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Time Course Studies on Impact of Low Temperature Exposure on the Levels of Protein and Enzymes in Fifth Instar Larvae of Eri Silkworm, *Philosamia ricini* (*Lepidoptera: satuniidae*)

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Abstract

Lactate dehydrogenase (LDH; EC 1.1.1.27) and malate dehydrogenase (MDH; EC 1.1.1.37) are the enzymes involved in energy metabolism of Eri silkworm, *Philosamia ricini*. However, no previous study has been reported about effect of low temperature exposure on their levels in different tissues of Eri silkworm. The present study was aimed to the time-course effects of low temperature (~10°C) exposure of 5th instar *P. ricini* on the levels of protein and energy metabolism enzymes of three major tissues (haemolymph, silk gland and fat body). The Eri silkworm larvae, reared on fresh leaves of castor-oil-plant (*Ricinus communis*), were divided into 4 groups: a control group reared at 25 ± 2°C along with three experimental groups reared at 10 ± 1°C containing 50 larvae in each, for varying durations (2, 4 and 7 days). The cell free extract was prepared by centrifuging the tissue homogenate at 9000 g and used for biochemical estimations (total protein content, lactate dehydrogenase and malate dehydrogenase activities**).** For isozyme analysis, another set of homogenates (20% w/v) was prepared in buffer (0.2 M Tris HCl, pH 7.0) containing 0.2 M sucrose and 10 mM EDTA, and analyzed by native-PAGE followed by activity staining. The activities of lactate dehydrogenase and malate dehydrogenase showed significant decrease in haemolymph, whereas in fat bodies both enzymes showed increased activity. In silk gland, Lactate dehydrogenase activity decreased uniformly, whereas malate dehydrogenase activity increased at all exposure durations. The isozyme analysis revealed significant perturbations in their expression profiles. The low temperature exposure resulted into accumulation of protein content in haemolymph and depletion in silk gland and fat body tissues. Fat bodies emerged as the main energy producing organ under this condition. Lactate dehydrogenase and malate dehydrogenase displayed presence of only one isozyme in all the tissues tested. The isozyme behaviour of lactate dehydrogenase and malate dehydrogenase towards low temperature varied in different tissues. These results suggested that alterations in expression and functions of these enzymes might be associated to the acclimation of larvae at low temperature.

Keywords: Lactate dehydrogenase; Malate dehydrogenase; Isozyme; Native-PAGE; Eri silkworm; Silk gland and Fat body tissues

Abbreviations: LDH: Lactate Dehydrogenase; MDH: Malate Dehydrogenase; PAGE: Polyacrylamide Gel Electrophoresis; EC: Enzyme Commission; NAD: Nicotinamidde Dinucleotide; TCA: Tricarboxylic Acid; APS-Ammonium Persulfate; SE: Standard Error; TBE: Tri-Borate Buffer; TEMED: N,N,N',N'-Tetramethylethylenediamine; KCl: Potassium Chloride, NADH: Nicotinamide Adenine Dinucleotide; EDTA: Ethylene Diamine Tetraacetic Acid; NaCl: Sodium Chloride; $CaCl_2$: Calcium Chloride; FCR: Folin–Ciocalteu Reagent; $CuSO_4$: Copper Sulphate; NaOH: Sodium Hydroxide; HCl: Hydrochloric Acid.

Introduction

Many insects are ecological (predators) and economically beneficial for example silkworms have been used to produce silk. Insects in their life history are encounter adverse climate conditions, such as scorching heat in summer and terrifying cold in winter season. It is necessary for the insects to evolve corresponding strategies against such adverse conditions to protect themselves [1,2]. *Philosamia ricini* (eri silkworm), a non-mulberry silkworm, is known to produce the silk with high thermal property, strength and durability. The eri silkworm can be blended with many other materials for textile and non-textile applications. It mainly feeds on castor leaves (*Ricinus communis*). It has been observed that the quality of host plant and the low temperature exposure may influence the production of silk.

