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A STUDY ON ELECTROPHORESIS ANALYSIS OF ALPHA ESTERASE ISOZYMES DURING DIFFERENT DEVELOPMENTAL STAGES OF KALIMPONG-A (KA), NEW BIVOLTINE-18 (NB_{18}), AND PURE MYSORE (PM) LINES OF BOMBYX MORI L

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ABSTRACT

A science concerned with establishing durable classification has itself undergone tremendous changes in the last three decades. Traditional approach still is the basis of all taxonomical studies. The molecular data, in particular gel electrophoresis of enzymes and numerical methods of analysis have proven useful in many groups of insects and will see much wider use in future. Therefore, present study was designed with the main purpose to analyze the activities of alpha esterase isozymes by electrophoresis method during different developmental stages of new breeding lines viz. Kalimpong-A (KA), NB_{18}, and Pure Mysore (PM) of Bombyx mori L.standardized disc electrophoresis method was performed. Esterase isozymes form distinct enzymes zones in the photographs and in the zymogram and these have been numbered in cathodal to anodal sequence. These isozyme patterns have been established after repeated runs. The total isozymes of different developmental stages of KA, NB_{18}, and PM have been grouped into different zones. The nomenclature of enzyme banding pattern has been followed. Results demonstrated that total number of 21 esterase bands were found in pure races and the isolated lines. High esterase activity was noticed in pure races than the isolated lines. The esterase activity was high in pupal stage followed by larval stage. Specific bands responsible for molecular differentiation for sexual dimorphism as well bands characteristic of bivoltine and multivoltine races have been identified.

KEYWORDS: *Bombyx Mori L, Alpha Esterase, Electrophoresis, Pupa, Larva*

Article History

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INTRODUCTION

A study on the taxonomy of closely related species is important though difficult. Earlier studies on the entomological taxonomy were based on morphologically defined species, the degree of morphological difference being the essential criterion. This topological approach still dominates date to day practices of most systematics. However, this concept has failed to deal adequately with sibling or cryptic species (Davidson et al., 1967). In such cases the use of biochemical study has been considered of some use in the separation of closely related species of insects.

Reliable methods for distinguishing members of the insect complex by chromatographic studies of pteridine species have been attempted which help to identify the members of the complex (Davidson et al., 1967, Micks et al., 1966 a, b Micks 1968). Immuno diffusing techniques have been used to separate insect species in particular mosquitoes (Cupp

and Ibrahim, 1973, Cupp et al., 1970). Schumann (1973) analysed through geldiffusion techniques different strains of mosquitoes of different origin and identified them.

A science concerned with establishing durable classification has itself undergone tremendous changes in the last three decades. Traditional approach still is the basis of all taxonomical studies. The molecular data, in particular gel electrophoresis of enzymes (Yashitake 1963 1968, Eguchi 1965, 1968 Kai and Nishi 1976 Takeda et al., 1992), and numerical methods of analysis have proven useful in many groups of insects and will see much wider use in future (Berlacher 1984). With this background, we designed the present study with the main aim to analyze the activities of alpha esterase isozymes by electrophoresis method during different developmental stages of new breeding lines KA, NB_{18}, and PM of *Bombyx mori* L.

MATERIALS AND METHODS

Silkworm Varieties and Rearing

The pure races of bivoltine Kalimpong-A (KA) spinning oval white cocoons, New Bivoltine-18 (NB_{18}) spinning dumbbell white cocoons and multivoltine Pure Mysore (PM) spinning pointed yellow cocoons of mulberry silkworm Bombyx *mori* L. were selected for the present breeding programme. These races were obtained from their respective seed areas and are reared in cytogenetics laboratory, Jnana Bharathi, Bangalore University.

The disease free layings were prepared as described by Krishnaswamy, and were incubated at 25°C and relative humidity of 60-70 %. On 8th day composite layings were prepared (10-20 layings were prepared 100-200 eggs were collected from each laying). The hatched worms were reared according to the method described by Krishnaswamy (Krishmaswamy, 1978). MS variety of mulberry leaves was used in rearing. The worms were reared in mass upto III instar, after III moult 300 worms were collected in three replicates in order to evaluate the rearing performance. Standard temperature and humidity were maintained in the rearing house.

Breeding

Single and three way crosses were made by using the above said three races. The first single cross involved KA females and PM males. The second single cross involved NB_{18} females and PM males. During the course of breeding selection was made at the egg, larva, pupa and cocoon stages to fix the desirable traits. F_5 progenies of the respective crosses were back crossed to their respective bivoltine males to improve commercial characters.

