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Studies on the Correlation between Protein, Amylase, Succinate Dehydrogenase, Esterase and Alkaline Phosphatase of Silkworm *Bombyx mori* L.

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ABSTRACT

Four pure mulberry silkworm breeds viz., Pure Mysore, Nistari, NB_4D_2 & CSR_2 and two hybrid (Pure Mysore x CSR_2 and Nistari x NB_4D_2) silkworms were selected for the present study. The total soluble proteins, specific activity levels of amylase, succinate dehydrogenase, esterase and alkaline phosphatase in haemolymph, mid gut as well as fat body tissues were estimated during fifth instar with a regular interval of 24 h. The average values of biochemical studies were subjected to regression analysis against each other individually to know the level and kind of correlation between them. Of the 91 results of regression analysis, 48 exhibited positive, 40 revealed negative and 3 showed neutral status. Of the positive correlation, 7 exhibited highly positive correlation.

***Keywords**: Proteins, succinate dehydrogenase, esterase, alkaline phosphatase, correlation.*

INTRODUCTION

Ever since its inception, more than four thousand years ago, Sericulture is playing an important role in the economic life of man[1]. Due to their great economic value, more than 3000 genetically different silkworm (*Bombyx mori*) strains are maintained in Europe and Asia[2]. To improve the quantity and quality of silk, many attempts are being made to improve the silkworm stocks through genetic manipulation. The conventional breeding programmes have contributed substantially by the introduction of improved silkworm breeds[3]. In conventional breeding, the parental selection and performance prediction is on the basis of either their performance[4] or performance of the progeny[5, 6]. Recent advances in plant and animal breeding have highlighted the prospects of using linked molecular markers (Isozyme/DNA) for improvement of desirable traits. Therefore, identification of suitable markers, holds the key to successful implementation of marker assisted selection (MAS) which is gaining ground fast in other fields of breeding (C.F.)[3]. Such supportive correlation studies in silkworm *Bombyx mori* were reported by several scientists *viz.*, correlation between yield and biochemical parameters[7], between commercial characters with proteins[8], commercial characters with amylase[9], commercial characters with esterase[10], commercial characters with alkaline phosphatase[11], commercial characters with succinate dehydrogenase[12] and commercial characters with fat body biomolecules[13]. Also, correlation studies between DNA, RNA & proteins of silkworm *Bombyx mori*[14] were reported. However, correlation studies among biomolecules like proteins, amylase, succinate dehydrogenase, esterase and alkaline phosphatase of silkworm *Bombyx mori* are rather scarce. Hence, the present investigation was undertaken.

MATERIALS AND METHODS

Four pure mulberry silkworm breeds *viz.*, Pure Mysore, Nistari, NB_4D_2 & CSR_2 and two hybrid (Pure Mysore x CSR_2 and Nistari x NB_4D_2) silkworms were selected for the present investigation.

The silkworm rearing was conducted in the laboratory following the method described by Krishnaswamy[15]. All experimental batches were maintained in triplicate. In each replication 500 larvae were kept after third moult.

The silkworm larvae from first day of fifth instar were collected daily with a regular interval of 24h till the end of fifth instar. The abdominal legs were punctured and haemolymph was collected in a pre cooled micro centrifuge tubes containing 1 mM thiourea as described by Mahesha *et al.*[16], centrifuged at 3000 rpm for 5 minutes in a cooling centrifuge at 5°C and preserved in a deep freezer at -20°C as stock and it was used whenever required. The midgut and fat body tissue were obtained from five larvae daily with a regular interval of 24h till the end of fifth instar by dissecting the larvae in ice cold water. The tissue was thoroughly washed in sterile distilled water. A 10% (w/v) homogenate of the tissue was prepared separately in pre cooled distilled water using mortar and pestle. The homogenate was centrifuged at 3000 rpm for 10 minutes in a cooling centrifuge at 5ºC. The clear supernatant was used for the assay of total proteins and enzymes.

The total soluble proteins present in the haemolymph, midgut and fat body tissues were estimated by following the method of Lowry *et al.*[17]. Bovine Serum Albumin was used as standard protein. The values were expressed as µg of protein/ µl and µg of protein/mg for haemolymph and midgut as well as fat body tissues respectively.

Quantitative analysis of amylase activity was done by following the method of Noelting and Bernfeld[18] using 3, 5-dinitrosalicylic acid reagent, as modified by Ishaaya and Swirski[19]. The amylase activity was expressed as µ moles of glucose generated/mg protein/min at 37ºC.

