CHAPTER - FIVE

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

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CHAPTER FIVE

I. INTRODUCTION :

1. Metamorphosis :

One of the most characteristic features of insects is the fact that they almost always hatch in a condition morphologically different from that of the imago. In order to reach the later instar they consequently have to pass through changes of form which are collectively termed metamorphosis; these are usually most pronounced towards the end of postembryonic development and they are accompanied by physiological and biochemical changes (Chen, 1971). Metamorphosis includes a series of ontogenetic events through which the insect transforms from the larva to the adult. The transformation process involves mainly the destruction of most larval tissues and organs (histolysis) and the differentiation of imaginal structures (histogenesis) (Chen, 1966).

Histolysis and Histogenesis :

Metamorphosis is a very complicated affair to deal with, not only because it represents in its most advanced state such a profound conversion of the living system, but also because there exists within the insect groups so many gradations in the transforming changes, when the larva develops into the imago. Specific larval organs representing adaptations to the larval feeding habits or other demands upon the environment always degenerate. The fat body, often the largest organ, by far in the larva, belongs to these specific larval organs. It represents a tissue for a storage of nutrients and it consistently histolysed during advancing metamorphosis and its contents are utilized for the imaginal growth (Agrell and Lundquist, 1973).

Besides the development of wings, compound eyes and of external and internal genitalia, one of the other main changes that occurs in metamorphosing insects may be in their feeding habits. Thus a caterpillar and a moth or butterfly, or a fly maggot and the adult fly, may have completely different habits and these are reflected in structural changes in the mouth parts, gut, salivary glands, and gut muscles. In a fly, the gut shortens, and the larval crop, larval caecae, and larval salivary glands all disappears. The very much shorter adult gut develops a crop and an elaborate rectum with characteristic rectal papillae. Changes in mouth parts accompanying changes in feeding habits, as can be seen in a chewing caterpillar and a sucking moth or butterfly. Also, changes in feeding habits and mouth parts are accompanied by changes in gut musculature.

In the pterygote or winged insect there are changes in the respiratory system of the terrestrial larval form during metamorphosis to the flying adult form. The elaborate adult thoracic musculature is heavily tracheated, often with an equally elaborate system of air sacs. A simple system of air sacs is also found in aquatic forms, where one function would be to provide buoyancy. With the acquisition of a flying habit the sensory environment is completely different so that larval sensillae are broken down and new adult ones appear. Thus, drastic internal changes are reflected in changes in the respiratory and nervous systems, and recent evidence shows equally elaborate change occurring in the haemocytes, associated with changes in other tissues. In fact there would appear, in the specialized endopterygote, to be no system that remains unaffected by the metamorphic changes occurring during the pupal stages (Whitten, 1968).

Insects that lack metamorphosis belong to ametabola. In these insects, the moulting may continue throughout their adult life. These insects on emergence from the eggs are essentially like the adults for all the matters except that they are smaller in size. They become adult undergoing a series of moults at certain intervals. After each moult they increase in size until the sexually mature stage is reached. This type of metamorphosis is found in wingless insects of the order Thysanura and Collembola, the former includes silver fish and fire brats and later the springtails.

Insects that undergo a gradual or incomplete metamorphosis belong to hemimetabola. It is also referred as simple metamorphosis. These insects on emergence from the egg shell do resemble the adults but do not possess the wings. They have the same habits, food, appendages and structure in general as the parents except some aquatic insects. The change that the insect undergoes on emergence from egg to adult is a relatively gradual change in external appearance in the moulting steps. The young of terrestrial forms are called nymphs. The functional wings develop in the final moult in those insects that possess wings. In nymphs wing pads can be seen even before the functional wings of adult are developed. The common examples of insects having incomplete metamorphosis are grass hopper, termites, stoneflies, true bugs etc.

The holometabolous insects include the Endopterygota and pass through an indirect or complete metamorphosis. The life-cycle incorporates a sequence of larval instars, successive larvae usually resembling each other closely but differing considerably from the adult. The last larval instar is followed by a more or less quiescent pupal instar, within whose cuticle most of the external an internal transformation to adult occurs (Richards and Davies, 1977).

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Pupal Development :

The term prepupa is used in more than one sense but it refers in most cases to the pharate pupa. Near the end of larval life the insect prepares for transformation into the pupa, usually constructing a cocoon, cell or other form of protection. Meanwhile the larval cuticle becomes separated from its epidermis and the developing pupa, with everted wings and appendages, lies within the unshed larval cuticle. The changes in form which accompany the development of the pupa often cause the deformation of this larval cuticle so that the prepupal phase differs somewhat in external appearance from the earlier larva. The body is commonly more depressed and the abdomen is relatively shorter. The insect at this time is usually quiescent and does not feed. The prepupal phase does not, however, constitute distinct instar and when the old larval skin is ultimately cast off it reveals the pupa (Richards and Davies, 1977).

The term pupa denotes the relatively inactive instar that intervenes between the larva and adult in Endopterygote insects, though it may be extended to include comparable stages in the life-cycle of aberrant holometabolous Exopterygotes like the Thysanoptera and male Coccoidea. During the pupal stage the insect is usually incapable of feeding and is quiescent. It is to be regarded as a transitional instar during which the larval body and its internal organs are remodelled to the extent necessary to produce the future imago though some of these processes begin in the larva, continue into the pharate adult and may not be complete until after the pupal-adult ecdysis. As often used, the term refers to the phase between the larval-pupal and pupal-adult ecdyses, but it must now be recognized that for a varying period before adult emergence the 'pupa' so defined is actually a pharate adult i.e. the more or less fully formed adult lies separate within the old pupal cuticle and the limited degree of locomotion which such 'pupae' undertake is usually the result of movements by the pharate adult (Richards and Davies, 1977).

The pupa in *M. separata* are obtect pupae in which the appendages of the pupa are firmly pressed against its body and are soldered down to it by a secretion produced at the last larval moult; the exposed surfaces of the appendages are more heavily cuticularized than those adjacent to the body. Such pupae are best known from all higher Lepidoptera and in some Coleoptera and Diptera (Richards and Davies, 1977).

2. <u>Metabolism during Metamorphosis</u> :

Insects grow in a series of moults in which there are alternate periods of growth and breakdown of the exoskeleton, and discontinuous growth of other tissues. Further specialization occurs in holometabolous insects where there is a major restructuring of nearly all tissues during metamorphosis. These events have considerable implications for the metabolism of tissues since there are extensive changes in degradative and synthetic pathways at different stages of development (Candy, 1985).

