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A STUDY ON ELECTROPHORESIS ANALYSIS OF BETA 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

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, we in the current study we aimed to analyze the activities of alpha esterase isozymes by electrophoresis method during different developmental stages Kalimpong-A (KA), B_{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. The relative front (Rf) of the esterase and phosphatase bands of all the developmental stages with reference to known indicator dye was calculated. Results demonstrated that beta-esterase zymograms during development revealed 16 bands in KA, 18 bands in NB_{18}, and 19 bands in PM were observed. However, 10 bands in KA, 12 bands in NB_{18} are strongly resulted in KA during embryogenesis. 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, Beta Esterase, Electrophoresis, Pupa, Larva*

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INTRODUCTION

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).

In recent years and electrophoretic techniques have been extensively used to study enzymatic variations in numerous organisms for various purposes, with reference to the developmental and tissue or organ specific difference of

enzymes in Drosophila, silkworm and other insect species. Different methods involving mainly paper, starch, agar gel and disc gel have been employed (Eguchi, 1964, Revanasiddaiah and Chowdaiah 1982).

A number of electrophoretic studies on the esterases have been made in recent years during development (Menzel gal., 1963, Ozita 1962, Lanfer 1961, Revanasiddaiah and Chowdaiah, 1988, Revanasiddaiah et al., 1989, Revanasiddaiah and Manjula, 1992). Eguchi gal. (1964) studied blood esterases of 161 silkworm strains through agar gel. Nine esterase bands were observed of various tissues of the silkworm Bombyx mori during development. They observed eight bands migrated towards the anode and one towards the cathode. Whittaker and West (1962) studied the insect haemolymph protiens through starch gel electrophoresis.

With this background, in the current study we aimed to analyze the activities of beta esterase isozymes by electrophoresis method during different developmental stages of new breeding lines 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 were 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.

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}, 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 allowed to photo polymerise 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 H_3PO_4+ 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 Photo polymerise 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 beta esterase constituted 25 mg of beta 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. 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}, and PM 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:

Rf = (Length gel before staining/ Length gel after staining)

X

(Distance moved by band/Distance moved by marker dye)

Results

The beta-esterase zymograms of KA revealed a total of 8-esterase zones with clear variations in mobility during different phases of development. Est-1 zone consists of 2 bands (182). Band 1 is absent and band 2 is moderately stained in I instar larvae. Est-2 zone consists of 2 bands (3 and 4) these are absent. Est-3 zone consists of 3 bands (5,6 and 7). Band 5 and 6 are absent and band 7 is moderately stained in 144h female pupae and darkly stained in 120h egg, 24h female pupae. Est-4 zone consists of 2 bands (8 and 9). Band 8 is faintly stained in 144h male pupae, moderately stained in I and II instar larvae, darkly stained in 24h female pupae. Band 9 is faintly stained in 24h male pupae, moderately stained in I, II instar larvae, 288h female pupae and male adult before copulation and darkly stained in 288h male pupae and 24h female pupae. Est-5 zone consists of 3 bands (10, 11 and 12). Band 10 is darkly stained in 288h male pupae, 24h female pupae. Band 11 is moderately stained Vth instar larvae, male adult before copulation and darkly stained in 120 eggs, III instar larvae 288h male pupae, 24-288h female pupae. Band 12 is moderately stained in IV instar larvae, darkly stained in 120h eggs, III and V instar larvae. Est-6 zone consists of 3 bands (13, 14 and 15). Band 13 is darkly stained in 120, 216h eggs, I, IV and V instar larvae and female pupae of 124, 288h and female adult after oviposition. Band 14 is moderately, stained in female adult before oviposition and darkly stained in 120, 216h eggs, I instar larvae, 24h, 144h male pupae and 288h female pupae. Band 15 is moderately stained in V instar larvae and darkly stained in 120h eggs.Est-7 zone consists of 3 bands (16, 17 and 18). Band 16 is darkly stained in 120h, 216h eggs, III, V instar larvae 144h female pupae and female adult after oviposition. Band 17 is moderately stained in 24h male pupae, male adult before oviposition and darkly stained in II, III, IV and V instar larvae, male pupae of 144h, female adult before and after oviposition. Band 18 is moderately stained in male adult before copulation, darkly stained in II, III and IV instar larvae 144h male pupae and female adult before and after oviposition.

