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Isolation and Purification of Lipase from the Midgut of Fifth Instar Larvae of *Antheraea mylitta drury*

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Authors' contributions

Present work was carried out in collaboration between both the authors. Author LM managed the literature searches, designed and performed all the experimental work and wrote the draft of manuscript. Author GB reviewed the work and Manuscript. Both the authors read and approved the final manuscript.

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ABSTRACT

Lipases obtained from healthy and pebrinised larvae were purified by 45-85% $(NH4)_2SO_4$ fractionation followed by Sephadex S-100 gel filtration and CM-Sepharose. In both the samples final enzyme purification reported was 19.02 and 17.7 folds(magnitude) and the recovery of final purified enzyme was 19.32% and 17.14% with a specific activity of 7.87 and 7.52 µmol/min/mg. Results also show that activity of purified lipase was highest at pH 8 in both the samples. Highest lipase activity was recorded between 37℃ to 40℃ tem perature and lipase activity was maximum at 37℃ temperature in healthy sample and 38℃ tempe rature in pebrinised sample. The enzyme activity reduced with addition of NaCl, Urea and $MgCl_2$ whereas EDTA and $CaCl_2$ increased the activity. The molecular weight of the purified enzyme was 30 kDa as determined by SDS-polyacrylamide gel electrophoresis.

Aim: The present study was conducted to isolate, purify and characterize lipases from the midgut of both healthy and pebrinised fifth instar larvae of *Antheraea mylitta drury*.

Study Design: Study involves dissection of midgut from the fifth instar larvae of fourth day of both healthy and pebrinised larvae, Lipases obtained from both the samples were purified by 45-85%

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$(NH4)_2SO_4$ fractionation followed by Sephadex S-100 gel filtration and CM-Sepharose and specific activity was measured at various temperatures and different pH. Molecular weight of lipase was measured by SDS PAGE.

Place and Duration of Study: Healthy and pebrinised fifth instar larvae of fourth day were collected from the forest patches of Jakaram (18.1E and 79.8 N), Warangal in August 2015.

Methodology: Lipase was isolated from the midgut of both healthy and pebrinised larvae and purification of enzyme was done by $(NH4)_2SO_4$ fractionation, Sephadex S-100 gel filtration and CM-Sepharose. Temperature, pH suitable for highest lipase activity was measured and specific activity against various chemicals was also measured. Kinetic parameters like Km and Vmax were estimated by Sigmaplot software version 11. SDS Polyacrylamide gel was used to determine the molecular weight of lipase.

Results: In both healthy and pebrinised larvae final enzyme purification reported was 19.02, 17.7 folds and the recovery % of final purified enzyme was 19.32, 17.14 with a specific activity of 7.87, 7.52 µmol/min/mg. Results also show that activity of purified lipase was highest at pH 8 in both the samples. Highest lipase activity was recorded at temperatures between 37 and 40℃ with a remarkable activity at 37℃ and 38℃ in healthy and pebrinised samples. The enzyme activity reduced with addition of NaCl, Urea and $MgCl_2$ whereas EDTA and $CaCl_2$ increased the activity. The molecular weight of the purified enzyme was 30 kDa as determined by SDS-polyacrylamide gel electrophoresis.

Conclusion: Pebrine disease has reduced the recovery percentage and also the specific activity of the enzyme. Maximum activity was recorded at high temperature in both the samples. During pebrine infection the midgut of fifth instar larvae has got influenced highly with a significant variation in many biochemical components including enzymes. Pebrine spores are the indicators of disease incidence.

Keywords: Antheraea mylitta drury; lipase; midgut; purification; characterisation.

