CHAPTER - THREE

PROTEIN AND PROTEASES ACTIVITY DURING EMBRYOGENESIS OF ARMYWORM *MYTHIMNA SEPARATA* (WALKER)

CHAPTER - THREE

PROTEIN AND PROTEASES ACTIVITY DURING EMBRYOGENESIS OF ARMYWORM *MYTHIMNA SEPARATA* (WALKER)

CHAPTER THREE

I. INTRODUCTION :

The eggs of *M. separata* are laid into clusters or in rows on rolled leaves, which are spherical and greenish white in color. Hatching of young larva requires 4 to 5 days incubation period. (Nayar *et al.,* 1976). Terrestrial propagation requires protection of the egg stages against desiccation and it requires well provisional eggs because only such eggs can produce larvae capable of coping with food much coarser than the plankton or micro organism on which so many minute aquatic larvae thrive.

The development of egg at the time of oogenesis is considerably due to the deposition of yolk. The egg nucleus occupies only a very small fraction of the egg volume, often located in the anterior half of the egg and surrounded by a small island of cytoplasm. Outside the oolemma the egg is surrounded by two membranes, an inner vitelline membrane and an outer chorion or egg-shell. The egg is covered with a layer of cement secreted by the colleterial glands of the female. This secretion secures the eggs to the surface on which they are laid (Wigglesworth, 1972). The egg is permeable to gases, and respiratory exchange between the interior of the egg and the environment occurs (Agrell and Lundquist, 1973).

1) Protein Metabolism during Embryogenesis :

The fertilized egg cell contains stored macromolecules providing both the raw material and the energy for building the larval body. The insect egg is a self-directing as well as self sustaining developmental system capable of functioning autonomously within a wide range of environmental conditions (Sander *et al.*, 1985).

The developing organism represents a dynamic system, which changes continuously in its physiological and biochemical properties as morphogenesis proceeds (Chaubey and Bhatt, 1988). During embryogenesis an intensive metabolism takes place which involves mainly breakdown of pre-existing yolk reserves and the conversion of these into tissue and organ (Premkumar *et al.*, 1991). The physiological and biochemical changes during insect embryonic development have been reviewed by Agrell and Lundquist (1973). The Morphological, Physiological, Genetical and Molecular aspects of insect embryogenesis have been reviewed by Sander *et al.*(1985).

The insect yolk is chemically constisuted by include proteins especially mucoproteins and lipoproteins, lipids mostly neutral lipids with triacylglycerol as their main component but phospholipids, sterol and sterol esters may also be present, free fatty acids, mono and diglycerides are usually present in small amount, carbohydrates such as glycogen and other polysaccharides.

During embryogenesis the major biochemical process underlying cellular differentiation is protein synthesis and analysis of the level and utilization of free amino acids in the course of development may provide valuable insight into the basic events that underlie embryonic differentiation. In general, all amino acids which are commonly found in protein occur in insect eggs and there are significant qualitative and quantitative changes of the free amino acid pool during embryonic development. At the onset of embryogenesis a rapid increase in the level of all major amino acids at the onset of embryogenesis has been reported in *Bombyx, Drosophial* and *Culex*. This corresponds to the periods of blastoderm formation and germ band elongation. The total concentration remains high at the beginning of blastokinesis and then declines during

later embryonic differentiation. The overall variation represents no doubt the balance between release of amino acids from yolk degradation and their utilization for *de novo* protein synthesis (Chen, 1978).

Colombo *et al.*(1961) studied the protein metabolism in eggs of *Schistocerca gregaria.* Chaubey and Bhatt (1988) have been made to understand the protein distribution during embryonic development. Protein is a very minor or negligible source of energy during embryogenesis (Agrell and Lundquist, 1973).

2) Review of Literature on Study of Egg Proteins and Proteases:

There have been substantial advances during the last decade, in our understanding of yolk protein biosynthesis and its hormonal control however, despite the importance of the process, the regulatory mechanism of its degradation still remains to be solved (Yamashita and Indrasith, 1988). According to Holzer and Heinrich (1980) it is now evident that proteolytic reactions play a key role not only in the regulation of intracellular protein turnover, but also in the control of many other physiological functions such as translocation and maturation of protein, fertilization, germination, oncogenic transformation and other morphogenetic process.

The literature on the protin metabolism in relation to insects or *M. separata* is reviewed below.

