Journal of Entomology and Zoology Studies

Available online at www.entomoljournal.com

E-ISSN: 2320-7078
P-ISSN: 2349-6800
JEZS 2015; 3(5): 325-328

Received: 29-08-2015
Accepted: 02-10-2015

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Studies on non-specific α-esterases in two major ecoraces of tropical tasar silkworm *Antheraea mylitta* Drury

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Abstract
Tropical tasar silkworm *Antheraea mylitta* exhibits diversity in the phenotypic, physio-genetic, behavioural and commercial characters due to diverse habitats. The variability in four populations belonging to two major ecoraces of *A. mylitta* viz., Daba and Laria was analysed by quantitative assay and electrophoretic profile of non-specific α-esterases in haemolymph samples, also thermo-stable esterases were explored in these populations. An apparent variability was recorded, indicating high heterogeneity with respect to the food plant, region and sex. Higher esterase activity was found in Laria female (4.96±0.63 µmole $min^{-1}g^{-1}$) and lowest activity (2.68±0.43 µmole $min^{-1}g^{-1}$) was recorded in the male Daba. Comparative analysis of α-esterase in two populations, showed a variation in number of bands and in the staining intensity. The three esterase bands with R_f values 0.18, 0.44 & 0.49 were prominently appeared when the gels were incubated at 35 °C followed by staining. This appears to be the heat tolerant esterase.

Keywords: α-esterases, *Antheraea mylitta,* PAGE, Thermo-stable, Ecoraces

Introduction
Tropical tasar silkworm *Antheraea mylitta* Drury is an economically important sericigenous insect producing non-mulberry tasar silk. The tasar culture is essentially forest and agro-based activity that covers agricultural and industrial applications. It covers cultivation of host plants, production of silkworm eggs, silkworm rearing, cocoon production, reeling, spinning, weaving and utilization of silk waste and other by-products. The end product of the industry is tasar fabric. *Antheraea mylitta* which belongs to the superfamily Bombycoidea and family Saturniidae has a wide distribution within the country. In India the range of distribution of this species covers Assam, Himachal Pradesh, Sikkim, Meghalaya, Manipur, Nagaland, West Bengal, Orissa, Bihar, Jharkhand, Chhattisgarh, Madhya Pradesh, Karnataka, Maharashtra, Rajasthan, Tamilnadu, Pondicherry, Kerala, Uttar Pradesh, Jammu & Kashmir and Andhra Pradesh [1].

The tropical tasar silkworm populations inhabiting different ecological and geographical regions, exhibit extensive phenotypic variability due to which they are known as 'eco-races'. About 44 ecoraces have been recognized throughout the India. Distribution of ecoraces in relation to the forest type indicates that the races are restricted mainly in the tropical moist deciduous forest area, where the average rainfall varies between 1200 and 2000 mm. The geographic range of population distribution is limited mainly in five types of soil viz., red loamy, sandy red, black clayey, laterite and forest hill. However, majority of the ecoraces are distributed in red loamy and black clayey soil. *A. mylitta* spawns diversity in the phenotypic, physio-genetic, behavioural and commercial characters due to diverse ecological niches [2-5].

Different approaches were used to evaluate genetic diversity between and among different insect species including morphological traits, isozyme profile and PCR based molecular markers. Enzyme markers have been found to be especially suitable to differentiate between individuals with respect to their genetic structure as they are less changeable between individuals than other biochemical constituents of haemolymph or other tissues [6, 7]. This characteristic makes them good biochemical markers [8].

An isozyme study offers a promising tool to investigate morpho-genetic variation within the population [9]. The genotype of individual is apparent from its isozyme phenotype.

Moreover gene activity at the level of polypeptide product gives more precise genetic analysis. Hence, the genetic loci of the marker isozymes can be of immense help not only to distinguish the race but also to establish the genetic distance and evolutionary divergence of the ecoraces.

The esterases (EST, 3.1.1.2) are a very large class of enzymes and occur in numerous isoforms expressed by distinct gene loci that generally have a high degree of genetic variability. It is used as bio-indicators to measure the toxic potency of pesticide residues usually applied in agriculture. Also these enzymes are associated with stress and tolerance of some organisms [10, 11]. In the present study, for the first time, the variability in the esterase profile of two major populations of Daba and Laria ecoraces of tropical tasar silkworm *A. mylitta* D is reported.

Materials and Methods

Experimental samples

Experiments were carried out at Silkworm Breeding and Genetics laboratory of Central Tasar Research & Training Institute, Ranchi. The cocoons of four populations such as semi-domestic Daba & Laria collected from harvested cocoons at CTR&TI field Germplasm and cocoons of wild population *i.e.*, Daba and Laria were collected from West Singhbhum forest area and Peterbar forest area of Jharkhand respectively, subsequently preserved in the Grainage (place where cocoons are preserved for subsequent preparation of disease free layings). The haemolymph samples from male and female pupae of different populations were collected separately for further biochemical analysis.