Preparation of Enzyme Extract

The different developmental stages such as 1st day, 5^{th} day and 9th day eggs, five larval instars (I, II, III, IV, and V instars), early, middle and late stages of male and female pupae, male moths before and after copulation. Female moths before and after egg laying of bivoltine races KA, NB_{18}, and multivoltine race PM were selected.

Electrophoresis

Disc electrophoresis was performed essentially according to Davis (1964) and Ornstein (1964). A discontinuous gel system consisting of 7.5% lower gel and 3.12 5% spacer gel was used. The lower gel consisted of one part of Tris-hydrochloric acid buffer (36.g Tris+ 48.0 ml of N HCl + 0.46 ml of TEMED, diluted to 100ml. pH 8.9), two parts of cyanogum 41 (3.08 g of cyanogum in 10ml of water), two parts of Ammonium persulphate (140mg of APS in 100ml of water) and three parts of distilled water. 1.2 ml of this solution was poured into clean, dry glass tubes (7 cm x 0.7 cmdia) held vertically. The

solution was carefully overlayered with distilled water and allowedto photopolymerise for 15 minutes under fluorescent lamp or day light. After polymerisation, the water layer was removed from the top and the spacer gel was added. The spacer gel consisted of 1 part of Tris phophoretic acid buffer (5.7 gtris + 25.6 ml of 1M H3Po4 + 0.46 ml of TEMED diluted to 100ml with distilled water pH 6.9) 2 parts of cyanogen 41 (1.25 g cyanogum 41 in 10ml of Distilled water) 1 part of APS (70 mg in 100 ml) and four parts of water. 0.2 ml of spacer gel was poured on the top of the lower gel each tube layered with a drop of water and allowed to Photopolymerise for 15 minutes. After polymerization the water was blotted off and the tubes with spacer gel were inserted into the rubber connectors of the upper electrode vessel. The electrode chambers were filled with electrode buffer (0.3 M boric acid and sodium hydroxide buffer pH 8.65). The sample, suitably diluted with 20% sucrose containing bromophenol blue, was carefully layered on to each gel and subjected to electrophoresis in cold (4°C) imposing a current of 2mA per tube for 2 hours.

Staining Procedure

The staining techniques of Ayala et al. (1972) was followed with a slight modification. The stain used for esterases constituted 25 mg of alpha naphthyl acetate dissolved in 2 of 1:1 acetone water and the same was added to 12.5 ml of 0.1 M phosphate buffer pH 5.9 to which 25 mg of Fast blue RR salt and 12.5 ml of 0.1 M phosphate buffer pH 6.5 were added. For beta-esterase the same incubating medium was used except for the substrate where beta-naphthyl acetate was substituted in place of alhpa salt. The gels were incubated in the stain for 30 minutes until the bands appeared. The gels were then stored in 6% acetic acid.Esterase isozymes form distinct enzymes zones in the photographs and in the zymogram and these have been numbered in cathodal to anodal sequence. These isozyme patterns have been established after repeated runs. The total isozymes of different developmental stages of KA, NB_{18}, PM, R_1 and R_2 have been grouped into different zones. The nomenclature of enzyme banding pattern has been followed after Ayala et al. (1972). The relative front (Rf) of the esterase and phosphatase bands of all the developmental stages with reference to known indicator dye was calculated as follows:

$$\text{Rf} = \frac{\text{(Length gel before staining/ Length gel after staining)}}{\text{(Distance moved by band/Distance moved by marker dye)}}$$

RESULTS

Electrophoretic analysis of alpha-esterase isozymes in different developmental stages of silkworm Bombyx mori races NB_{18}, KA, and PM have revealed marked variation in the number of isozymes. The photographs and zymograms reveal the presence of 21 isozymes based on their relative front values. The alpha-esterase isozymes found in different developmental stages of the above species are classified into eight esterase zones. They are Est-1, Rf 0.06-0.12, Est-2 Rf 0.2-0.23; Est-3 Rf 0.30; Est-4 0.41-0.51; Est-5 Rf 5.5-6.2; Est-6 Rf 0.65-0.71; Est-7 Rf 0.76-0.83; Est-8 Rf 0.91-1.0. The isozyme patterns are numbered in a cathodal to anodal sequence from 1-21.