Investigations of the nitrogen metabolism indicate constancy in the amount of total nitrogen during the pupal life. The main excretory product during the pupal life is uric acid, which accumulates, especially during histolysis (Russo-Caia, 1960). The increase in structural protein seems related to the respiratory increase. An intense formation of muscle mitochondria was observed to occur in parallel as was also an incorporation of protein into the muscle mitochondria at the expense of soluble proteins (Lennie and Birt, 1967). Therefore, this later part of the metamorphosis should represent differentiation during which period the efficiency of the respiratory system increases (Agrell and Lundquist, 1973).

The electrophoretic pattern of pupal proteins in *Calliphora*, as well as the behaviour of different protein fractions, indicates a possible splitting and subsequent synthesis in time with histolysis – histogenesis. Considering the profound changes which the developing insect undergoes at the time of metamorphosis, only minor changes in the pool size of free amino acids during this period have been detected. In *Calliphora* slight reduction in the total concentration of these compounds occur at the initiation and during the later half of pupal life. It is thought that the first decline is related to the histogenesis of the hypodermal tissue, especially the formation of the imaginal buds, and that second one coincides with the differentiation of muscles in the thorax. The highest concentration is found at about the early middle of the pupal development at which stage the breakdown of larval tissues reaches maximum (Chen, 1966).

3. <u>Review of Literature on Pupal Proteins and Proteases</u>:

The culminating feature of metamorphosis is the formation of the imago, which differs between the hemi and holometabolous insects. In hemimetabolous forms it is accomplished through a gradual series of external and internal changes and alterations of form, which may be traced back to simple growth during the immature stages, though the changes at last moult, are generally greater than those at previous ones. In holometabolous insects the transformation from larva to the adult is accomplished largely during the quiescent pupal instars and a more detailed consideration of the complex changes which occur there, is required. The simplest transition is effected when the cells of a particular larval tissue are carried over to form the corresponding tissue of the adult, the process being accomplished with relatively minor alterations due to differentiation. In other cases the larval tissue is largely or completely destroyed, a process known as histolysis, and the adult tissue which replaces it is developed by histogenesis from certain masses of formative cells know variously as imaginal buds, imaginal disks or histoblasts.

Inspite of radical processes of histolysis and histogenesis, in the pupa there are only comparitively small changes in the amount of protein and low molecular weight nitrogen compounds irrespective of insect group (Pant and Lal, 1970). The increase in stuctural protein seems related to the respiratory increase. An intence formation of muscle mitichondria was observesd to occur in a parallel as was also an incarporation of protein into muscle mitocondria at the expence of soluble protins (Lennie and Birt, 1967). Williams and Birt (1972) studied the quantitative significance of protein synthesis during metamorphosis of the sheep blowfly, *Lucilia.* Houlihan and Neutan (1976) studied the muscle growth and protein synthesis in the puparia of *Calliphora vomitoria.* Haemolymph protein patterns during the spinning stage and metamorphosis of *Rhynchsciara americana* was studied by De- Bianchi and Terra (1976). Verma and Nath (1991) studied the changes in haemolymph protein picture during late larval and pharate pupal stages of *Spodoptera litura.*

Yano *et al.*(1995) worked on regulation of the expression of Cathepsin B in *Sarcophaga peregrina* (Flesh fly) at the translational level during metamorphosis. Pupal haemocytes participates in dissociation of fat body of *Sarcophaga* larvae at metamorphosis. In this study authors demonstrated that the translational level, that is larval haemocytes stored a sinificant amount of untransnslated Cathepsin B in mRNA. Yang and Davies (1968) studied Trypsin and Chymotrypsin during metamorphosis in *Aedes aegypti* and properties of Chymotrypsin. Proteases activity and cell death during metamorphosis in the salivary gland of the *Chironomous tentans* was studied by Henrikson and Clever (1972). The chestnut seed cystatin differentially effective against cysteine proteinases in closely related pests was studied by Pernas *et al.*(1998).

During transformation into the pupa and throughout the pupal and pharate adult instars insects are particularly vulnerable. Since at these periods they are provided with very limited powers of movement and defence, special methods of protection are necessary. Most pupae are concealed in one way or another from their enemies, and also from such adverse influences as excess of moisture, sudden marked variations of temperature, shock and other mechanical disturbances. Provision against such influences is usually made by the larva in its last instar. Many Lepidopteran and Coleopteran larvae burrow beneath the ground and there construct earthen cell in which to pupate. Most insects, however, construct cocoons which are special envelopes formed either of silk alone, or of extraneous material bound together by silken threads. In these instances the materials are held together by a warp of silk and worked up to form cocoons. Many other insects, including some Neuroptera and Trichoptera, many Lepidoptera and Hymenoptera and the Siphonaptera, use only silk in forming their cocoons (Richards and Davies, 1977).

II. MATERIAL AND METHODS :

1. Material :

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

A. Pupal Stages for Study :

The sixth instar larva generally enter into the fold of leaf, form a case with the help of its fross and pupate naked therein. Pupation usually occurs during night time. At the time of pupation the larva become restless when the skin ruptured from the body and larva transformed into pupa inside the pupal case. The pupa was coppery, obtect and showed blunt cephalic portion and tapering posterior end. The length of the pupa ranged from 14 to 18 mm with an average of 16.4 mm. The sexes were easily differentiated at the pupal stage itself based on the position of genital opening. In case of male the genital slit was situated on the ninth abdominal segment ventrally, while in case of female it was situated on the eighth abdominal segment. The female pupa was differentiated from the male by presence of characteristic elevated eighth sternal plate on which the genital slit is situated.

The metamorphosis period of *M. separata* is of 9 to 11 days with an average of 10 days. The pupal developmental stages from 1-day to 10-day pupae ($P_1 - P_{10}$) 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 in chapter II (Material and Methods)

III RESULTS :

1. Metamorphosis Period :

The metamorphosis period of *M. separata* is of 10 days.

2. Pupal Proteins :

The changes in proteins during metamorphosis of male and female pupae of *M. separata* are represented in Table No. 3 and illustrated graphically in fig. No. 9. The change in proteins in male and female pupae follow same pattern. Gradual decrease in the amount of proteins was observed from 1^{st} to 4^{th} -day pupae, increase in amount of proteins was observed in 4^{th} to 6^{th} -day pupae and then it remained relatively constant upto 10-day pupae. In the later stages of metamorphosis the amount of proteins in female pupae was more as compared to male pupae.