Preparation of Samples for enzymatic studies:

The haemolymph was collected by cutting the pupa at head region and collected in a pre-cooled micro-centrifuge tube containing a pinch of phenylthiourea as an anti-melanizing agent [12]. Further, it was centrifuged at 5,000 rpm for 5 min at 4 °C. The supernatant was collected and stored at -20 °C until further use.

Estimation of Esterase

Esterase activity was measured, using 1- naphthylacetate as broad spectrum substrate for α-esterases [13]. The esterase activity was determined spectrophotometrically at room temperature (23 °C) by measuring the increase in absorbance at 322 nm. The reaction solution was containing 1.5 ml 0.1 M Tris/HCl buffer pH 7.4 and 30 µL of 1-naphthylacetate (100 mM 1-naphthylacetate prepared by dissolving in absolute methanol). For each solution, 200µL of crude enzyme extract was added. Spectrophotometric measurements were performed in 1.0 cm cuvettes for every 15 seconds (time-scale) over a three-min-period at fixed wave length of 322 nm. The esterase activities were corrected for spontaneous hydrolysis of 1-naphthylacetate. The activities were calculated using the molar absorption coefficients of $\varepsilon_{1\text{-naphthol (322 nm)}} = 2 \times 10^3\ M^{-1}\ cm^{-1}$ [9]. The absorption coefficients for 1- naphthol were corrected for the absorption coefficients of the acetate esters measured at the same wavelengths. The blank contained the buffer and corresponding naphthylacetate. The activity was expressed as units of hydrolysed substrate per min and per mg of protein (units min^{-1} mg^{-1} protein).

Electrophoretic study of esterase

Non-specific α-esterase was studied in the pupal haemolymph by native gel electrophoresis [14]. The relative mobility (R_f value) of esterase bands was calculated using the formula, R_f =distance of protein migration/distance of dye migration [15].

Study of Heat Stable esterases (HsEST)

Heat stable esterases (HsEST) were analysed in the pupal tissue and hemolymph samples [16]. After electrophoresis the normal gel were incubated separately in different temperatures 35 ºC, 45 ºC and 60 ºC for 15 minutes each in a hot water bath. The esterases maintaining the activity after incubation were regarded as HsEST. The gel was immersed in 0.5M Boric acid for 30 minutes and rinsed twice in ice-cold distilled water. The staining of esterases was done by using α-naphthyl acetate as substrate and fast blue RR. Subsequently, documentation and relative mobility was calculated for each esterase band.

Results

Quantitative assay of Esterases

The esterase activity was recorded in the pupal haemolymph of different ecoraces. Comparatively higher esterase activity was found in Laria wild female pupae followed by Daba wild female pupae (4.96±0.63 and 4.92±0.55 µmole $min^{-1}g^{-1}$ respectively). Lowest esterase activity (2.68±0.43 µmole $min^{-1}g^{-1}$) was recorded in the male pupa of Daba (Table 1).

Table 1: Quantitative analysis of esterase activity in the non-diapause pupae of different population of *Antheraea mylitta* D.

Tasar silkworm population	Enzyme activity (µmole $min^{-1}ml^{-1}$)
Daba reared male	2.68±0.43
Daba reared female	3.37±0.58
Daba wild male	2.90±0.49
Daba wild female	4.92±0.55
Laria reared male	3.10±0.19
Laria reared female	4.77±0.95
Laria wild male	3.42±0.44
Laria wild female	4.96±0.63

Qualitative profile of Esterases by PAGE

Comparative Electrophoretic analysis of alpha-esterase was studied using α-naphthyl acetate as substrate in the diapause pupal haemolymph samples of two populations of Daba and Laria ecoraces. Localization of esterase with α-naphthyl acetate showed variability among the different groups. Three bands are appeared prominently and the R_f value has been measured as R_f: 0.20 to 0.22, 0.50 to 0.58 and 0.90 to 0.96. More number of Esterase bands was noticed in the female hemolymph sample of Daba (wild) with four esterase bands. Three esterase bands each in Daba (semi-domestic) and Laria (reared). Esterase band with R_f value 0.93 appeared in all the batches but with the differential staining intensity (Figure 1).

Variability in the electrophoretic esterase profile was also studied in the non-diapause populations and presented in Fig 2. Appearance of esterase bands was less polymorphic and very few number of esterase bands were recorded. Bands with R_f value 0.42 and 0.83 was found to appear commonly in all the batches but with the variation in the staining intensity. Bands with R_f values 0.18, 0.22 & 0.33 showed variation among different samples.

