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Transaminases activity in the hemolymph: Biomarkers determining the thermal stress in the new bivoltine lines of *Bombyx mori*

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ABSTRACT

Varying atmospheric temperatures invariably induce thermal stress in the silkworm, *Bombyx mori*, which influence the functionality of aspartate aminotransferase (AST) and alanine aminotransferase (ALT). Thus, we have investigated AST and ALT activity in the new bivoltine lines (NBL). Interestingly, day-3 fifth instar larvae of NBL-5 being more susceptible to the thermal stress of 45°C showed highest level of AST (11.64 µm/mL) and ALT (9.83 µm/mL) activity in their hemolymph. Consequently, on day-5, AST (3.54 µm/mL) and ALT (1.39 µm/mL) activity were lower than day-3. Comparatively, between NBL and its parental breeds, the AST and ALT activity were found to be higher in day-3 (12.41 and 10.90 µm/mL) and day-5 (4.02 and 1.69 µm/mL) larvae of CSR_2, while it was 12.93 and 10.14 µm/mL in CSR_{27} on day-3, and 4.31 and 2.03 µm/mL on day-5, respectively. This salient finding validates the linkage between the rate of cytotoxicity and AST and ALT enzyme activity in relation to the thermotolerance in NBL and its parents. Thus, the silkworm larvae can be considered as a model system for rapid evaluation of cytotoxicity and varied levels of AST and ALT activity as one of the biomarkers to correlate well with the tolerance level to critical temperature.

1. INTRODUCTION

Tolerance to thermal stress is often referred to ability of an organism survive under extreme temperature stress by synthesis of heat shock proteins (HSPs) [1]. Due to involvement in several physiological activities including synthesis, transport, and folding of proteins, HSPs are generally referred to as molecular chaperones [2]. The silkworm *Bombyx mori*, being domesticated over 7500 years [3], has become more susceptible to the fluctuating environmental temperature. Moreover, environmental changes, such as global warming, not only adversely affect silkworm viability but also unfavorable rearing temperatures impede the larval growth and development, and outbreak of several diseases. Unlike polyvoltine strains, lack of tolerance to such thermal stress is accountable for poor performance of the bivoltine strains under tropical conditions or censorious environmental conditions [4]. Interestingly, changes occur during thermal stress with the production of HSPs insulate and minimize the harsh effect induced by the temperature in the silkworm [5].

Basically, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are the two important components of amino acid catabolism, mainly involved in transferring an amino group from one amino acid to another keto acid. In addition, the activity of AST and ALT is under the influence of various physiological and pathological conditions and serves as a strategic link between the carbohydrate and protein metabolism [6]. To diagnose the tissue damage in heart and the liver in humans, the level of AST and ALT in the serum has been considered as a prominent biomarker [7]. Thus, bacterial infection is determined with the increased transaminase activity in the silkworm hemolymph [8].

Although, the thermal stress known to induce tissue damage in the silkworm, especially in the fat body, midgut, silk gland, and cuticle but no empirical evidences are available to emphasis altered transaminases activity in the new bivoltine lines (NBL) that are developed by induction of heat shock at 45°C. Thus, this investigation assess the variation in the transaminase activity in response to the induced thermal stress in different lines of NBL and its parentage to develop them as a pertinent biomarkers to identify not only temperature tolerant breeds but also screen potent breeds for tropics.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Silkworm

Six NBL of *B. mori* – NBL-2, NBL-4, NBL-5, NBL-9, NBL-10A, and NBL-10B developed employing heat shock technology (HST) [5,9] along with their parental breeds $CSR_2 \times CSR_{27}$ were used in the present

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investigation. The NBL used in the present study were stabilized over ten generations by inducing multigenerational thermal stress at 45°C. The disease free layings of NBL were incubated under optimum environmental conditions (25 ± 1°C temperature and 75 ± 5% relative humidity) followed by black-boxing and till hatching. The larvae were reared on mulberry leaves following standard rearing procedure [10].

2.2. Methods

2.2.1. Induction of thermal stress condition

The larvae of fifth instar day-3 and day-5 were selected for the induction of thermal stress. The larvae placed in an open plastic Petri dishes were exposed to 45°C heat shock with 75 ± 5% relative humidity in a water bath for 2 h, followed by 2 h of incubation at room temperature as a recovery period. The hemolymph was drawn from the experimental larvae (10 larvae per replication) soon after 2 h of recovery period into a prechilled Eppendorf tube mixed with thiourea and preserved at −20°C for ALT and AST assays.