Table No. 3

Period of Metamorphosis (days)	Amount of Protins (Mg/gm of body wt.)	
	Male	Female
1	39	43
2	37	40
3	35	36
4	33	32
5	32	35
6	35	37
7	36	37.5
8	36.5	38
9	37.2	38.3
10	37.7	38.5

3. <u>Pupal Proteases</u>:

A. Male Pupal Proteases:

a. Acidic Protease :

Partial characterization of acidic proteases:

The partial characterization of acidic proteases during metamorphosis *of M. separata* revealed the maximum activity at pH 3.4 (Cathepsin D like enzyme) and pH 4.6 (Cathepsin B like enzyme), temperature 37^0C, 30 min. incubation time, 1% enzyme concentration and 2% substrate concentration.

Acidic protease activity:

i. Cathepsin D like enzyme:

Changes in cathepsin D like enzyme activity during metamorphosis of *M. separata* are illustrated graphically in fig 10.

During early period of metamorphosis the gradual increase in activity of enzyme was observed on 1^{st} to 6^{th} day in pupae while decreases in enzyme activity was observed in the pupae on 6^{th} to 8^{th} day. During later period of metamorphosis i.e. from 8^{th} to 10^{th} day the gradual increase in activity of enzyme was observed. Maxmium activity of enzyme was observed on 6^{th} day while minimum enzyme activity was observed on 1^{st} day of development in pupae.

ii. Cathepsin B like enzyme :

Changes in cathepsin B like enzyme activity during metamorphosis of *M. separata* are illustrated graphically in fig 10.

During early period of metamorphosis of pupae the gradual increase in enzyme activity was observed on 1^{st} to 6^{th} day and decrease in activity of enzyme was observed on 6^{th} to 8^{th} day of metamorphosis of pupae. During later period of metamorphosis activity gradual increase in enzyme activity was observed on 8^{th} to 10^{th} day. Maximum and minimum activity was observed respectively on 6^{th} day and 1^{st} day of metamorphosis in pupae.

b. Neutral protease :

Partial characterization of neutral protease ;

The partial characterization of neutral protease during metamorphosis of *M. Separata* reveled the maximum activity at pH 7, temperature 37^0C, 30 min. inclubation time, 1% enzyme concentration and 5% substrate concentration.

Neutral protease activity :

Changes in neutral protease activity during metamorphosis of *M. separata* are illustrated graphically in Fig 11.

During early period of metamorphosis i.e. 1^{st} to 5^{th} day the gradual increase in enzyme activity was observed and decrease in activity was observed on 5^{th} to 8^{th} day of metamorphosis in pupae. During later period of meamorphosis gradual increase in enzyme activity was observed on 8^{th} to 9^{th} day of pupae Maximum and minimum activity was observed respectively on 5^{th} day and 1^{st} day of metamorphosis in pupae.

c. Alkaline Protease :

Partial characterization of alkaline protease :

The partial characterization of neutral protease during metamorphosis of *M. separata* reveled the maximum activity at pH 7.7 (Chymotrypsin like enzyme) and pH 8.4 (Trypsin like enzyme), temperature 37^0C, 30 min. incubation time, 1% enzyme concentration and 2% substrate concentration.

Alkaline protease activity :

i. Chymotrypsin like enzyme :

Changes in Chymotrypsin like enzyme during metamorphosis of *M. separata* are illustrated graphically in Fig 12.

During early period of metamorphosis of pupe the gradual increase in enzyme activity was observed 1^{st} to 5^{th} -day pupae and decrease in enzyme activity was observed on 5^{th} to 8^{th} -day of metamorphosis in pupe. During later period of metamorphosis of pupe gradual increase in enzyme activity was observed 8^{th} to 10^{th} -day. Maximum and minimum activity of metamorphosis of pupe was observed on 5^{th} and 1^{st} day pupae respectively.

ii. Trypsin like enzyme :

Changes in trypsin like enzyme during metamorphosis of *M. separata* are illustrated graphically in Fig 12.

During early period of metamorphosis the gradual increase in enzyme activity was observed on 1^{st} to 5^{th} -day pupae and decrease in enzyme activity was observed on 5^{th} to 8^{th} -day of metamorphosis in pupe. During later period of metamorphosis gradual increase in enzyme activity was observed 8^{th} to 10^{th} –day pupae. Maximum and minimum enzyme activity was observed respectively on 5^{th} and 1^{st} day of metamorphosis in pupe.

B. Female pupal Protease:

a. Acidic Protease :

Partial characterization of acidic proteases:

The partial characterization of acidic proteases during female pupal development of *M. separata* revealed the maximum activity at pH 3.4 (Cathepsin D like enzyme) and pH 4.6 (Cathepsin B like enzyme), temperature 37^0C, 30 min. incubation time, 1% enzyme concentration and 2% substrate concentration.

Acidic protease activity:

i. Cathepsin D like enzyme:

Changes in Cathepsin D like enzyme activity during metamorphosis of *M. separata* are illustrated graphically in fig 13.

During early period of metamorphosis of pupae the gradual increase in enzyme activity was observed 1^{st} to 6^{th} day pupae and decrease in enzyme activity was on 6^{th} to 8^{th} day of metamorphosis in pupae. During later period gradual increase in enzyme activity was observed 8^{th} to 10^{th} day in pupe. Maxmimum and minimum enzyme activity was observed respectively on 6^{th} and 1^{st} day of metamorphosis in pupae.

ii. Cathepsin B like enzyme :

Changes in Cathepsin B like enzyme activity during metamorphosis of *M. separata* are illustrated graphically in fig 13.

During early period of metamorphosis the gradual increase in enzyme activity from 1 to 6-day pupae and decrease from 6 to 8-day pupae was observed. During later period of metamorphosis gradual increase in enzyme activity from 8 to 10-day pupae was observed. Maximum activity was observed in 6-day pupae and minimum in 1-day pupae.

b. Neutral protease :

Partial characterization of neutral protease ;

The partial characterization of neutral protease during metamorphosis of *M. Separata* reveled the maximum activity at pH 7, temperature 37^0C, 30 min. inclubation time, 1% enzyme concentration and 5% substrate concentration.

Neutral protease activity :

Changes in neutral protease activity during metamorphosis of *M. separata* are illustrated graphically in Fig 14.

