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A STUDY ON ELECTROPHORESIS ANALYSIS OF ACID PHOSPHATASE ISOZYMES DURING DIFFERENT DEVELOPMENTAL STAGES OF EVOLVED RACES R_1 AND R_2OF *BOMBYX MORI* L

Manjula A.C[1] & Keshamma E[2]

[1]*Research Scholar, Department of Sericulture, Maharani Cluster University, Palace Road, Bengaluru, Karnataka, India*

[2]*Research Scholar, Department of Biochemistry, Maharani Cluster University, Palace Road, Bengaluru, Karnataka, India*

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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, present study was designed with the main purpose to analyze the activities of alkaline phosphatase isozymes by electrophoresis method during different developmental stages of newly evolved races R_1 and R_2 of Bombyx mori L. Standardized disc electrophoresis method was performed. Acid phosphatase (ACPH) 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 R_1 and R_2 have been grouped into different zones. The nomenclature of enzyme banding pattern has been followed. Results delineated that changes in ACPH zymograms during development revealed a total of 17 bands in R_1 and 21 bands in R_2 recorded for ACPH. From the present results it was clear that ACPH activity was low in R_1 and R_2. ACPH activity was higher in R_1. Whereas, in R_2 moderate ACPH activity was exhibited in larval as well pupal stages. In pupal stage the activity was less in male and female pupae of R_1& R_2 races.

KEYWORDS: *Bombyx Mori L, Acid Phosphatase, Electrophoresis, Race 1 (R_1), Race 2 (R_2)*

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 geldiffusion 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]Withthis background, current study was planned with the main purpose toanalyze the activities of alkaline phosphatase isozymes by electrophoresis method during different developmental stages of new breeding lines and races 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.[15]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.

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.

Evolutions of New Lines R_1 and R_2

Females of KA and NB_{18} were crossed with males of PM. The composite layings of F_1 hybrid were brushed and reared under standard laboratory conditions. The selection parameters explained earlier were applied to choose the seed cocoons for the preparation of F_2 layings. The replicates showing higher pupation rate were selected for intra family selection of cocoons. Further, segregation with respect to cocoon colour and built was noticed. Only white oval in case of KAxPM and dumbbell white in case of NB_{18}xPM qualifying the parameter of selection were choosen for breeding in subsequent generations. The females of F_5 were backcrossed to the males of KA and NB_{18} respectively in both the lines and reared up to 11 generations. At the end of the 11th generation the lines R_1 and R_2 were extracted with higher ERR than their respective better parents, with shorter larval period and with moderate cocoon productivity character in case of R_1 and R_2.

Table 1

Breeding Plans I and II													
				I							II		
	KA	O	O	x	PM	Cto		NB18	O	O	x	PM	Cfo
		+	i-						+	+			
				F1							F1		
				F2							F2		
				F3							F3		
				F4							F4		
F5	x	KA	O	↘	er'		F5x	NB18	Cta	+			
				F1							F1		
				F2							F2		
				F3							F3		
				F4							F4		
				F5							F5		
				F6	(R1)						F6	(R2)	

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 evolved races Race-1 (R_1) and Race-2 (R_2) 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 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 stain of acid 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 0.125 M acetate buffer at pH 5.0. 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.