2.2.2. Estimation of AST activity

The silkworm larval hemolymph AST assay was performed following the protocol prescribed by Reitman and Frankel [11] using pyruvic acid as standard. After incubating at 37°C for 1 h, the enzyme substrate mixture was treated with 2, 4-dinitrophenyl hydrazine solution and then 0.4 N Sodium hydroxide. 10 min after, the intensity of the color developed was measured at 520 nm in a spectrophotometer, wherein the amount of oxaloacetate formed as an index of AST level. The final estimation was done by plotting a standard curve and the results were expressed in micro mole (µm) of oxaloacetate per ml of hemolymph [12].

2.2.3. Estimation of ALT activity

ALT assay was conducted using hemolymph of silkworm larvae as a sample following the protocol as described by Reitman and Frankel [11], wherein pyruvic acid was standard. After incubating at 37°C for 1 h, the enzyme substrate mixture was treated with 2, 4-dinitrophenyl hydrazine solution followed by 0.4 N sodium hydroxide. The intensity of color developed after 10 min was measured at 520 nm in a spectrophotometer which represents the amount of pyruvate formed as an index of ALT level. The final estimation was done by plotting a standard curve and the results were expressed in micro mole (µm/mL) of pyruvate per ml of hemolymph.

2.2.4. Statistical analysis

The data derived from all the experiments were subjected for one-way ANOVA analysis ($P \leq 0.05$) employing SPSS statistical package (Ver. 28.0).

3. RESULTS

3.1. AST Activity in Fifth Instar Larvae after HS at 45°C

An elevation of 11.64 µm/mL AST level in the hemolymph of day-3 fifth instar larvae of NBL-5 exposed to a thermal stress of 45°C was reported, which is much higher than the AST level of 6.00 µm/mL observed in the respective control. In NBL-9, NBL-10A, NBL-4, and NBL-2, it was 9.18, 8.78, 8.67, and 7.39 µm/mL, respectively, which is higher compared to their respective control groups. Further, a lowest of 5.75 µm/mL was observed in NBL-10B subjected to thermal stress at 45°C against its control measuring 3.70 µm/mL [Figure 1].

Similarly, highest AST activity measuring 3.54 µm/mL was recorded on day-5 of fifth instar NBL-5 against its respective control accounting to 0.85 µm/mL. The activity level of AST in NBL-9 HS, NBL-10A HS, NBL-4 HS, and NBL-2 HS was 3.10, 2.16, 1.97, and 1.77 µm/mL, respectively, which is higher than their respective control batches. Further, the lowest AST activity of 1.09 µm/mL was observed in NBL-10B against its control measuring 0.40 µm/mL.

On the other hand, on exposure to thermal stress at 45°C, the parental breeds CSR_2 and CSR_{27} exhibit higher levels 12.41 and 12.93 µm/mL on day-3 against their control group of 6.65 and 7.74 µm/mL. Day-5 fifth instar HS larvae exhibit 4.02 and 4.31 µm/mL against their respective control group with 1.07 and 1.20 µm/mL, respectively. The data presented are statistically significant at $P < 0.05$.

3.2. ALT Levels on Day-3 and 5 of Fifth Instar Larvae after HS at 45°C

Exposure to the thermal stress at 45°C on day-3, the fifth instar larvae exhibit highest ALT activity accounting to 9.83 µm/mL in NBL-5 HS against control of 5.08 µm/mL. However, increased ALT activity of 7.70, 6.70, 5.94, and 5.84 µm/mL in the HS-induced larvae of NBL-9, NBL-10A, NBL-4, and NBL-2 against their respective control batches, respectively [Figure 2]. Lowest of 5.40 µm/mL was observed in NBL-10B HS against its control (3.30 µm/mL).

On day-5 of fifth instar, the ALT activity in thermal stress (45°C) induced larvae was low compared to day-3. 1.39 µm/mL ALT activity, which is highest, was recorded in NBL-5 HS compared to 0.59 µm/mL in control. Enhanced AST activity was also recorded

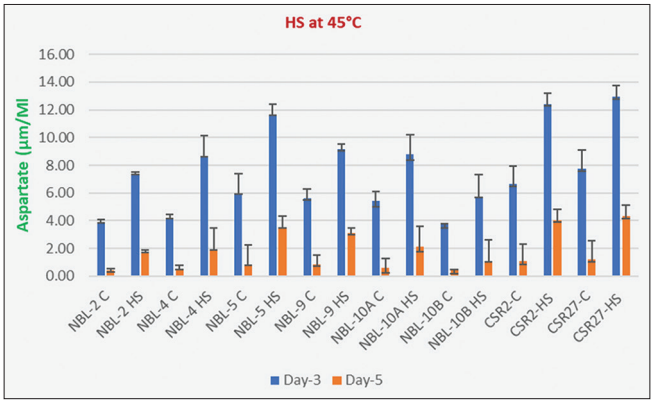

Figure 1: Impact of heat shock on hemolymph aspartate aminotransferase activity in the new bivoltine lines, and its parental breeds larvae of *Bombyx mori* heat shocked at 45°C. C: Control, HS: Heat shock.