Elliot and Gillott (1979) studied an electrophoretic study of proteins of the ovary, fat body and haemolymph in the migratory grasshopper, *Melanoplus sanguinipes.* The changes in vitelline and other yolk proteins during embryonic development in the *Bombyx mori* were studied by Irie and Yamashita (1980). An immunotitration experiments demonstrated that vitelline was not utilized during embryonic

differentiation but was consumed during larval differentiation. Such a prolonged persistence of vitelline is discussed in relation to protein metabolism during embryonic development in silkworm. According Oliverira *et al.*(1986) the ability of oocytes to take up yolk protein at different stages of development in *Rhodnius prolixux* increases with their size upto the time of chorion formation. Medina and Vallejo (1989) studied the contents of proteins, carbohydrates, lipids and DNA during embryogenesis of *Drosophila.* Sinha *et al.* (1991) studied the changes in concentration of proteins and carbohydrates in the developing healthy and pebrine infected embryos of tropical Tasar Silkworm, *Antheraea mylittad.* Ibanez *et al.*(1992) studied the characterization of vitellogenins in *Spilostethus pandurus* (Hemiptera). Kuk Meiri *et al.* (1966) investigated Cathepsin type proteolytic activity in the developing eggs of the American migratory Locust (*Locust migratoria* migratorioides). They suggested that the eggs contained one or more Cathepsin type enzyme, differing from the trypsin like enzymes of the gastroalimentary system of insects. Cathepsin B like proteinase in *Drosophila* and its role in yolk degration was investigated by Medina *et al.* (1988). The results suggest that the Cathepsin B like proteinase is implicated in yolk degradation in *Drosophila.* Medina and Vallejo (1989) studied the aspartic proteinase in *Drosophila.* According to them proteinase is maximally active at pH 3.5 and has been characterized by its sensitivity to specific inhibitors. They further explained that this proteinase is detected in mature oocytes and remain essentially constant during embryogenesis. This suggests that in the *Drosophila* aspartic proteinase function mainly before embryogenesis. They also worked on a serine proteinase in *Drosophila* embryos. They suggested that the activity of serine proteinase increases exponentially during embryogenesis. The subcellular localization changes from the yolk granules, in oocytes, to the soluble fraction, in late embryos. They also

studied the maternal origin of acid hydrolases in *Drosophila* and their relation with yolk degradation and explained that the maternal origin of the acid hydrolases suggests their role during embryogenesis and are involved in yolk degradation.

Identification of yolk platelet associated hydrolases in the oocytes of blood sucking bug, *Rhodnius prolixus* was studied by Nussenzeig *et al.* (1992). They suggested that the yolk platelets of this insect are composed mostly of vitelline and they contain at least two hydrolytic enzymes, a phosphtase and Cathepsin D like proteinase, where both the enzymes have an acid pH optima. Takahashi *et al.* (1992) studied the cysteine proteinase from *Bombyx* eggs and its role in degradation of yolk proteins during embryogenesis. Ribolla and De Bianchi (1995) studied the processing of procathepsin from *Musca domestica* eggs. They proposed that the major source of amino acids for insect embryos is yolk proteins, which accumulated in developing oocytes and is hydrolyzed during embryogenesis. In *Musca domestica* cathepsin zymogen activation occur in two steps. First an intramolecular cleavage of the procathepsin polypeptide which then undergoes autolysis to produce the mature enzyme.

II. MATERIAL AND METHODS :

1. Material:

The culture of *M. separata* and chemicals used were same as in Chapter II (Material and methods).

A. Egg Stages for Study :

The number of eggs laid per female has been recorded to be 500 to 600. The eggs were laid in rows between the folds of paper fastened by sticky secretion. The freshly laid eggs were bright yellowish in colour and

spherical in shape with flattened base without any sculptures. The diameter of the egg ranged from 0.5 to 0.7 mm with an average diameter being 0.62 mm.

For incubation studies freshly laid eggs were kept in petridishes provided with wet blotting paper at the bottom which protected the eggs from desiccation. After three days when the eggs turned to black purple colour, they were transferred to a clean jar. When the eggs were ready to hatch, they changed initially to dull brown and later dark brown. In majority of the cases hatching took place during night hours. The incubation period of the eggs ranged from 4 to 5 days. The embryonic developmental stages from 1-day to 5-day eggs (E_1 – E_5) were taken for the study of proteins and proteases activity.

2) Methods :

Estimation of proteins and proteases:

The estimation of proteins and enzyme assay of proteases were same as in chapter II (Material and Methods).

III. RESULTS :

1) Embryogenesis Period :

The number of eggs laid per female has been recorded to be 500 to 600. The embryogenesis period is of 5 days.

2) Egg Proteins :

The changes in proteins during embroygenesis of *M. separata* are recorded in Table No. 1 and illustrated graphically in fig.No. 1. The gradual increase in the amount of proteins was observed upto 4^{th} day of

embroygenesis. Where as slight decrease in protein content was observed on 5th day. Maximum and minimum amount of proteins were observed respectively on 4th and 1st day of embroygenesis.

