A Study on Electrophoresis Analysis of Alkaline Phosphatase Isozymes During Different Developmental Stages of Kalimpong-A (KA), New Bivoltine-18 (NB18), 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 alkaline phosphatase isozymes by electrophoresis method during different developmental stages of Kalimpong-A (KA), B18, and Pure Mysore (PM) of Bombyx mori L. Standardized disc electrophoresis method was performed. Alkaline phosphatase 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, NB18, 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 changes in APH zymograms during development revealed a total of 18 bands in KA, NB18, and PM. From the present results it was clear that APH the activity in eggs is high in KA, NB18 and low in PM. In the larval stage. In pupal stage the activity was less in male pupae of all the races and it was found to be high in female pupae of all the races. Furthermore, phosphatases. take part in the digestion and assimilation, histolysis. They are mostly present in the lysosymes.**

Index Terms **- Bombyx mori L, Alkaline phosphatase, Electrophoresis, Kalimpong-A (KA), New Bivoltine-18 (NB18), Pure Mysore (PM).**

I.INTRODUCTION

A study on the taxonomy of closely related species is important though difficult. Earlier studies on the entomological taxonomy was 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.[1] 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.[1-4] Immuno diffusing techniques have been used to separate insect species in particular mosquitoes.[5,6] Schumann (1973) analysed through gel diffusion techniques different strains of mosquitoes of different origin and identified them.[7]

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,[8-13] and numerical methods of analysis have proven useful in many groups of insects and will see much wider use in future.[14] With this background, current study was planned with the main purpose to analyze the activities of alkaline phosphatase isozymes by electrophoresis method during different developmental stages of new breeding lines and races of *Bombyx mori* L.

II. 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.[15] MS variety of mulberry leaves were used in rearing. The worms were reared in mass up to 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, 5th 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, NB18, and multivoltine race PM were selected.

Electrophoresis

Disc electrophoresis was performed essentially according to Davis (1964) and Ornstein (1964).[16,17] 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 cm dia) held vertically. The solution was carefully over layered with distilled water and allowed to 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 g tris + 25.6 ml of 1M H3Po4 + 0.46 ml of TEMED diluted to 100 ml 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 stain of alkaline phosphatases (APH) constituted sodium 1-naphthyl phosphate 100 mg, polyvnylpyrrolidone 500 mg. Fast blue RR salt 100 mg. Manganese chloride 60 mg. Magnesium chloride 60 mg and sodium chloride 2 gms dissolved in 100 ml of 0.05 M Tris - HCl buffer at pH 8.5. The gels were incubated in the stain for 10-20 minutes until the bands appeared. The gels were then stored in 6% acetic acid. All pertinent gels were photographed and diagrammatic representation of the gels was presented in the form of zymograms as deeply stained, moderately stained and faintly stained bands.

Phosphatase 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).[18] 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)

III. RESULTS

The zymograms of the alkaline phosphatase of the race KA revealed 8 APH zones. APH-1 zone consists of 1 band. It is moderately stained in 24h female pupae. APH-2 zone consists of 2 bands (2 & 3). Band 2 is absent and band 3 is moderately stained in 120h eggs. APH 3 zone consists of 3 bands (4, 5 & 6). Band 4 is faintly stained in 24h male pupae, moderately stained in I instar larvae, 24h female pupae and darkly stained in 288h male pupae. Band 5 is darkly stained in 288h male pupae. Band 6 is darkly stained in 288h male pupae. APH-4 zone consists of 3 bands (7, 8 and 9). Band 7 is moderately stained in 216h egg, darkly stained in male adult before copulation. Band 8 is moderately stained in 24h, 144h male pupae, darkly stained in II instar larvae. Band 9 1s darkly stained in II instar larvae, female pupae of 144h. APH-5 zone consists of 3 bands (10, 11 and 12). Band 10 1s moderately stained in 144h female pupae, darkly stained 1n 120h eggs, II instar larvae, 24h, 288h female pupae. Band 11 is moderately stained in IV instar larvae, darkly stained in 24h, 120h eggs, I, V instar larvae 24h, 144h male pupae and 288h female pupae. Band 12 is darkly stained 1n 24h eggs, I, III, IV, V instar larvae, 144h male pupae, 288h female pupae. APH-6 zone consists of 3 bands (13, 14 and 15). Band 13 1s darkly stained in 24h, 120h, 216h eggs, III, IV, V instar larvae, 24h, 144h female pupae. Band 14 is darkly stained in 24h, 288h female pupae and female adult after oviposition. Band 15 is faintly stained in 288h female pupae moderately stained in IV instar larvae, darkly stained in 120h eggs and female adult before oviposition. APH-7 zone consists of 3 bands (16, 17 and 18). Band 16 1s moderately stained in IV and V instar larvae, darkly stained in II, III instar larvae. Band 17 1s moderately stained in 120h eggs, V instar larvae. Band 18 is moderately stained in 288h female pupae, darkly stained in 216h eggs III and IV instar larvae. APH-8 zone consists of 3 bands (19, 20 and 21). Band 19 is moderately stained in 120h eggs, darkly stained in III and IV instar larvae. Band 20 is absent band 21 is present in all the developmental stages (Figure 1, 2, and 3).

