphosis exhibited that massive degeneration of midgut tissues. The present study clearly indicated that apoptosis and autophagy responsible for larval to pupal metamorphosis.

ACKNOWLEDGMENTS:

Authors are thankful to DST, Inspire programme New Delhi and UGC New Delhi for financial support.

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