Table No. 1

Period of Embryogenesis (days)	Amount of Proteins (mg/gm of body wt.)
1	15
2	21
3	25
4	32
5	30

3) <u>Egg Proteases</u>

A) Acidic Proteases :

Partial characterization of acidic proteases :

The partial characterization of acidic proteases during embryogenesis revealed the maximum activity at pH 3 (Cathepsin D like enzyme) and pH 4.5 (Cathepsin B like enzyme), temperature 37^{0}C, 30 min. incubation time, 1% enzyme concentration and 2% substrate concentration.

Acidic Proteases Activity:

a) Cathepsin D like enzyme :

The changes in activity of cathepsin D like enzyme during embryogenesis of *M. separata* are illustrated graphically in Fig. 2.

Gradual increase in enzyme activity was observed from 1st to 3rd day of embryogenesis; whereas sharp decrease was observed on the 4th day, comparatively activity was decreased slowly on the 5th day.

Maximum and minimum activity of cathepsin D like enzyme was observed respectively on 3rd and 1st day of embryogenesis.

b. Cathesin B like enzyme :

The changes in cathepsin B like enzyme activity during embryogenesis of *M. Sepaeta* are illustrated graphically in Fig 2.

Sharp increase in activity cathepsin B like enzyme was observed on 2nd day in comparison with the activity of enzyme on 1st day of embryogenesis. Then activity was observed to be sharpely decreased on the 3rd day of embryogenesis. Then enzyme activity was remained constant on 4th and 5th day as compaired to the activity of the enzyme on 3rd day of embryogenesis.

Maximum and minimum activity of cathepsin B like enzyme was observed on 2nd and 6th day of embryogenesis respectively.

B. Neutral Protease :

Partial characterization of neutral protease :

The partial characterization of neutral protease during embryogenesis revealed the maximum activity at pH 7, temperature 37^0C, 30 min. incubation time, 1% enzyme concentration and 5 % substrate concentration.

Neutral protease activity :

The changes in neutral protease activity during embryogenesis of *M. separata* are illustrated graphically in Fig 3.

Activity of enzyme, neutral protease on second day is increased slightly more than 1st day of embryogenesis, but it decreases sharply on 3rd day of embryogenesis. Then it is observed that the enzyme activity is increased steadily on the 4th and 5th day of embryogenesis.

Maximum and minimum activity of enzyme neutral protease is observed on 2nd and 3rd day of embryogenesis respectivily.

C. Alkaline Proteases :

Partial characterization of alkaline proteases:

The partial characterization of alkaline proteases during larval development revealed the maximum activity at pH 7.8 (Chymotrypsin like enzyme) and pH 8.2 (Trypsin like enzyme), temperature 37^0C, 30 min. incubation time, 1% enzyme concentration and 2% substrate concentration.

Alkaline protease activity :

a) Chymotrypsin like enzyme :

Changes in chymotrypsin like enzyme activity during embryogenesis of *M. seperata* are illustrated graphically in Fig 4.

2nd day activity of chymotrypsin like enzyme was observed to be increased sharply than the activity of enzyme of 1st day of embryogenesis, where as sharp decrrease in activity of chymotrypsin like enzyme was observed on the 3rd day of embryogenesis. Further activity of enzyme decreased very slowly on 4th and 5th day of embryogenesis.

Maximum and minimum activity of chymotrypsin like enzyme was observed respectively on 2nd and 1st day of embryogenesis.

b) Trypsin like enzyme :

Changes in trypsin like enzyme activity during embryogenesis of *M. seperata* are illustrated graphically in Fig 4.

Sharp increase in enzyme activity of trypsin like enzyme was observed on 2nd day than 1st day of embryogenesis, whereas the activity of enzyme was observed sharply decreased on 3rd day of embryogenesis, whereas gradual increase was observed on 4th and 5th day of embryogenesis.

Maximum and minimum activity of trypsin like enzyme was observed respectively on 2nd and 5th day of embryogenesis.

IV. DISCUSSION:

1. Egg Proteins :

Protein is an essential organic constituent which plays important role in cellular metabolism. The embryogenesis is characterised by an intensive protein metabolism takes place which involves mainly the breakdown of pre-existing yolk reserves and its conversion into tissue- and organ- specific proteins (Chen, 1966).

Premkumar *et al.*(1991) studied the biochemical changes during embryonic development in the aquatic hemipteran bug, *Loccotrephes griseus*. They found marked increase in the protein level from 2 to 6-day eggs. Chaubey and Bhatt (1988) studied the changes in the level of nucleic acid protein, total free amino and glycogen, and activity of acid phosphatase in the eggs, during normal embryonic development of rice moth, *Corcyra cephalonica* (Stainton). They observed the increase in RNA content in the early phase of embryonic development which indicates that, during this period active protein synthesis occurs. Such an increase in the protein synthesis seems to be necessary for synthesizing a

number of tissue- and organ- specific new proteins to fulfill the demand of the developing embryo.

