Proc. Indian Acad. Sci. (Anim. Sci.), Vol. 94, No. 5, October 1985, pp. 567–572.

Alkaline protease in the midgut of the silkworm *Bombyx mori* L: changes during metamorphosis

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MS received 13 February 1985; revised 22 April 1985

Abstract. The protease activity in the midgut of bivoltine and multivoltine races of the silkworm, *Bombyx mori*, was studied. The enzyme activity increased during fifth instar, reached a peak, and decreased significantly through pre-pupal stage to the lowest level at the pupal stage. During pharate adult period, the protease activity increased to reach another peak just before emergence of the moth, and decreased thereafter. The enzyme activity in bivoltines was about 2–3 times higher than multivoltines at the peak levels. Bivoltines showed a sex difference in midgut protease activity while no significant difference was observed in multivoltines. Larvae and pharate adults showed a difference in the pH optima for the enzyme activity. From the results the possible role of midgut protease during the process of metamorphosis is discussed.

Keywords. *Bombyx mori*; bivoltine; multivoltine; midgut protease; metamorphosis.

1. Introduction

The most common proteolytic enzymes in the alimentary canal of insects have long been recognised as trypsin-like enzymes (Day and Waterhouse 1953; House 1974; Law *et al* 1977; Jany *et al* 1978). Eguchi and Iwamoto (1982) have also established that midgut protease, the activity of which has been earlier demonstrated in the alimentary canal of the larva of the silkworm, *Bombyx mori* (Horie *et al* 1963; Eguchi and Yoshitake 1967; Hamano and Mukaiyama 1970; Eguchi and Iwamoto 1976) is a trypsin-like enzyme possessing certain properties (Eguchi and Arai 1983). However, most of the studies on the midgut protease of silkworms are restricted to the larval stage mainly because of its important role in protein digestion. Eventhough Eguchi *et al* (1972) have studied the proteolytic enzymes in pharate adults, very little information is available on the pattern of change in total protease activity during larval-pupal-adult transformations in silkworms. It has already been reported that the trypsin-like enzyme in mosquitoes changes significantly during metamorphosis (Chen 1978). The present communication deals with the pattern of change in total protease activity of the midgut, a comparison of the enzyme activity in different races and sexes and the pH optima for midgut protease at different stages of metamorphosis.

2. Materials and methods

2.1 *Animals*

Bivoltine (NB_{18} and NB_4D_2) and multivoltine (Pure Mysore) silkworm races were maintained under standard laboratory conditions at 25–28°C and a relative humidity

of 70–90% on mulberry leaves (M_5 variety). Silkworms were used from fifth larval instar upto the adult stage for the present experiment. The midgut was excised along with its contents after freezing the animals at −20°C for about 12 hr, to avoid any loss of protease activity as most of it is detected in the digestive juice. A 10% (W/V) homogenate of the tissue was prepared in ice-cold borate buffer, pH 9·0 and pH 11·0 for adults and larvae respectively, with glass homogenizer. The homogenate was centrifuged in an IEC refrigerated centrifuge at 3000 RPM for 15 minutes and the supernatant was used as the enzyme source. Four to five individuals were taken at each time point for independent determination and only the mean value of four independent determinations is presented in the graph. Further, student's *t* test was conducted to assess the level of significance.

2.2 *Enzyme assay*

Protease activity was assayed according to Eguchi and Iwamoto (1982) with slight modifications. The pH optima were 11 and 9 respectively for the larval and pharate adult stages. The time of incubation was 30 min for enzyme from the larva and 60 min for enzyme from the pharate adult. Half ml of 1% casein and 2 ml of 0·1 M borate buffer were incubated with 0·5 ml of appropriately diluted enzyme solution at 30°C. The reaction was stopped by adding 2 ml of 10% TCA and centrifuged. The concentration of digested protein in the supernatant was determined colorimetrically with Beckman DU2 spectrophotometer using Folin's reagent at 660 nm. Protein concentration was determined according to the method of Lowry *et al* (1951). The specific activity of the enzyme was expressed as μmol of tyrosine formed per minute per mg of protein.

