CHAPTER - FOUR

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

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CHAPTER FOUR

I. INTRODUCTION :

The transformation of stored yolk components into active protoplasm results in the inner growth of egg. The growth during insect development is restricted to the larval development. The mass necessary for the final adult will be deposited during the feeding period of larva. In most organisms growth occurs through cell multiplication. However, in the insect larva, the general principle of growth by cell multiplication is modified. In endopterygote groups the growth of many organs may be attained through an enormous enlargement of the single cells. Often, but not obligatorily, this growth by increase in the dimensions of the cells concerns the specific larval organs which undergo breakdown at metamorphosis (Agrell and Lundquist, 1973).

The rigid integument cannot easily accommodate itself to the increasing size of a growing insect and must therefore be shed and renewed periodically. At each moult there is cast off not only the general cuticle that invests the body and its appendages externally, but also the various endoskeletal structures and the intima or lining of most of the tracheal system, fore and hind gut, ectodermal glands and different reproductive ducts. All these, together with hairs, scales and cuticular sensilla, are renewed by the underlying epidermal cells (Richards and Davies, 1977). The moulting is controlled by ecdysteroid and follow a similar course in all insects (Sehnal, 1985).

1. Metabolism during Larval Growth :

A number of substances, particularly amino acids and vitamins, are essential for any development to take place; others while not essential,

are necessary for optimal development. The balance between different constituents is also important (Chapman, 1969). Dipteran larvae are known to accumulate lipids, glycogen and proteins during development (Pearincott, 1960; Wigglesworth, 1972). The reason for storing these constituents in larva is fairly obvious, this material later on can be used during metamorphosis.

A certain amount of protein is stored in the fat body and much is deaminated or converted into carbohydrate or fat and used for energy production (Wigglesworth, 1972). Proteins provide the chief structural elements of the muscles, glands and other tissues. During larval development fat body is responsible for the synthesis of various major haemolymph proteins and serves at the same time as a place of storage of these components, in addition to carbohydrates and lipids (Chen, 1978). The physiological role of the fat body is very much dependent upon the developmental stage and the actual time within instars. For example in *Calliphora erythrocephala* larvae, the fat body in the early third larva synthesizes and releases protein into haemolymph, whereas, in late larvae it sequesters proteins back from the haemolymph (Price, 1973), whereas protein is stored in the larval fat body to be used later in the assembly of adult tissues at the time of metamorphosis (Rees, 1977).

2. Review of Literature on Larval Proteins and Proteases:

The developing insect larva, in contrast to egg and pupa, depends on a continoues supply of food for energy production and growth.The growth and development of insect proceeds discontinuously through a series of programmed stages. The existence of such a controlled discontinuous program of growth and development requires very specialized metabolic processes occur during defined periods. Many

possibly all of these processes involve the action of specialized proteases and peptidases.

The haemolymph proteins and lipoproteins in Lepidoptera were studied by Whitmore and Gilbert (1974); Firling (1977) studied the amino acid and protein changes in the haemolymph of developing fourth instar, *Chironomous tentanus.* Tysel and Butterwarth (1978) studied rate of protein granule formation in the larval fat body of *Drosophila melanogaster.* Tojo *et al.* (1985) studied the hormonal regulation of phase polymorphism and storage protein fluctuation in the common cutworm, *Spodoptera litura.* Jindra and Sehnal (1989) studied the larval growth, food consumption and utilization of dietary protein in *Galleria mellonella.* Sinha *et al.*(1991) studied the changes in protein content in the haemolymph of healthy and pebrine infected larvea and pupae of *Antheraea myllita.*

Hagenmaier (1971) studied by purification and characterization of trypsin like proteinase from midgut of larva hornet *Vespa orientalis.* He reported that, hornet protease is homologous with the other serine proteases. According to experimental results the pH profile, temperature activity, the mode of action as shown by inhibitors and the cleavage specificity on ß-chain of insulin are all quite similar to mammalian trypsin. Ahmad *et al.*(1976) studied the alkaline protease in the larvae of the armyworm, *Spodoptera litura* and reported that the alkaline protease activity in the gut was found to increase with the onset of pupation. Eguchi and lwamoto (1976) studied the alkaline protease in the midgut tissue and digestive fluid of silkworm, *Bombyx mori.* According to their results, the proteolytic enzymes in the alimentary canal are predomiantly localized in the gut contents. These proteases, however, are somewhat different in thermostability and in other properties of enzymes. The bound form of tissue proteases may be a source of digestive fluid