In the present work gradual increase in the amount of proteins from 1 to 4-day eggs indicates synthesis of new proteins which provides the structural components to the developing embryo. Our results are in good agreement with Chen (1966), Chaubey and Bhatt (1988) and Premkumar *et al.*(1991).

2. Egg Proteases :

A. Acidic Proteases:

According to Sander *et al.,* 1985 the terrestrial propogation requires protection of the egg stages against desiccation and it requires well-provisioned eggs. An insect egg can be considered as a closed system and its supply of amino acids for protein synthesis depends on degradation of yolk reserves. The amino acids are used in the synthesis of proteins for developing embryo and their concentration falls as the rate of protein synthesis increases (Chen, 1966). During embryogenesis an intensive protein metabolism takes place which involves mainly the breakdown of pre-existing yolk reserves and conversion of these into tissue and organ specific proteins (Chen, 1978). Enzymes responsible for the hydrolysis of proteins down to amino acids are the acid proteases.

The proteolytic activity in homogenates of eggs of *Locusta migratorioides* at various embryonic stages Kuk Meiri *et al.*(1966). Experiments were carried out mostly within the acid range of hydrogen ion concentration. Homogenates of 3-day-old eggs showed slight but distinct proteolytic activity. Proteolytic activities markedly increased in homogenates of 4-day-old eggs and reached maximum in homogenates of 5 and 7-day eggs. There after the activity of enzyme decreased slightly in

homogenates of 8- day old eggs and remained almost constant upto 13-day-old eggs, but increased again slightly at the end of the embryonic development. It was concluded that the eggs contained one or more cathepsin type enzymes, differing from the Trypsin like enzymes of the gastroalimentary system of insects.

As per the findings of Colombo *et al.*, 1962 the simultaneous sudden increase of the free amino acids in the eggs of *Schistocerca gregaria* and of the Cathepsin like activity in the eggs of *Locusta migratoria* migratorioides which occur in the same embryonic stages suggests that the Cathepsin like enzymes play a catabolic role, at least in the yolk of the eggs.

During development of emryos Cathepsins play the general, long-term role of assisting in the breakdown of storage proteins. On the other hand, as per openion of Roonwal(1936) the sudden increase in the Catheptic activity of eggs containing embryos may be co-related with the secondary yolk cleavage, which is the most important morphological change of yolk in the eggs of *Locusta migratoria* migratorioides.

The yolk platelets from *Rhodnius prolixus*, a blood sucking bug are composed of vitelline and they contain at least two hydrolytic enzymes, a phosphate and Cathepsin D like proteinase. The proteinase has acidic pH optima. The proteinase appears to be bound to the yolk platelet membrane. (Nussenzeig *et al.*, 1992).

As per the finding of Ribolla and De-Bianchi, 1995, the studies on *Musca domestica* embryogenesis indicated that a Cathepsin B like proteinase is responsible for yolk protein degradation.

A cysteine Cathepsin B like protease activity has been found in *Drosphila* embryo. It appears associated with yolk granules and its activity during embryogenesis corelates well with the degradation of these organelles. In mature oocytes the enzyme is found in an inactive

form, which may be activated by limited proteolysis, by a serine proteinase also present in oocyte. The proteinase activity increased during early embryogenesis and decreases to low value in late embryo. The results altogether suggest that the Cathepsin B like proteinase is implicated in yolk deradation in *Drosophila.* (Medina *et al.,* 1988; Medina and Vallejo, 1989).

According to Kageyama *et al.,* 1981; Takahashi *et al.,* 1992 the several proteinases catalyzing the degradation of yolk proteins are detected in eggs of arthropod very recently from silkmoth eggs, at least 4 kinds of proteinases have been found and purified, they are 3 seryl trypsin like proteinases and 1 Cysteine proteinase. Cysteine proteinase is major proteinase in early developing eggs. In many cases, the conversion of precursors to active forms represents an important part in physiological regulation (Bond and Butler, 1987). Activated form of the proteinase is detected in developing *Bombyx* eggs. (Takahashi *et al.,* 1992) indicating that activation of the enzyme at low pH might be important in the regulation of protein degradation.

In the present work sharp increase in Cathepsin D like enzyme activity during embryogenesis of *M. separata* from 1 to 3-day the sharp decrease in enzyme activity was observed up to 4-day and then it remained almost constant upto 5-day eggs. Maximum enzyme activity was observed in 3-day and minimum in 5-day eggs. In case of Cathepsin B like enzyme gradual increase in enzyme activity from 1 to 2-day egg and gradual decrease from 2 to 4-day eggs was observed. After 4-day the enzyme activity was low, remained almost constant upto 5-day eggs. Maximum enzyme activity was observed in 2-day and minimum in 5-day eggs.

