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Role of alkaline phosphatase in development and pathogen response in the tropical Tasar silkworm (*Antheraea mylitta*)

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Abstract
This study investigates the activity of alkaline phosphatase (ALP) in the tropical tasar silkworm, *Antheraea mylitta*, across different developmental larval stages during normal and infected conditions. The research highlights the enzyme's role in metabolic processes, particularly during larval development and in response to bacterial and viral infections. ALP activity was measured in the midgut and haemolymph, revealing a clear pattern of increasing enzyme activity from the first to the fifth instar larvae. The findings indicate that ALP plays a crucial role in nutrient absorption and metabolic regulation, with significant variations observed during infection.

Keywords: Alkaline phosphatase, *Antheraea mylitta*, nutrient absorption

Introduction

Alkaline phosphatase (ALP) is a hydrolase enzyme that catalyzes the hydrolysis of phosphate esters, resulting in the release of inorganic phosphate and alcohol. This enzyme is widely distributed across various tissues in animals, plants, microorganisms, fungi, and bacteria, playing crucial roles in numerous biological processes (Eguchi, 1995; Oshima, 1997; Lee *et al*., 1999) [4, 14, 20].

ALP operating optimally at an alkaline pH, typically between 10.1 and 11.3 (Eguchi *et al*., 1972a; Eguchi, 1975) [3, 4].

In mammals, alkaline phosphatase is primarily found in the liver, bones, kidneys, and bile ducts, with distinct isoforms corresponding to different tissues. For instance, bone alkaline phosphatase is involved in bone mineralization and growth, while liver alkaline phosphatase is associated with bile secretion and liver function. Elevated levels of ALP in the bloodstream can indicate various pathological conditions, including liver disease, bone disorders, and certain cancers, making it a valuable biomarker in clinical diagnostics (Van Hoof and De Broe 1994) [28].

The phosphatase activity in insects was first reported by Nakamura (1940) [19] in *Bombyx mori*. The phosphatase activity in the silkworm egg during the incubation period was studied. A rapid increase in both alkaline and acid phosphatase activities was observed just prior to hatching (Ito *et al*., 1954; Sugai, 1957a) [11, 25]. Furthermore, Ishihara (1957) [10] demonstrated the presence of both acid and alkaline phosphatases in the Malpighian tubules of the silkworm larva. A series of studies by Terra *et al*. (1979) [27] explored the distribution of digestive enzymes along the alimentary canal of *Rhynchosciara americana* larvae found that alkaline phosphatase (ALP) was localized exclusively in the midgut cells, where it co-localized with acid phosphatase. Furthermore, ALP activity was shown to be dependent on the presence of Mg^{2+} ions. Building on this work, Ferreira and Terra (1980) [7] provided evidence that ALP was associated with the plasma membrane of these midgut cells. Ferreira *et al*. (1988) [8] expanded on these findings by studying the posterior midgut cells of *Rhodnius prolixus* nymphs, identified several distinct digestive enzymes in this region, with ALP specifically associated with the inner microvillar membranes of the posterior midgut cells. In *bombyx mori*, understanding the biochemical properties of ALP can provide insights into the physiological status of silkworms and their productivity traits, such as silk yield and quality (Kasmaei and Mahesha, 2012) [12]. ALP activity observed in certain conditions, such as during JH (juvenile hormone) treatment or in response to Bacillus thuringiensis (Bt) infection in *Bombyx mori* (Tang and Yang, 2020) [26]. Manjula and Keshamma (2021) [16] the activities of alkaline and acid phosphatases during different developmental stages of new breeding lines and races of the silkworm *Bombyx mori* L

Lokesh *et al*., (2014) [15] The study examined the genetic variability and enzyme activity in different ecoraces and reciprocal crosses of the tropical tasar silkworm, *Antheraea mylitta* D. Three reciprocal crosses were made between semi-domestic Daba, Laria, and wild Daba ecoraces. The activities of digestive enzymes like amylase, protease, and alkaline phosphatase were measured in the 5th instar larval midgut.