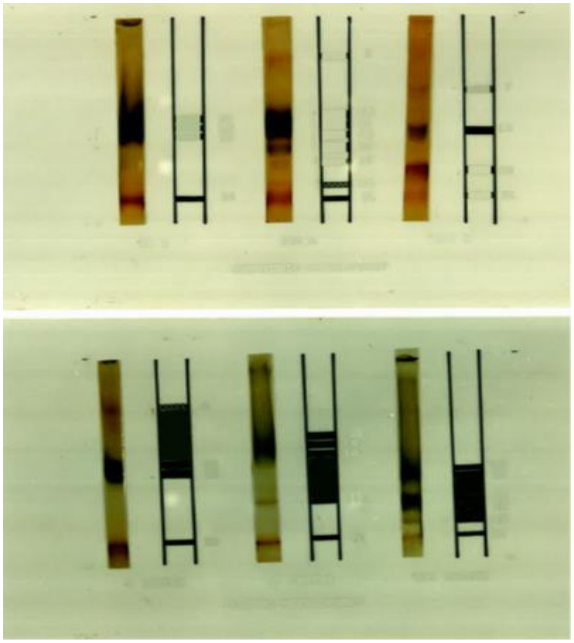

Figure 1: Alkaline phosphatase zymograms of KA

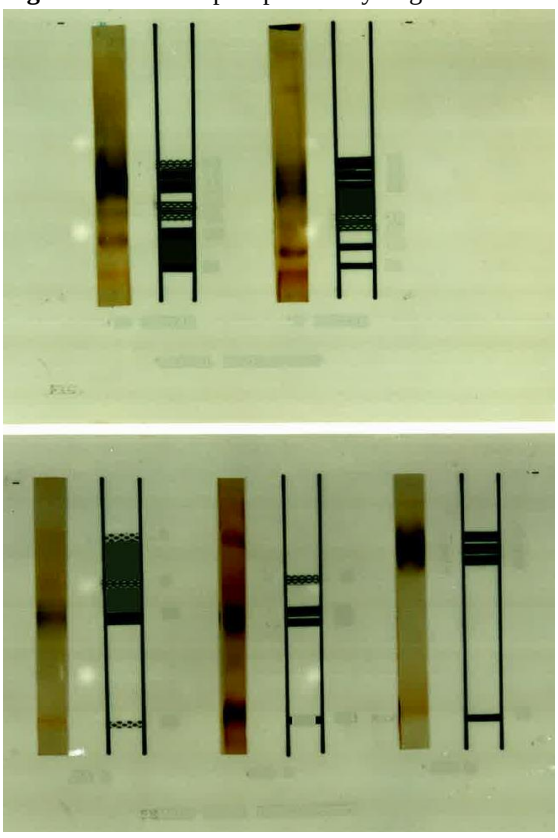

Figure 2: Alkaline phosphatase zymograms of KA

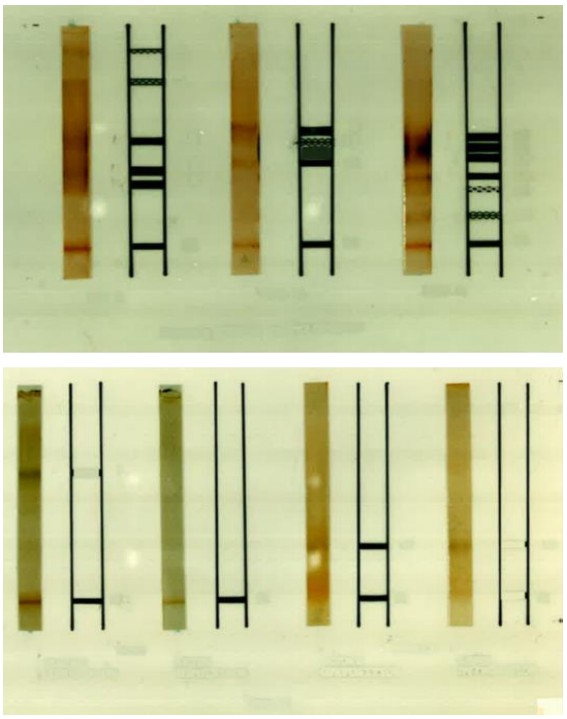

Figure 3: Alkaline phosphatase zymograms of KA

The zymograms of the alkaline phosphatase of the race NB_{18} revealed 8 APH zones. APH-1 zone consists of no bands. APH-2 zone consists of 2 bands (2 and 3). Band 2 is darkly stained in 124h female pupae. Band 3 is faintly stained in 120h, 216h eggs and male adults before copulation. APH-3 zone consists of 3 bands (4, 5 and 6). Band 4 is faintly stained in 216h eggs, male adult before copulation, moderately stained in female adult before oviposition, darkly stained in III instar larvae, 24h female pupae. Band 6 is darkly stained in 216h eggs, III and V instar larvae. APH-4 zone consists of 3 bands (7, 8 and 9). Band 7 is faintly stained in 144h female pupae, moderately stained in 24h, 288h male pupae, female adult after oviposition, darkly stained in I, V instar larvae and 288h female pupae. Band 8 is moderately stained in 288h male pupae, female adult