The high activity of enzymes during early embryonic development suggests the active role of these enzymes in degradation of yolk proteins

and supply of amino acids for protein anabolism during early embryogenesis of *M. Separata.* The low enzyme activity during later period of embryogenesis of *M. Separata* indicates the decrease of catabolism role of these enzymes. Our findings coincide with Kuk-Meiri *et al.* (1966), Ribollo *et al.* (1993), Medino *et al.* (1988) and Medina and Vallejo (1989).

B. Netural protease :

Chen, 1966; 1971 had reported the rapid increase in the level of all major amino acid at the onset of embryogenesis in *Bombyx, Drosophila, Spharodema, Culex, Schistocerca, Teleogryllus* and *Aulocara.* This corresponds to the periods of blastoderm formation and germ band elongation. The total concentration remains high at the beginning of blast kinesis and then declines during later embryonic differentiation. The overall variation represents no doubt the balance between release of amino acids from yolk degradation and their utilization for *de novo* protein synthesis.

In the present work sharp increase in neutral protease activity from 1 to 2-day and sharp decrease from 2 to 3-day eggs was observed. After 3-day steady increase in enzyme activity was observed upto 5-day eggs. Maximum activity was observed in 2- day eggs and minimum in 3-day eggs.

Our results are in good agreement with the findings of Chen (1966, 1971). The highest activity of neutral proteases during early embryonic development of *M. separata*, suggests the maximum catabolic role of the neutrl protease in degradation of yolk and their utilization for *de novo* protein synthesis. The low and steady increase in enzyme activity during later embryonic development of *M. separata* indicates the role of neutral

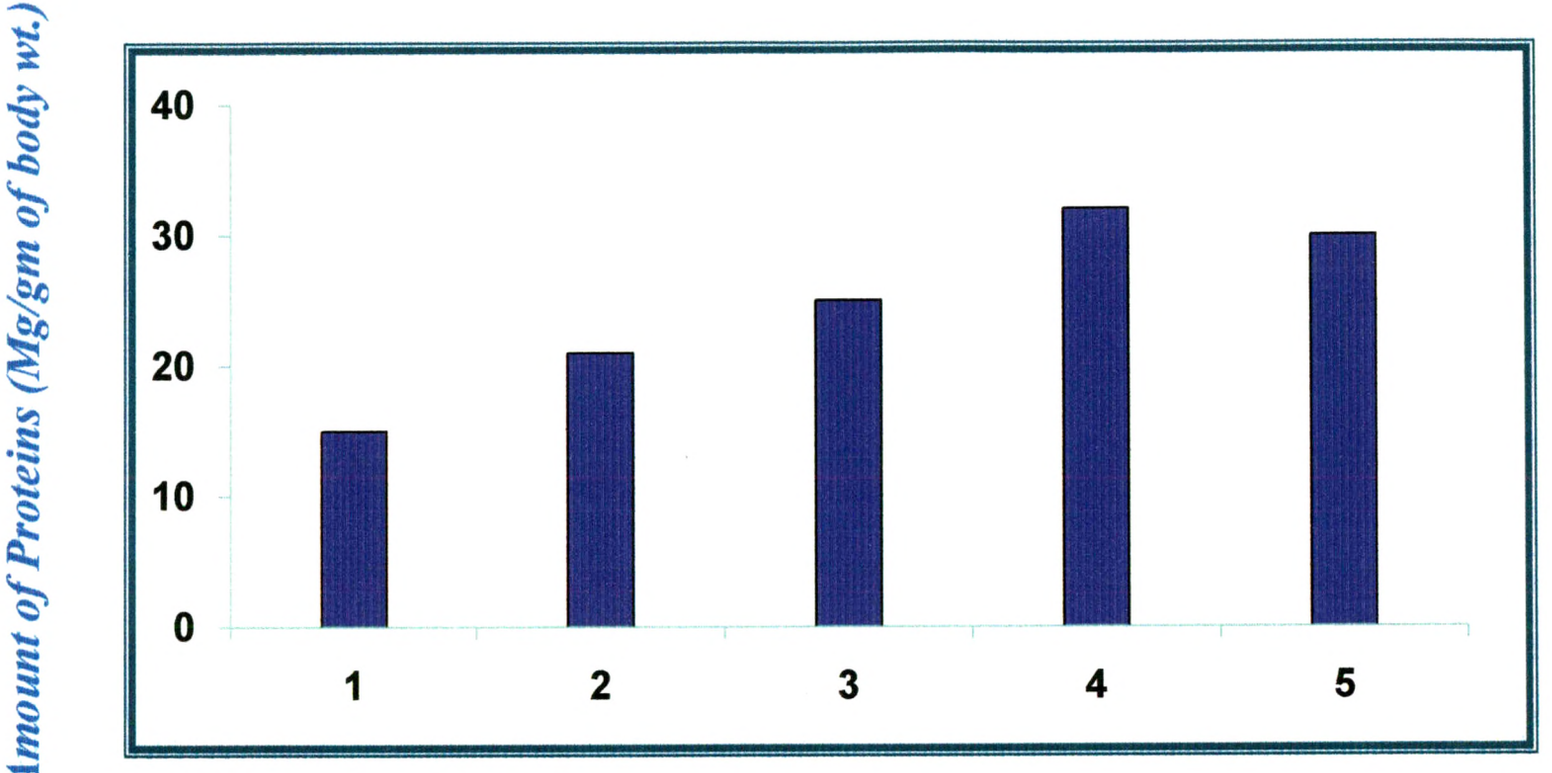
protease to provide essential amino acids during late embryonic development.