protease. Proteolytic activity in the digestive fluid of larvae of *Trichoplusia* ni was investigated by Prichett *et al.*(1981). They observed both tryptic and chymotryptic activities in the digestive fluid of *Trichoplusia ni.* larvae. Baker (1976) studied the properties of midgut protease in larveae of *Attagenus megatoma.* According to him the midgut protease actvity exhibited high temperature and alkaline pH optima. The total protease levels declined in starved larvae but increased after 48 hr of feeding. Charcterization of an acidic proteinase from the posterior midgut of *Rhodnius prolixus* stal was carried out by Houseman and Downe (1982). They reported that the *Rhodnius prolixus* stal contained Cathepsin D in the posterior midgut to breakdown ingested blood proteins. The presence of Cathepsin B and lysosomal carboxypeptidase B, which have also been detected in the postorior midgut of *R. prolixus* and other blood sucking Hemiptera. Christeller *et al.*(1989) studied the purification and characterization of the major midgut proteases of grass grub larveae, *Constelytra zealandica.* They reported that trypsin can be considered the major target in attempts to interfere with protein digestion. Meenakshisundaram and Gujar (1998) worked on alkaline proteases from some Lepidopteran larvae. They reported that the Lepidopteran larvae possess alkaline proteases in the midgut region which are almost having similar properties with respect to the optmum conditions of pH, temperature, time and differing in their substrate and inhibitor specificity which probably make the insects to thrive on certain selected host plants utilizing different plant proteins qualitatively and survival of herbivore insects.

II MATERIAL AND METHODS :

1. Material :

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

A. Larval Stages for Study :

The embryogenesis period of *M. separata* is of 5 days. The newly hatched larva was tiny, cylindrical and active. The larval skin was soft and larva was pale brown coloured with black head. The length of the larva ranged from 0.9 mm to 1.3 mm with an average of 1.27 mm. The larval instars were studied by transferring freshly hatched larvae into the specimen jars. The cut pieces of the fresh and tender maize leaves were provided as food. The food was changed after every 24 hours. Number of days covered by each larval instar were recorded.

The larval developmental period is of 22 to 27 days. The larvae moult five times thus had six larval instars. The larval developmental stages from 1-day to 25-day larvae ($L_1 - L_{25}$) were taken for the study of enzyme activity.

2. Methods :

Estimation of Proteins and Proteases:

The preparation of larval homogenate and estimation of proteins and enzyme assay of proteases were same as in chapter II (Material and methods)

III. RESULTS :

1. Larval Developmental Period :

The total development of larvae is marked by six instars as a developmental stages. From first to sixth instar each stage lasts for 4, 5, 5, 3, 3 and 5 days respectively. The moulting in larvae is observed after 4, 9, 14, 17, 20 and 25 days of development.

2. Larval Proteins :

Changes in proteins during larval growth are represented in Table No. 2 and illustrated graphically in Fig. No. 5. Gradual increase in the amount of proteins from 1 to 17- day larvae was observed. After 17- day it remained relatively constant upto 25-day larvae.

Table No. 2

Period of larval Growth (days)	Amount of Proteins (mg/gm of body wt.)
1	18
2	21
3	26
4	28
5	29
6	36
7	38
8	40
9	41
10	42
11	47
12	49
13	53
14	56
15	58
16	63
17	64
18	61
19	60
20	58
21	59
22	60
23	61
24	60
25	58

3. Larval Proteases :

A. Acidic Proteases :

Partial characterization of acidic proteases :

The partial characterization of acidic proteases during larval development of *M. Separata* revealed the maximum activity at pH 3.2 (Cathepsin D like enzyme) and pH 4.2 (Cathepsin B like enzyme), temperature 37^0C, 30 min. incubation time, 1% enzyme concentration and 2 % substrate concentration.

Acidic Protease Activity:

a. Cathepsin D like enzyme activity :

The observations on chages in the cathepsin D like enzyme activity of *M. separata* during larval growth are illustrated graphically in Fig.6.

Gradual increase in enzyme activity is observed in the larvae after one to two days of developmental period, while it is observed decreased in the larvae having developmental period of 2-4 days, after 4 days of development remains constant up to 5-day larvae. Sharp increase in enzyme activity was observed on 6^{th} day, while it decreases sharply on 7^{th} day of development of larva. Then gradual decrease in the enzyme activity was observed from 7^{th} to 10^{th} days of development. The enzyme activity was remained constant from 11^{th} to 25^{th} day of development of larva with exception of slight increase in activity on the 20^{th} day. Maximum enzyme activity was observed on 6^{th} day and minimum enzyme activity was observed on 23^{rd} day of development of larva in *M. separata.*

b. Cathepsin B like enzyme activity :

Changes in cathepsin B like enzyme activity of *M. Separata* during larval growth are illustrated graphically in Fig 6.

The enzyme activity was observed to be increased on 2nd to 3rd day of development of larva, while it decreases on the 4th day of larval development. Further some increase in the enzyme activity was observed on 5th day, but it increases sharply on the 6th day of development of larva. The enzyme activity observed on 6th day is found to be maximum acivity in *M. separata.* The activity of enzyme again falls on 7th day and it decreases slowly upto the 10th day of larval development. This enzyme activity increases slowly on 11th to 12th day of development and further more again this activity decreases slowly upto the 16th day. This activity of enzyme them remain constant upto the 23rd day of larval development, on 24th day of development of larvae increase in the activity was observed which decreases sharply on the 25th day of larval development, which is found to be minimum activity of enzyme in *M. separata.*

B. Neutral Protease :

Partial characterization of neutral protease :

The partial characterization of netural protease during larval development of *M. Separata* revealed the maximum activity at pH 7, temperature 37^{0}C, 30 min. incubation time, 1% enzyme concentration and 5% substrate concentration.