Est-8 zone consists of 3 bands (19,20 and 21). Band 19 is darkly stained in 120h egg, II and V instar larvae, male adult before and after copulation and female adult before oviposition. Band 20 is moderately stained in V instar larvae and darkly stained in 216h egg and IV instar larvae. Band 2 is present in all the developmental stages (Figure 1, 2, and 3).

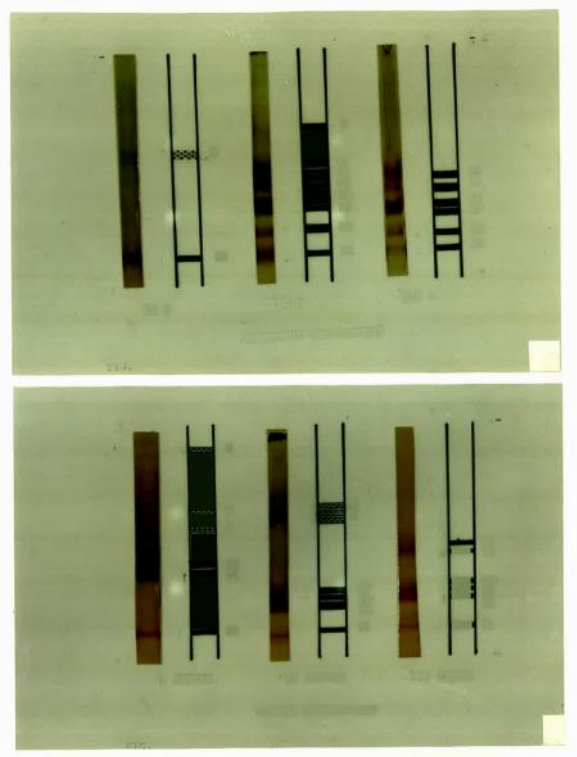

Figure 1: Beta Esterase Zymograms of KA

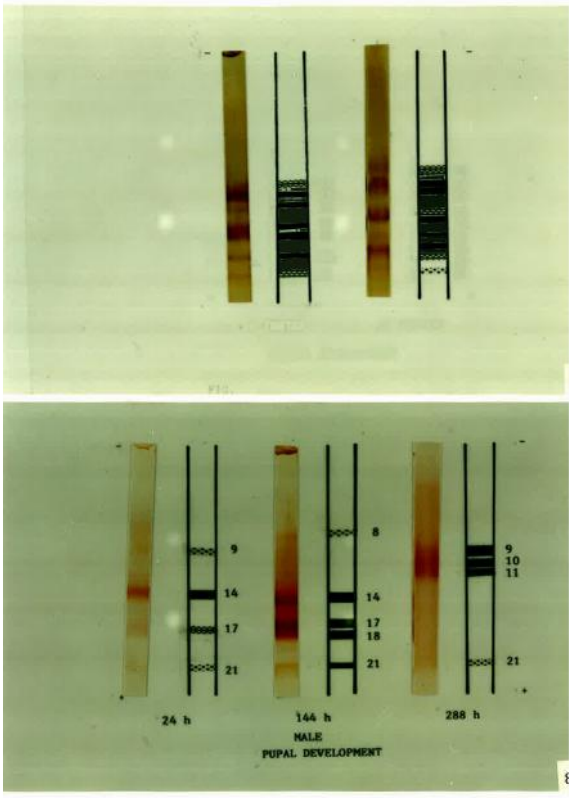

Figure 2: Beta Esterase Zymograms of KA

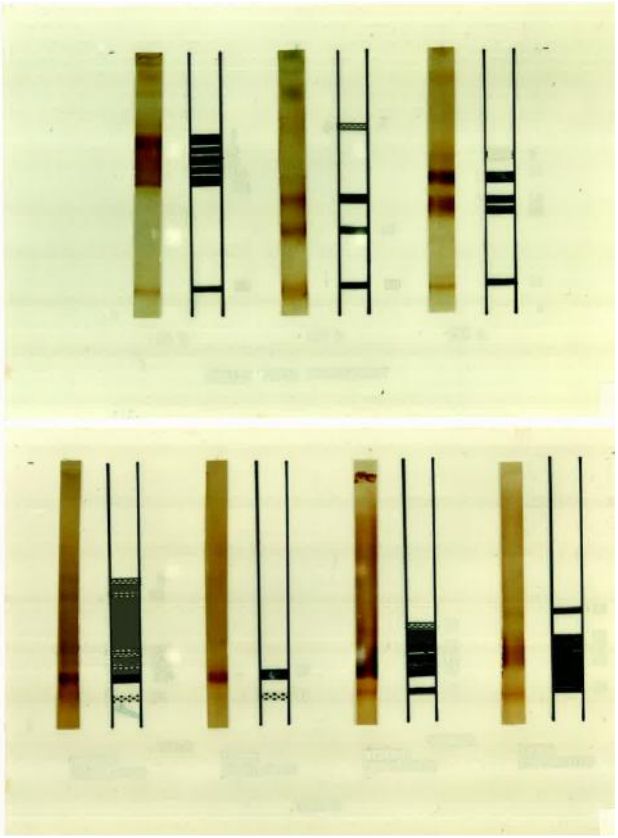

Figure 3: Beta Esterase Zymograms of KA

The zymograms of NB_{18} revealed 8, B-Est zones. Est-1 zone consists of 2 bands and they are absent. Est-2 zone consists of 2 bands (3 and 4). Band 3 is darkly stained in III instar larvae. Band 4 is absent. Est-3 zone consists of 3 bands (5,6 and 7). Band 5 is faintly stained in IV instar larvae, moderately stained in 24h, 216h eggs, II instar 24h male pupae and darkly stained in 288h female pupae. Band 6 is faintly stained in 144h female pupae, moderately stained in II instar larvae and darkly stained in 120h egg. Band 7 is faintly stained in 144h female pupae moderately stained in 24h, 216h egg. Est-4 zone consists of 2 bands (8 and 9). Band 8 is moderately stained in 24h eggs, II instar larvae, 288h male pupae and darkly stained in III and IV instar larvae and 24h male pupae. Band 9 is moderately stained in 144h female pupae and darkly stained in I, IV instar, larvae female adult before oviposition. Est-5 zone consists of 3 bands (10,11 and 12). Band 