It has been reported that insects display an extraordinary array of adaptations that allow them to sustain metabolic activity during such harsh conditions [3]**.** A large number of insects usually meet with harsh low temperature in winter season. They change physiologically, biochemically and behaviorally for increasing the capacity of cold-hardiness [2,4]**.** Some studies have been conducted to study the acclimation of various organisms to low temperature and hypoxic conditions in order to understand the role of few intermediary metabolites and enzymes. The enzymes concerned with energy metabolism can be divided into three groups, those related to anaerobic metabolism, aerobic metabolism and the pentose phosphate pathway [5]**.** However, studies are required to understand the role of energy metabolism enzymes in insects including Eri silkworm exposed to low temperature [6]**.**

There are many important enzymes involved in energy metabolism of insects out of which lactate dehydrogenase (LDH, lactate; NAD-oxidoreductase, E.C. 1.1.1.27) and malate dehydrogenase (MDH, L-malate: NAD'-oxidoreductase, E.C. 1.1.1.37) are the most studied.

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LDH is one of the enzyme involved in Glycolytic pathway [5,7,8] which catalyses a reversible reduction of pyruvate into lactate using NADH as a cofactor under anaerobic conditions. MDH is involved in TCA cycle and known to catalyze the reversible oxidation of malate to oxaloacetate requiring NAD as a cofactor. It is also involved in gluconeogenesis, lipogenesis and in malate-aspartate shuttle during aerobic glycolysis [9].

Literatures suggested that there is no report available to explain the roles of LDH and MDH and their behaviour of corresponding isozymes in different tissues of the Eri silkworm species particularly under low temperature stress. Therefore, the present study was envisaged to investigate the impact of low temperature (10°C ± 1°C) exposure for different time durations on the levels of LDH and MDH activity in the Indian silk producing insect, Eri silkworm. The present work also illustrated the perturbations in the behaviour of isozymes of LDH and MDH from different tissues of Eri silkworm insect exposed to low temperature using native-polyacrylamide gel electrophoresis (native-PAGE) [9-12].

We focused on changes in the behaviour of different isozymes of LDH and MDH and total protein content in silkworm, *Philosamia ricini*. For the first time, we studied the changes in activity pattern of energy metabolizing enzymes due to the exposure of low temperature *in vivo*. In this study, the effects of exposure of the silkworm to the low temperature on the levels of biochemical, and physiological indices have been investigated. This study provides evidence that low temperature exposure may induce acclimation in eri silkworm.

Materials and methods

Animals

The Eri silkworm, *Philosamia ricini* used in all the experiments is a continuously breeding lepidopteran. This insect mainly feeds on castor-oil-plant leaves. The life cycle of *P. ricini* passes through five instar stages. At the culmination of fifth instar stage, it spins silk cocoon and changes into the pupa [13]. Disease-free egg laying Eri silkworms were obtained from Mangaldoi Seri culture farm of Assam. The silkworms were reared at Sericulture/entomology laboratory, Department of Zoology, University of Allahabad, Allahabad, under recommended condition at 25 ± 2°C temperature [14] 75 ± 5% relative humidity and 12 h:12 h of Light: Dark photoperiod. The larvae were fed with *Ricinus communis* (castor) leaves harvested from the garden of Department of Botany, University of Allahabad campus. The fifth instar larvae were reared in isolated Petri dishes and used for the study. The standard rearing methods were adopted as recommended by Sarkar. The leaves were exposed to the low temperature and fed ad libitum. All the experiments were performed three times in triplicates (50 larvae per replication).

Reagents and chemicals

Oxaloacetate, acrylamide, N,N'-Methylenebisacrylamide, Ammonium Persulfate (APS), glycine, N,N,N',N'-Tetramethylethylenediamine (TEMED), bromophenol blue and **sodium bicarbonate** were purchased from Sigma-Aldrich. Potassium chloride (KCl), sodium pyruvate, nicotinamide adenine dinucleotide (NADH), t*ris* base, Ethylene Diamine Tetra Acetic acid (EDTA) was purchased from E. Merck, Darmstadt, Germany. Bovine serum albumin, were purchased from Loba Chemie Pvt. Ltd. Sucrose, sodium potassium-tartrate, n-butanol, boric acid, sodium chloride (NaCl), calcium chloride ($CaCl_2$), Folin–Ciocalteu reagent (FCR), copper sulphate ($CuSO_4$) and sodium hydroxide (NaOH) were purchased from Sisco Research Laboratory Pvt. Ltd, Mumbai, India. Hydrochloric acid (HCl) was from Qualigens Fine Chemicals Pvt. Ltd. India.