Neutral protease activity :

Changes in the neutral protease enzyme activity of *M. Separata* during larval growth are illustrated graphically in Fig 7.

The enzyme activity is increased slightly on 2nd day of development of larva and then it was found decreased on the 3rd day. Whereas the enzyme activity was increased gradually on 4th and 5th day and sharply on 6th day. Then the activity of enzyme decreased gradually up to the 13th day of larval development after word again enzyme activity

was increased and then decreased gradually on 14th - 15th day and 16th - 17th day respectively. Furthermore enzyme activity increased up to the 20th day and then decreased again gradually up to the 23rd day of larval development, on 24th day sharp increase in enzyme activity was observed and then it falled again on 25th day of development of larva. Maximum and minimum enzyme activity was observed respectively on 6th and 25th day of larval development in *M. separata.*

C. Alkaline Proteases :

Partial characterization of alkaline proteases :

The partial characterization of alkaline protease during larval development of *M. Separata* revealed the maximum activity at pH 7.9 (Chymotrypsin like enzyme) and pH 8.1 (Trypsin like enzyme), temperature 37^{0}C, 30 min. incubation time, 1% enzyme concentration and 2 % substrate concentration.

Alkaline protease activity :

a) Chymotrypsin like enzyme activity :

Changes in the Chymotrypsin like enzyme activity of *M. Separata* during larval growth are illustrated graphically in Fig 8.

The enzyme activity was increased on 2nd day of larval development and then decreased gradually on 3rd and 4th day of larval development. The activity found sharply increased on 6th day. Then enzyme activity was slowly decreased up to 10th day and slowly increased upto 14th and 15th day of larval development, with very slight changes activity of enzyme remained constant from 16th to 23rd day and then it was increased gradually on 24th to 25th day of larval development. Maximum and minimum enzyme activity was observed respectively on 6th and 1st day of larval development in *M. separata.*

b. Trypsin like enzyme activity :

Changes in the trypsin like enzyme activity of *M. Separata* during larval growth are illustrated graphically in Fig 8.

Steady decrease in enzyme activity was observed from 1st to 4th day larval development. where as sharp increase is observed on 4th to 6th day. The enzyme activity was gradually decreased from 6th to 10th day of development of larva. Further more activity of enzyme was found increased gradually up to the 16th day and there is sudden fall in the enzyme activity was observed on the 17th day. Then again gradual decrease in the enzyme activity was observed up to the 22nd day which was decreased on 23rd day and then increased slowly on 24th and 25th day of larval development. Maximum and minimum enzyme activity was observed respectively on 6th and 10th day of larval development in *M. separata.*

IV. DISCUSSION :

1. Larval Proteins :

According to Wigglesworth, 1972 the proteins provide the chief structural elements of the muscles, glands and other tissues. Various reports are avaiable on showing that the total content of haemolymph proteins increases during larval development and decline rapidly with the advance of larval life with a concomitant appearance of protein granules in the fat body cells.

According to Chen, 1966 in some insects the increase is most rapid during the time approaching pupation. Wyatt *et al.*(1956) reported that the blood protein of *Bombyx* rises from 1.2 per cent in early third instar to 5.3 per cent in the late fifth instar. Apparently the same is true for *Samia cynthia* whose protein concentration, according to Laufer (1960)

increases rapidly from the third instar to a maximum in the spinning fifth larval instar.

In present work proteins are found to be increased in the early development of larva upto seventeen days and after words they remain constant upto 25th days. Our results are in good agreement with the findings of Wyatt *et al.*(1956), Laufer (1960), Chen (1966) and Wigglesworth (1972). Increased amount of proteins upto 17 days of development suggests the synthesis of proteins required for the development of larva after words amount of proteins remained constant up to 25 days which indicates that there is less utilization of proteins for structural elements required for the further development of larva.

2. <u>Larval Proteases</u>:

Insect proteases are important enzymes existing freely in the lumen or bound to the microvillar membrane that proteolyze different kinds of proteins in a variety of insect species (Applebaum, 1985; Terra, 1988).

Development of insects is punctuated by shedding of the integument. During a moult the insects digest most of its exoskeleton, synthesizes a new one that may be morphologically quiet different and sheds the remains of the old. The moulting cycle commences with a period of mitotic cell division in the epidermis after which the cells become closely packed and columnar. Following cell division the cutical becomes detached from the epidermal cells (apolysis), production of a space that is soon filled with a mixture of inactive proteolytic enzymes called the moulting gel. The moulting gel that first fills the exuvial space after apolysis contains no proteases or chitinase activity. However after deposition of a new cuticulin layer, which propects the underlying epidermal cells both proteolytic and chinolytic activities appear as the

moulting gel is transformed into moulting fluid (Passonneau and Williams, 1953; Bade, 1975).