During early period of metamorphosis of pupae the gradual increase in enzyme activity was observed 1^{st} to 5^{th} day pupae and decrease in enzyme activity was observed on 5^{th} to 8^{th} day of metamorphosis in pupae. During later period gradual increase in enzyme activity was observed 8^{th} to 10^{th} day in pupė. Maxmimum and minimum activity was observed respectively on 5^{th} and 1^{st} day of metamorphosis in pupae.

c. Alkaline Protease :

Partial characterization of alkaline protease :

The partial characterization of neutral protease during metamorphosis of *M. separata* reveled the maximum activity at pH 7.7 (Chymotrypsin like enzyme) and pH 8.4 (Trypsin like enzyme), temperature 37^0C, 30 min. incubation time, 1% enzyme concentration and 2% substrate concentration.

Alkaline protease activity :

i) Chymotrypsin like enzyme :

Changes in Chymotrypsin like enzyme during metamorphosis of *M. separata* are illustrated graphically in Fig 15.

During early period of metamorphosis of pupae the gradual increase in enzyme activity was observed 1^{st} to 5^{th} day pupae and decrease in enzyme activity was on 5^{th} to 8^{th} day of metamorphosis in pupae. During later period gradual increase in enzyme activity was observed 8^{th} to 10^{th} day in pupe. Maxmimum and minimum activity was observed respectively on 5^{th} and 1^{st} day of metamorphosis of pupae.

ii. Trypsin like enzyme :

Changes in trypsin like enzyme during metamorphosis of *M. separata* are illustrated graphically in Fig 15.

During early period of metamorphosis of pupae the gradual increase in enzyme activity was observed on 1^{st} to 5^{th} day pupae and decrease in enzyme activity was observed on 5^{th} to 8^{th} day of metamorphosis in pupae. During later period gradual increase in enzyme activity was observed 8^{th} to 10^{th} day in pupe. Maxmimum and minimum activity was observed respectively on 5^{th} and 1^{st} day of metamorphosis in pupae.

IV. DISCUSSION :

1. <u>Pupal Proteins</u> :

In spite of the radical processes of histolysis and histogenesis in pupa there are only comparatively small changes in the total amount of proteins (Agrell and Lundquist, 1973). In *Calliphora* the highest concentration of free amino acids is found at about early middle of the pupal development at which stage the breakdown of larval tissues reaches maximum (Chen, 1966). The developing pupa represents a closed system and thus its concentration levels of free amino acids could be considered as indicating the balance between histolysis and histogenesis. In general however, the fluctuation is too small to account for such drastic morphological changes, unless it is assumed that the amino acids produced by histolysis are immediately used for synthetic purpose (Chen, 1966).

In the present work there was gradual decrease in the amount of proteins from 1 to 4-day pupae indicates the histolysis of most of the larval organs. Gradual increase in proteins from 4 to 6-day pupae indicates gradual increase in protein synthesis and utilization of such new proteins as structural components for development of imaginal organs of adult. After 6-day the amount of proteins remains constant indicates completion of protein synthesis and gradual histogenesis. In later stages of metamorphosis the amount of proteins in female pupae was more as compared to male, indicates storage of proteins (vitellogenin) in the fat body which were utilized during oogenesis. Our results are in good agreement with Chen (1966) and Agrell and Lundquist (1973).

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

The developing pupa represents a closed system and its concentration levels of free amino acids could be considred as indicating the balance between histolysis and histogenesis. In ganeral however the fluctuation is too small to account for such drastic morphological changes unless it is assumed that the amino acids are produced by histolysis are immediately used for synthetic purpose (Skinner, 1960).

Trypsin and Chymotrypsin occurred in the midgut of all stages of *Aedes agypti.* The low activity of Trypsin and Chymotrypsin between larval, pupal and pupal adult ecdyses presumably relates to the lack of feeding during this period (Yang and Davies, 1971).

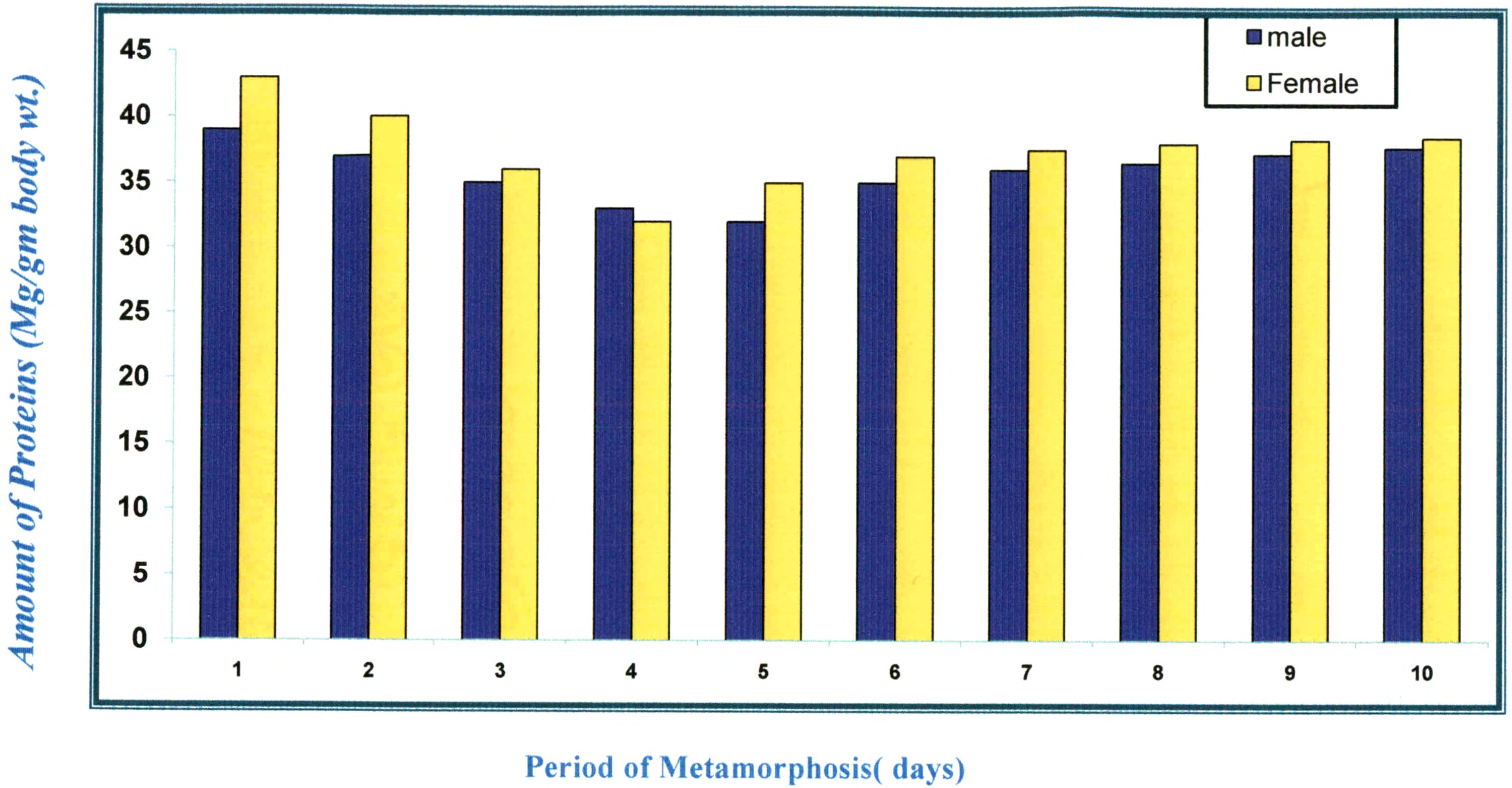
During pupal stage of insect processes like destruction of certain larval tissues and reconstruction of various tisues into adult takes place.