before oviposition, darkly stained in 24h female pupae, darkly stained in I, II, IV and V instar larvae and 144h fa ale pupae. APH-5 zone consists of 3 bands (10, 11 and 12). Band 10 1s moderately stained in 120h eggs, roiled pupae 24h, darkly stained in I, V instar larvae, 124h female pupae. Band 11 1s moderately stained 1n 24h female pupae. Band 12 1s moderately stained in 24h female pupae, darkly stained 1n II, IV instar larvae and 288h male pupae. APH-6 zone consists of 3 bands (13, 14 and 15). Band 13 1s darkly stained 1n 216h eggs, I instar larvae 144, 288h male pupae. Band 14 1s moderately stained 1n 24h fen ale pupae, darkly stained 1n I, IV instar larvae and 144h female pupae. Band 15 is faintly stained 1n 24h male pupae, 120, 216h eggs and 144h female pupae. APH-7 zone consists of 3 bands (16, 17 and 18). Band 16 1s absent. Band 17 is darkly stained is 24h female pupae. Band 18 is darkly stained in 24h, 288h male pupae. APH-8 zone consists of 3 bands (19, 20 and 21). Band 19 1s faintly stained in 24h female pupae. Band 20 is absent. Band 21 is commonly present in all the developmental stages (Figure 4, 5, and 6).