Houseman and Downe (1982) studied the characterization of an acidic proteinase from the posterior midgut of *Rhodnius prolixus* stal and stated that Cathepsin D in the posterior midgut involve in breakdown of ingested blood proteins. The presence of Cathepsin D as an extra cellular digestive proteinase is consistent with the presence of Cathepsin B and lysosomal carboxypeptidase B, that have also been detected in the posterior midgut of *R. prolixus* and other blood sucking Hemiptera.

Comparison between the levels of aspartic and cysteine proteinases of the larval midguts of *Callosobruchus maculaatus* and *Zabrotes subfasciatus* (BOH) was studied by Silva and Filho (1991). The utilization of aspartic and cysteine protenases similar to cathepsins for extracellular digestion in insects was first described in Hemiptera feeding on blood or seeds (Houseman and Downe 1980b, 1981a, 1982a, 1982b, 1983;). Later, Cathepsin B like proteinases were described in the bruchids *C. maculatus* (Kitch and Murdock, 1986; Campos *et al.*, 1989) and *A. obtecta* (Wieman and Nielsen, 1987), but now the presence of both kinds of proteinases seems to be much more widespread than was originally suggested (Murdock *et al.*, 1987).

After detection and partial characterization of cysteine and aspartic proteinases in the larval midgut of both *C. maculatus* (Campos *et al.*, 1989) and *Z. subfasciatus* (Lemos *et al.*, 1990), the differences found between the proteolytic complement of the midgut of *C. machulatus* and *Z. subfasciatus*, particularly the great difference in aspartic proteins content, suggests that the larvae of the last bruchid have a greater potential for protein digestion.

Ahmad *et al.*(1976) investigated alkaline proteases in the larvae of the armyworm, *Spodoptera litura*. The alkaline protease activity in the

gut of *Spodoptera litura* was found to increase with the development of larvae and decreased with the onset of pupation fasting of the fifth instars lavae cause to a slight increase in protease activity at 4 hr which declined consistently on further starvation.

Changes in protein concentration and protease activity during further starvation also suggest a relationship between them. It is of interest to mention here that feeding of the larvae with high protein diet causes a marked stimulation in protease activity (Ishaaya *et al.,* 1971). The proteolyic activity of several insects decreases during starvation and increases again on reseeding (Dadd, 1956; House, 1965; Engelmann, 1966, 1969; Janda and Krieg, 1969) also suggests the influence of the protein on gut proteolytic activity.

According to Eguchi and Iwamoto (1976) the proteases in the midgut tissue and digestive fluid increase with increasing feeding from 1 to 9-day of fifth instar. The results suggest that the secretion of protease occur in response to feeding. As inferred by Dadd (1956) from experimental results in *Tenebrio,* the synthesis and discharge of the midgut proteases may be interdependent, since little enzyme is accumulated in the epithelial tissue when the total midgut enzyme is greatly increased and tissue proteases may reasonably be considered an index of the rate of synthesis of enzyme in the secretary cells.

Baker (1977) has studied midgut proteolytic activity in the black carpet beetle, *Attagenus megatoma.* The control of proteolytic digestive enzyme in the larvae of *A. megatoma* was not regulated by the amount of food being consumed but rather by the amount of protein present in midgut. When starved larvae of the black carpet beetle, *Attagenus megatoma,* were fed on selected diets, increases in proteolytic trypsin and chymotrypsin activity correlated with total midgut protein and not with the amount of food consumed.

Garcia and Garcial (1977) studied the protease secretion in the intestine of fifth instar larvae of *Rhodnius prolixus*. In the protein fed larvae the protein content and the specific protease activity increased. These findings exclude neurosecretory control and that indicate ingested protein stimulate the proteolytic activity of Rhodnius prolixus midgut.

Baker (1976) reported that the total protease levels declined in staved larvae but increased after 48 hr of feeding. These *in vitro* studies indicate that midgut homogenates of *A. Megatoma* possess total protease activity with an optimum pH range in the alkaline region similar to that of many other insect species. Protease activity in *A. megatoma* larvae starved for extended periods declined to immeasurable levels. When these larvae were given a food source, feeding occur immediately, and guts were nearly full after 24 hr. However no protease activity was detected until 48 hr. Such delays between the onset of feeding and a measurable increase in protease activity in insects are common (Engelmann, 1969) and have been used to argue for a distinct synthesis of enzyme response to ingested food rather than activation of a zymogen already present in gut lumen.