Since proteins are the first biological factors making their manifestation during development (Schmidt and Schwakl, 1975).

In the present work gradual increase in protease activity during early metamorphosis from 1 to 6-day pupae of *M. separata* suggests histolysis in which active role of these enzymes in degradation of proteins.

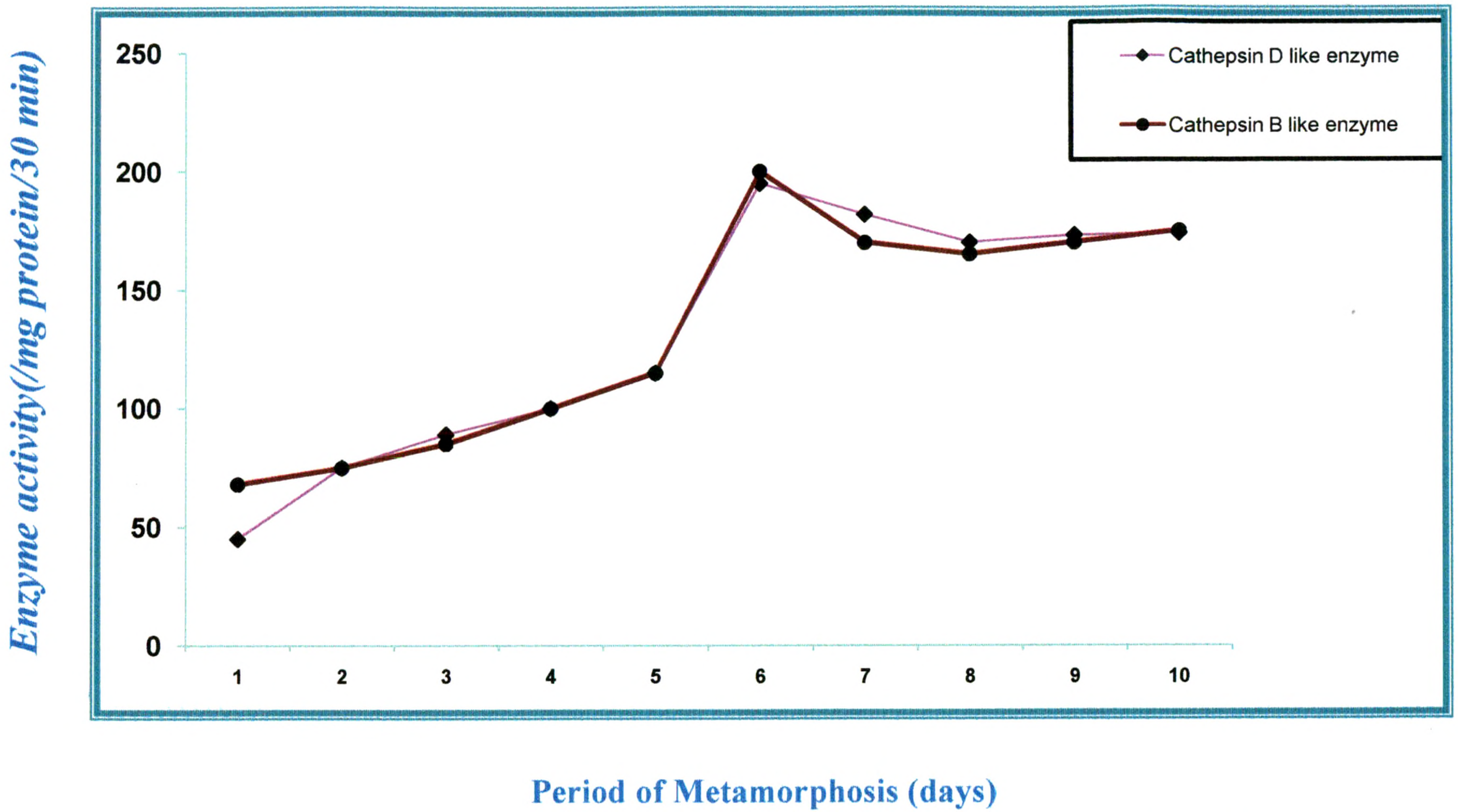
Our results are in good agreement with the findings of Skinner (1960) and Schmidt and Schwakl (1975).

Gradual decrease in protease activity during middle period of metamorphosis from 6 to 8-day pupae indicates histogenesis in which synthesis of protein occurs. Gradual increase in enzyme activity from 8 to 10-day pupae suggests degradation of some proteins for the synthesis of secretary proteins in male and yolk proteins in female adult.