Exposure to low temperature

The newly hatched larvae of Eri silkworm were reared on fresh leaves of castor-oil-plant (*Ricinus communis*). The fifth instar larvae of Eri silkworm insect were divided into 4 groups; each containing an equal number (50) of larvae in plastic trays. One of the groups was served as a control (reared at 25 ± 2°C) and the other three were exposed to low temperature (10 ± 1°C) for varying exposure durations (2, 4 and 7 days).

Preparation of cell-free extract from the homogenates of different tissues (silk glands and fat body tissues)

The fifth instar larvae of Eri silkworm insect were dissected in ice-cold Bodenstein's Ringer solution. Their silk glands and fat body tissues were excised out [13]. The homogenates (20% w/v) of tissues were prepared in 50 mM Tris-HCl buffer (pH 7.0) using Potter-Elvehjam homogenizer with teflon coated pestle under ice cold condition. The cell free extract was prepared using cooling centrifuge. The homogenates were centrifuged at 9000 g and supernatants were used as cell free extract for further biochemical estimations. For isozyme analysis, another set of homogenates (20% w/v) were prepared in sucrose buffer containing 0.2 M sucrose, 10 mM EDTA, and 0.2 M Tris HCl, pH 7.0.

Estimation of total protein

The protein contents of cell free extracts in different tissues from Eri silkworm insect was determined according to the method of Lowry et al. [15,16] using bovine serum albumin (BSA) as standard.

Assay for the activity of enzymes

The activities of LDH and MDH were measured by NADH-linked optical assay following the method of Horecker and Kornberg (1948). In case of LDH, sodium pyruvate (0.5 mM) was used as substrate whereas in case of MDH, oxaloacetate (0.6 mM) served as substrate. The spectrophotometric measurements were done using ELICO SL 164 UVD model double beam spectrophotometer using quartz cuvette (1.0 cm light path length) against enzyme blank at room temperature (28 ± 2°C). The measurements were made in duplicate in each tissue homogenate. The reaction velocity is determined by decreasing absorbance resulting from oxidation of NADH at 340 nm. One unit of enzyme activity causes the oxidation of one micromole of NADH per minute at 25°C; pH 7.4.

Native-polyacrylamide gel electrophoresis (native-PAGE) and activity staining of isozymes

The activity staining of isozymes from different tissues from Eri silkworm insect exposed to low temperature, were analyzed by native-polyacrylamide gel electrophoresis (native-PAGE) [11,16]. Isozyme variations were investigated using equal amount of proteins from different samples loaded to 7% polyacrylamide gel and resolved in cold for 2 h [17]. The native PAGE gels were conducted at constant voltage (150 V) in Tris-Borate-EDTA (TBE) buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.6). The isozyme bands in the gel were visualized using activity staining methods of Ayala.

Statistical analysis

Each assay was replicated four times. Values were expressed as mean ± SE of replicates and Student's t-test was applied to locate significant ($p<0.05$) differences between treated and control groups.

Tissues	Control (mg/g wet weight of tissue)	Experimental (mg/g wet weight of tissue)		
	0 Day	2 Days	4 Days	7 Days
Haemolymph	15.1 ± 0.29[a]	17.2 ± 0.31[a] (+13.9)	20.4 ± 0.18[a] (+35.1)	25.5 ± 0.77[a, b] (+68.9)
Silk gland	108.0 ± 0.70	95.5 ± 0.34 (-11.5)	86.2 ± 0.32[b] (-20.2)	74.0 ± 0.31 (-31.5)
Fat body tissues	92.0 ± 0.31	82.6 ± 0.16 (-10.2)	74.5 ± 0.17[b] (-19.0)	64.5 ± 0.19 (-29.9)

P. ricini 5th instar larvae was exposed for seven days at 10 ± 1°C for the experimental set whereas the control group insects were reared at 25 ± 2°C. The total protein content was estimated using procedures as described in materials and methods. The values presented in the parentheses indicate the percent increase (+) or decrease (-) over control. [a] Values in mg/ml, [b] Significantly different at $p \leq 0.05$ (Student's 't' test).