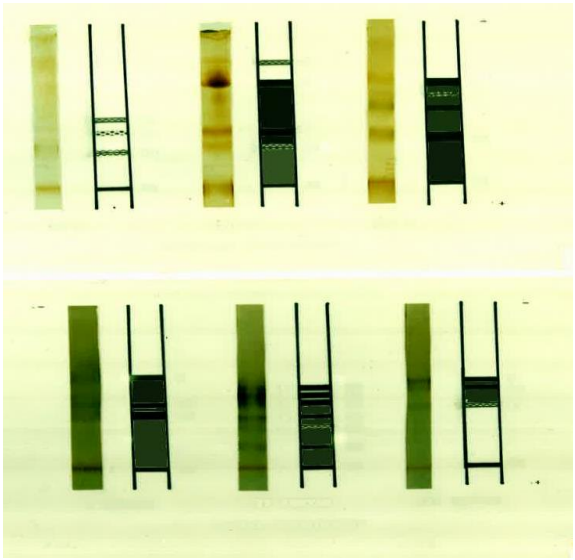

Figure 4: Alkaline phosphatase zymograms of NB_{18}

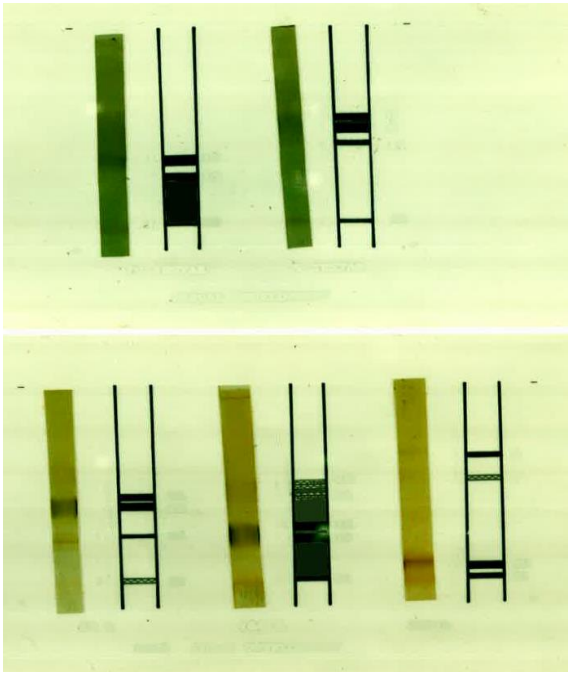

Figure 5: Alkaline phosphatase zymograms of NB_{18}

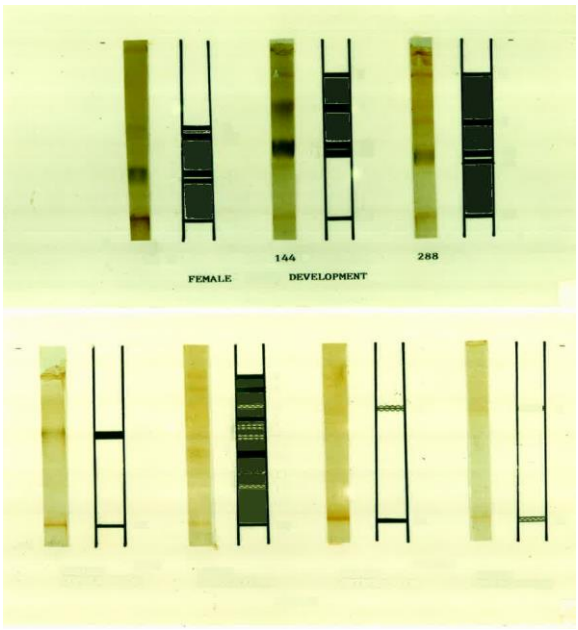

Figure 6: Alkaline phosphatase zymograms of NB_{18}

The zymograms of the alkaline phosphatase of the race Pure Mysore (PM) revealed 8 APH zones. APH-1 zone consists of one band. Band 1 1s moderately stained in 144h male pupae. APH-2 zone consists of 2 bands (2 and 3). Band 2 1s moderately stained in I instar larvae, 144h male pupae, 24h female pupae, darkly stained in 24h male pupae. Band 3 1s faintly stained in 288h female pupae, moderately stained 1n V instar larvae, 144h male pupae, darkly stained 1n 144h female pupae. APH-3 zone consists of 3 bands (4,5 and 6). Band 4 1s moderately stained in 144, 288h male pupae, darkly stained 1n 24h eggs, 24h female pupae. Band 5 is absent. Band 6 1s moderately stained in IV instar larvae, darkly stained 1n I, III instar larvae, 24h male pupae. APH-4 zone consists of 3 bands (7, 8 and 9). Band 7 1s darkly stained in I, III instar larvae, 288h male pupae. Band 8 is moderately stained in male adult after copulation, darkly stained 1n I, III instar larvae. Band 9 1s moderately stained in 216h eggs, V instar larvae, darkly stained in I instar larvae, 144h male pupae. APH-5 zone consists of 3 bands (10, 11 and 12). Band 10 1s faintly stained in 24h male pupae, moderately stained in I instar larvae, male adult after copulation, darkly, stained in III, IV instar larvae, 144h male pupae. Band 11 is darkly stained in 120h, 216h eggs, II, IV instar larvae, 144h, 288h male pupae. Band 12 is darkly stained in 120h eggs, V instar larvae, 144h, 288h male pupae, 24h, 288h female pupae, male adult before and after copulation, female adult before oviposition. APH-6 zone consists of 3 bands (13, 14 and 15). Band 13 is moderately stained in I instar larvae, darkly stained in II, IV, V instar larvae, male adult after copulation, female adult before and after oviposition. Band 14 is darkly in V instar larvae. Band 15 is darkly stained in IV instar larvae. APH-7 zone consists of 3 bands (16, 17 and 18). Band 16 is darkly stained in II instar larvae. Band 17 is dark stained in II instar larvae. Band 18 is absent. APH-8 zone consists of 3 bands (19, 20 and 21). Band 19 is moderately stained in V instar larvae. Band 20 is absent. Band 21 is commonly present in all the developmental stages (Figure 7, 8, and 9).