1. INTRODUCTION

Living organisms depend on proteins, lipids and carbohydrates for their physiological activities. But the proportion of requirement varies from species to species. Storage lipids and membrane lipids are two types of lipids in insects. Storage lipids are triglycerides that are converted to mono and di glycerides in the midgut and stored for further metabolic processes [1]. Although lipids produced from carbohydrates, dietary lipids are the most important parts of ingested food [2]. Phospholipids and glycolipids are the membrane lipids digested by phospholipases [2]. Triacylglycerol lipases (EC3.1.1.3) can hydrolyse the fatty acid ester bonds in presence of organic solvents like propanol so they can be widely used in industrial areas like dairy, food, detergent and biofuel [3]. Till now lipases were isolated and extracted mainly from microorganisms, fish, fungi, milk and plants [3-4]. Lipid biochemistry studies in insects are time-consuming due to high diversity of insects and changes in lipid composition of haemolymph during metamorphosis from larva to pupa [4]. Insects rely on lipid reserves like lipophorins to survive during physiological non feeding periods or during egg development, flight and starvation [5]. In insects most of the stored fatty acids are released as 1,2-diacylglycerols and mobilization of lipid reserves from insect fat body is under the control of adipokinetic hormone [1].

Studies on enzyme catalysis explains insect physiology during stress and also help to develop new stress management strategies [6-8]. Pebrine is one of the dreadful disease seen in *Antheraea mylitta Drury (Daba TV ecorace),* caused by intracellular parasite *Nosema species*. The infection accounts for 20-25% yield loss in *Antheraea mylitta* [9]. Biochemical compounds like aminoacids, proteins and enzymes like alanine amino transferase and aspartate amino transferase was reported in the pebrinised fifth instar larvae of *Antheraea mylitta drury* [10]. During pebrine infection midgut is highly affected. As it is known that infection affects various biochemical parameters we tried to identify whether there would be any change in lipase activity The present work has taken up to understand purified midgut lipase activity (as purification explains the specific activity and yield) of fifth instar larvae of pebrinised *Antheraea mylitta drury (Daba T.V)* under different pH, temperatures and chemical compounds exposure.

2. MATERIALS AND METHODS

2.1 Sample Preparation

Fifth instar larvae of fourth day were selected and midgut was dissected in ice-cold buffer (6 µM NaCl). A 10% (w/v) homogenate of the midgut tissue of fifth instar larvae was prepared using mortar and pestle with 1 ml of universal buffer (0.2 ml of Disodium phosphate (0.2 M) +0.8 ml of Citric acid- 0.1 M). The homogenate was transferred to 2 ml centrifuge tubes and centrifuged at 10,000 rpm for 15 min. The supernatant was pooled and stored at -20℃ for subsequent purification steps and lipase assay [1].

2.2 Lipase Assay

Assay was carried out according to [11]. Mid gut extract of 20 µl and 100 µl of p-nitrophenyl butyrate (50Mm) were mixed and incubated at 35℃ temperature for 20 min. In case of control midgut extract was placed in boiling water bath for 20 min to destroy enzyme activity and then cooled. After 5 min, 200 µl of distilled water was added and absorbance was recorded at 405 nm. One unit of enzyme release one nano moles of p-nitrophenol per minute using p-nitro phenyl butyrate as substrate. Standard curve was used to measure the specific activity of enzyme (Fig. 1).

2.3 Purification of Lipase

Purification of midgut lipase of fifth instar larvae was performed in three steps as described by Orscelk et al. [12].

2.4 Ammonium Sulphate Treatment

Midgut samples were first subjected to ammonium sulphate precipitation by 40 and 80% fractions. The precipitated fractions were centrifuged at 10000 rpm for 10 min and supernatant of each fraction was suspended in 1 ml universal buffer (pH 10).

2.5 Gel Filtration

Sephadex S-100 column (2 cm × 100 cm) equilibrated with 0.02 M concentration of universal buffer (pH 10) was used on which last ammonium sulphate fraction of fifth instar larvae was subjected for further purification. Same buffer was used to collect enzyme fractions of 5 ml at a flow rate of 30 ml/hr. Protein content and lipase activity of both samples were measured in all fractions to identify the fraction with highest activity.