The zymograms of KA revealed 8 esterase zones. Est-I consists of two bands (1 and 2). Band 1 is absent and band 2 is present in 24h eggs, which is darkly stained and moderately stained in 144h male pupae. Est-2 consists of 2 bands (3 and 4). Band 3 is present in 24h eggs, I instar larvae, 288h male pupae and 24h female pupae and is darkly stained. Band 4 is moderately stained in 144h male pupae, 144h female pupae darkly stained in I instar larvae and 24h female pupae. Est-3 zone consists of 3 bands (5,6 and 7). Band 5 is moderately stained in 120h eggs, 144h female pupae darkly stained in I instar larvae, 288h male pupae. Band 6 is present in 120h, 216h male pupae. Band 6 is present in 120h, 216h, eggs I instar

larvae, 288h male pupae, 288h female pupae and male adult before copulation and is darkly stained. Band 7 is moderately stained in V instar larvae, and is darkly stained in 120h, 216h eggs, I instar larvae, 24h male pupae, 24h, 144h, and 288h female pupae and male adult before copulation.

Est-4 zone consists of 3 bands (8, 9 and 10). Band 8 is faintly stained in IV instar larvae moderately stained in female adult after oviposition darkly stained in 24h eggs, I instar larvae V instar larvae, 24h male pupae, 144h and Est-5 zone consists of 3 bands (11,12 and 13). Band 11 is moderately stained in female adult after oviposition darkly stained in IV instar larvae, 144h, 288h male pupae, female pupae male adult before copulation and female pupae before oviposition. Band 12 is darkly stained in 120h, 216h eggs, II, III, V instar larvae, 288h male pupae, 24h female male, adult before copulation. Band 13 is darkly stained in 24h, 216h eggs, II, III, IV, V instar larvae, 24h male pupae, male adult before copulation. Est-6 zone consists of 2 bands (14 and 15). Band 14 is darkly stained in 216h eggs, II instar larvae, 24h male pupae, 288h female pupae, male adult before and after oviposition. Band 15 is darkly stained in II instar, V instar larvae, 144h male pupae, 288h female pupae.

Est-7 zone consists of 3 bands (16,17 and 18). Band l6 is darkly stained in 120h eggs, I instar larvae, III instar larvae, 144h male pupae and female adult before and after oviposition. Band 17 is darkly stained in 216h eggs, I, III, IV, V instar larvae, 144h male pupae. 288h female pupae, male adult before and after copulation and female adult before and after oviposition. Band 18 is likely stained in 216h eggs, I, III, IV, V instar larvae, 24h, 144h, male pupae, 288h female pupae, male adult before and after copulation, female adult before and after oviposition. Est-8 zone consists of 3 bands (19,20 and 21). Band 19 is darkly stained in V instar larvae, 288h female pupae, male adult before and after copulation, female adult before oviposition. Band 20 is darkly stained in III, IV and V instar larvae, female adult after oviposition. Band 21 is present in all the stages of development (Figures 1, 2, and 3).

The zymograms of NB_{18} revealed a total of 8 Esterase zones with clear variation in mobility during different phases of developmen. Esterase--1 zone consists of 2 bands (1 and 2). Band 1 is moderately stained in the male adult after copulation, band 2 is moderately stained in IV instar larva and 24h male pupae. Esterase-2 zone consists of 2 bands (3 and 4). Band 3 is darkly stained in the male adult before copulation and female adult after oviposition and it is moderately stained in V instar, 144h male pupae, 288h male pupae and male adult after copulation.Band 4 is faintly stained in 24h eggs, moderately stained in 120h eggs, 144 male pupae and is darkly stained in 288h female pupae, male adult before copulation, female adult before oviposition and after oviposition.

Est-3 zone lightly stained consists of 3 bands (5,6 and 7). Band 5 is in 24h eggs, moderately stainedin 216h eggs, V instar larvae and female adult before oviposition. It is darkly stained in 120h eggs IV instar larvae. Band 6 is moderately stained in V instar larvae and V larvae, 144h male pupae, 288h male pupae, 144h male and darkly stained in 216h eggs, IV instar larvae is faintly stained in 24h female pupae, moderately instar pupae Band 7 stained in II and III instar and darkly stained in 24h male pupae. Est-4 zone consists of 3 bands (8,9 and 10). Band 8 is faintly stained in IV instar larvae, 24h female pupae moderately stained in II instar larvae, 288h female pupae and darkly stained in III instar V instar larvae and female adult after oviposition. Band 9 is moderately stained in 288h female pupae darkly stained in III, IV instar larvae, 24h male pupae and female adult after oviposition. Band 10 is faintly stained in 24h, 120h, 216h eggs, moderately stained in I instar, 144h female pupae darkly stained in IV, V instar larvae before and after oviposition in female adult.