There is limited information on the activities of alkaline phosphatase, during viral and bacterial infections in *A. mylitta*. While these enzymes have role for metabolic processes, their specific roles and changes during infections are not well understood. The highlights the need for further research to explore how infections may affect these enzymes activities in *A. mylitta.*

Materials and Methods

Collection of normal and infected larvae

In the present study healthy (Fig 1), CPV (Fig. 2) and bacterial infected (Fig 3.1) *Antheraea mylitta* larvae were collected from government tasar silkworm field located in Arjuni Morgaon, Dist. Gondia, based on the characteristic symptoms of Cytoplasmic Polyhedrosis Virus (CPV) and bacterial infected larvae were collected and maintain in

separate insectaries on basis of visible signs of CPV and bacterial were selected for further analysis.

Polyhedral Inclusion Bodies (PIBs): The presence of polyhedral inclusion bodies typical of CPV infection was confirmed through microscopy (Fig. 2.2).

Bacterial Identification: The gram-negative bacteria in infected larvae, gram staining was performed. (Fig 3.2). Five days after molt larvae take for experiment.

Sample Preparation and Haemolymph Collection

In the laboratory, infected larvae were anesthetized by placing them on ice pack to reduce activity. Following anesthesia, the proleg of larvae was carefully punctured to collect haemolymph and add pinch of phenyl thiourea. The midgut was then dissected from the larva, and weighing 50 mg on weighing machine of midgut tissue for further processing.

Homogenization and Centrifugation

The dissected midgut tissue was immediately placed in 1 ml of buffer solution and homogenized using a mortar and pestle. The resulting homogenate was then subjected to centrifugation at 3000 rpm for 3 minutes to separate cellular debris. The supernatant, which contained the soluble components, was carefully collected for use in subsequent enzymatic assays.

Alkaline Phosphatase Activity Assay

Alkaline phosphatase activity was measured using ALP 60 kit (Accurex) which is based on kinetic method and p-NPP used as substrate in ALP activity buffer. ALP phosphatase activity was monitored by measuring absorbance of p-nitrophenol (pNP) at 405 nm for 60 or 30 seconds in a microplate reader (UV 1800 Shimadzu UV Spectrophotometer Devices). Specific activity is expressed as IU/L.at room temperature. Experiments contained 20 µl of final extracted from haemolymph and midgut tissue with 1 ml working solution (Diethanolamine buffer, pH 9.8-1 mol/l, p-nitrophenyl phosphate- 10 mmol/l, magnesium chloride- 0.5 mmol/l). Optimal pH was determined by using $MgCl_2$ 0.5 mmol/l, Diethanolamine buffer 1 mol/l (pH 9.8) [Henry, 1974]. The extract was separated from haemolymph and midgut tissue. For the extract from haemolymph, pinch of phenyl thiourea used as anticoagulant. Alkaline phosphatase is stabilized for 4 days at 28 °C. To analyze thermal stability of ALP, it was incubated at 37 °C temperature for 60 seconds and 30 seconds of time intervals before enzymatic assay

Calculation: ALP (IU/L) = Abs. / min x 2720

Statistical Analysis

Each determination was the mean (± SE) of at least three assays, and statistical analyses were performed to evaluate the significance of the results.

Observation and result:

ALP Levels in Normal Midgut and Haemolymph:

Midgut ALP levels showed a steady increase from 750.50 ± 12.58 IU/L in the 1st instar to 2081.67 ± 75.74 IU/L in the 5th instar (fig. 4). This increase was consistent across developmental stages, suggesting an overall increase in metabolic activity and enzymatic processes associated with growth. Haemolymph ALP levels also exhibited a gradual increase from 612.50 ± 23.42 IU/L in the 1st instar to 1008.17 ± 48.73 IU/L in the 5th instar (Fig. 4). The increase in haemolymph ALP was less pronounced than in the midgut, indicating that the midgut plays a more active role in enzymatic processes during larval development.