2.6 CM-Sepharose Separation

Final high active fractions were applied to a CM-Sepharose column (3 cm × 30 cm) equilibrated with 0.02 universal buffer (pH 10). Same buffer was used for first wash and in presence of sodium chloride, bound proteins were eluted. Fractions of 5 ml each were collected at a flow rate of 60ml/h. Highest lipase activity fractions were pooled and stored at -20℃ for further analysis.

2.7 Kinetic Parameters

Every assay was carried out with thirty microliter of diluted enzyme. Final concentrations of substrate were 20, 30, 40, 50 and 60 mM. Michaelis Menten constant (Km) and the maximal velocity (Vmax) were estimated by Sigmaplot software version 11. Km and Vmax are the means of ±SE of three replicates.

2.8 Effect of Various Compounds on Enzyme Activity

To test the effect of various compounds on the midgut lipase activity of fifth instar larva CM-Sepharose final active fractions were used. Enzyme assay was performed in the presence of different concentrations of sodium chloride (10, 20, 30, 40 mM respectively), Urea (10, 20, 30 and 40 mM respectively), calcium chloride (10, 20, 30 and 40 mM respectively), magnesium chloride (10, 20, 30 and 40 mM respectively), and EDTA (1.0, 2.0, 3.0. and 4.0 mM respectively). The compounds were added to assay mixture and activity was measured after 45 minutes.

2.9 Effect of Temperature on Enzyme Activity

Temperature impact was determined by incubating CM Sepharose final active fractions of fifth instar larvae at 20, 25, 30, 35, 37, 38 40, 45, 50, 55, 60 and 65℃ for 24 hours followed by enzyme assay.

2.10 Effect of pH on Enzyme Activity

pH effect on lipase activity was measured using CM Sepharose final active fractions of fifth instar

larvae. Optimal pH for activity was estimated using universal buffer with pH set at 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 and 13.

2.11 Protein Determination

Concentration of protein was measured according to the method of using Bovine serum albumin as standard [13].

2.12 Polyacrylamide Gel Electrophoresis (PAGE)

Molecular mass of lipase was carried out using 2.5% and 6.5% polyacrylamide for the stacking and resolving gels [14]. The gel was stained with 1.5% (w/v) Coomassie Brilliant Blue G-250.Later gels were de stained in a solution containing 5% (V/V) acetic acid and 20% methanol (V/V).

2.13 Statistical Analysis

Each assay was replicated 3 times. Values were expressed as mean ±SE of replication and data was compared by one way analysis of variance (ANOVA). Students t-test was applied to locate significant ($p<0.05$) differences.

3. RESULTS AND DISCUSSION

Results showed that lipase is present in the midgut of both healthy and pebrinised fifth instar larvae of *Antheraea mylitta drury*. Tables 1 and 2 depicts purification steps of lipase of both healthy and pebrinised fifth instar larvae. Lipases obtained from the midgut of larvae were purified by 45-85% $(NH4)_2SO_4$ fractionation followed by Sephadex S-100 gel filtration and CM-Sepharose. Protein concentration has reduced by 89% from crude extract to final Ammonium sulphate extraction step in healthy larvae whereas a reduction of 84.5% was recorded in pebrinised larvae which denotes high protein purification level in healthy larvae in comparison to pebrinised larvae. The Standard curve was used to measure specific activity of lipase (Fig. 1). In case of healthy and pebrinised larvae, final enzyme purification reported was 19.02, 17.7 folds and the recovery of final purified enzyme was 19.32%, 17.14% with a specific activity of 0.41, 0.42 µmol/min/mg of crude extract which has increased to 7.87, 7.52 µmol/min/mg in CM Sepharose fraction. During infection specific activity of lipase generally increases [15]. Metabolite depletion causes physiological imbalances in the host which lead to reduction in lipid concentrations and increase in lipase activity [15].