Est-5 consists of 3 bands (11,12 and 13). Band 11 1s faintly stained 1n 120h eggs moderately stained in II instar larvae, 24h female pupae, darkly stained 1n 216 h eggs V instar larvae 288h female pupae Band 12 1s faintly stained in

24h eggs, moderately stained in 216 h eggs, 144h female pupae darkly stained in II, III instar larvae, 24h, 144h, 288h male pupae and 288h female pupae. Band 13 is present in 200h eggs I instar, III instar, IV instar, V instar larvae 24h, 144h female pupae. Est-6 consists of 2 bands (14 and 15). Band 14 1s present 1n 216h eggs, 24h, 144h, 288h male pupae, 24h female pupae, male adult after copulation, before and after oviposition 1n case of female adult. Band 15 1s faintly stained in 288h male pupae moderately stained in 120h, 24h and 216h eggs darkly stained in 144h male pupae 144h female, pupae male adult before copulation, female adult before oviposition. Est-7 zone consists of 3 bands (16,17 and 18). Band 16 1s faintly stained in III instar larvae moderately stained 1n II instar larvae darkly stained in I instar larvae, 288h male pupae and 144h female pupae. Est-8 zone consists of 3 bands (19,20 and 21). Band 19 1s faintly stained in 24h male pupae moderately stained 1n 288h female pupae and darkly stained in I instar larvae. Band 20 is moderately stained in 144h female pupae, darkly stained in I instar larvae. Band 21 is present in all the developmental stages (Figures 4, 5, and 6).

The alpha-esterase zymograms of PM revealed 8 esterase zones. Est-1 zone consists of two enzymes (1 and 2). Band one is darkly stained in 144h male pupae Band 2 is absent. Est-2 zone consists of 2 bands (3 and 4). Band 3 is faintly stained in female adult after oviposition, is moderately stained in I instar larvae and darkly stained in 24h female pupae. Band 4 is moderately stained in 24h male pupae and darkly stained in 144h male pupae, 24h female pupae. Est-3 zone consists of 3 bands (5,6 and 7). Band 5 is faintly stained in 24h egg darkly stained in IV instar larvae, 24h, 144h, male pupae and 288h female pupae. Band 6 is faintly stained in 24h eggs and female adult after oviposition, moderately stained in 120h eggs, darkly stained in IV instar larvae, 24h, 144h, 288h male pupae, 288h female pupae and male adult after copulation. Band 7 is faintly stained in female adult before oviposition, moderately stained in 24h male pupae and female adult after oviposition, darkly stained in I and II and IV instar larvae, 288h male pupae, 24h female pupae and male adult after copulation.

Est-4 zone consists of 3 bands (8,9 and 10). Band 8 is faintly stained in 120h eggs, moderately stained in 24h male pupae, female adult before oviposition, darkly stained in I, II, IV instar larvae, 144h female pupae. Band 9 is darkly stained in 144h male pupae. Band 10 is faintly stained in I instar larvae, moderately stained in female adult before oviposition, darkly stained in III instar, 144h male pupae, 24h female pupae, male adult after copulation. Est-5 zone consists of 3 bands (11,12 and 13). Band 11 is moderately stained in 216h eggs, darkly stained in II, IV instar larvae, 144h male pupae, 144h 288h female pupae, male adult before and after copulation, female adult after oviposition. Band 12 is moderately stained in I instar larvae, female adult before oviposition and darkly stained in III instar larvae, V instar larvae, 24h male pupae, 288h male pupae, 144h, 288h female pupae, male adult before and after copulation and female adult after oviposition. Band 13 is faintly stained in III instar larvae, darkly stained in 120h eggs, 160h eggs, IV, V instar larvae, 288h female pupae, male adult before copulation, female adult after oviposition.

Est-6 zone consists of 2 bands (14 and 15). Band 14 is faintly stained in 24h, 216h eggs, moderately stained in I instar larvae, darkly stained in III, IV and V instar larvae, 288h male pupae, 288h female pupae, male adult before copulation. Band 15 is faintly stained in 24h egg, and darkly stained in 24h male pupae. Est-7 zone consists of 3 bands (16,17 and 18). Band 16 is moderately stained in 24h male pupae, darkly stained in 216h eggs, III instar larvae, female adult before oviposition. Band 17 is faintly stained in 24h eggs, moderately stained in IV, V instar larvae, 24h female pupae, darkly stained in 288h male pupae, 144h, 288h female pupae, male adult after copulation, female adult before oviposition. Est-8 zone consists of 3 bands (19,20 and 21). Band 19 moderately stained in male adult before copulation, darkly stained in 144h female pupae, female adult before oviposition. Band 20 is moderately stained in female adult before oviposition. Band 21 is present in all developmental stages (Figure 7, 8, and 9).