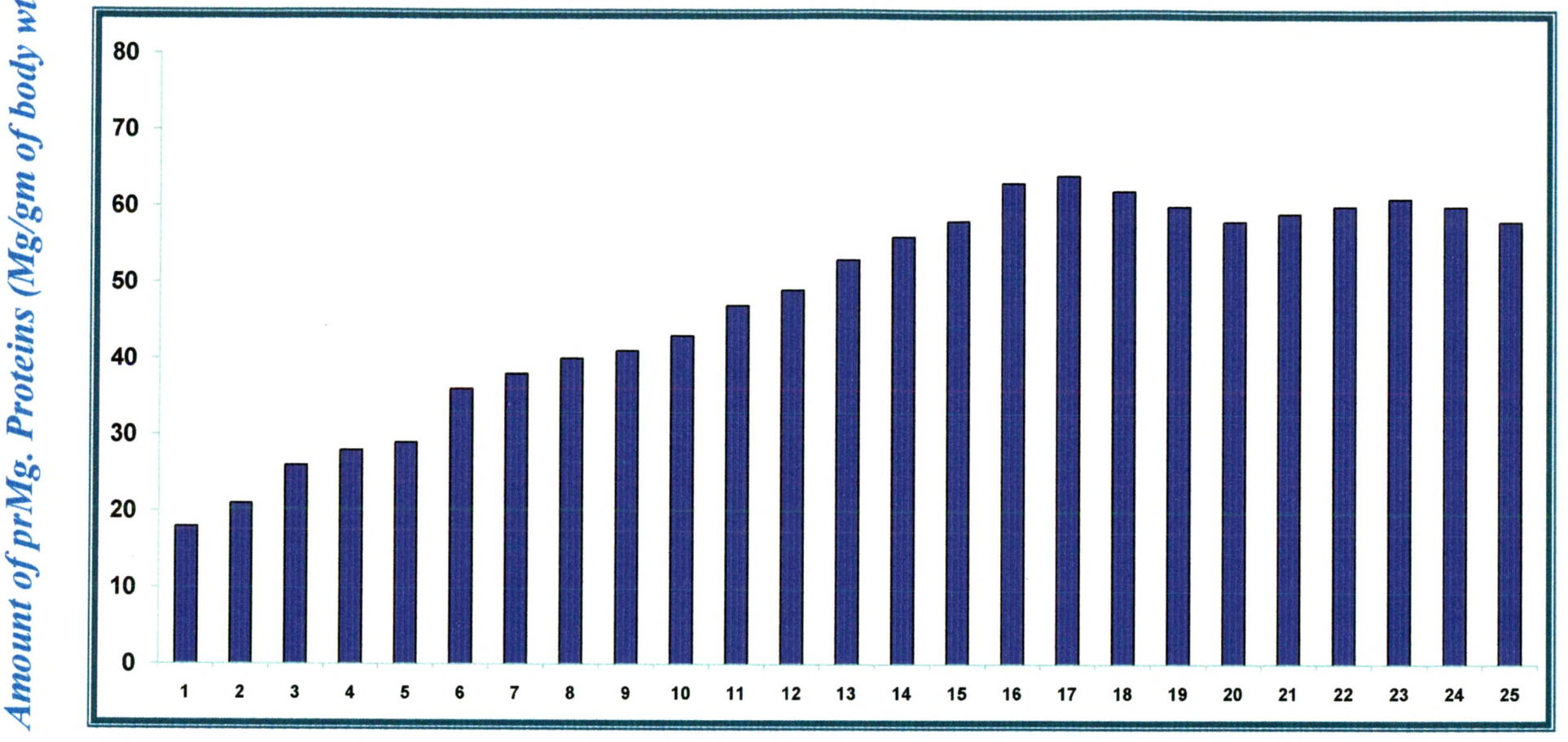
Meenakshisundaram and Gujar (1998) studied on alkaline proteases in Lepidoptera larvae. It was observed that the initial protein peaks had very low protease activity. The protease activity however showed rising trend and then reached at the peak. It is evident from above results that the alkaline midgut region of the test insects is conductive to proteases to degrade efficiently the protinacious substrate present in the food to drive the required amino acids after digestion for the growth and development.

In the present investigation gradual increase in proteases activity of *M. Separeta* from 1 to 2-day larva suggests the degradation of yolk proteins and ingested proteins after hatching for growth of larva. Gradual

decrease in enzyme activity from 2 to 4-day larvae suggests depletion of yolk proteins. Sharp increase in enzyme activity from 4 to 9- day and high activity in 6-day larvae suggests the active feeding stage of the larva and degradation of proteins. Sharp fall in enzyme activity from 6 to 7-day and decrease from 7 to 10-day larvae suggests the low degradation of protein and synthesis of proteins. Gradual increase in enzyme activity from 10 to 12-day larvae indicates the active feeding stage of larva and degradation of proteins. Fall in enzyme activity from 12 to 13-day larvae indicates quiescent stage of larva in which degradation of protein is low and synthesis and accumulation of proteins may be high. After 13-day the enzyme activity remained constant up to 17-day indicates the larva enters in to the third molt and protein synthesis during this period. Slow but low increase in enzyme activity from 17 to 20-day larvae indicates feeding stage of larva with protein catabolism. Decrease in enzyme activity from 20 to 23-day larvae indicates the synthesis and storage of proteins in the haemolymph and fat body. Slow but low increase in enzyme activity from 23 to 25-day indicates slow feeding larval stage with very very low protein catabolism.