C. Alkaline proteases :

Ribolla and De Bianchi, 1995 reported the major sources of amino acids for insect embryos are yolk proteins, which accumulate in developing oocytes and are hydrolysed during embryogenesis, whereas Medina *et al.*, 1988 reported the proteinase activity increases during early embryogenesis and decrease in late embryo. A serine protease has been found in *Drosophila* oocyte. The detected activity increases exponentially during embryogenesis. The subcellular localization changes from the yolk granules in oocytes, to the soluble fraction in late embryos (Medina and Vallejo, 1989).

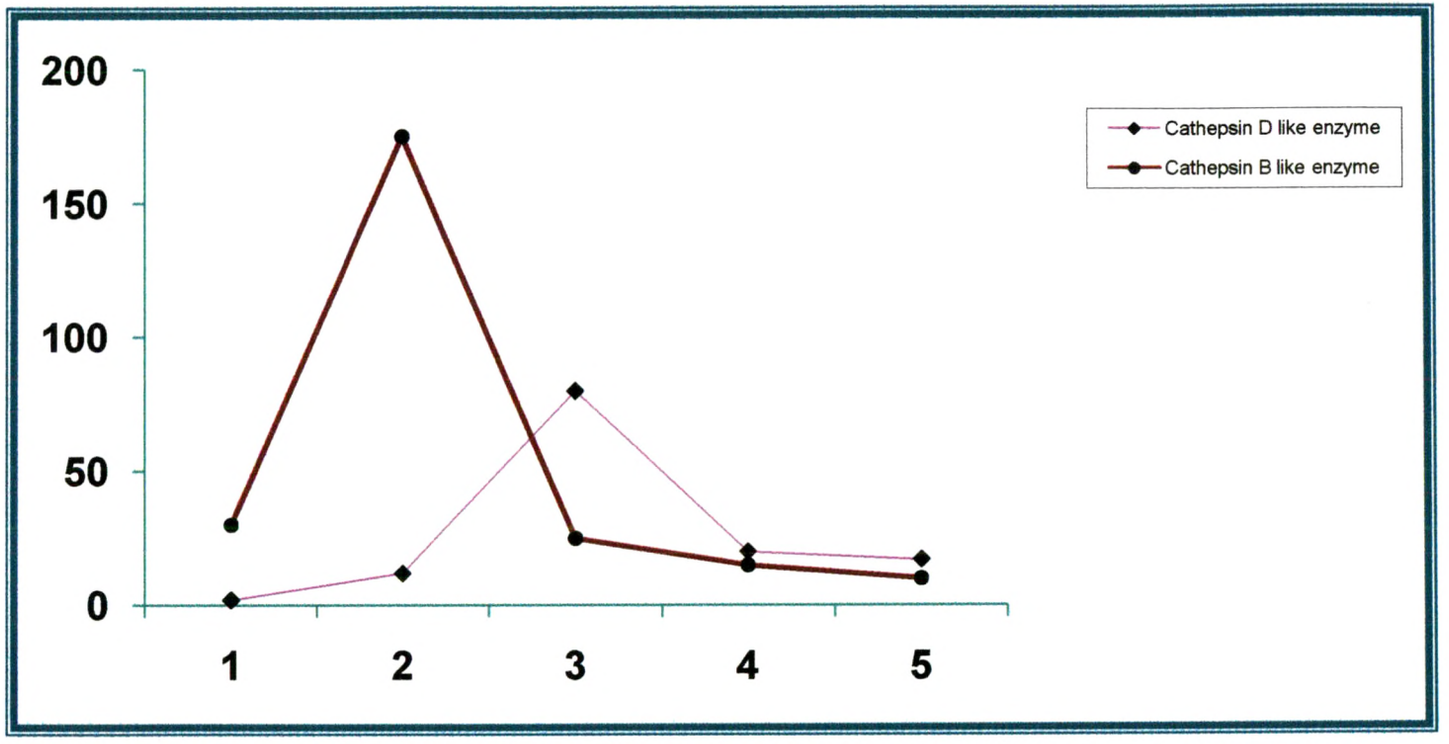
In the present work sharp increase in alkaline protease enzyme (Chymotrypsin and Trypsin like) activity from 1 to 2-day and sharp decrease from 2 to 3- day eggs was observed. After 3-day the steady increase in enzyme activity was observed upto 5-day eggs. Maximum enzyme activity was observed in 2-day and minimum in 3-day eggs.