10 is moderately stained in 120h egg, II instar larvae, darkly stained in I, III instar larvae and 288h male pupae. Band 11 is faintly stained in 24h male pupae, moderately stained in 120h egg, 144h male pupae and darkly stained in 24h egg, 288h male pupae and 288h female pupae. Band 12 is moderately stained in 288h female pupae and darkly stained in III, V instar larvae. Est-6 zone consists of 3 bands (13, 14 and 15). Band 13 moderately stained in 24h egg, 144h male pupae and darkly stained in 216h egg, III instar larvae, 24, 288h male pupae and female adult before oviposition. Band 14 is moderately' stained in 288h female pupae male adult before copulation and darkly stained in III, V instar larvae and female adult before oviposition. Band 15 is moderately stained in 24h, 120h, eggs, IV instar larvae, 288h male pupae and male adult after copulation and darkly stained in 144h, 288h female pupae. Est-7 zone consists of 3 bands (16, 17 and 18). Band 16 is faintly stained in III instar larvae and darkly stained in III instar larvae and darkly stained in 288h female pupae. Band 17 is faintly stained in III instar larvae, moderately stained in 24h eggs, female adult after oviposition and darkly stained in 216h eggs, 144h, 288h male pupae, 24h female pupae. Band 18 is darkly stained in 216h eggs, V instar larvae, 24h and 288h

female pupae. Est-8 zone consists of 3 bands (19,20 and 21). Band 19 is moderately stained in IV instar larvae. Band 20 is faintly stained in 288h female pupae. Band 21 is present in all developmental stages (Figures 4, 5, and 6).