Table 3 shows that several chemicals affect the activity of lipase in midgut. With the increase in chemical concentration lipase activity found increased due to $CaCl_2$ and EDTA but decreased in presence of NaCl, Urea and $MgCl_2$ in both the samples. This is because high concentration of monovalent ions neutralise protein charges and changes protein structure and thus decreases enzyme activity. Whereas divalent ions form salt bridges between carboxylic groups of amino acids and reduces enzyme activity. High concentration of some salts decrease water activity and thus reduces enzyme activity. The role of lipase inhibitors will provide a better perceptive of their mechanism of action and successful identification of potent and specific inhibitors which results in their application in certain treatments [16]. Lipases are metalloproteins which require Ca^{2+} ions as their activators. Elevation of calcium concentration in the reaction solution of lipases extracted from *R. prolixus* increases the enzyme activity [3].

Table 1. Purification procedure of the lipase from the midgut of fifth instar larvae of healthy *Antheraea mylitta*

Purification step	Total protein (mg)	Total enzyme activity µmol/min	Specific activity µmol/min/mg	Yield (%)	Purification (fold)
Crude extract	0.786±0.18	0.326±0.028	0.414±0.058	100	1
$(NH_4)_2SO_4$ (0-40%)	0.375±0.12	0.308±0.015	0.821±0.085	94.47	1.98
$(NH_4)_2SO_4$ (40-80%)	0.12±0.08	0.218±0.021	1.816±0.068	66.87	4.39
Sepharyl G-100	0.065±0.006	0.165±0.025	2.538±0.055	50.61	6.13
CM-Sepharose	0.008±0.002	0.063±0.018	7.875±0.075	19.32	19.02

Table 2. Purification procedure of the lipase from the midgut of fifth instar larvae of pebrinised *Antheraea mylitta*

Purification step	Total protein (mg)	Total activity µmol/min	Specific activity (µmol/min/mg)	Yield (%)	Purification (fold)
Crude extract	0.741±0.18	0.315±0.015	0.425±0.058	100	1
$(NH_4)_2SO_4$ (0-40%)	0.364±0.12	0.242±0.014	0.658±0.085	76.82	1.55
$(NH_4)_2SO_4$ (40-60%)	0.115±0.006	0.202±0.025	1.756±0.068	64.12	4.13
Sepharyl G-100	0.061±0.005	0.142±0.015	2.325±0.055	45.07	5.47
CM-Sepharose	0.07±0.004	0.054±0.012	7.523±0.075	17.14	17.7

Table 3. Activity of midgut lipase of healthy and pebrinised *Antheraea mylitta* larvae towards various compounds

Type of compounds	Concenteration (mmol/l)	Midgut lipase activity in healthy larvae µmol/min/mg	Midgut lipase activity in pebrinised larvae (µmol/min/mg)
NaCl	10	110.35±4.5	95.85±7.5
	20	98.95±3.6	63.25±5.6
	30	47.85±2.8	43.65±4.2
	40	12.65±1.2	10.48±1.2
EDTA	1.0	48.66±2.4	37.45±2.6
	1.5	96.54±3.5	78.95±6.5
	2.0	143.56±6.2	125.63±11.4
	2.5	218.55±12.8	197.64±12.6
UREA	10	85.75±4.8	75.86±8.6
	20	68.48±3.8	53.24±6.5
	30	45.76±2.6	35.63±2.5
	40	26.35±1.8	18.22±3.6
$CaCl_2$	10	48.56±2.5	38.95±2.6
	20	68.75±3.6	56.85±4.5
	30	95.45±8.6	87.65±6.5
	40	112.65±10.5	105.65±8.6
$MgCl_2$	10	225.66±12.8	218.23±10.8
	20	145.55±10.4	123.45±12.5
	30	102.34±8.5	98.46±9.5
	40	35.21±3.4	22.18±2.2

Table 4 shows that, the optimum temperature range for midgut lipase activity of both healthy and pebrinised fifth instar fourth day larvae were 37℃ to 40℃. Whereas maximum activity was recorded in-vitro at 37℃ in healthy and 38℃ temperature in case of pebrinised larvae. [17] Working on lipase activity in *Cirrhinus reba* reported that the optimum temperature of lipase for the hydrolysis is 35℃. Extreme temperatures will disrupt the hydrogen bonds that hold the enzyme in its three dimensional structure finally denaturing the proteins [18].