Table 1: Effect of low temperature (10 ± 1°C) exposure on the levels of protein content in three major tissues of 5th instar larvae of *P. ricini* exposed for different days.

Enzymes	Control (U/mg protein)	Experimental (U/mg protein)		
	0 Day	2 Days	4 Days	7 Days
[a]MDH	11.7 ± 0.08	9.8 ± 0.06 (-16.2)	8.2 ± 0.09 (-29.9)	7.3 ± 0.06[b] (-37.6)
[a]LDH	7.9 ± 0.3	5.2 ± 0.12 (-34.2)	4.3 ± 0.17 (-45.6)	2.8 ± 0.09[b] (-64.6)

P. ricini 5th instar larvae were exposed for 7 days at 10 ± 1°C for experimental set whereas control group insects were reared at 25 ± 2°C. One unit of enzyme activity has been defined as the amount of enzyme required to catalyze the transformation of one µmol of substrate or the formation of one µmol of product per min under specified experimental conditions. The specific activity has been expressed as U/mg protein.[a] The values are to be multiplied by a factor of 10^{-2} for MDH and by a factor of 10^{-3} for LDH. The results represent the average values of three independent experiments. The values in parentheses indicate percent increase (+) or decrease (-) over control. [b] Significantly different at $p \leq 0.05$ (Student's 't' test).

Table 2: Effect of low temperature (10 ± 1°C) exposure on specific activities of MDH and LDH in haemolymph of 5th instar larvae of *P. ricini* exposed for different days.

Enzymes	Control (U/mg protein)	Experimental (U/mg protein)		
	0 Day	2 Days	4 Days	7 Days
[a]MDH	7.9 ± 0.06	8.7 ± 0.15 (+10.1)	9.8 ± 0.12 (+24.0)	11.5 ± 0.17 (+45.6)
[a]LDH	1.8 ± 0.12	1.6 ± 0.06 (+11.1)	1.4 ± 0.08 (+22.2)	1.2 ± 0.09[b] (-33.3)

P. ricini 5th instar larvae were exposed upto seven days at 10 ± 1°Cfor experimental set whereas the control group insects were reared at 25 ± 2°C. One unit (U) of enzyme activity has been defined as the amount of enzyme required to catalyze the transformation of one µmol substrate or the formation of one µmol product per min under specified experimental conditions. The specific activity has been expressed as U/mg protein. [a] The values are to be multiplied by a factor of 10^{-2} for MDH and by a factor of 10^{-3} for LDH. The results represent average values of three independent experiments. The values in parentheses indicate percent increase (+) or decrease (-) over control. [b] Significantly different at $p \leq 0.05$ (Student's 't' test).

Table 3: Effect of low temperature (10 ± 1°C) exposure on the levels of specific activities of MDH and LDH in silk gland of 5th instar larvae of *P. ricini* exposed for different days.

Results

Effect of low temperature on the level of total protein content in different tissues of eri silkworm *(Philosamia ricini)*

The exposure of low temperature results is shown in Table 1, on the levels of protein content in three different organs of Eri silkworm insect larvae. The maximum protein content was observed in silk gland (108 mg/g wet weight of the tissue) followed by fat body tissues (92 mg/g wet weight of the tissue) and haemolymph (15 mg/g wet weight of the tissue) in control. After exposure to low temperature, the insect larvae haemolymph exhibited elevation in the level of protein content (14% to 69%) over different periods of exposure (2 to 7 days). However, a reverse pattern was observed in the silk gland and fat body tissues. The low temperature stress caused decrease in protein content of silk gland and fat body tissues to almost similar extent and the values being 12% to 32% and 10% to 30% in silk gland and fat body tissues, respectively (Table 1). The impact of low temperature stress on the levels of protein content was more pronounced at higher exposure duration (7 days) than that at lower exposure durations (2 or 4 days).