RESULTS

The zymograms of alkaline phosphatase isozymes of the race R_1 showed 8 ACPH zones. ACPH-1 zone consists of only one band and it is absent. ACPH-2 zone consists of 2 bands (2 and 3). Band 2 1s doubly stained in 24h, male pupae. Band 3 1s faintly stained in 144h male pupae, moderately stained in 144h female pupae. ACPH-3 zone consists of 3 bands (4,5 and 6). Band 4 1s darkly stained in I instar larvae. Band 5 1s moderately stained in 24h, eggs, doubly stained in 216h eggs. Band 6 is faintly stained 1n 24h male pupae. ACPH-4 zone consists of 3 bands (7,8 and 9). Band 7 1s moderately stained in 144h male pupae, darkly stained 1n 216h eggs, 288h male pupae, 288h female pupae. Band 8 1s moderately stained in 24h, female pupae, darkly stained in 120h eggs, 216h eggs. Band 9 is moderately stained in 24h female pupae, darkly stained in 120h, 216h eggs. ACPH-5 zone consists of 3 bands (10, 11 and 12). Band 10 is moderately stained in 24h female pupae, darkly stained in 120h eggs, III, IV and V instar larvae, 144h, 288h male pupae, 144h female pupae. Band 11 is moderately stained in 288h female pupae, female adult before oviposition, darkly stained in I, III, IV and V instar larvae, 144h, 288h male pupae, 144h female pupae, male adult before copulation. Band 12 is darkly stained in II, III, IV, V instar larvae, 144h male pupae, 144h female pupae, male adult before copulation. ACPH-6 zone consists of 3 bands (13, 14 and 15). Band 13 is darkly stained in V instar female pupae 288h, male adult before copulation. Band 14 is faintly stained in II instar larvae, 24h male pupae, darkly stained in V instar larvae. Band 15 is faintly stained in I instar larvae, doubly stained in I instar larvae, doubly stained in 288h female pupae. ACPH-7 zone consists of 3 bands (16, 17 and 18). Band 16 is moderately stained in female adult before oviposition, darkly stained in 288h female pupae. Band 17 is absent. Band 18 is faintly stained in 24h male pupae, male adult after copulation, moderately stained in 288h male pupae. ACPH-8 zone consists of one band (21) which is commonly presently in all the developmental stages (Figures 1, 2, and 3).

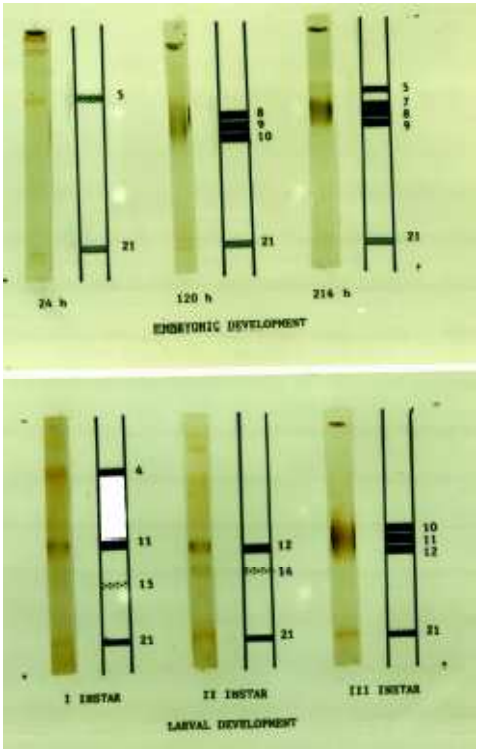

Figure 1: Acid Phosphatase of Zymogram R_1

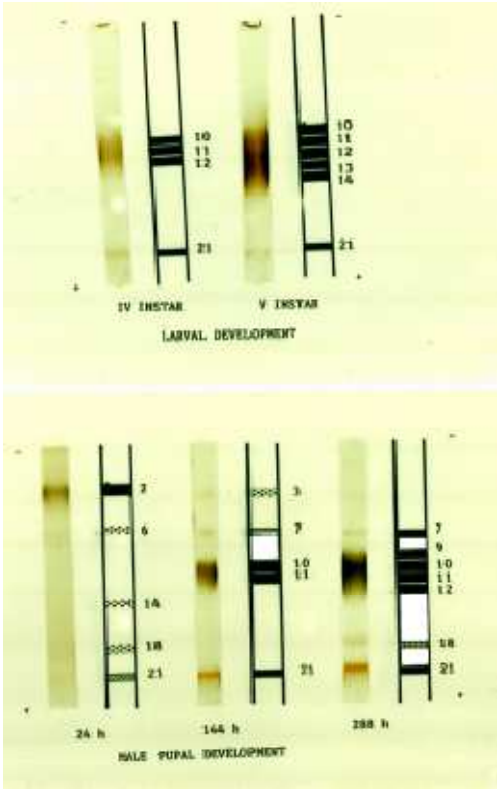

Figure 2: Acid Phosphatase of Zymogram R_1.