Specific activity of succinate dehydrogenase was estimated by the method of Nachlas *et. al.*[20] with modifications[21,22]. The succinate dehydrogenase activity levels were expressed in micromoles of farmazan formed / mg protein / min at 37ºC.

The quantitative analysis of non specific esterase activity was done by following the method of Valdes and Chambers[23] with slight modifications[10]. The esterase activity levels were expressed as µmoles of paranitrophenol released /mg protein/min at 37ºC. Paranitrophenol was used as standard.

The quantitative analysis of alkaline phosphatase was done by following method. The reaction mixture contained 1ml of 0.1M sodium carbonate buffer (pH 10) containing 50 mM paranitrophenol phosphate was incubated at 37ºC for 5 min. After this pre incubation, appropriately (1:10) diluted 10 µl haemolymph for haemolymph alkaline phosphatase assay and 10 µl tissue (0.5 %) extract for midgut and fat body alkaline phosphatase assay respectively. Incubation of this mixture was carried out for 30 min at 37°C in a water bath. After 30 min, 2 ml of 0.1 N NaOH was added and the contents were mixed thoroughly. Then the volume was made up to 4 ml with buffer. The contents were shaken vigorously and the optical density was measured at 540 nm setting the spectrophotometer to zero with blank consisted of incubation mixture to which enzyme sample was added after termination of the reaction. The alkaline phosphatase activity levels were expressed as µmoles of paranitrophenol released /mg protein/min at 37ºC. Paranitrophenol was used as standard.

The experimental data were statistically analyzed through SPSS by one way ANOVA[24], Scheffe's post hoc test[25] and linear regression analysis using the formula $Y = b X + a$ [26] wherever they were applicable.

RESULTS AND DISCUSSION

The concentration of protein and specific activity levels of amylase, succinate dehydrogenase, esterase and alkaline phosphatase in silkworm haemolymph is presented in table 1. In case of haemolymph proteins, highest concentration was observed in Pure Mysore x CSR_2 (45.62 µg/µl haemolymph was the average during fifth instar) followed by CSR_2 (44.03 µg/µl), Nistari x NB_4D_2 (43.28 µg/µl), NB_4D_2 (42.8 µg/µl), Nistari (39.93µg/µl) and Pure Mysore (36.85µg/µl). In case of amylase, the CSR_2 larvae showed highest activity levels (0.156 µM/mg protein/min at 37°C was the average during fifth instar), followed by NB_4D_2 (0.151 µM/mg/min at 37°C), Pure Mysore x CSR_2 (0.117 µM/mg/min at 37°C) , Pure Mysore (0.116 µM/mg/min at 37°C), Nistari x NB_4D_2 (0.112 µM/mg/min at 37°C) and Nistari (0.109 µM/mg/min at 37°C).

The highest succinate dehydrogenase activity was observed in Pure Mysore x CSR_2 (2.72 µM/mg protein/min at 37°C was the average during fifth instar), followed by NB_4D_2 (2.68 µM/mg/min at 37°C), Pure Mysore (2.58 µM/mg/min at 37°C), CSR_2 (2.57 µM/mg/min at 37°C), Nistari x NB_4D_2 (2.46 µM/mg/min at 37°C) and Nistari (1.87 µM/mg/min at 37°C). In case of esterase, the highest activity was observed in Pure Mysore (8.92 µM/mg protein/min at 37°C was the average during fifth instar) followed by CSR_2 (8.60 µM/mg/min at 37°C), Pure Mysore x CSR_2 (8.53 µM/mg/min at 37°C), NB_4D_2 (7.59 µM/mg/min at 37°C), Nistari x NB_4D_2 (7.33 µM/mg/min at 37°C) and Nistari (6.55 µM/mg/min at 37°C). The alkaline phosphatase activity in haemolymph was found to be nil.