Fig 1.1: Healthy fifth instar larva of tasar silkworm *Antheraea mylitta*

Fig 2.1: Viral Infected fifth instar larva of tasar silkworm *A. mylitta*

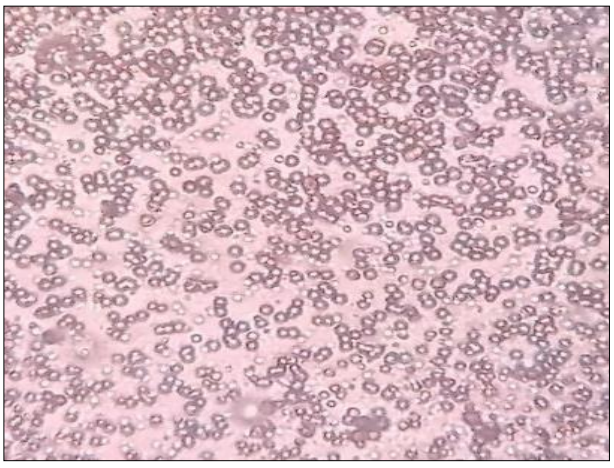

Fig 2.0: Viral Infected fifth instar larva of tasar silk worm Isolated cytoplasmic polyhedrosis viruses showing pure polyhedral inclusion body (40 X)

Fig 3.1: Bacterial infected of tasar silkworm *A. mylitta* showing continuous attached fecal matter

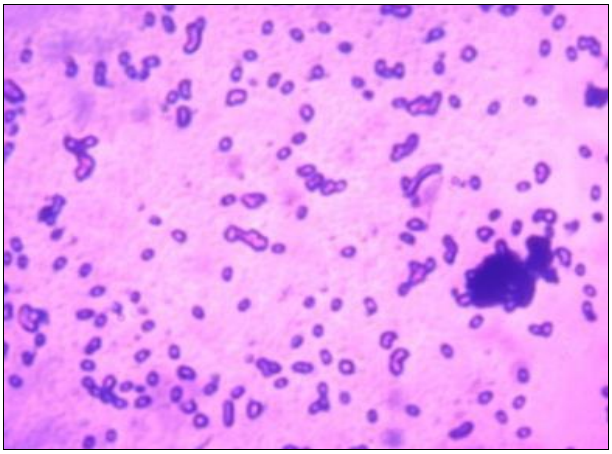

Fig 3.2: Culture of bacteria stain grown in nutrient agar culture showing Gram negative by gram stating (40X)

ALP Levels in Midgut During CPV and Bacterial Infections

In the healthy control, midgut ALP levels remained relatively stable, with early phase levels of 2049.57 ± 39.32 IU/L and late phase levels of 2058.02 ± 65.20 IU/L (Table 2). This suggests that the larvae maintained normal physiological activity during uninfected conditions. Following CPV infection, there was a slight decrease in midgut ALP activity, from 1978 ± 56.05 IU/L in the early phase to 1915.25 ± 23.75 IU/L (Table 2): in the late phase. This decrease suggests a potential suppression of metabolic activity or immune response, possibly due to viral interference. Bacterial infection initially caused a slight increase in midgut ALP activity (2060.30 ± 20.41 IU/L in the early phase), but this was followed by a decline in the late phase (1958.25 ± 30.87 IU/L) (Table 2). This pattern may indicate an early immune response followed by a reduction in enzyme activity due to tissue damage or immune system exhaustion.

ALP Levels in Haemolymph During CPV and Bacterial Infections

In the healthy control, haemolymph ALP levels increased slightly from 1005.21 ± 54.29 IU/L in the early phase to 1062 ± 19.42 IU/L (Table 3) in the late phase. This increase may reflect normal physiological and immune activities. During CPV infection, haemolymph ALP levels decreased from 980.32 ± 56.69 IU/L in the early phase to 948.33 ± 43.93 IU/L (Table 3) in the late phase. The reduction in ALP activity in the haemolymph could indicate suppression of the immune response or a lack of sufficient immune activation to counteract the viral infection. In bacterial infection, haemolymph ALP levels decreased more significantly, from 1020 ± 56 IU/L in the early phase to 920.05 ± 20.32 IU/L (Table 3) in the late phase. This larger decrease could suggest a stronger immune response to bacterial pathogens, potentially leading to greater tissue damage and a decline in ALP activity as a consequence.