Effect of low temperature on the activity level of energy metabolism enzymes (LDH and MDH) in different tissues of eri silkworm (*Philosamia ricini*)

While comparing the LDH activity with control tissues, haemolymph displayed maximum specific activity (7.9 U/mg protein, Table 2) followed by fat body tissues (3.0 U/mg protein) and silk gland (1.8 U/mg protein, Table 3). However, the activity of MDH in the control tissues was recorded to be maximum in the fat body tissues (12 U/mg protein, Table 4) followed by haemolymph (11.7 U/mg protein, Table 2) and silk gland (7.9 U/mg protein, Table 3). The activity of MDH in fat body tissues (Table 4) and haemolymph (Table 2) of control Eri silkworm insect larvae appeared to be almost similar. When the insect larvae were exposed to low temperature, the activities of LDH and MDH exhibited significant ($p \leq 0.05$) decrease of 34% to 65% and 16% to 38%, respectively in haemolymph (Table 2). The impact of low temperature stress on the levels of the enzymes activity was found to be higher at maximum exposure duration (7 days) than the others (2 or 4 days).

Similar experiments were conducted in silk gland of Eri silkworm insect larvae. In this, the low temperature stress resulted into drastic increase (10% to 46%) in MDH activity, whereas the activity of LDH displayed a different behaviour (Table 3). The trend was not uniform over different exposure durations (2, 4 and 7 days). The activity of LDH increased (11% to 22%) upto 4th day and then after it significantly ($p \leq 0.05$) decreased (33%) as compared to control due to further increase in exposure duration (7th day) (Table 3). Thus, the effect of low temperature exposure on the activity of LDH from silk gland of Eri silkworm insect larvae appeared to be biphasic in nature.

The results of low temperature exposure on the activity levels of these two enzymes (LDH and MDH) from fat body tissues of 5th instar larvae of Eri silkworm insect are shown in Table 4. The data reflected that the low temperature stress induced significant ($p \leq 0.05$) elevations in activity of LDH (40% to 23%) and MDH (50% to 170%) from fat body tissues of Eri silkworm insect larvae over varying periods of exposure. Rise in the activity levels of these enzymes were found to be consistently enhanced up to 7th day of exposure (Table 4). The data also demonstrated that percent increase in MDH activity was much higher than LDH activity due to low temperature stress along

Enzymes	Control (U/mg protein)	Experimental (U/mg protein)		
	0 Day	2 Days	4 Days	7 Days
[a] MDH	12.1 ± 0.17	18.2 ± 0.52 (+50.4)	26.4 ± 0.64 (+118.2)	32.7 ± 0.7[b] (+170.2)
[a]LDH	3.0 ± 0.12	4.2 ± 0.17 (+40.0)	5.6 ± 0.15 (+86.7)	6.7 ± 0.23 (+123.3)

P. ricini 5th instar larvae were acclimated for seven days at 10 ± 1°C for the experimental set whereas the control group insects were reared at 25 ± 2°C . One unit (U) of enzyme activity has been defined as the amount of enzyme required to catalyze the transformation of one µmol substrate or the formation of one µmol product per min under specified experimental conditions. The specific activity has been expressed as U/mg protein. [a] the values are to be multiplied by a factor of 10^{-2} for MDH and by a factor of 10^{-3} for LDH. The results represent average values of three independent experiments. The values in parentheses indicate percent increase (+) or decrease (-) over control. [b] significantly different at $p \leq 0.05$ (Student's 't' test).

Table 4: Effect of exposure to low temperature (10 ± 1°C) on the levels of specific activities of MDH and LDH in fat body tissues of 5th instar larvae of *P. ricini* exposed for different days.