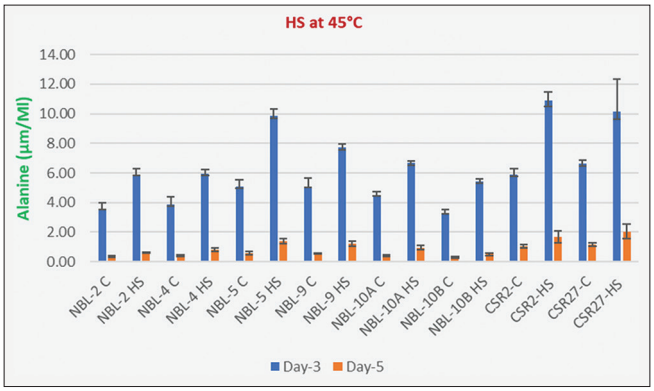

Figure 2: Impact of heat shock on hemolymph alanine aminotransferase activity in the new bivoltine lines, and its parental breeds larvae of *Bombyx mori* heat shocked at 45°C. C: Control, HS: Heat shock.

from NB lines HS at 45°C accounting to 1.23 (NBL-9), 0.95 (NBL-10A), 0.80 (NBL-4), and 0.61 µm/mL (NBL-2) being higher level over their respective control batches. Lowest of 0.51 µm/mL was observed in NBL-10B HS against their control of 0.29 µm/mL. On day-3 on exposure to 45°C thermal stress, CSR_2 and CSR_{27} exhibited 10.90 and 10.14 µm/mL, respectively, against their respective control (5.91 and 6.61 µm/mL). Similarly, on day-5 of fifth instar, it was 1.69 and 2.03 µm/mL against their respective control (1.02 and 1.15 µm/mL). The data presented are statistically significant at $P < 0.05$.

4. DISCUSSION

Insects being ectothermic organisms are significantly affected by the changes in their ambient temperature. Hence, it is worthwhile to understand the impact of thermal stress on some key enzymes, such as AST and ALT, in the intermediary metabolism of proteins. By and large, in human blood, ALT activity levels are considered as a highly sensitive and fairly specific preclinical and clinical biomarker of cytotoxicity or hepatotoxicity. Therefore, ALT activity levels in the blood of mammals are measured in many pharmaceutical tests to determine the hepatotoxic effects induced by natural products or newly synthesized chemicals. Similarly, the drug-induced tissue injury was also evaluated based on the measurements of ALT activity in the hemolymph of *B. mori* [13]. In this context, this study was undertaken to investigate the effect of thermal stress on the hemolymph transaminases of grown (fifth instar) silkworm larvae with an aim to develop AST and ALT as one of the biomarkers to diagnose the thermotolerance in NBL of *B. mori*, which are developed with the application of HST. Thus, the present investigation is unique in this respect.

Significant differences observed in AST and ALT activity revealed varied levels of response to thermal stress in the NBL and its parental breeds. Notably, exposure of day-3 and day-5 fifth instar larvae to a thermal stress of 45°C elevated the AST and ALT levels in the hemolymph as an indicator of physiological imbalance. Comparatively, among NBL, 3-day old NBL-5 exhibits highest AST and ALT levels in their hemolymph due to thermal stress 45°C, but it was even lesser than its parental breeds CSR_2 and CSR_{27}. This increased AST and ALT levels in the hemolymph are considered as a sign of severe tissue damage due to abiotic and/or biotic stress. For instance, the parasitism of the fifth instar larvae of *B. mori* by Uzi fly increased the amount of both transaminases in the hemolymph [14].

However, less AST and ALT activity was observed in day-5 of fifth instar NBL compared to that of day-3 on exposure to 45°C HS. This clearly indicates that there is an obvious enhancement in the resistance capacity of fifth instar larvae of all NBL and its parental breeds against thermal stress of 45°C as larval development precedes [9]. However, death of the larvae recorded might be due to over-expression of a large number of HSPs as a fact of autophagy [5] and thermal stress (45°C) induced severe cytotoxicity or hepatotoxicity.

More interestingly, CSR_2 and CSR_{27} being parentage of NBL exhibits highest AST and ALT activity than NBL [Figures 1 and 2]. This clearly indicates that the rate of tissue damage is less as evidenced with low AST and ALT activity in all NBL, and it depicts that NBL possesses higher acquired tolerance compared to that of the parent breeds not only in non-HS induced but also as a response to induced thermal stress at 45°C.