Table 1: Show the ALP level of development stages of larvae of normal gut and haemolymph

Sr. no	Larval stages	ALP (Normal Midgut) (IU/L)	ALP (Normal Haemolymph) (IU/L)
1	I	750.50 ± 12.58	612.50± 23.42
2	II	798.33±36.55	615± 28.25
3	III	814.67± 37.36	663.17 ±41.92
4	IV	1287.33 ±37.19	749.67 ±35.89
5	V	2081.67±75.74	1008.17± 48.73

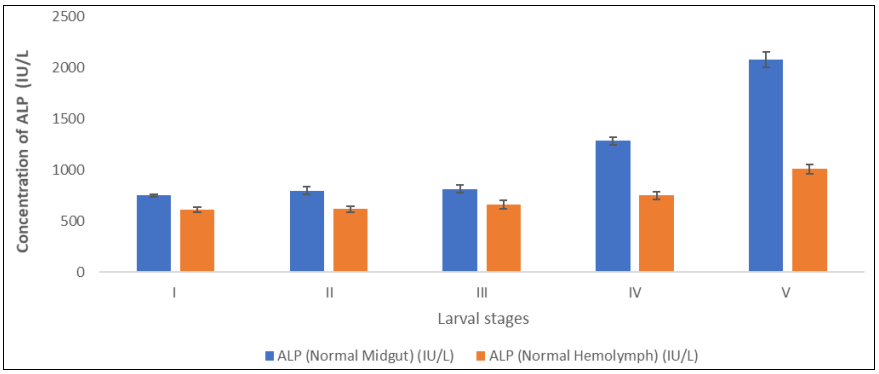

Fig 4: Show the ALP level of development stages of larvae of normal gut and haemolymph

Table 2: Show the ALP level in midgut of 5th instar larvae during CPV and Bacterial Infection

ALP (IU/L) 50 mg Midgut	Healthy Control		CPV infected		Bacterial Infected	
	Early	Late	Early	Late	Early	Late
	2049.57 ± 39.32	2058.02± 65.20	1978 ± 56.05	1915.25± 23.75	2060.30± 20.41	1958.25± 30.87

Table 3: Show the ALP level in haemolymph of 5th instar larvae during CPV and Bacterial Infection

ALP(IU/L) Haemolymph	Healthy Control		CPV infected		Bacterial Infected	
	Early	Late	Early	Late	Early	Late
	1005.21± 54.29	1062± 19.42	980.32 ±56.69	948.33± 43.93	1020 ± 56	920.05 ± 20.32

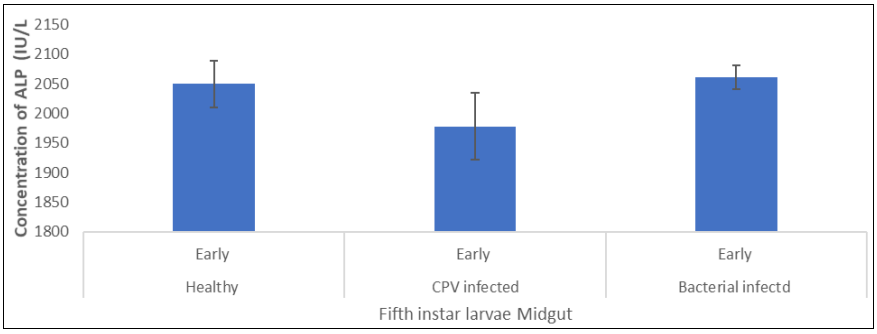

Fig 7: ALP Levels in the midgut in early healthy, CPV infected and Bacterial infected of Fifth Instar Larvae

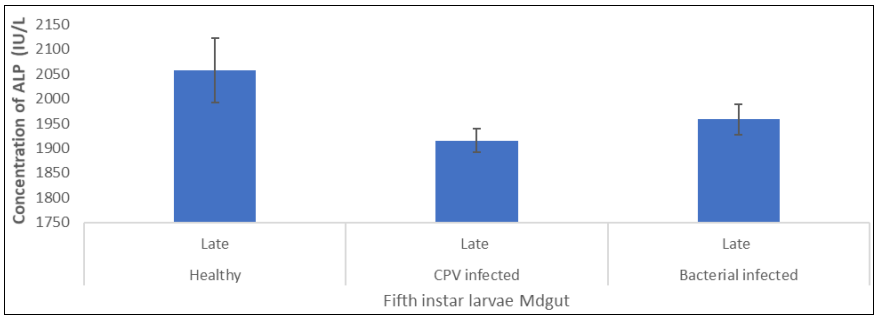

Fig 8: ALP Levels in the midgut in late healthy, CPV infected and Bacterial infected of Fifth Instar Larvae