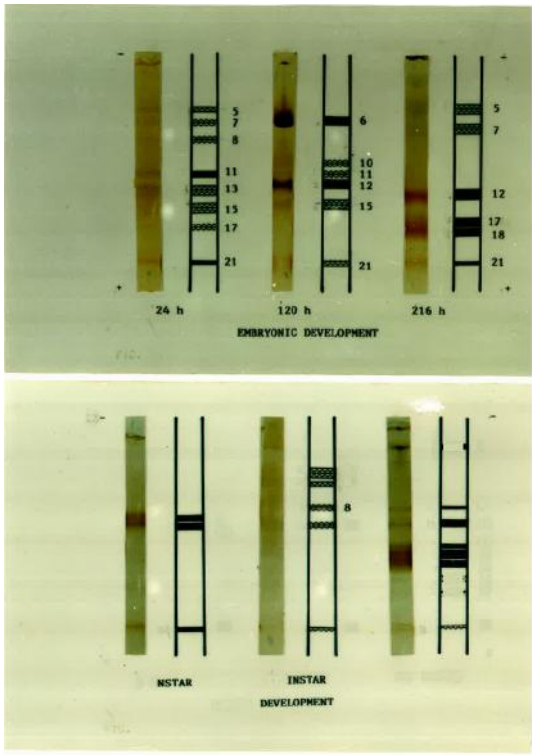

Figure 4: Beta Esterase Zymograms of NB_{18}

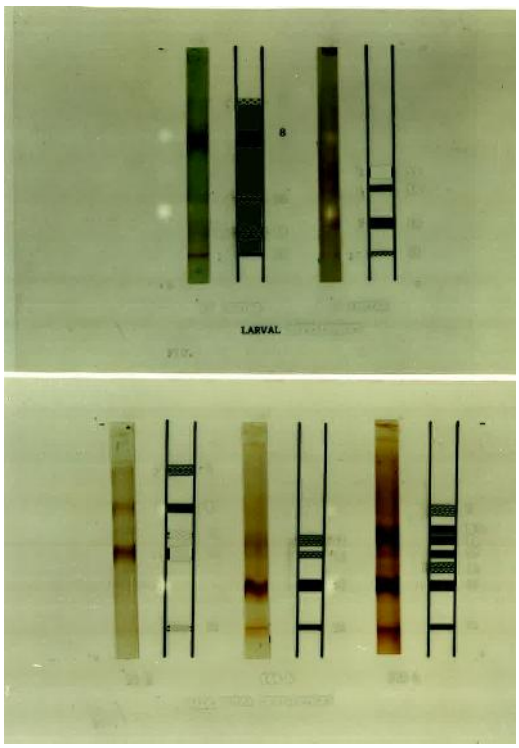

Figure 5: Beta Esterase Zymograms of NB_{18}

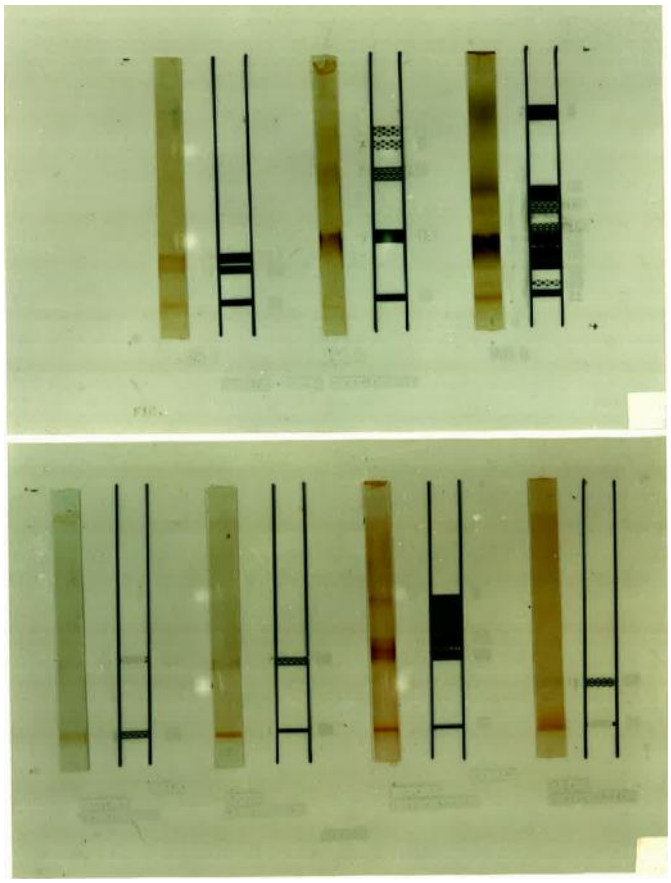

Figure 6: Beta Esterase Zymograms of NB_{18}

The zymograms of Pure Mysore (PM) beta-esterase revealed 8 esterase zones. Est-1 zone consists of 2 bands. Band I is absent. Band 2 is moderately stained in 216h egg. Est-2 zone consists of 2 bands (3 and 4). Band 3 is moderately stained in 216h eggs, II, IV and V instar larvae. Band 4 is faintly stained in III instar larvae, 144h male pupae, male adult after copulation and darkly stained 24, 144h and 288h female pupae. Est-3 zone consists of 3 bands (5,6 and 7). Band 5 is darkly stained in 24h, 288h male pupae and 144h female pupae. Band 6 is faintly stained in 120h eggs, 144h male pupae, darkly stained in 24h male pupae, 144h female pupae, male adult before copulation. Band 7 is faintly stained in V instar larvae, moderately stained in 24h, 120h eggs, I instar larvae, female adult before oviposition and darkly stained in II instar larvae, 144h female pupae, male adult before copulation and female adult after oviposition. Est-4 zone consists of 2 bands (8 and 9). Band 8 is faintly stained in 120h egg, male adult after copulation, female adult before oviposition, moderately stained in 24h, 216h eggs, I instar larvae, 24h, 288h, male pupae, 24h, female pupae, darkly stained in IV instar larvae and female adult after oviposition. Band 9 is faintly stained in 24h egg, III instar larvae, moderately