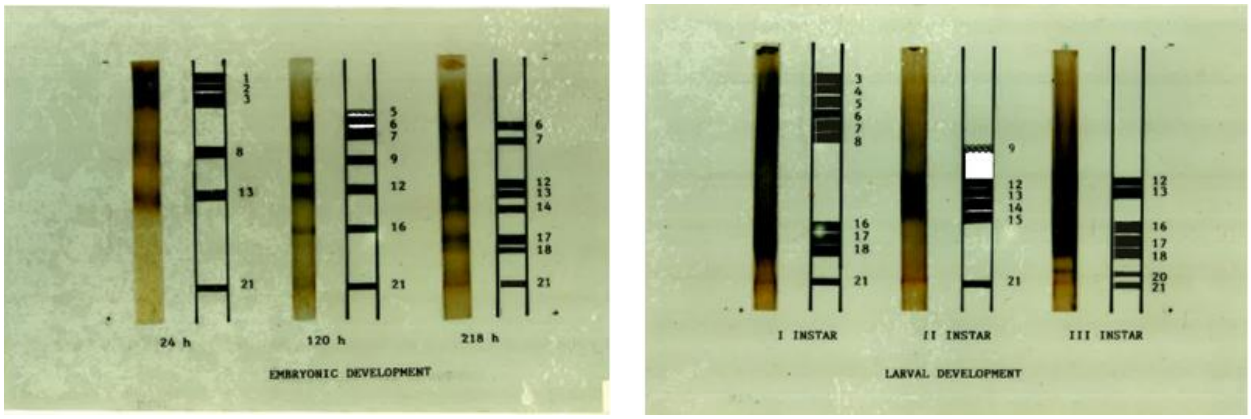

Figure 1: Alpha Esterase Zymograms of KA.

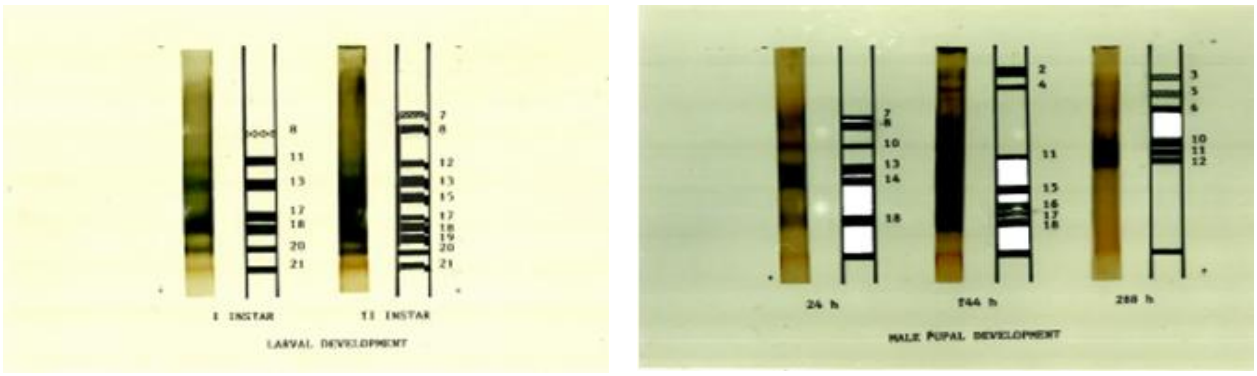

Figure 2: Alpha Sterase Zymograms of KA.

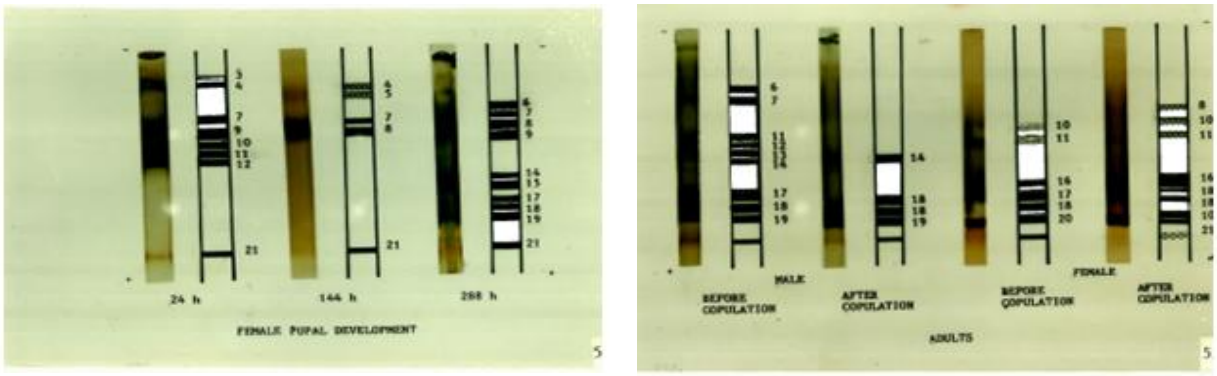

Figure 3: Alpha Esterase Zymograms of KA.