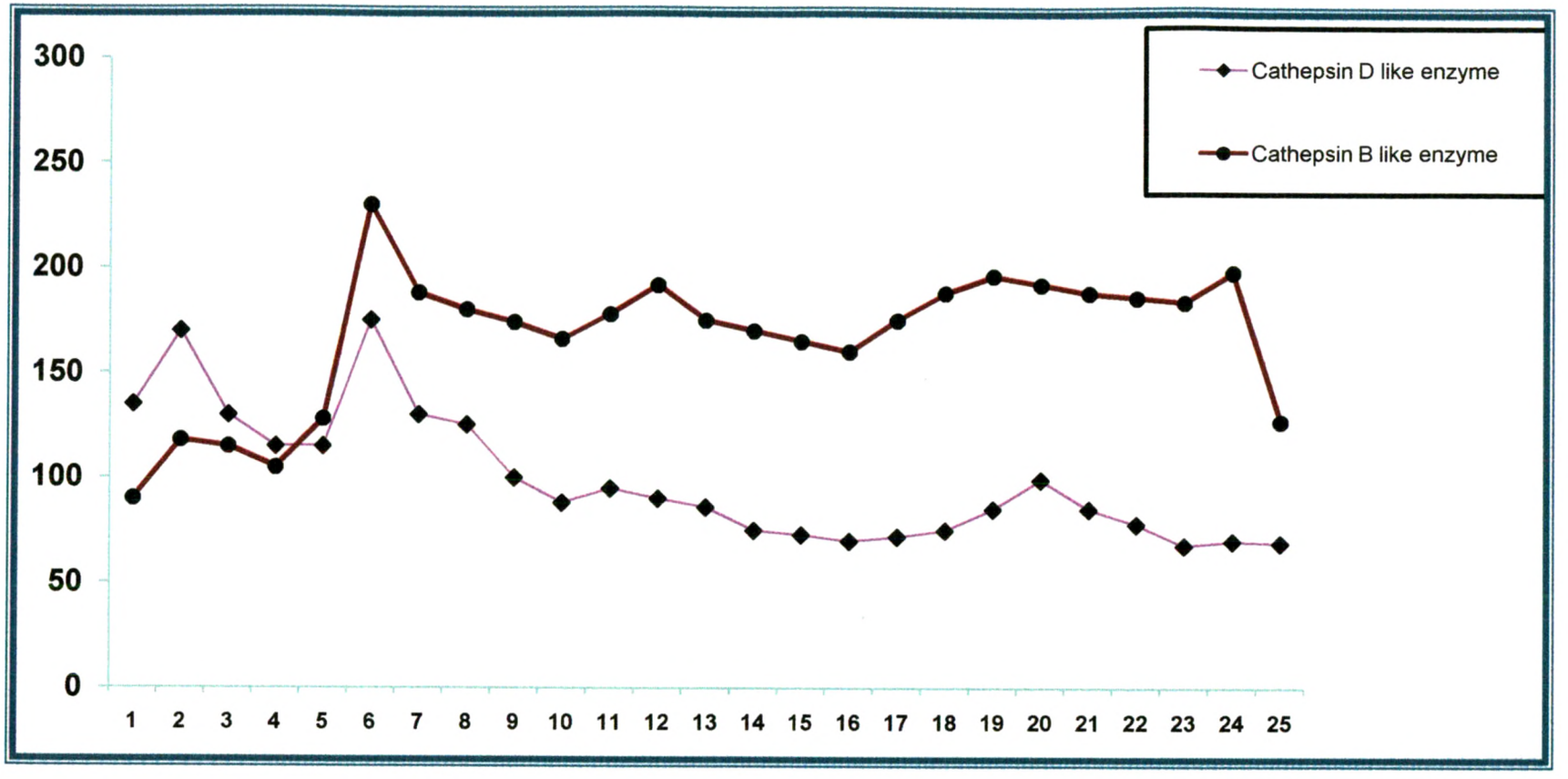
Our results are in good agreement with Ahmad et al.(1976); Fujii and Kato (1930); Matsumura and Oka (1936); Ishaaya et al.(1971); Dadd (1956); House (1965); Engelmann (1966, 1969); Janda and Krieg (1969); Eguchi and Iwamoto (1976); Baker (1976, 1977) and Meenakshisundaram and Gujar (1998).

The decline in the enzyme activity on 4, 10, 17and 23-day larva indicates first, second, third and fourth larval-larval moults and fifth larval-pupal moult respectively.