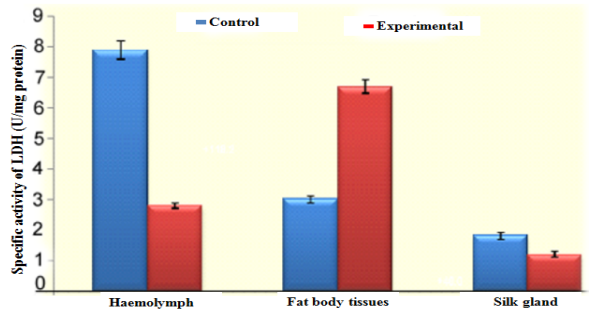

Figure 1: Effect of low temperature (10 ± 1°C) exposure on the levels of LDH activity in three different tissues of 5th instar larvae of *P. ricini* exposed for 7 days. The histogram was prepared with the data presented into Tables 1, 2 and 3 for the effect of low temperature exposure on the levels of LDH activity in different tissues of 5th instar larvae of *P. ricini* for maximum duration (7 days) of exposure for organwise comparison.

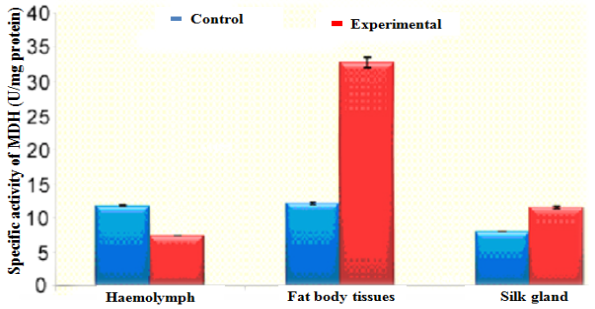

Figure 2: Effect of low temperature (10 ± 1°C) exposure on the levels of MDH activity in three different tissues of 5th instar larvae of *P. ricini* exposed for 7 days. The histogram was prepared with the data presented in Tables 1, 2 and 3 for organwise comparison upto maximum period (7 days) of exposure.

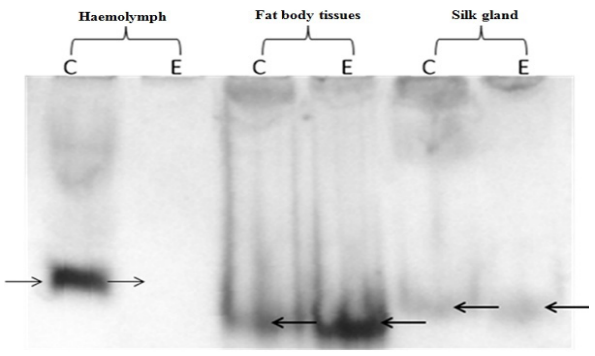

Figure 3: Isozyme activity pattern of LDH in 5th instar *P. ricini* larvae exposed up to 7 days. C=Control, reared at temperature (25 ± 2°C), E=Experimental, exposed at temperature (10 ± 1°C) for 7 days. The arrow (→) represents the position of isozyme bands.

all of three different exposure durations. Similarly, the effect of low temperature exposure in the haemolymph and fat body tissues of Eri silkworm insect larvae were also found to be dependent on period of exposure.

Since the impact of low temperature stress on the activity levels of LDH and MDH in different tissues of Eri silkworm insect larvae was found to be highest after the maximum exposure duration (7 days), the data for LDH and MDH from Tables 2, 3 and 4 for this maximum exposure duration (7 days) period was presented in Figures 1 and 2 showing the marked perturbations in all the three organs of Eri silkworm insect larvae as compared to control. The data presented in Figure 1 indicated that haemolymph and silk glands exhibited decrease in LDH activity by 65% and 33%, respectively whereas fat body tissues registered sharp increase (123%) in LDH activity due to low temperature stress after 7 days of exposure. On the other hand, the data presented in Figure 2 demonstrated that the activity of MDH significantly increased in silk gland (46%) and fat body tissues (170%) whereas significant decrease by 38% was recorded in haemolymph due to low temperature stress after 7 days of exposure.