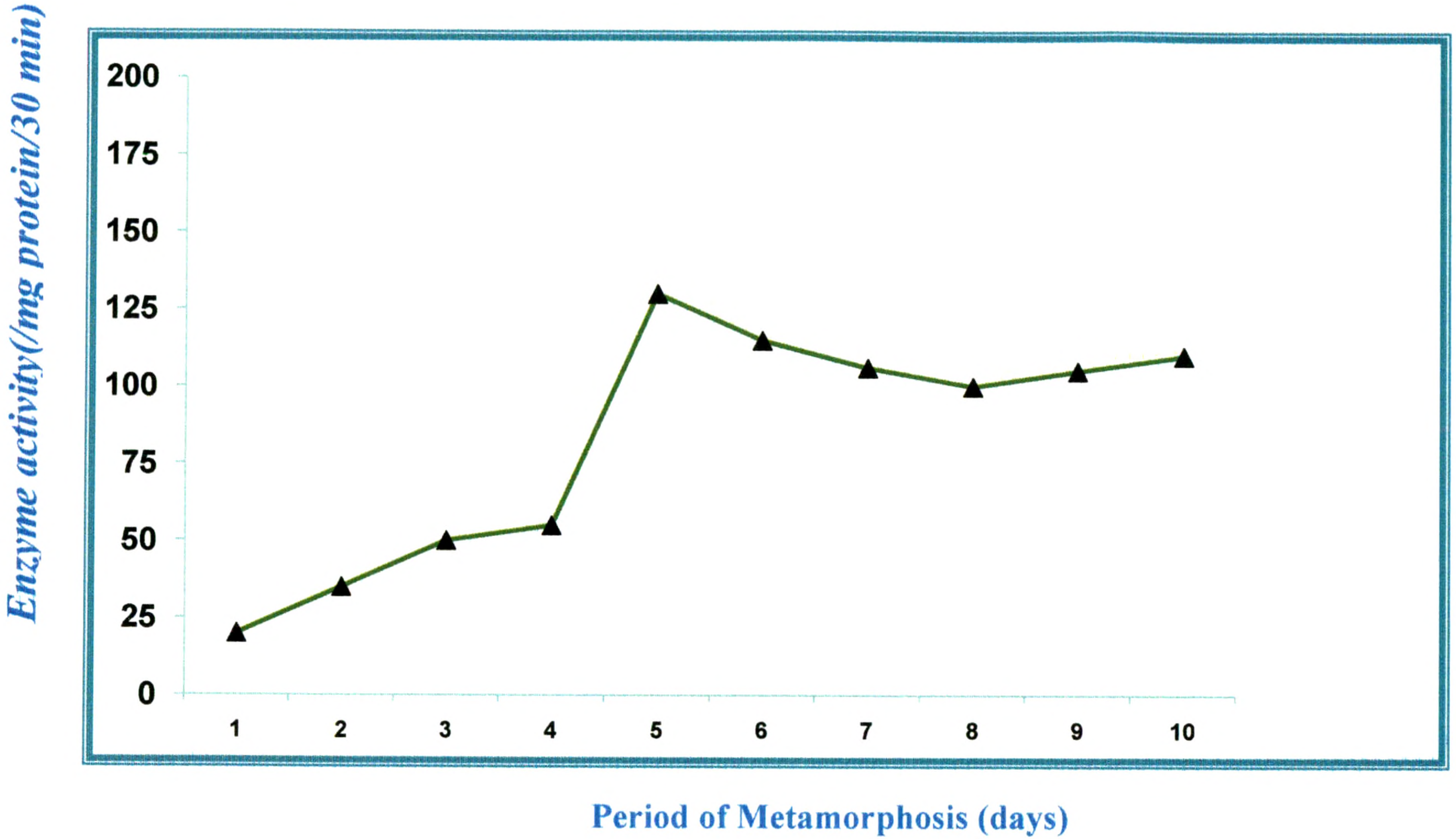
Changes in protein during metamorphosis of *Mythimna separata*