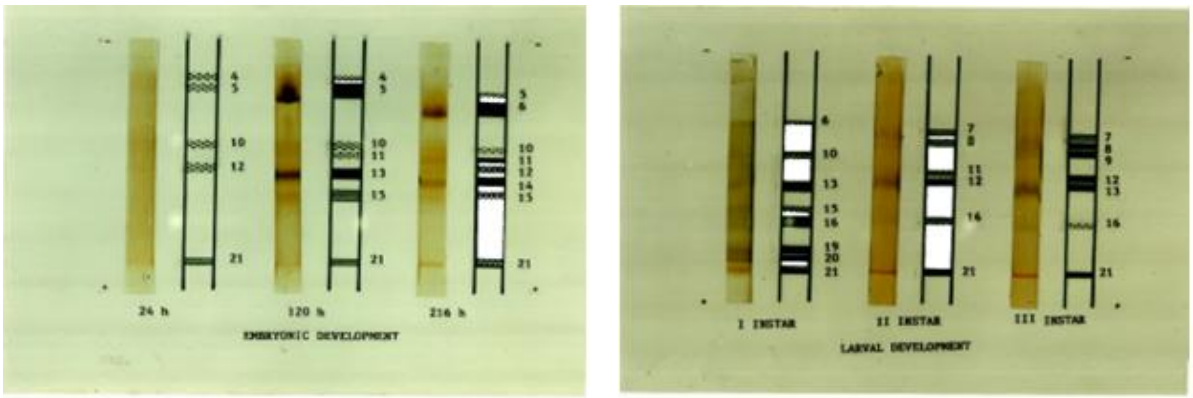

Figure 4: Alpha Esterase Zymograms of NB_{18}.

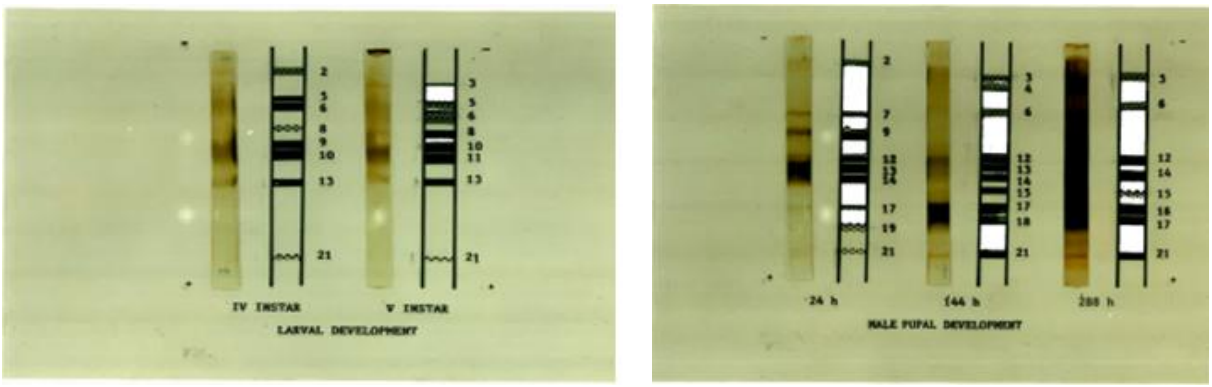

Figure 5: Alpha Esterase Zymograms of NB_{18}.

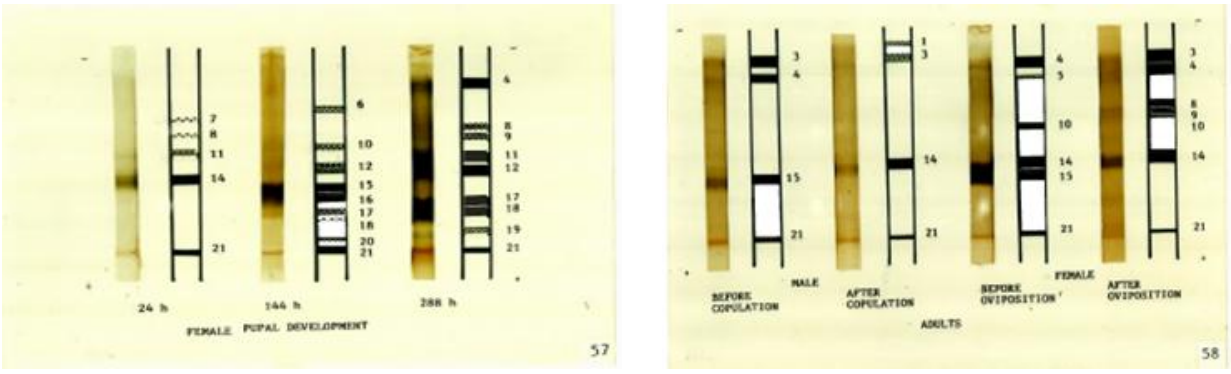

Figure 6: Alpha Esterase Zymograms of NB_{18}.