Effect of low temperature on the expression level of energy metabolism enzymes (LDH and MDH) in different tissues of eri silkworm *(Philosamia ricini)*

Results from the effect of low temperature stress on the isozymes expression of LDH and MDH in Eri silkworm insect larvae after 7 days of exposure are presented in Figures 3 and 4, respectively. As it is evident from the Figures 3 and 4 of native-PAGE, only one isozyme band was obtained from each of these cases. The comparative isozyme pattern of LDH from all the three major tissues of Eri silkworm insect larvae as shown in Figure 3 indicated maximum activity of LDH in haemolymph of control group (lane C) which could not be detected in gel after low temperature exposure (Lane E). The silk gland tissues showed the lower LDH activity in low temperature exposed group (lane E) as compared to that of the control group (Lane C). Interestingly, the isozyme pattern of LDH in fat body tissues was unique in a manner that the low temperature stress caused significant increase in the intensity of LDH isozyme band (lane E) as compared to control (lane C). The LDH isozyme bands from silk glands and fat body tissues migrated longer as compared to that of haemolymph. The order of migration of LDH isozymes in tissues tested was as following: haemolymph>silk gland >fat body tissues. However, the gel pattern of MDH isozyme activity (Figure 4) reflected rise in the silk gland and fat body tissues of the Eri silkworm insect larvae exposed with low temperature (lane E) when compared with that of control (lane C). In contrast, the activity of MDH isozyme from haemolymph was observed to be diminished, due to exposure of low temperature (lane E). Similar to the migration of LDH isozyme bands, the distance traveled by the MDH isozyme bands from silk gland and fat body tissues were more than the MDH isozyme from haemolymph (Figure 4). The MDH isozyme migration order of bands

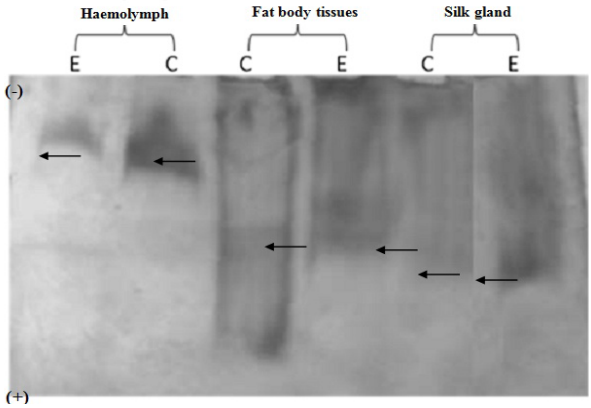

Figure 4: Isozyme activity pattern of MDH in 5th instar *P. ricini* larvae exposed for 7 days. C=Control, reared at temperature (25 ± 2°C), E= Experimental, exposed at temperature (10 ± 1°C) up to 7 days. The arrow (→) represents the position of isozyme bands.

in these tissues was as following: haemolymph>fat body tissues>silk gland.

Discussion

The impact of low temperature (10 ± 1°C) exposure to 5th instar larvae of Eri silkworm, *P. ricini,* were resulted into some changes in its biochemical constituents, so that it can cope up with such harsh conditions. Here, the emphasis was given to evaluate the impact of low temperature exposure on activity levels of two enzymes (LDH and MDH) which are involved in energy metabolism of Eri silkworm insect larvae. The results shown in Tables 1-4, indicated that low temperature stress affected the levels of total protein content and activity of enzymes (LDH and MDH) in different tissues of Eri silkworm insect larvae.

Earlier it has been reported that some organisms, when subjected to low temperature stress, exhibited a marked alteration in their metabolism by changing the levels of certain enzyme(s) activity [3,5,18,19]. In present study, we have shown that the level of total protein content increases consistently in hemolymph of Eri silkworm insect larvae due to low temperature stress over increasing period of exposure. It is important to note that the level of protein gradually decreases over increasing exposure period in silk gland as well as in fat body tissues. Since, haemolymph of the insects acts as a reservoir of various metabolites, from different organs [20]. It is quite likely that the decreasing pattern of total protein content in silk gland and fat body tissues may be due to the mobilization of the proteins from these two organs to haemolymph and/or utilization of protein for generation of energy, to survive and mitigate adverse impact of low temperature exposure. The over wintering insects have to contend with osmotic stress [21,22]. It has been reported that the rapid cold-hardening is associated with increase in concentration of some metabolites and osmolality of haemolymph [23]. While studying low temperature stress tolerance in *Drosophila melanogaster,* Misener et al., have explained their results in the haemolymph of low temperature stressed insect in similar manner [24].