Our results are in good agreement with the findings of Ribolla and De Bianchi (1995), Medina *et al.*(1988) and Medina and Vallejo (1989). The highest activity of alkaline proteases (Chymotrypsin and Trypin like) during early embryonic development of *M. Separata* suggest the maximum role of these proteases in hydrolysis of the yolk proteins and conversion of these into tissue and organ specific proteins. The steady increase in enzyme activity during later embryonic development of *M. separata* suggests active role of these enzymes to provide essential amino acids during later embryonic development.



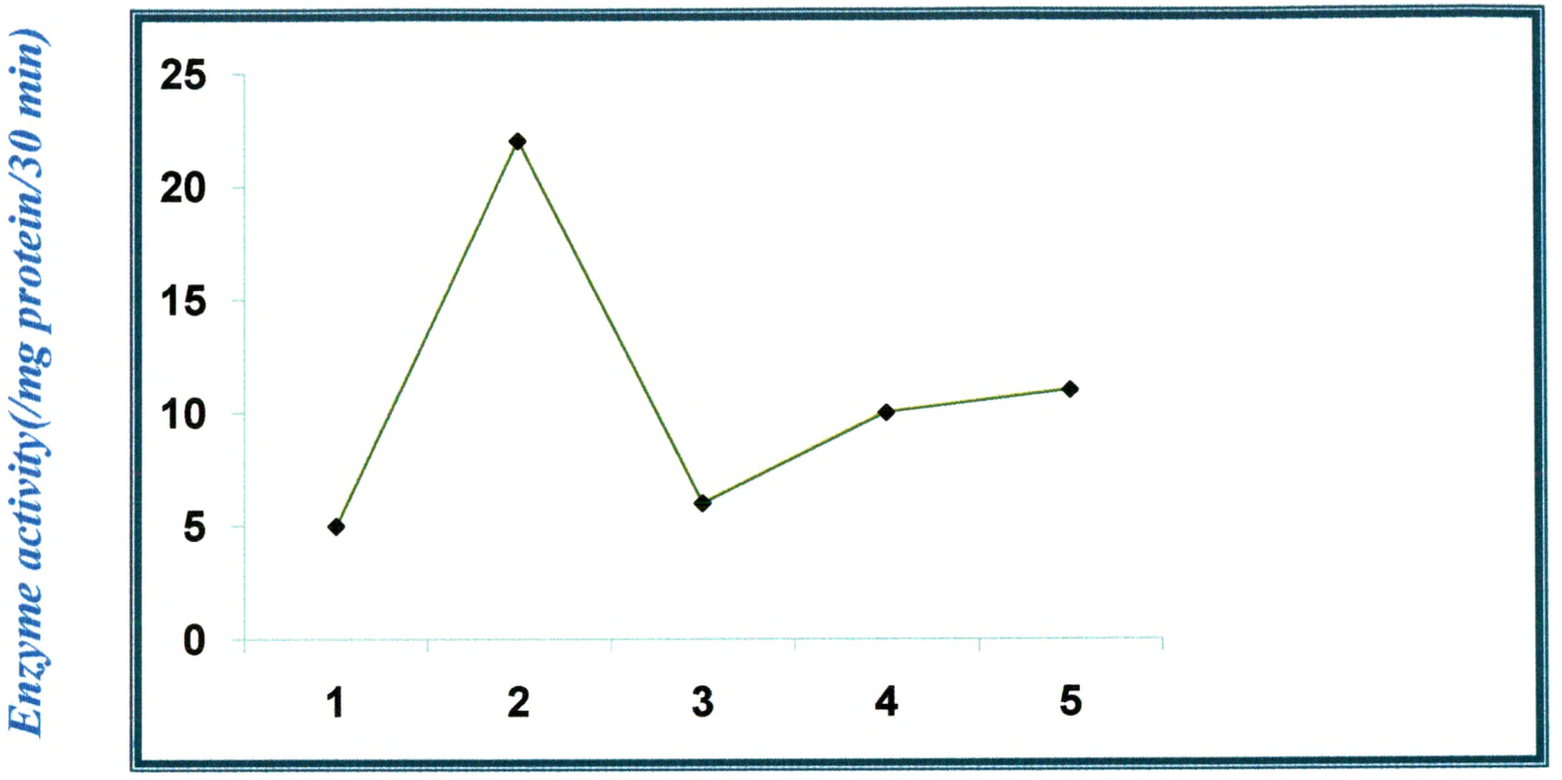
Changes in protein during embryogenesis of *Mythimna separata*