3. Results

In bivoltines, males and females showed a significant difference ($P = < 0{\cdot}001$) in midgut protease activity during development except the pupal stage (figure 1). In males, the peak enzyme activity was observed around the third day which significantly decreased ($P = < 0{\cdot}001$) thereafter during the fifth instar development. While in females, the enzyme activity increased significantly ($P = < 0{\cdot}001$) to reach a peak around the sixth day and decreased thereafter. In general, midgut protease activity was high in females compared to males during the larval stage. In both males and females, there was about 50–70 fold decrease in total protease activity from larval to pre-pupal stage which decreased further to attain the lowest level during pupal stage. During pharate adult stage, protease activity increased gradually to attain another peak just before emergence of the moth and decreased thereafter during adult period. The enzyme activity in males during pharate adult stage was about 2 times higher than that of females.

In Mysore race, there was no significant difference in midgut protease activity between male and female throughout the development except the pharate adult stage ($P = < 0{\cdot}025$) (figure 2). Peak activity of the enzyme was observed around the sixth day of fifth instar development in both the sexes. There was about 75–90 fold decrease in total protease activity from larval to pre-pupal stage which reached its minimum value

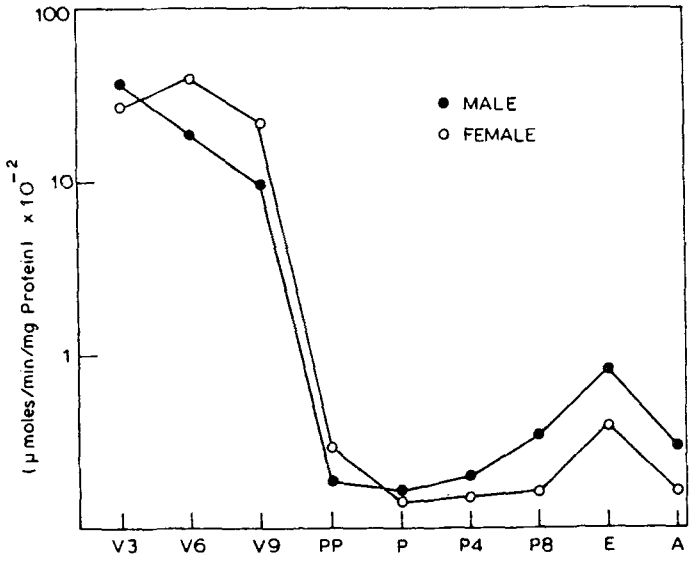

Figure 1. Changes in midgut protease activity during development of bivoltines (V) fifth instar in days (PP) pre-pupa (P) pupa in days (E) before emergence (A) adult.

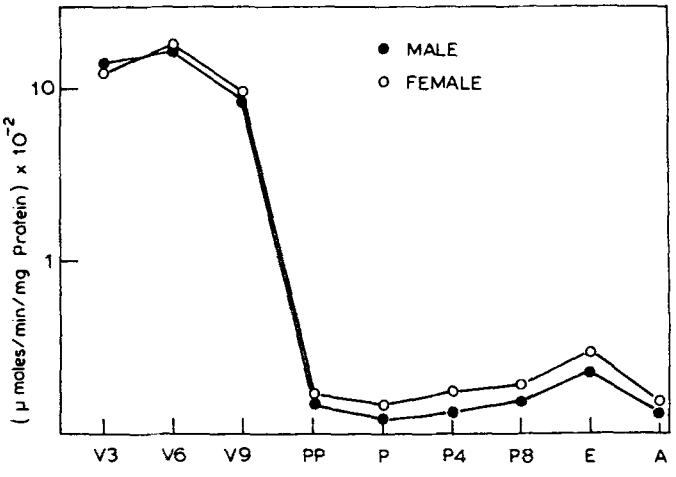

Figure 2. Changes in midgut protease activity of pure Mysore during development (V) fifth instar in days (PP) pre-pupa (P) pupa in days (E) before emergence (A) adult.

during the pupal stage in both males and females. During pharate adult stage, the enzyme activity increased slowly to reach another peak just before emergence and decreased rapidly ($P = < 0{\cdot}001$) in both the sexes during adult stage.