In present study, the activities of LDH and MDH were expressed differently in different tissues of Eri silkworm insect larvae, when subjected to low temperature exposure for 2, 4 and 7 days. The activity levels of these enzymes decreased in haemolymph with maximum reduction in LDH activity as compared to MDH, thereby showing the energy metabolism system in haemolymph of Eri silkworm insect larvae ceases to support physiological activities at low temperature. In fat body tissues their activities sharply increased across all exposure periods. The increase in LDH activity have been reported to be associated with an increase in anaerobic catabolism leading to production of ethanol, lactate and alanine as end-products in *C. riparius* larvae when exposed to anoxia condition [3,9,25].

The present study indicated that during low temperature exposure, the extent of elevation in MDH activity was significantly higher than LDH at all the three durations. These findings suggested that under low temperature stress condition, the energy metabolism system in fat body tissues may get activated to support the functions of Eri silkworm insect larvae by supplying required amount of energy. Though, in silk gland Eri silkworm insect larvae, the activity of MDH increased consistently with increasing exposure durations, the extent of elevation in MDH activity was not as high as it was recorded in fat body tissues. The results suggested that under low temperature exposure condition, fat body tissues served as a primary energy producing organ. Just like other organisms, these findings also indicated that the low temperature exposure induce aerobic respiration with greater extent as compared to anaerobic respiration, thereby suggesting the main energy supplying process in Eri silkworm insect larvae, while anaerobic respiration may play auxiliary roles in energy metabolism as reported in *T. granosa* [6].

Insects have been reported to exploit several biochemical mechanisms to survive during low temperature stress conditions [26,27]. The increased MDH activities of *P. ricini* larvae (present investigation) suggested that this enzyme contribute to reducing power in form of NADH in fat body tissues and silk gland due to low temperature exposure, which may also lead to anoxia condition [9,26,27]. Under these conditions, pyruvate produced by the oxidative decarboxylation of malate and converted to alanine in *C. riparius* larvae exposed to anoxia, a consequence of low temperature stress [3].

The expression patterns of LDH and MDH isozymes from different tissues of Eri silkworm insect larvae revealed greatly influenced by low temperature exposure. The control (C) and experimental (E) lanes corresponds to the activity values indicated in Tables 2-4. In native-PAGE gel, the migration of isozyme bands of LDH and MDH from haemolymph was relatively smaller than silk gland and fat body tissues. These findings suggest that LDH and MDH may have different molecular composition and/or conformation in haemolymph as well as in other tissues of Eri silkworm insect larvae tested. However, a detailed study is required to characterize different molecular forms of these isozymes from different tissues of Eri silkworm insect larvae before reaching to any definitive conclusion.

Conclusion

The 5th instar larvae of Eri silkworm *P. ricini* displayed accumulation of protein content in haemolymph and depletion of the same in silk gland and fat body tissues due to low temperature stress. Under this condition, fat body tissues emerged as the main energy supplying organ of Eri silkworm insect larvae, thus supporting their survival under low temperature stress. In low temperature stressed Eri silkworm insect larvae, aerobic respiration was augmented to act as main energy supplying pathway. LDH and MDH displayed the presence of only one isozyme in all tissues of Eri silkworm insect larvae tested. The behaviour of the LDH and MDH isozymes towards low temperature stress response and migration varied in different tissues of Eri silkworm insect larvae. The molecular forms of LDH and MDH present in haemolymph seem to be distinct from rest of the tissues. The alterations in biochemical characteristics of these biomolecules may be attributed

to the acclimation of low temperature in 5th instar larvae of Eri silkworm insect, *P. ricini.*

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