Fig. No.9



Acidic proteases activity during male metamorphosis of *Mythimna separata*

Fig. No. 10



Neutral proteases activity during male metamorphosis of *Mythimna separata*

Fig. No. 11

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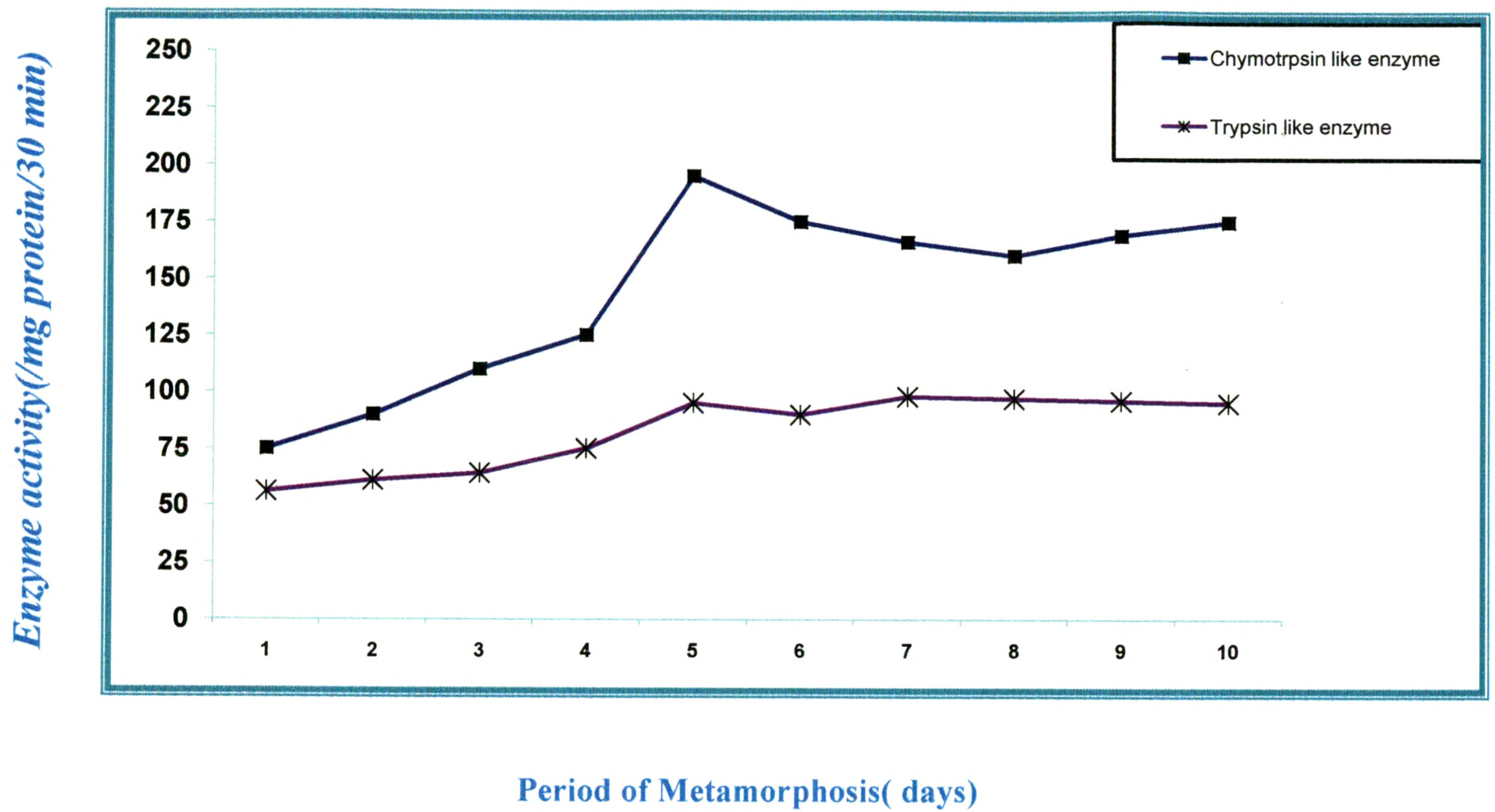
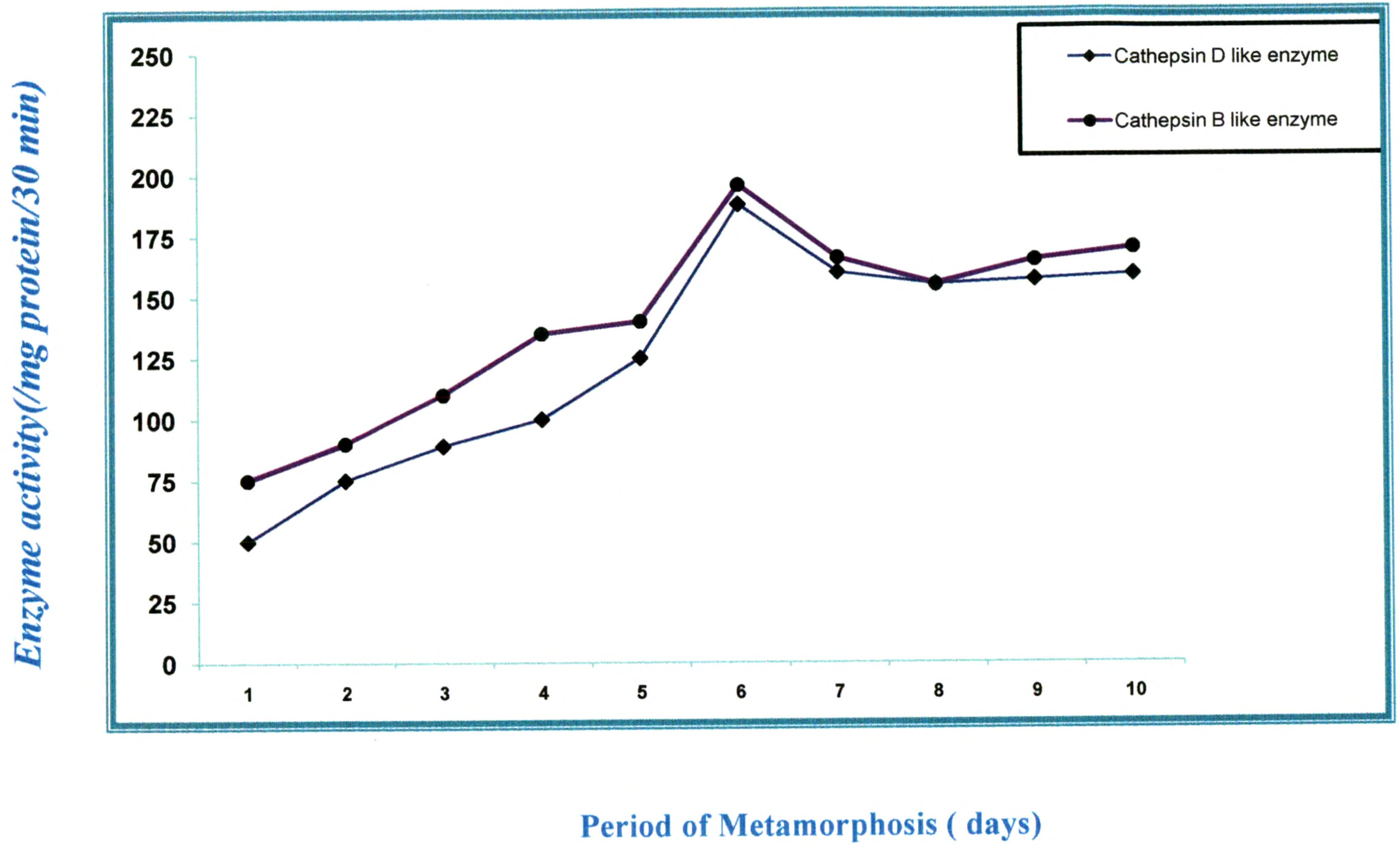