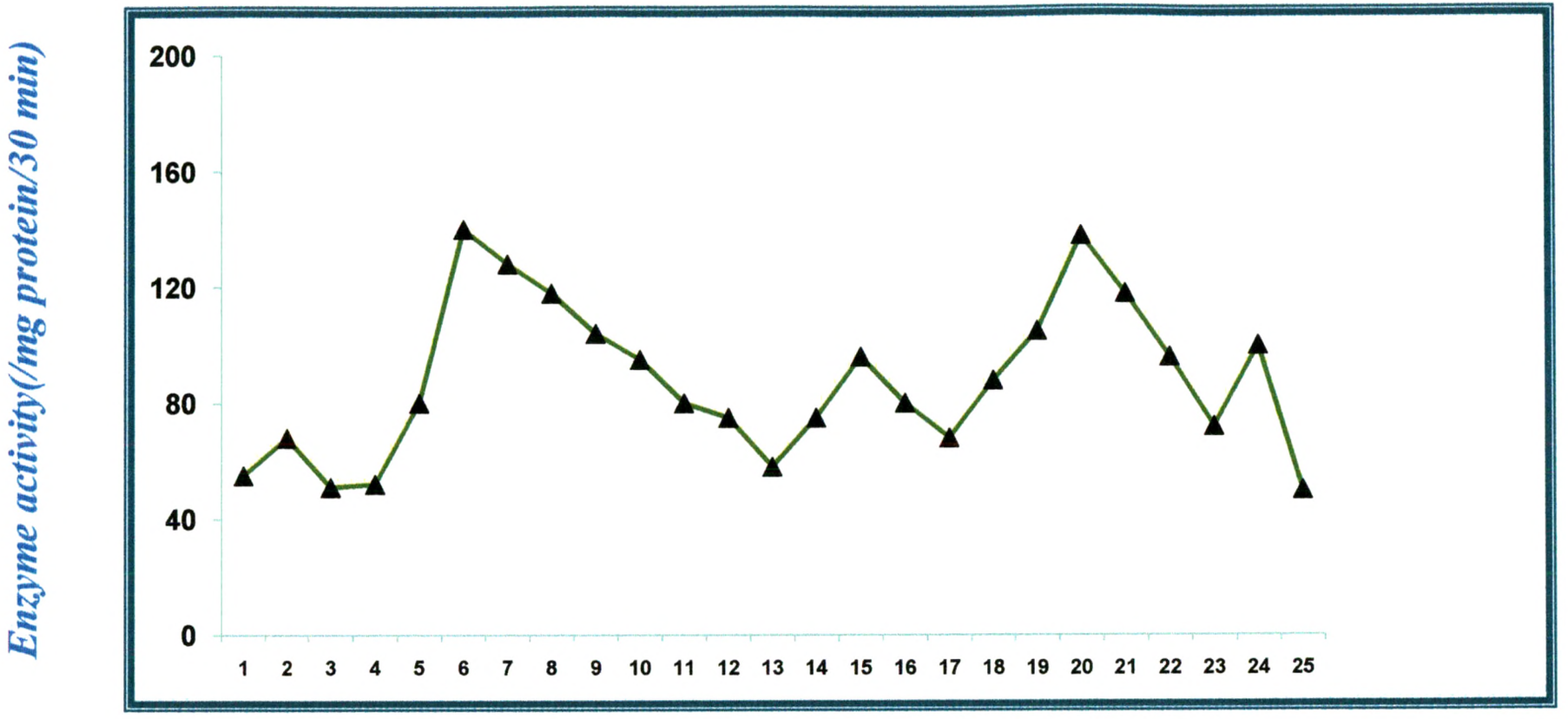
Changes in protein during larval growth of *Mythimna separata*

Fig. No.5



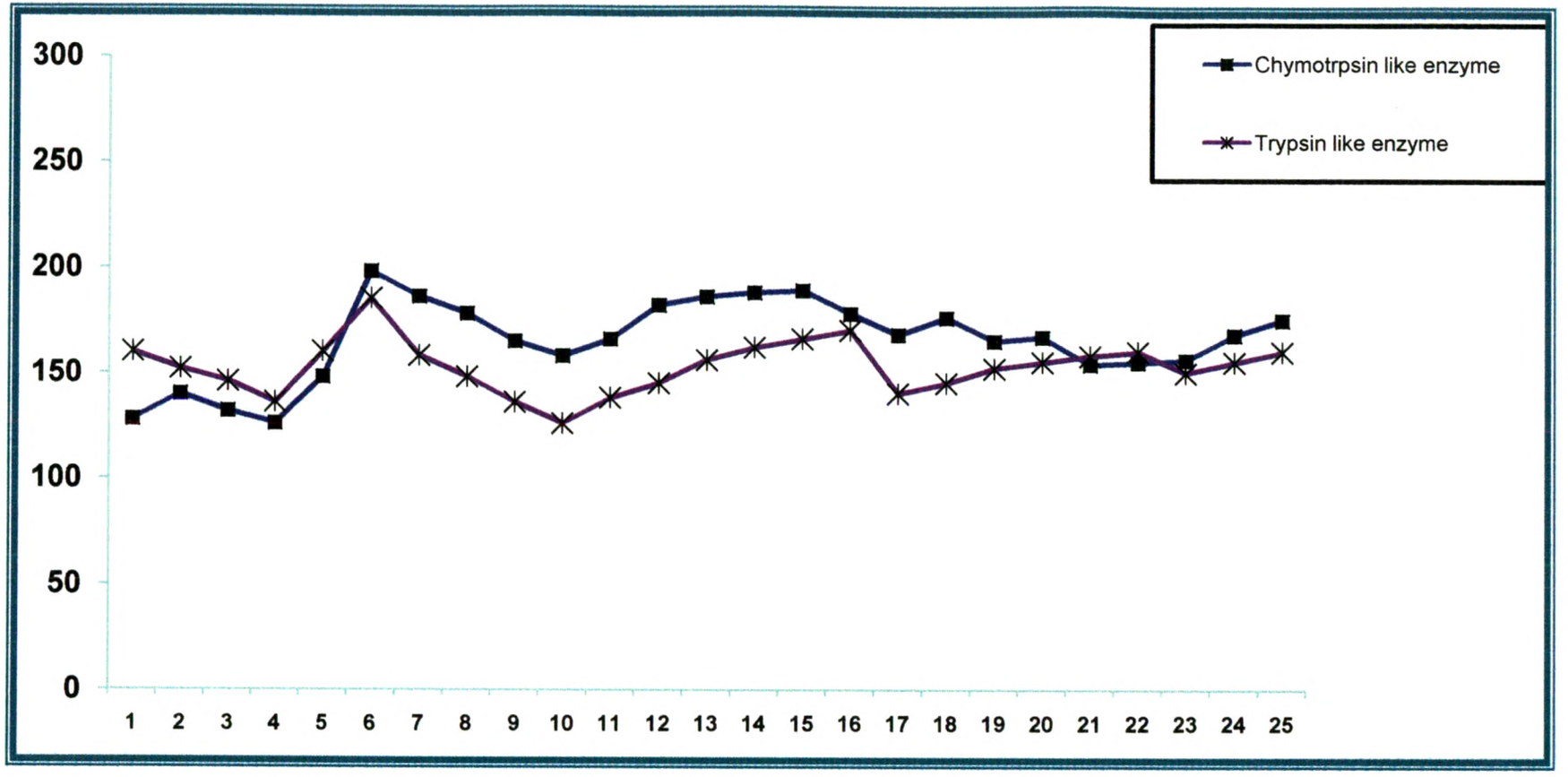
Acidic proteases activity during Larval growth of *Mythimna separata*