stained in 120h egg, 24h male pupae darkly stained in IV instar larvae. Est-5 zone consists of 3 bands (10,11 and 12). Band 10 is faintly stained in 216h eggs and darkly stained in 24h female pupae. Band 11 is faintly stained in 288h male pupae, male adult before copulation, and darkly stained in IV instar larvae, 24h male pupae. Band 12 is moderately stained in V instar larvae and darkly stained, II, III and IV instar larvae, 288h female pupae. Est-6 zone consists of 3 bands (13, 14 and 15). Band 13 is moderately stained in 144h female pupae and darkly stained in 216h eggs, I and II instar larvae, 144h, 288h male pupae and 288h female pupae. Band 14 is faintly stained in V instar larvae and darkly stained in IV instar larvae and female adult after oviposition. Band 15 is moderately stained in 288h female pupae, male adult before copulation and darkly stained in 288h male pupae, female adult before oviposition. Est-7 zone consists of 3 bands (16,17 and 18). Band 16 is faintly stained

in 120h eggs, moderately stained in III instar larvae, 24h female pupae, darkly stained in 216h eggs, 24h, 288h male pupae, 144h female pupae and female adult before oviposition. Band 17 is faintly stained in 144h male pupae an darkly stained in 144h female pupae, male adult before and after copulation. Band 18 is darkly stained in V instar larvae, male adult after copulation. Est-8 zone consists of 3 bands (19,20 and 21). Band 19 is faintly stained in 144h male pupae. Band 20 is absent. Band 21 is commonly present in all developmental stages. (Figure 7, 8, and 9).