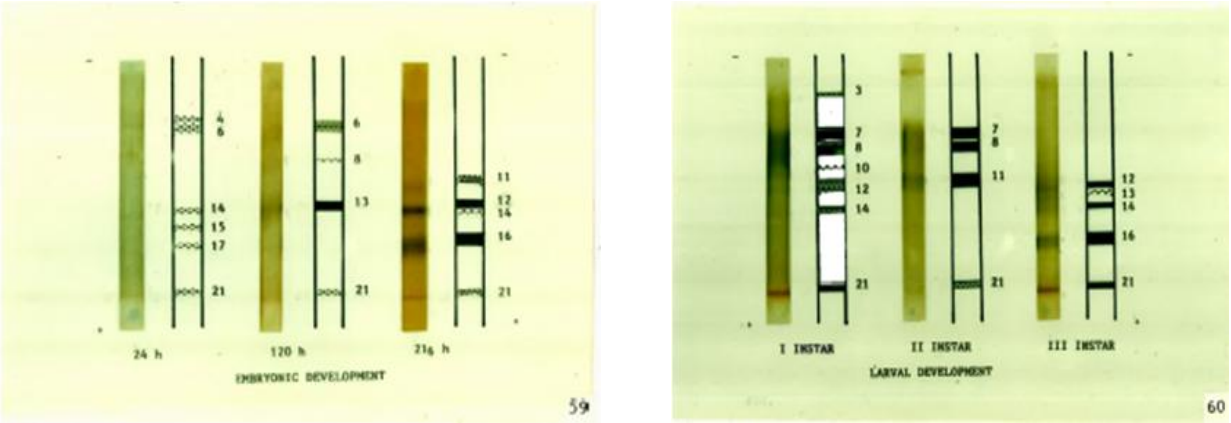

Figure 7: Alpha Esterase Zymograms of PM.

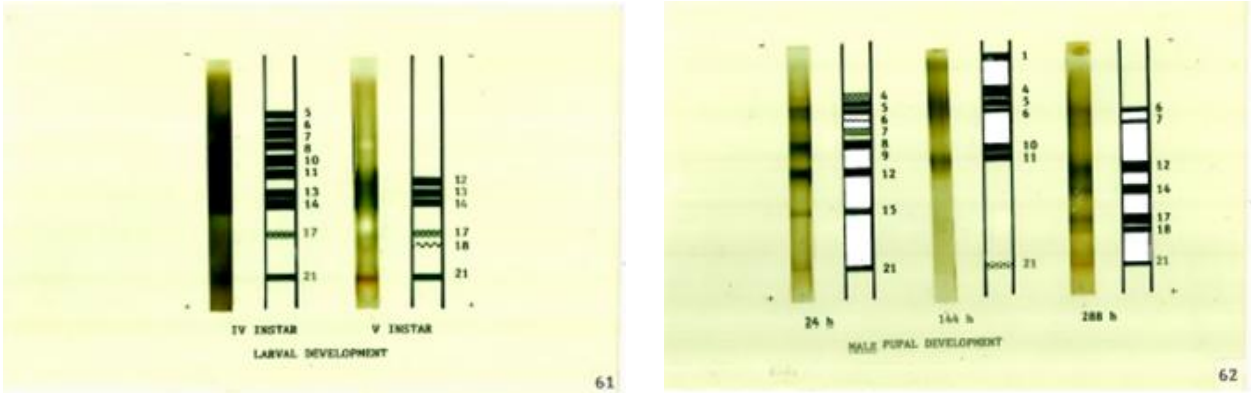

Figure 8: Alpha Esterase Zymograms of PM.