Studies on heat stable esterases by PAGE

The presence of bands prominently after the gel treated with high temperature followed by non-specific staining designated as heat-stable esterases. The esterase activity sustained the high temperature conditions and stains with α-naphthyl acetate and fast blue RR. Three esterase bands (Fig 3) with R_f values 0.18, 0.44 & 0.49 were prominently appeared when the gels were incubated at 35 °C followed by staining in the almost all the batches studied with variability in the intensity of staining. The samples of wild population were stained with high

intensity compared to semi-domestic populations. Also a similar observation was made in the 65 °C temperature treatment with marginal changes in the appearance and R_f values except for Rf value 0.18 (Fig. 4). Other bands recorded at 0.24, 0.49, 0.50 & 0.52 with significant variations between the batches studied.

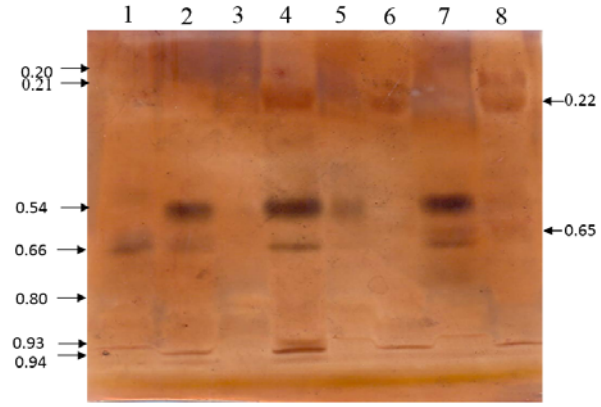

Fig 1: Native PAGE analysis of diapause haemolymph esterase isozyme of different population of *Antheraea mylitta* D. Lane: 1- Daba reared male, 2- Daba reared female, 3- Daba wild male, 4- Daba wild female, 5- Laria wild male, 6- Laria wild female, 7- Laria reared female and 8- Laria reared male

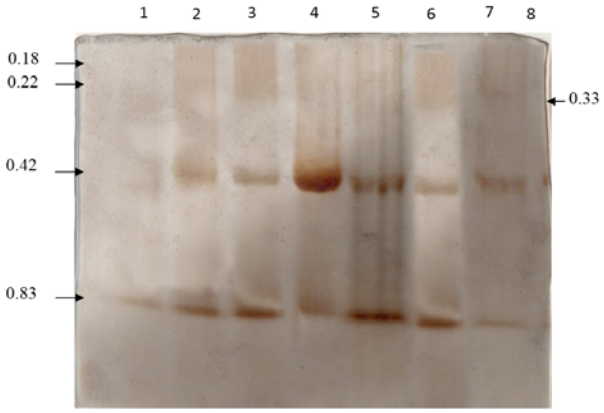

Fig 2: Native PAGE analysis of non-diapause haemolymph esterase isozyme of different population of *Antheraea mylitta* D. Lane: 1- Daba reared male, 2- Daba reared female, 3- Daba wild male, 4- Daba wild female, 5- Laria wild male, 6- Laria wild female and 7- Laria reared male and 8-Laria reared female

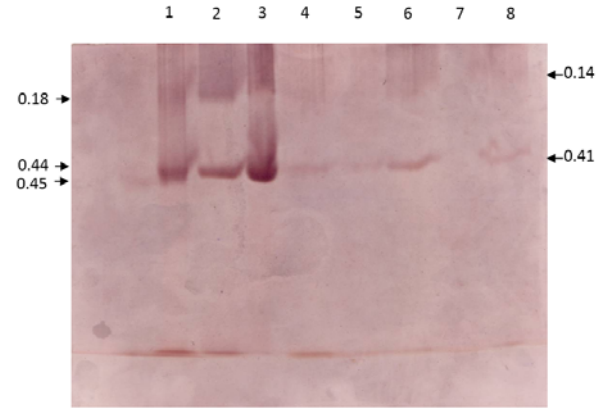

Fig 3: Native PAGE analysis of non-diapause haemolymph heat stable (at 35 ^{0}C) esterase isozyme of different population of *Antheraea mylitta* D. Lane: 1- Daba reared male, 2- Daba reared female, 3- Daba wild female, 4- Daba wild male, 5- Laria wild male, 6-Laria wild female, 7-Laria reared male and 8-Laria reared female