Fig. No. 1



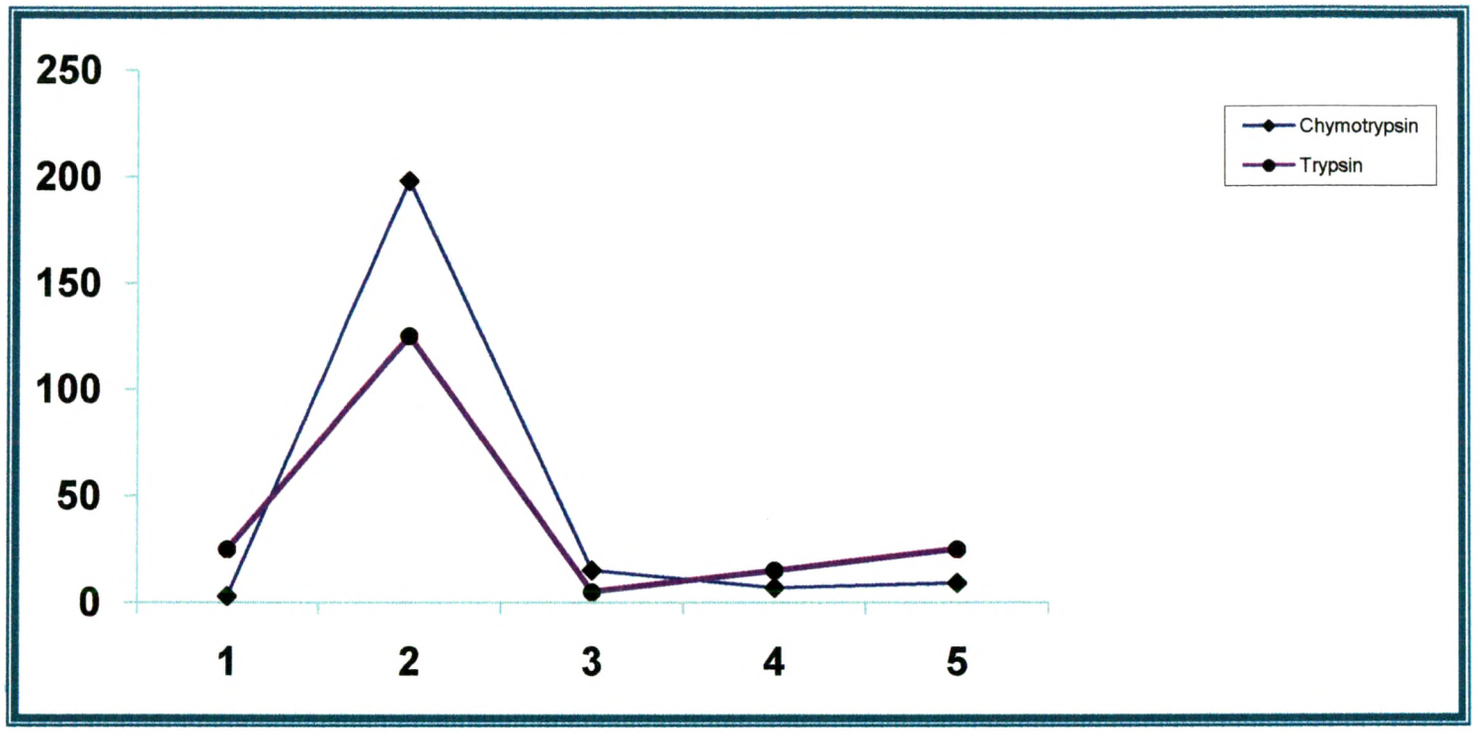
Acidic proteases activity during embryogenesis of *Mythimna separata*

Fig. No. 2



Neutral proteases activity during embryogenesis of *Mythimna separata*

Fig. No. 3



Alkaline proteases activity during embryogenesis of *Mythimna separata*

Fig. No. 4

Fig 1: Freshly laid eggs (1-day eggs)

Fig 2: 2-day eggs

Fig 3: 3-day eggs (Pale brown colour)

Fig 4: 4 –day eggs (Brown colour)

Fig 5: 5-day eggs before hatching (Black colour)

Plate No. 2: Stages of embryogenesis of *M. separate*