The *in vitro* evaluation of midgut lipase indicated that enzyme activity increased steadily from pH 2 to 8 in both healthy and pebrinised fifth instar larvae (Table 5). Enzyme gave maximum activity at pH 8 and after this threshold level, activity got decreased with the increase of pH in both the samples. More than 50% activity was decreased below pH 4 and beyond pH 11in both the samples. Immobilization and stability studies of lipase from thermophilic Bacillus *sp.* have shown that lipase activity was maximum at p^H 8 [19]. Vmax and Km of midgut lipase recorded were 8.2±0.56 µmol/min/mg and 26.4±1.45 Mm. In

case of pebrinised larvae, Vmax and Km got decreased to 0.5±0.05 mol/min/mg and 8.2±1.2 mM (Figs. 2, 3 and Table 6).

Table 4. Temperature effect on midgut lipase activity of healthy and pebrinised *Antheraea mylitta* larvae

Temperature (℃)	Midgut lipase activity in healthy larvae (µmol/min/mg)	Midgut lipase activity in pebrinised larvae (µmol/min/mg)
20	0.1±0.08	0.05±0.004
25	0.18±0.05	0.10±0.08
30	0.25±0.06	0.16±0.06
35	0.42±0.12	0.35±0.12
37	0.54±0.15	0.42±0.15
38	0.48±0.12	0.50±0.18
40	0.45±0.13	0.40±0.16
45	0.32±0.14	0.30±0.14
50	0.30±0.15	0.28±0.12
55	0.26±0.05	0.22±0.14
60	0.23±0.08	0.18±0.05
65	0.22±0.05	0.12±0.06
70	0.17±0.05	0.08±0.005

Table 5. Impact of pH on midgut lipase activity of healthy and pebrinised *Antheraea mylitta* larvae

pH	Midgut lipase activity in healthy larvae (µmol/min/mg)	Midgut lipase activity in pebrinised larvae (µmol/min/mg)
2	0.08±0.004	0.05±0.006
3	0.16±0.06	0.10±0.04
4	0.28±0.05	0.18±0.06
5	0.32±0.12	0.26±0.05
6	0.43±0.16	0.35±0.12
7	0.58±0.22	0.44±0.15
8	0.82±0.24	0.68±0.14
9	0.74±0.18	0.52±0.18
10	0.68±0.14	0.43±0.16
11	0.42±0.16	0.32±0.15
12	0.33±0.12	0.24±0.08
13	0.21±0.15	0.15±0.05

Fig. 4 shows that purified enzyme was used as an enzyme sample and was subjected to SDS-PAGE of which lipase showed a molecular weight of 30 kDa. The molecular weight was compared with the high range protein marker.