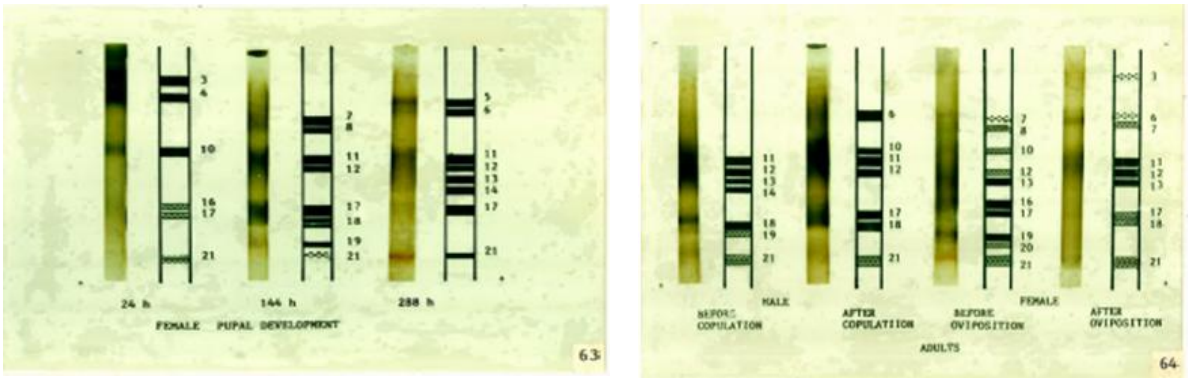

Figure 9: Alpha Esterase Zymograms of PM.

DISCUSSIONS

The changes in alpha-esterase zymograms during development revealed 21 bands in KA and NB_{18}, and the alpha-esterase activity is higher. These bands are not found in any single stage of development of *B. mori*. More or less same investigations have been conducted on the eri silkworm *Philosamia ricini* (Revanasiddaiah et al., 1989), and they reported 30 bands at various developmental stages. But their studies were conducted around egg development and they noticed twelve isozymes in embryogenesis. However, in the present study 14 bands in KA, 10 bands in NB_{18} and PM are recorded for alpha esterase respectively and all the bands are strongly resulted in KA. This also agrees with the report of Fei and Sheng (1987). They reported a total of 12 esterase isozymes during embryogenesis.

At larval stages 16, 13, and 14, bands are recorded for alpha-esterases in KA, NB_{18}, and PM breeding lines. In pupal stages more number of bands are recorded and in adults the numbers have been decreased. There is a gradual increase of esterase isozymes from eggs to pupae and they decreased in adults. This variation reflects regulation of gene activity so as to meet the den and of different metabolic activities (Revanasiddaiah et al., 1989, Krishnamurthy al., 1984).

The eggs show high esterase activity. This is due to the presence of large amounts of enzymes stored in the yolk which will be utilized during embryogenesis. The larval, pupal and adult stages reveal maximum heterogeneity like *P. ricini*. Esterase isozymes show sexual dimorphism in both pupal and adult stages. This shows stable difference in the expression of different genes in the same race of *B. mori* during ontogeny.

Thus the analysis of isozymes different at developmental stages of pure races and their isolated races suggest that the enzyme bands appear, disappear and reappear in different developmental stages. On the basis of their manifestation, the bands have been classified into two categories. One category persists throughout the developmental stages and called "generalized segregating and non-specific esterases" which include Est-3, Est-4, Est-5, Est-6 and Est-7 zones of alpha esterase. These are comparable to the results found in Drosophila 1mmigrans (Pautelouris and Downer, 1969).

The other category which is confined to some of the developmental stages is called "specific non segregating esterases" which include Est-1, Est-2 and Est-8 of alpha esterase. These are comparable to the ones found in *Zaprionus paravittiger* described by Kaur and Parkash (1979). The electrophoretic analysis has shown that the eggs of all the races contain alpha Est-3, Est-4, Est-5 zones. The developmental esterases show a gradual increase in the number of isozymes from I larval to V larval instar of all the races studied. Such an increase during development has been reported in Drosophila nastuta by Siddaveeregowda et al. (1977) and also insects in general by Laufer (1961). A comparative study pertaining to larval developmental stages of the races show a gradual increase in the number of isozymes from I larval to V

larval instar in all the races wherein very less number of bands are found in V instar larvae of PM. This may be due to the voltinism.

The results of the esterase activity in pupal stage of the three races studied indicate that alpha non-specific esterases are present in KA, NB_{18} and female pupae of PM, and high specific esterase activity was noticed in all the races. This observation coincides with that of Prakash and Reddy (1978) who have reported such high activity of alpha esterase in the pupal stage of fruit fly Drosophila rajashekari.

CONCLUSIONS

In conclusion, total number of 21 esterase bands were found in pure races and the isolated lines. High esterase activity was noticed in pure races than the isolated lines. The esterase activity was high in pupal stage followed by larval stage. Specific bands responsible for molecular differentiation for sexual dimorphism as well bands characteristic of bivoltine and multivoltine races have been identified.

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