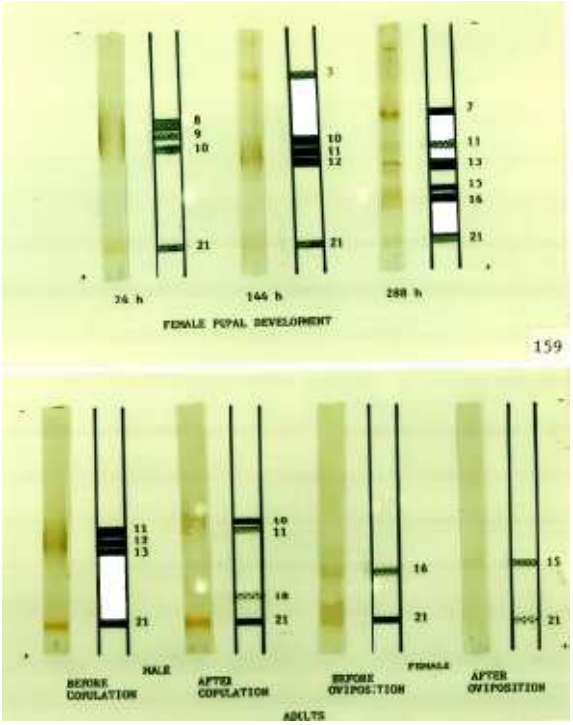

Figure 3: Acid Phosphatase of Zymogram R_1.

The zymograms of the enzyme acid phosphatase of the race R_2 revealed 8 ACPH zones. ACPH-1 zone consists of 1 band (1). It is moderately stained in IV instar larvae. ACPH-2 zone consists of 2 bands (2 and 3). Band 2 is moderately stained in IV instar larvae, female adult before oviposition. Band 3 is darkly stained in III instar larvae, 24h female pupae.

ACPH-3 zone consists of 3 bands (4, 5 and 6). Band 4 is moderately stained in the male pupae, 144h, female pupae 144h female adult after oviposition, darkly stained in I instar larvae. Band 5 is moderately stained in288h female pupae, darkly stained in 24h male pupae. Band 6 is moderately stained in V instar larvae, female adult after oviposition, darkly stained in 288h male pupae. ACPH-4 zone consists of 3 bands (7, 8 and 9). Band 7 is moderately stained in 288h female pupae, darkly stained in 288h male pupae. Band 8 is moderately stained in 144h female, pupae, darkly stained in 288h male pupae. Band 9 is moderately stained in III instar larvae 288h female pupae darkly stained 1n IV, V instar larvae. ACPH-5 zone consists of 3 bands (10, 11 and 12). Band10 is moderately stained 1n female adult before oviposition. Band 11 is darkly stained in 24h, 144h female pupae. Band 12 is darkly stained in 144h male pupae, 24h, 144h, 288h female pupae. ACPH-6 zone consists of 3 bands (13, 14 and 15). Band 13 is darkly stained ln III instar larvae, 144h male pupae, 288h female pupae. Band 14 is darkly stained ln male adult before copulation. Band 15 lS faintly stained 1n male adult after copulation, darkly stained 1n IV, V instar larvae, 288h female pupae. ACPH-7 zone consists of 3 bands (16, 17 and 18). Band 16 is moderately stained in 144h male pupae, darkly stained 1n 216h eggs. Band 18 is faintly stained in female adult after oviposition, darkly stained in 120h, 216h eggs, II instar larvae. ACPH-8 zone consists of 3 bands (19, 20 and 21). Band 19 is darkly stained in 120h, 216h eggs. Band 20 1s darkly stained in 24h, 120h, 216h eggs. Band 21 1s present 1n all, the developmental stages (Figures 4 and 5).