Notably, highest of 44.25% in non-HS groups and 53.64% in HS groups with respect to acquired tolerance was recorded in NBL-10B based on AST activity, while it was low in NBL-5. Concomitantly, ALT activity also substantiated it with highest acquired tolerance of 44.16% and 50.40% in non-HS and HS larvae, while it was low again in NBL-5 [Tables 1 and 2]. This tolerance acquired might be due to continuous exposure of silkworm larvae to thermal stress of 45°C over ten consecutive generations, which influences activation of HSP gene expression to acquire tolerance to high thermal stress with less tissue damage.

One of the most typical responses to tissue, particularly liver injury in humans, is a rise in blood AST and ALT levels. Hence, the blood AST and ALT levels are a common clinical measure used to evaluate liver-related anomalies and diseases. Silkworms infected with *Streptococcus mutans* exhibit damage caused to fat body cells and a drastic hike in the hemolymph AST and ALT levels [8]. Further, increase in the AST and ALT level in hemolymph and fat body of silkworm exposed to different doses of insecticides indicates an active transportation of amino acids which provide keto acid to serve as precursors in the synthesis of essential constituents under stress conditions [15].

Taken together, it is obvious from the present investigation that the elevated AST and ALT levels in the NBL and its parents are not only a response to thermal stress of 45ºC but also substantiate different rates of tissue damage and tolerance level. Interestingly, NBL has shown less AST and ALT activity compared to its parents, that is, CSR_2 and CSR_{27} indicate a higher level of acquired tolerance to thermal stress. Thus, we not only recommend the AST and ALT assays as one of the potential biomarkers to identify the thermotolerant breeds but also to

Table 1: Percent of change in tolerance acquired by new bivoltine lines over its parentage to thermal stress based on aspartate aminotransferase activity.

Aspartate	CSR_2				CSR_{27}			
	Day-3		Day-5		Day-3		Day-5	
	Control	HS	Control	HS	Control	HS	Control	HS
NBL-2	40.85	40.45	58.82	56.07	49.19	42.82	63.54	59.01
NBL-4	36.32	30.15	51.76	51.09	45.30	32.93	57.29	54.36
NBL-5	9.72	6.21	20.00	12.15	22.45	9.94	29.17	18.02
NBL-9	15.66	26.06	22.35	23.05	27.55	29.00	31.25	28.20
NBL-10A	18.49	29.29	41.18	46.42	29.98	32.10	47.92	50.00
NBL-10B	44.25	53.64	62.35	72.90	52.11	55.48	66.67	74.71
F-value	4.176	5.955	2.167	6.050	1.824	99.084	2.157	102.777
Significance	NS	*	NS	*	NS	**	NS	**

*Significant, **Highly significant, NS: Non-significant. NBL: New bivoltine lines, HS: Heat shock.

Table 2: Percent of change in tolerance acquired by new bivoltine lines over its parentage to thermal stress based on Alanine aminotransferase activity.

Alanine	CSR_2				CSR_{27}			
	Day-3		Day-5		Day-3		Day-5	
	Control	HS	Control	HS	Control	HS	Control	HS
NBL-2	39.07	46.38	67.90	63.70	45.54	42.40	71.74	69.75
NBL-4	34.82	45.45	59.26	52.59	41.75	41.41	64.13	60.49
NBL-5	14.01	9.78	41.98	17.78	23.15	3.09	48.91	31.48
NBL-9	14.23	29.34	46.91	27.41	23.34	24.10	53.26	39.51
NBL-10A	23.78	38.55	59.26	43.70	31.88	33.99	64.13	53.09
NBL-10B	44.16	50.40	71.60	69.63	50.09	46.72	75.00	74.69
F-value	9.883	48.402	11.043	1.496	7.162	6.017	6.296	5.931
Significance	**	**	**	NS	*	*	*	*

*Significant, **Highly significant, NS: Non-significant. NBL: New bivoltine lines, HS: Heat shock.

use all NBL developed with the application of HST for commercial exploitation in the field.

However, further studies in thermal stress induced histotoxicity are needed to clarify the mechanism of tissue injury induction in silkworm, but we aptly demonstrated varied level of AST and ALT activity in the silkworm referring different rate of tissue injury induction as indicated by elevated levels of these enzymes. Thus, the silkworm can be used as a model system to rapidly evaluate histotoxicity caused by not only abiotic and biotic stresses but also by novel drugs.

5. CONCLUSION

Considering the significance of AST and ALT activity in the serum as bio-markers to diagnose the histotoxicity in heart and liver of human (7), the elevated level of these enzymes explicit heat shock induced tissue injury leading to death of silkworm larvae. Hence, we suggest AST and ALT assays shall be considered as one of the bio-markers not only to evaluate the rate tolerance to harse climate but also identification of thermotolerant silkworm breeds/hybrids of *B. mori*.

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7. AUTHORS' CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

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9. CONFLICTS OF INTEREST

The authors declare that there is no financial or any other conflicts of interest in this work.

10. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

11. DATA AVAILABILITY

All data generated and analyzed are included within this research article.

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