Figure 3 shows the effect of pH on the total protease activity from larval and pharate adult stages of both bivoltines and multivoltine (Pure Mysore). During larval stage, only one peak was observed at pH 11·0 in both the races and the enzyme activity in

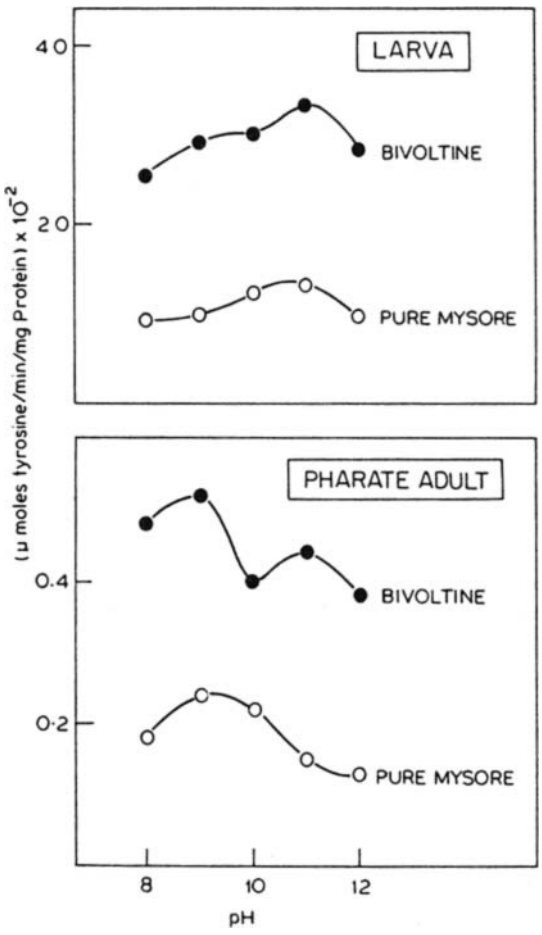

Figure 3. Effect of pH on the alkaline protease of the midgut.

bivoltines was about 2·5 times higher than that of multivoltine. During pharate adult stage, the bivoltines interestingly showed two peaks of enzyme activity at pH 9 and pH 11. But in case of pure Mysore only one peak was observed at pH 9. The enzyme activity in bivoltines was about 2·5 times higher than multivoltines during pharate adult stage.

4. Discussion

Midgut enzymes in insects are synthesized in the midgut epithelium and secreted into the gut lumen (Engelmann 1969; Engelmann and Geraerts 1980; Eguchi and Arai 1983). Thus changes in total protease activity of the midgut reflect changes in the overall production of the enzyme during metamorphosis. A significantly high activity of midgut protease during fifth instar larval development of silkworms shows a higher rate of enzyme synthesis corresponding to enhanced food intake (Waldbauer 1968). This might facilitate a greater utilization of proteins for larval growth and silk

production as well. Higher protease activity in bivoltines might result in better conversion of exogenous proteins which ultimately lead to production of more silk compared to multivoltines (Tanaka 1964). The sharp decrease in midgut protease activity from larval to pupal stage shows the difference between active feeding and non-feeding stages during metamorphosis. Thus it can be presumed that the rate of synthesis of midgut protease is dependent upon the rate of food intake. Furthermore, traces of protease activity during pupal stage might be the residual larval enzymes. In pharate adults, protease activity increases and reaches another peak just before emergence. This is in accordance with earlier reports (Eguchi *et al* 1972) and supports their view that midgut protease in pharate adult might contribute either in part or full towards the formation of cocoonase.

The present study shows that the optimum pH for total protease activity of the midgut is around pH 11·0 in the larval stage. Eguchi and Iwamoto (1976) have shown that the pH optima for protease from midgut tissue and digestive fluid are the same. Interestingly a shift in pH optima for protease activity is observed during metamorphosis from pH 11·0 at larval stage to pH 9·0 at pharate adult stage. It cannot be confirmed at present whether the larval protease is different from adult protease or whether they are two isoenzymes of protease active at different stages of metamorphosis. The presence of two peaks, each at pH 9·0 and pH 11·0, in midgut protease activity of bivoltine pharate adult, likewise, again provides a basis to speculate the existence of two isoenzymes of protease having different pH optima characteristics. However, the single peak at pH 9·0, in the enzyme activity of multivoltine pharate adult requires further studies to draw any conclusion.

Acknowledgement

Grateful acknowledgements are due to the World Bank for financial assistance and to the co-ordinator, R & D Programme in Sericulture, Bangalore University for facilities.

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