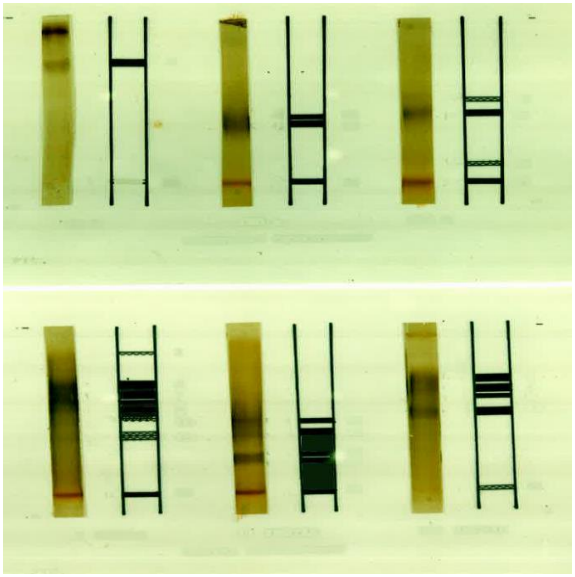

Figure 7: Alkaline phosphatase zymograms of PM

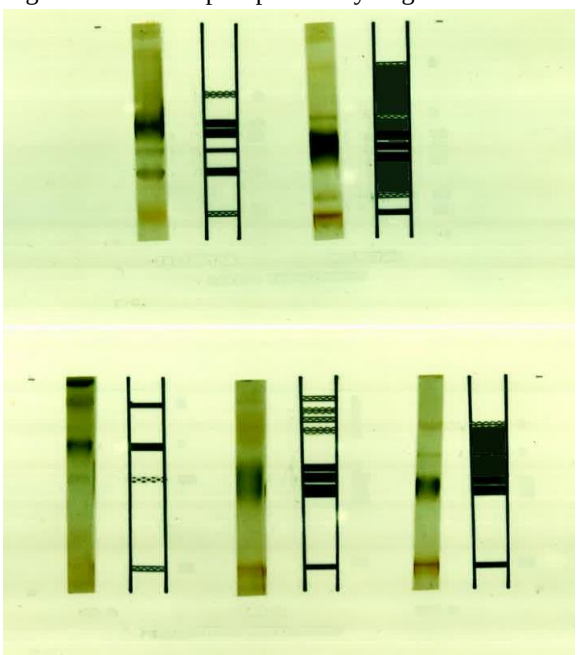

Figure 8: Alkaline phosphatase zymograms of PM

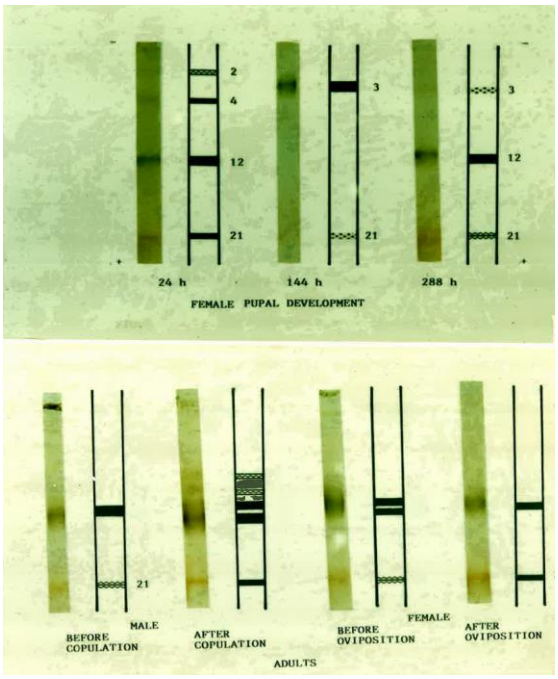

Figure 9: Alkaline phosphatase zymograms of PM

IV. DISCUSSION

The occurrence of phosphatases in silkworm *Bombyx mori* were first reported by Nakamura (1940)[19] later many workers studied these enzymes.[20-22] The results obtained showed increased activity from eggs to V instar larvae and it was decreased in pupae and adults. This is supported by other workers also.[23,24] The high activity of phosphatase during larval stage is due to the hydrolysis, histogenesis, cell differentiation and transformation. Low activity of the phosphatase in pupae and adult is because of the process of hydrolysis from V instar larval stage to pupae. This is also studied in Lepidopterous.[25] On the basis of above studies, it is suggested that during metamorphosis midgut tissues undergo gradual degradation under the presence of hydrolases like acid and alkaline phosphatases which are originated from lysosymes in degenerated cells. This is also studied in Anthereae myliltta, tasar silkworm by Sinha et al. (1991) where ACPH activity was maximum in 3rd instar and decreased in spinning stage. APH activity was minimum and decrease again in spinning stage.[26]

The changes in APH zymograms during development revealed a total of 18 bands in KA, NB_{18}, and PM. From the present results it was clear that APH the activity in eggs is high in KA, NB_{18} and low in PM. In the larval stage. In pupal stage the activity was less in male pupae of all the races and it was found to be high in female pupae of all the races. Here also sexual dimorphism has been observed. The present results also agree with the findings of Hedge and Krishnamurthy (1984) where the activity of APH was low in eggs but increased gradually towards V instar larvae and it was still decreased in later stages.[27]