The concentration of protein and specific activity levels of amylase, succinate dehydrogenase, esterase and alkaline phosphatase in silkworm midgut tissue is presented in table 2. In midgut tissue, the highest concentration of protein was observed in Pure Mysore x CSR_2 (33.82 µg/mg tissue was the average during fifth instar) followed by Nistari x NB_4D_2 (29.99 µg/mg), Pure Mysore (23.99 µg/mg), CSR_2 (23.24 µg/mg), NB_4D_2 (21.64 µg/mg/min) and Nistari (20.39 µg/mg). In the case of amylase, the highest activity was observed in Pure Mysore (0.066 µM/mg protein/min at 37°C was the average during fifth instar), followed by CSR_2 (0.064 µM/mg/min at 37°C), Pure Mysore x CSR_2 (0.062 µM/mg/min at 37°C), Nistari (0.060 µM/mg/min at 37°C), NB_4D_2 and Nistari x NB_4D_2 (0.058 µM/mg/min at 37°C). The highest specific activity levels of succinate dehydrogenase in midgut tissue was observed in NB_4D_2 (2.94 µM/mg protein/min at 37°C was the average during fifth instar), followed by CSR_2 (2.90 µM/mg/min at 37°C), Nistari (2.86 µM/mg/min at 37°C), Pure Mysore x CSR_2 (2.83 µM/mg/min at 37°C), Pure Mysore (2.76 µM/mg/min at 37°C) and Nistari x NB_4D_2 (2.53 µM/mg/min at 37°C). In the case of esterase, the highest activity was observed in NB_4D_2 (11.43 µM/mg protein/min at 37°C was the average during fifth instar) followed by CSR_2 (11.36 µM/mg/min at 37°C), Nistari (10.23 µM/mg/min at 37°C), Pure Mysore (9.32 µM/mg/min at 37°C), Nistari x NB_4D_2 (8.72 µM/mg/min at 37°C) and Pure Mysore x CSR_2 (8.22 µM/mg/min at 37°C). In the case of alkaline phosphatase, the highest activity was observed in Nistari (9.38 µM/mg protein/min at 37°C was the average during fifth instar) followed by Pure Mysore (8.41 µM/mg/min at 37°C), NB_4D_2 (8.38 µM/ mg/ min at 37°C), CSR_2 (8.15 µM/mg/min at 37°C), Pure Mysore x CSR_2 (7.17 µM/ mg/ min at 37°C) and Nistari x NB_4D_2 (6.79 µM/mg/min at 37°C).

The concentration of protein and specific activity levels of amylase, succinate dehydrogenase, esterase and alkaline phosphatase in silkworm fat body is presented in table 3. In the case of fat body tissue, the highest concentration of protein was observed in CSR_2 (44.03 µg/mg tissue was the average during fifth instar), followed by Pure Mysore x CSR_2 (34.07µg/mg), Nistari x NB_4D_2 (29.45 µg/mg), Pure Mysore (26.47 µg/mg), NB_4D_2 (26.26 µg/mg) and Nistari (22.63 µg/mg). The highest amylase activity in fat body was observed in Nistari x NB_4D_2 (0.065 µM/mg protein/min at 37°C was the average during fifth instar), followed by Pure Mysore x CSR_2 (0.058 µM/mg/min at 37°C), Pure Mysore (0.055 µM/mg/min at 37°C), NB_4D_2 (0.052 µM/mg/min at 37°C) and CSR_2 as well as Nistari (0.051 µM/mg/min at 37°C). In the case of fat body succinate dehydrogenase, the highest activity was observed in Nistari x NB_4D_2 (3.14 µM/mg protein/min at 37°C), followed by Pure Mysore X CSR2 (3.13 µM/mg/min at 37°C), Nistari (3.08 µM/mg/min at 37°C), Pure Mysore (2.83 µM/mg/min at 37°C), CSR_2 (2.73 µM/mg/min at 37°C) and NB_4D_2 (2.58 µM/mg/min at 37°C). In the case esterase, the highest activity was observed in Nistari x NB_4D_2 (7.76 µM/mg protein/min at 37°C) followed by Pure Mysore x CSR_2 (7.41 µM/mg/min at 37°C), NB_4D_2 (7.24 µM/mg/min at 37°C), CSR_2 (7.05 µM/mg/min at 37°C), Pure Mysore (6.77 µM/mg/min at 37°C) and Nistari (6.70 µM/mg/min at 37°C). The highest alkaline phosphatase activity in fat body was observed in Nistari x NB_4D_2 (6.72 µM/mg proetin/min at 37°C was the average during fifth instar), followed by NB_4D_2 (6.47 µM/mg/min at 37°C), Pure Mysore (6.24 µM/mg/min at 37°C), Nistari (6.2 µM/mg/min at 37°C), CSR_2 (6 µM/mg/min at 37°C) and Pure Mysore x CSR_2 (5.82 µM/mg/min at 37°C).

The summary of regression analysis among biomolecules is presented in table 4. Of the 91 analysis, 48 exhibited positive, 40 revealed negative and 3 showed neutral status. Of the positive correlation, 7 exhibited highly positive correlation *i.e.*, above 0.5 correlation co-efficient.