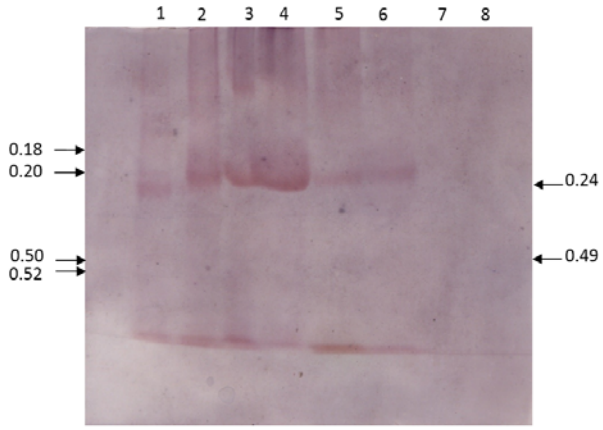

Fig 4: Native PAGE analysis of non-diapause haemolymph heat stable (at 65 ^{0}C) esterase isozyme of different population of *Antheraea mylitta* D. Lane: 1- Daba reared male, 2- Daba reared female, 3- Daba wild female, 4- Daba wild male, 5- Laria wild male, 6-Laria wild female, 7-Laria reared male and 8- Laria reared female

Discussion

Silkworms are generally phytophagous in nature and feeds on leaves of the food plants, which are the sole food of the silkworm. The food contains proteins to a major extent besides minor constituents; carbohydrates, lipids, sterols, vitamins etc. The consumption of leaf during final larval instar accounts for more than 80% of the total consumption during its larval life. Food consumed in this stage is effectively utilized for the production of silk proteins as well as to support its metabolism. Thus the energy acquired by the larvae as a consequence of feeding is utilized in the subsequent non-feeding stages. In insects the complex food molecules are utilized after they have been processed into simpler molecules through the action of digestive enzymes in the gut of the larva. Thus, the enzyme system in the silkworms play a vital role in determining the performance of the silkworm in terms of effective transformation of organic food molecules of the leaf into useful biomolecules of the silkworm.

The different races of silkworm of Indian origin, exotic, multivoltine, bivoltine and their hybrids have been tested for a few enzyme systems which bring about certain groups of chemical reactions among silkworm races, in which amylases, proteases, esterases and others have been detected by a technique specially developed for the purpose [17].

Esterases are more suitable biochemical markers of developmental processes and have greater number of expressed isoforms [9]. The variable esterase activity in different samples is in corresponding with the variability in the population. Since Laria is recognized as wild (Sal based) ecorace of *A. mylitta*, it has expressed higher esterase activity to cope up with several natural vagaries viz., digestion of complex nutritional materials, detoxification of xenobiotics [18, 10, 11] and in reproduction [19].

The esterase spectra in different tissues of silkworm *Bombyx mori* have been studied earlier [20, 21] but no report of electrophoretic analysis of esterases is available in tasar silkworm *Antheraea mylitta* D. In the present study, the comparative electrophoretic analysis of esterase activity in the pupal samples of different populations revealed polymorphic variations. The appearance of three constitutive bands of R_f values 0.20 to 0.22, 0.50 to 0.58 and 0.90 to 0.96 indicates the species specific esterases and this could also be homogeneous distribution of non-specific esterases. Similar findings were reported in the *Bombyx mori* [16, 22]. Esterase band with R_f value 0.96 was intensely stained which depicts the higher

expression level of the enzyme [23], this could be one of the markers to unravel the esterase polymorphism in different ecoraces of tasar silkworm. The esterase of hemolymph of silkworm is under the polygenic control and does not show sex specificity [8].

The appearance of bands after treating the gel with high temperature and subsequent staining with α/β –naphthyl acetate and fast blue RR designated to be the heat stable esterases [16]. In the present experiment, appearance of prominent bands with the R_f values 0.18, 0.44 & 0.49 after the gel was treated at 35 °C and 65 °C attributes the presence and expression of some heat-stable esterases in the pupae of tropical tasar silkworm. The differential intensity of the staining of these bands, wherein higher intensity recorded at 35 °C compared to 65 °C suggests that the esterase activity gets gradually degraded at higher temperatures. Also, variability in the esterase activity in response to heat treatment was recorded with different batches studied; this could be due to the genetic makeup of the ecoraces in withstanding the high temperature (Lokesh *et al*, 2013) [24].

It is evident that there is an apparent variability in different populations of *Antheraea mylitta*, expressing high heterogeneity with respect to the food plant, region and also in sexual dimorphism. Quantitative and qualitative analysis of esterase can be used as a tool for the characterization of different populations of *Antheraea mylitta.* Besides, Esterase was found to be a suitable marker to investigate population diversity, inter-ecoracial polymorphism and also to explore analysis of heat-stable properties in silkworms. Also, the esterase may be used as marker molecules in the evolution of new thermo tolerant breeds of *Antheraea mylitta* with better sustainability.

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