The phosphatases are mainly concerned with the digestion of the ingested food and degradation of the cellular substances.[28] The fact that the silkworm egg shows neither digestive activity nor degradation of cellular substances accounts for low activity of APH in the eggs. Further, the larval stage is the only feeding stage in silkworm after which feeding stops. In the pupal and moth stages there is a lot of resorption of cells and tissues. Hence high phosphatase activity is seen in larval stage which gradually declines in later stages. The genes which control this mechanism are active in the larval stages and are less active in pupal and get inhibited in adult stage.

Phosphatases also show specificity. The APh-5, APh-6 and APH-7 in case of KA, APH-4, APh-5, APH-6 in case of NB_{18}, APH-5, APH-6 in PM are non-specific but are stage specific. However, the specificity varies from one race to another race. Here some zones are stage specific also. This specificity/differences in the electrophoretic mobilities of both the enzymes implies divergence in the molecular properties of the protein.[13] Genetic studies made in Drosophila (Beckman and Johnson, show electrophoretic variations in larval APH controlled by a pair of codominant alleles. Subsequently genetic and developmental relationship between larval and pupal APH were investigated by.[29] Genetic inheritances of polymorphic m-APH in Bombyx midgut was studied by Takeda et al., (1992).[13] The functions of both APH have been well reported in the silkworm.[24]

V. CONCLUSION

In conclusion, changes in APH zymograms during development revealed a total of 18 bands in KA, NB18, and PM. From the present results it was clear that APH the activity in eggs is high in KA, NB18 and low in PM. In the larval stage. In pupal stage the activity was less in male pupae of all the races and it was found to be high in female pupae of all the races. Furthermore, phosphatases. take part in the digestion

and assimilation, histolysis. They are mostly present in the lysosymes.

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