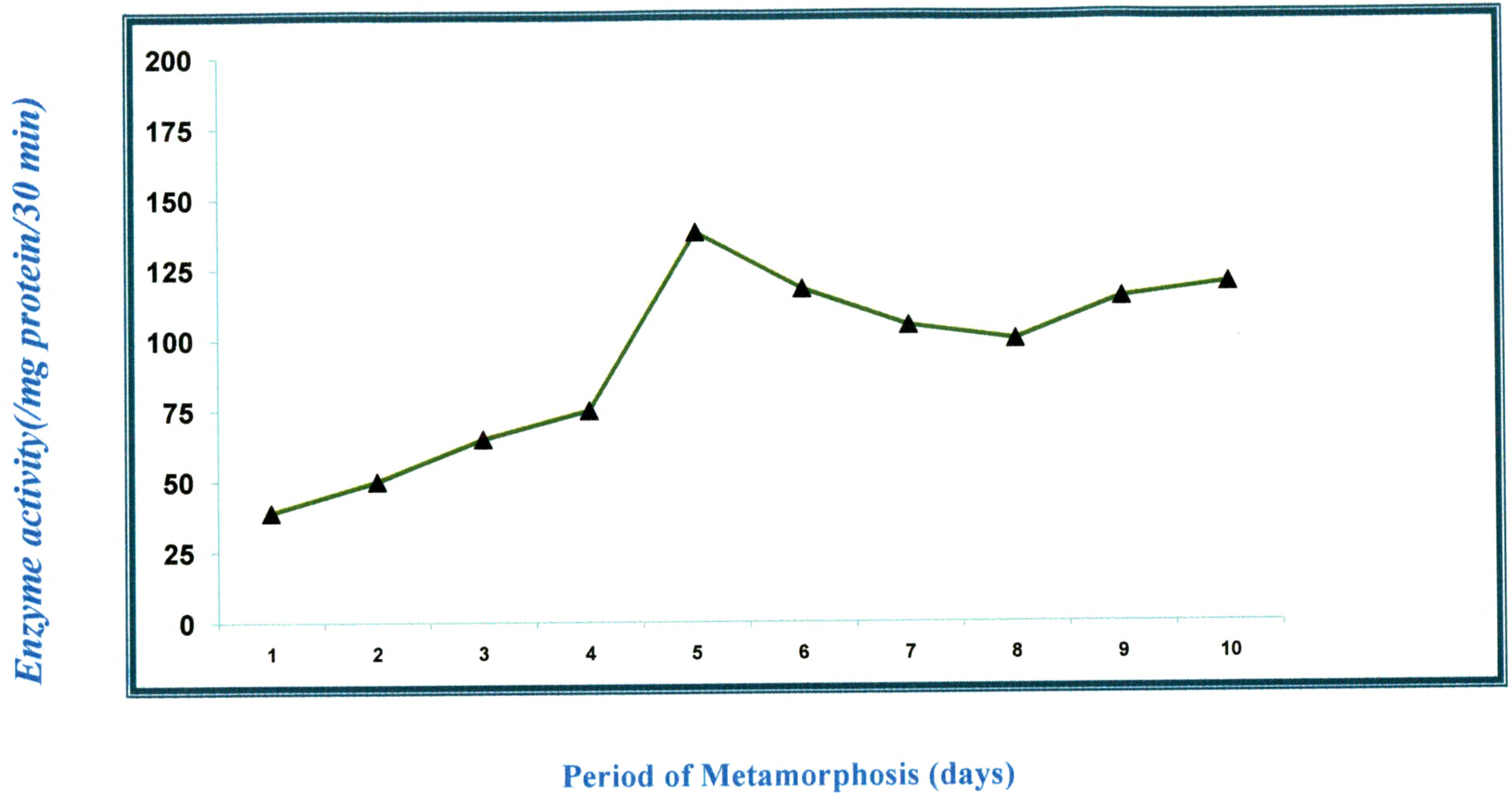
Alkaline proteases activity during male metamorphosis of *Mythimna separata*

Fig. No. 12



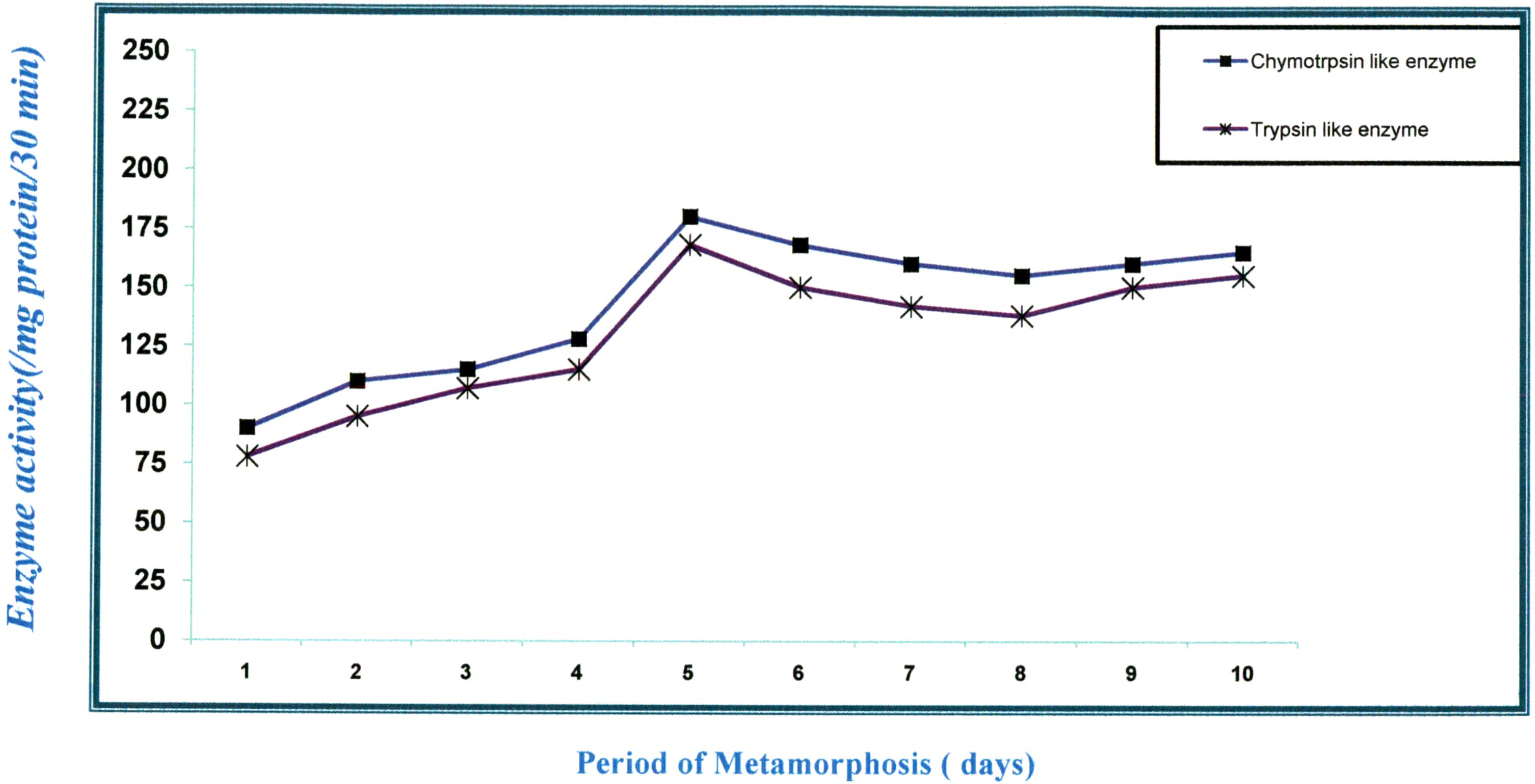
Acidic proteases activity during female metamorphosis of *Mythimna separata*

Fig. No. 13



Neutral proteases activity during female metamorphosis of *Mythimna separata*

Fig. No. 14



Alkaline proteases activity during female metamorphosis of *Mythimna separata*

Fig. No. 15

Fig 1:1-day pupae (male and female)

Fig 2: 6-day pupae (male and female)

Fig 3: 9-day pupae (female)

Plate No. 6: Pupae of *M.separata*