The proteins in all viable cells, as nucleoproteins, are essential to the cell division and as enzymes and hormones are essential to control many chemical reactions in the cell metabolism[27]. In other words, proteins enter at various reactions such as the hormonal regulation and they integrated in the cell as a structural element at the same time as the carbohydrates and the lipids[28, 29]. Such as an important biomolecule exhibited positive correlation with proteins of different sources. Also, haemolymph proteins showed positive correlation with haemolymph and fat body amylase. Midgut proteins showed highly positive correlation with fatbody amylase only. Fatbody protein exhibited positive correlation with haemolymph amylase and midgut amylase only. Proteins from all the three sources exhibited positive correlation with succinate dehydrogenase activity level except between midgut protein and midgut succinate dehydrogenase as well as fat body protein and fat body succinate dehydrogenase. In case of proteins and esterase activity levels also, positive correlation was noticed except between haemolymph proteins and haemolymph esterase as well as midgut protein and midgut esterase. The alkaline phosphatase activity in haemolymph was nil. However, the haemolymph proteins and fatbody proteins showed positive correlation with midgut alkaline phosphatase activity levels. Though the insect tissues *viz.*, midgut and fatbody are bathed in the haemolymph the source for the synthesis of these biomolecules in these tissues may be from different origin. Because haemolymph is the only extracellular fluid in insects has diverse functions[30] such as immunity, transport and storage[31] of the products required for cellular metabolism. However, some of the biomolecules exhibited statistically significant correlation with haemolymph proteins and some other showed self tissue correlations. Amylase is one of the key enzymes involved in digestion and carbohydrate metabolism in insects[32, 33, 34]. Such an enzyme showed statistically significant negative correlation between fat body and haemolymph as well as midgut amylase activity levels. Again the amylase enzyme showed statistically significant positive correlation with succinate dehydrogenase of all sources except between fat body succinate dehydrogenase and haemolymph as well as midgut amylase. However, midgut amylase expressed highly negative correlation with fat body succinate dehydrogenase. Such a correlation between digestive and oxidizing enzymes in silkworm larvae might help in the utilization of more food material and efficient conservation of digested food material, ultimately leading to superior economic traits[35]. The amylase activity levels showed statistically positive correlation with esterase from different sources except haemolymph esterase and fatbody amylase, midgut esterase and midgut as well as fat body amylase, fat body esterase and hamolymph as well as midgut amylase. As esterases are a group of enzymes which catalyze the hydrolysis of various types of acetyl esters[36] the correlation coefficient is found to be strong with exceptions. In case of alkaline phosphatase and amylase activity levels of the 6 analysis three showed positive and remaining three negative correlations. Succinate dehydrogenase is one of the Kreb's cycle enzymes and activities may be correlated with the level of oxidation in a particular tissue. Of the results of regression analysis among succinate dehydrogenase from different sources 2 results showed statistically significant negative correlations. Also, of the 9 correlation studies between succinate dehydrogenase and esterases of different sources 5 showed positive correlation and remaining 4 are negative. The correlation among esterase of different sources was found to be negative. In case of succinate dehydrogenase and alkaline phosphatase, the correlation between midgut alkaline phosphatase and midgut Succinate dehydrogenase found to be statistically significant. Esterases catalyze the hydrolysis of various types of acetyl esters. Among the esterases from different sources negative correlations were observed. Alkaline phosphatase is a set of hydrolytic enzymes, which hydrolyze phosphomonoesters under the alkaline condition. The activity level during various stages of ontogeny reflects the generation, utilization of energy for efficient assimilation. The correlation between midgut and fat body alkaline phosphates was found to be negative.

In view of the above, this study helps us to understand the biomolecular variations in some of the selected silkworm varieties. Also it is useful to reveal the relationship/correlation with each other from same as well as different tissues. The information gathered from this work will be useful during the breeding of new silkworm strains with better traits and also contributes to silkworm biochemistry and physiology in general.

Table 1: Average concentration of protein and specific activity levels of amylase, succinate dehydrogenase, esterase and alkaline phosphatase of silkworm haemolymph during fifth instar

Biomolecules / Silkworm Breeds	Proteins#	Amylase*	Succinate dehydrogenase*	Esterase*	Alkaline phosphatase*
Pure Mysore	36.85	0.116	2.58	8.92	Nil
Nistari	39.93	0.109	1.87	6.55	Nil
CSR_2	44.03	0.156	2.57	8.60	Nil
NB_4D_2	42.80	0.151	2.68	7.59	Nil
Pure Mysore x CSR_2	45.62	0.117	2.72	8.53	Nil
Nistari x NB_4D_2	43.28	0.112	2.46	7.33	Nil

The variation between the races is statistically significant at 0.1 % ($P<0.001$).

#-The values expressed as µg of protein/ µl of haemolymph.

*-The activity levels expressed as µ moles of product generated/mg protein/min at 37ºC.