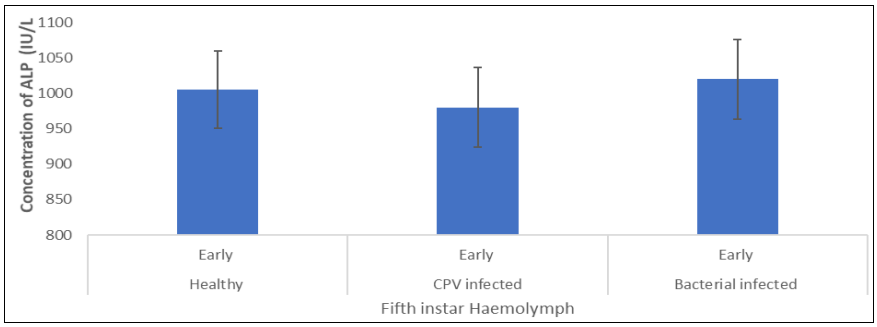

Fig 9: ALP Levels in the haemolymph in early healthy, CPV infected and Bacterial infected of Fifth Instar Larvae

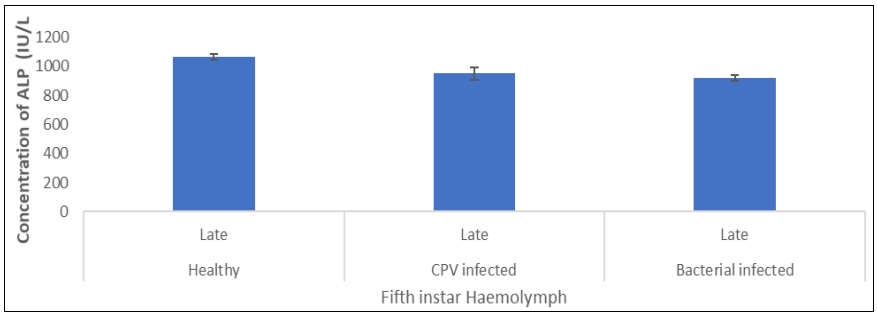

Fig 10: ALP Levels in the haemolymph in early healthy, CPV infected and Bacterial infected of Fifth Instar Larvae

Discussion

The results indicate that ALP activity in both the midgut and haemolymph of 5th instar larvae is influenced by developmental stages and infection conditions. In normal healthy larvae, both midgut and haemolymph ALP levels increase with development, suggesting that ALP plays an important role in growth and metabolism.

The midgut is more active in terms of ALP than the haemolymph, reflecting its central role in digestion and nutrient absorption. Infected larvae (both CPV and bacterial infections) exhibit decreased ALP activity, particularly in the late phases of infection. This decrease may be indicative of metabolic alterations, immune suppression, or tissue damage caused by the infections. The bacterial infection

resulted in a more significant decrease in haemolymph ALP levels compared to CPV infection, which may suggest a stronger or more rapid immune response to bacterial pathogens. Interestingly, bacterial infections appear to trigger an initial increase in midgut ALP activity, potentially reflecting an acute stress response or immune activation, followed by a decline as the infection progresses. This pattern was not observed in CPV-infected larvae, which showed a more consistent decline in ALP levels across both phases.

The study of alkaline phosphatase (ALP) activity in the velvet bean caterpillar (*Anticarsia gemmatalis*) reveals significant developmental stage-specific regulation of the enzyme. ALP activity was detected across all larval instars, from 1st to 5th, with the highest levels observed in the 2nd to 4th instars. De Silva *et. al*., (2019) [2].