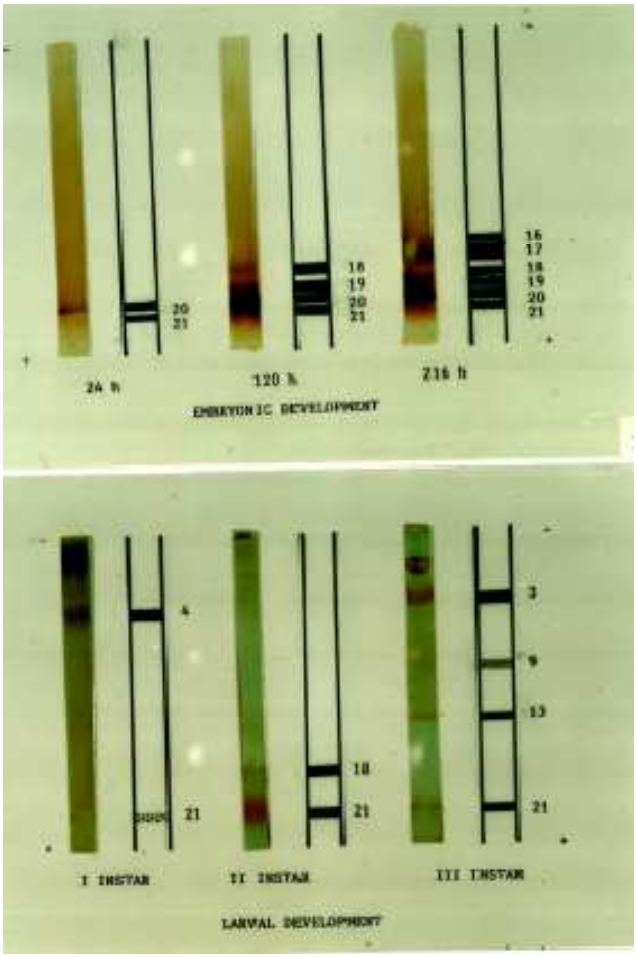

Figure 4: Acid Phosphatase of Zymogram R_2.

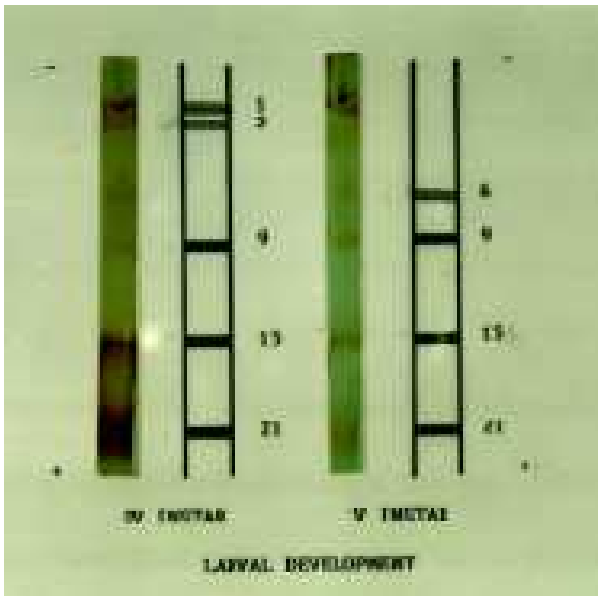

Figure 5: Acid Phosphatase of Zymogram R_2

DISCUSSION

The occurrence of phosphatases in silkworm Bombyx mori were first reported by Nakamura (1940),[18] later many workers studied these enzymes.[19-21] 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.[22,23] 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.[24] 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.[25]

The changes in ACPH zymograms during development revealed a total of 17 bands in R_1 and 21 bands in R_2 recorded for ACPH. From the present results it was clear that ACPH activity was low in R_1 and R_2. ACPH activity was higher in R_1. Whereas, in R_2 moderate ACPH activity was exhibited in larval as well pupal stages. In pupal stage the activity was less in male and female pupae of R_1& R_2 races. Here also sexual dimorphism has been observed. The present results also agree with the findings of Hedge and Krishnamurthy (1980) where the activity of ACPH was low in eggs but increased gradually towards V instar larvae and it was still decreased in later stages.[26]

The phosphatases are mainly concerned with the digestion of the ingested food and degradation of the cellular substances.[27]The fact that the silkworm egg shows neither digestive activity nor degradation of cellular substances accounts for low activity of ACPH 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. ACPH-4 and ACPH-5 zones in R_1 and ACPH-3, ACPH-4 and ACPH-6 zones in $R_2$are non-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 codominent alleles. Subsequently genetic and developmental relationship between larval and pupal APH were investigated by Willis and Fox (1968).[28] Genetic inheritance of polymorphic m-APH in Bombyx midgut was studied by.[13] The functions of both APH and ACPH have been well reported in the silkworm.[29]

CONCLUSIONS

In conclusion, changes in ACPH zymograms during development revealed a total of 17 bands in R_1 and 21 bands in R_2 recorded for ACPH. From the present results it was clear that ACPH activity was low in R_1 and R_2. ACPH activity was higher in R_1. Whereas, in R_2 moderate ACPH activity was exhibited in larval as well pupal stages. In pupal stage the activity was less in male and female pupae of R_1& R_2 races.

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