Table 2: Average concentration of protein and specific activity levels of amylase, succinate dehydrogenase, esterase and alkaline phosphatase of silkworm midgut during fifth instar

Biomolecules / Silkworm Breeds	Proteins#	Amylase*	Succinate dehydrogenase*	Esterase*	Alkaline phosphatase*
Pure Mysore	23.99	0.066	2.76	9.32	8.41
Nistari	20.39	0.060	2.86	10.23	9.38
CSR_2	23.24	0.064	2.90	11.36	8.15
NB_4D_2	21.64	0.058	2.94	11.43	8.38
Pure Mysore x CSR_2	33.82	0.062	2.83	8.22	7.17
Nistari x NB_4D_2	29.99	0.058	2.53	8.72	6.79

The variation between the races is statistically significant at 0.1 % ($P<0.001$).

#-The values expressed as µg of protein/ mg of midgut tissue.

*-The activity levels expressed as µ moles of product generated/mg protein/min at 37ºC.

Table 3: Average concentration of protein and specific activity levels of amylase, succinate dehydrogenase, esterase and alkaline phosphatase of silkworm fat body during fifth instar

Biomolecules / Silkworm Breeds	Proteins#	Amylase*	Succinate dehydrogenase*	Esterase*	Alkaline phosphatase*
Pure Mysore	26.47	0.055	2.83	6.77	6.24
Nistari	22.63	0.051	3.08	6.70	6.20
CSR_2	44.03	0.051	2.73	7.05	6.00
NB_4D_2	26.26	0.052	2.58	7.24	6.47
Pure Mysore x CSR_2	34.07	0.058	3.13	7.41	5.82
Nistari x NB_4D_2	29.45	0.065	3.14	7.76	6.72

The variation between the races is statistically significant at 0.1 % ($P<0.001$).

#-The values expressed as µg of protein/ mg of fat body tissue.

*-The activity levels expressed as µ moles of product generated/mg protein/min at 37ºC.

Table 4: Summary of the Regression Analysis among Biomolecules. The values expressed in r^2

		Protein			Amylase			Succinate dehydrogenase			Esterase			Alkaline phosphatase		
		H	M	F	H	M	F	H	M	F	H	M	F	H	M	F
Proteins	H	#	**+0.312**	**+0.358**	**+ 0.123**	-0.154	**+0.060**	**+ 0.152**	**+0.004**	**+0.019**	0.000	**+0.002**	**+0.495**	Activity-NIL	**+0.362**	-0.048
	M	*	#	**+ 0.080**	-0.132	-0.001	**+0.597**	**+0.225**	-0.261	**+0.376**	**+0.102**	-0.622	**+0.528**		-0.789	-0.030
	F	*	*	#	**+0.325**	**+0.146**	-0.002	**+0.222**	**+0.022**	-0.027	**+0.332**	**+0.025**	**+0.055**		**+0.148**	-0.229
Amylase	H	*	*	*	#	**+0.001**	-0.267	**+0.216**	**+0.559**	-0.723	**+0.087**	**+0.650**	0.000		**+0.011**	-0.012
	M	*	*	*	*	#	-0.089	**+0.044**	**+0.035**	-0.024	**+0.624**	-0.004	-0.302		**+0.030**	-0.377
	F	*	*	*	*	*	#	**+0.054**	-0.834	**+0.381**	0.000	-0.597	**+0.644**		-0.736	**+0.203**
Succinate dehydrogenase	H	*	*	*	*	*	*	#	**+0.001**	-0.156	**+0.582**	-0.009	**+0.222**		-0.367	-0.015
	M	*	*	*	*	*	*	*	#	-0.375	**+0.013**	**+0.455**	-0.316		**+0.399**	-0.330
	F	*	*	*	*	*	*	*	*	#	-0.092	-0.672	**+0.087**		-0.140	-0.003
Esterase	H	*	*	*	*	*	*	*	*	*	#	-0.02	-0.001		-0.092	-0.242
	M	*	*	*	*	*	*	*	*	*	*	#	-0.162		**+0.369**	**+0.003**
	F	*	*	*	*	*	*	*	*	*	*	*	#		-0.832	**+0.145**
Alkaline phosphatase	H	Activity – NIL														
	M	*	*	*	*	*	*	*	*	*	*	*	*		#	-0.024
	F	*	*	*	*	*	*	*	*	*	*	*	*		*	#

H- Haemolymph; M- Midgut; F- Fat body. * - values are presented in other direction; # - No Correlation with in the same biomolecule.

Positive Correlations are shown in bold letters. The underlined bold figure shows highly positive correlations.

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