In the context of viral infections of cytoplasmic polyhedrosis virus (CPV), on ALP activity provide further insights into the enzyme's role in midgut physiology. CPV, which specifically infects midgut epithelial cells, results in cellular damage and a reduction in ALP activity. Miao (2002) [17] reported that viral infections, particularly CPV, are associated with decreased ALP levels. The decreased ALP activity following CPV infection suggests that the viral-induced cellular damage impairs normal enzyme function, which aligns with previous observations in other lepidopteran species, including *Bombyx mori* (Miao, 2002; 2003) [17, 18].

On the other hand, the increase in ALP activity observed in certain conditions, such as during JH (juvenile hormone) treatment or in response to Bacillus thuringiensis (Bt) infection, offers further evidence of the dynamic regulation of this enzyme. For instance, treatment with JH and 20hydroxyecdysone (20E) in *Bombyx mori* resulted in elevated ALP levels in both the haemolymph and midgut (Tang and Yang, 2020) [26], indicating that hormonal regulation plays a key role in modulating ALP expression. These findings highlight the complex interactions between ALP activity and the various physiological processes involved in insect responses to environmental stressors and pathogens.

Additionally, the interaction between Cry1Ac toxins and ALP has been well documented, with studies showing that exposure to Cry1Ac leads to a reduction in ALP activity (English and Readdy, 1989) [6]. Similar reductions in ALP activity were reported for the midgut enzyme from *Helicoverpa armigera* (Sarkar *et al*., 2009) [23].

The significant increase in the activity of key hydrolases, such as acid and alkaline phosphatases, in the silk glands and fat body may be attributed to the rupture of cellular and lysosomal membranes that contain these hydrolytic enzymes. This disruption likely leads to the release of enzymes, resulting in an overall increase in their activity (Singh *et al*., 2010) [24].

The higher levels of acid and alkaline phosphatase activity observed in the midgut of F1 hybrid larvae are consistent with the activity of other digestive enzymes. Phosphatases catalyze the hydrolysis of a variety of phosphate monoesters from the consumed leaves within the larval gut, facilitating the transphosphorylation of these compounds into silkworm biomolecules. Furthermore, the level of phosphatase activity in the midgut is closely correlated with silk protein synthesis and the absorption capacity of digested food, highlighting its role in supporting nutrient assimilation during larval development (Gaikwad *et al*., 2010) [9].

Interestingly, a notable increase in alkaline phosphatase activity was recorded on day 5 prior to spinning in *Antheraea mylitta*, indicating a potential role for this enzyme in the preparation for pupation. This two-fold increase aligns with the physiological changes that occur as the insect transitions from larval to pupal stages, suggesting that ALP may be involved in the mobilization of metabolic resources necessary for this critical developmental shift (Pant and Lacy, 1969) [21]. I support the finding of ALP level high during the fifth instar larvae before going to spinning. During the pupal development, alkaline phosphatase activity was particularly low in groups exposed to continuous darkness (LD 0:24 h) and continuous light (LD 24:0 h), indicating that extreme photoperiods may suppress the enzymatic activity. Conversely, the highest levels of ALP activity were generally observed between days 82 and 96 across various light conditions, particularly in the LD 10:14 h group. This suggests that moderate light exposure may enhance the enzymatic activity, potentially facilitating metabolic processes that are crucial for the maturation of the pupae (Pant and Jaiswal, 1982) [22]

The midgut alkaline phosphatase activity levels showed a moderately high positive correlation with "renditta," which is a measure of silk yield quality, specifically the ratio of the weight of raw silk to the weight of the cocoon. A positive correlation means that as the activity of alkaline phosphatase in the midgut increases, the renditta also tends to increase. This suggests that higher levels of alkaline phosphatase activity may be associated with better silk production efficiency or quality, indicating that the enzyme could be a useful biochemical marker for selecting silkworm breeds with desirable commercial traits, Kasmaei, F. G., and Mahesha, H. B. (2012) [12]

Additionally, ALP may assist in the detoxification of xenobiotics, thereby enhancing the organism's survival in fluctuating environmental conditions (Ahlgren *et al*., 2009) [1]. This functional versatility underscores the importance of ALP in the biology of lepidopteran pests, particularly in managing their interactions with host plants and adapting to stressors.

Conclusion

In conclusion, the regulation of ALP activity in response to developmental stage, viral infection, and bacterial infection underscores the enzyme's significant role in insect physiology.

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