Fig. No. 6



Neutral proteases activity during Larval growth of *Mythimna separata*

Fig. No. 7



Alkaline proteases activity during Larval growth of *Mythimna separata*

Fig. No.8

Fig 1: Newly hatched larvae

Fig 2: First instar larvae during feeding

Fig 3: First instar larvae before and after moulting

Plate No .3: Early larval development stages of *M. separate*

Fig 1: Fourth instar larva

Fig 2: Fifth instar larva .

Fig 3: Fifth instar larva before moulting

Fig 4: Sixth instar larva

Plate No .4 : Later larval developmental stages

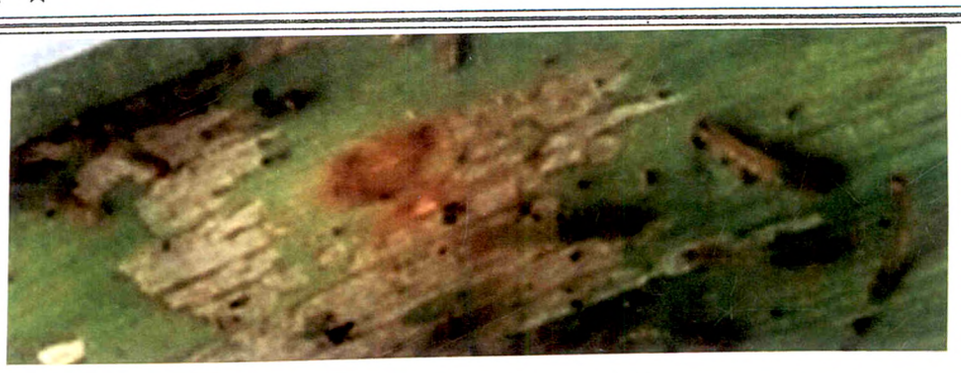


Fig 1: Feeding of first instar larvae(scar on leaf)

Fig 2: Feeding of second instar larvae

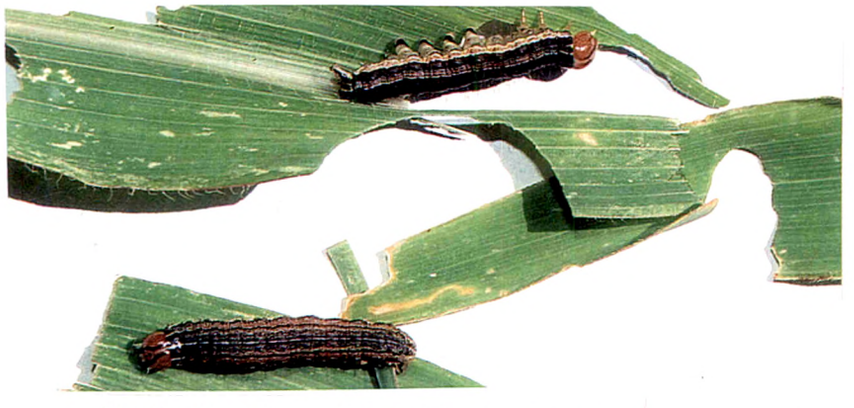


Fig 3: Feeding of third instar larvae

Fig 4: Infested maize plant

Plate No. 5: Feeding of larvae on maize leaves