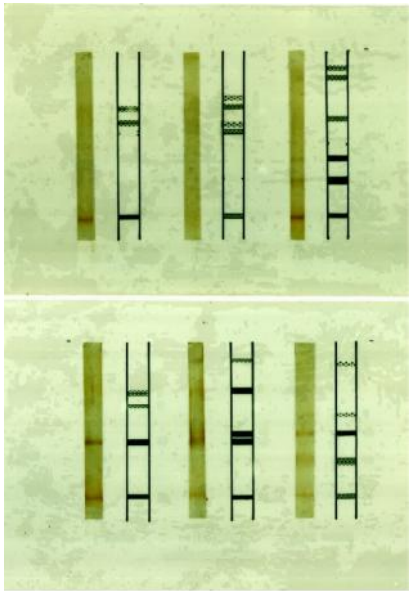

Figure 7: Beta Esterase Zymograms of PM

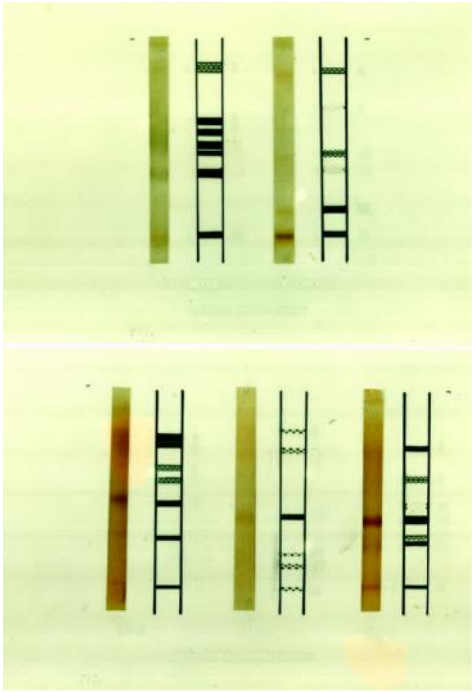

Figure 8: Beta Esterase Zymograms of PM

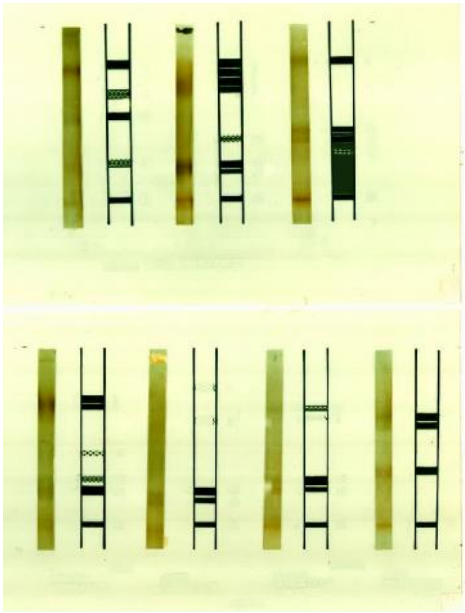

Figure 9: Beta Esterase Zymograms of PM.

DISCUSSIONS

The changes in beta-esterase zymograms during development revealed 16 bands in KA, 18 bands in NB_{18}, and 19 bands in PM were observed. However, 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. In a multivoltine strain of - mori (Pure Mysore) L- and beta- esterase isozyme patterns were reported by Krishnamurthy et al. (1984). But their studies were conducted around egg development and they noticed twelve isozymes in embryogenesis. However, in the present study 10 bands in KA, 12 bands in NB_{18} and PM are strongly resulted in KA during embryogenesis. This also agrees with the report of Fei and Sheng (1983). They reported a total of 12 esterase isozymes during embryogenesis.

At larvalstages 14, 14, 12, 11 & 12 bands are recorded for beta-esterase. 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 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 zones of Beta esterase. These are comparable to the results found in Drosophila immigrants (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, Est-7 and Est-8 of beta esterases. These are comparable to the ones found in Zaprionus paravittiger described by Kaur and Parkash (1979).

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 1sozymes 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 five races studied indicate that the beta specific esterases the activity is expressed only in PM the activity was expressed whereas it is absent in all the racesbut non-specific esterase activity is found in all the races. A comparison of the zymograms of adults of all the races indicate the activity of esterase in general is lower than that of larval instars and pupae. This may be because all the fatty acids are used up in histogenesis.

The functions of esterase isozymes in physiology of silkworm have been reported by many workers (Kai and Hasegawa 1972, Pant and Gupta 1980, Oberlander and Schaeiderman 1966, Kaur and Prakash 1979)that esterases help in breaking down lipids and fatty acids. The specific esterases found in various developmental stages could be involved in modifying hormones responsible for subsequently metamorphic events growth, moulting, pupation and differentiation into adult (Kaur and Prakash 1979).

CONCLUSION

In conclusion, beta-esterase zymograms during development revealed 16 bands in KA, 18 bands in NB18, and 19 bands in PM were observed. However, 10 bands in KA, 12 bands in NB18 are strongly resulted in KA during embryogenesis. 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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