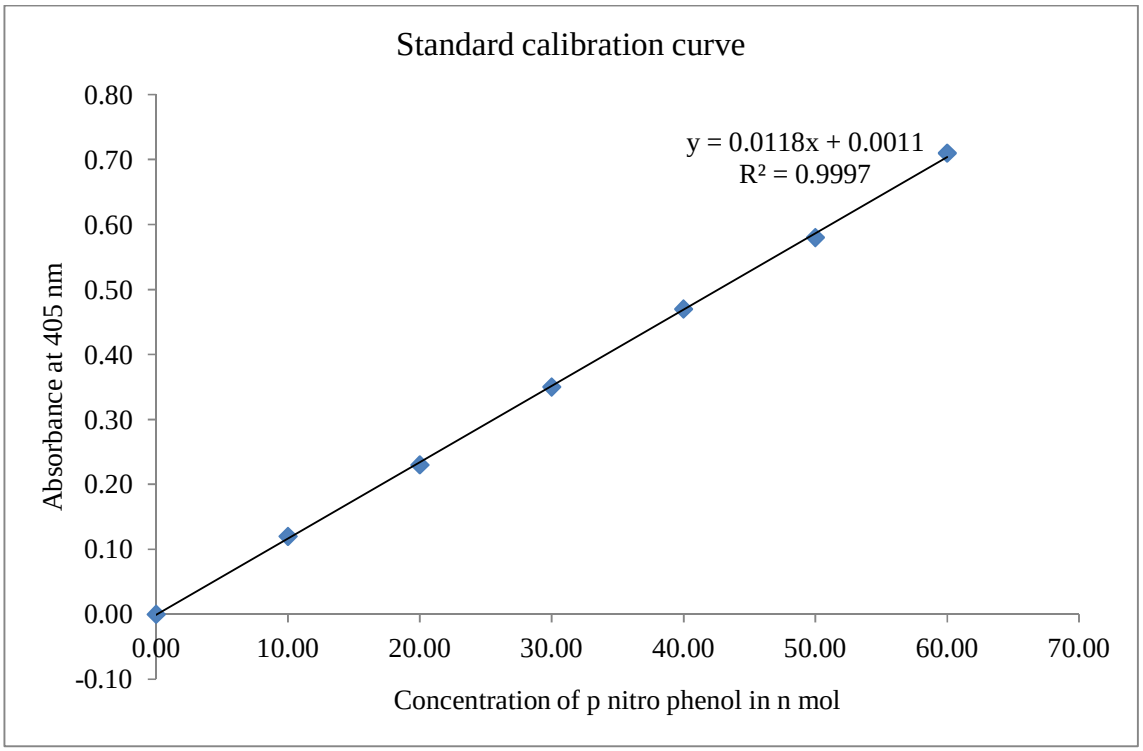

Fig. 1. Standard calibration curve for the determination of p-nitro phenol released in the lipase

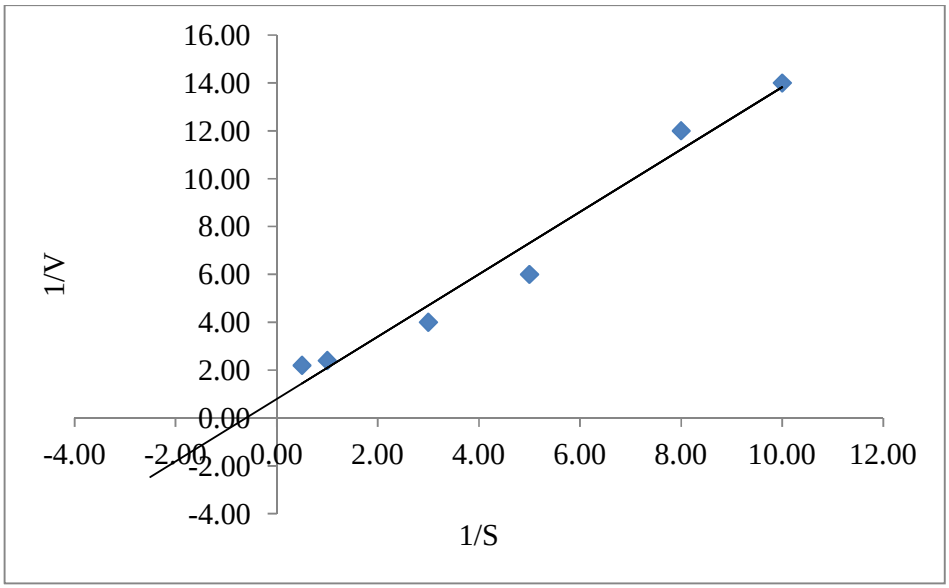

Fig. 2. Line Weaver -Burk plot (Vmax and Km) of lipase extracted from the healthy fifth instar *Antheraea mylitta drury* larvae

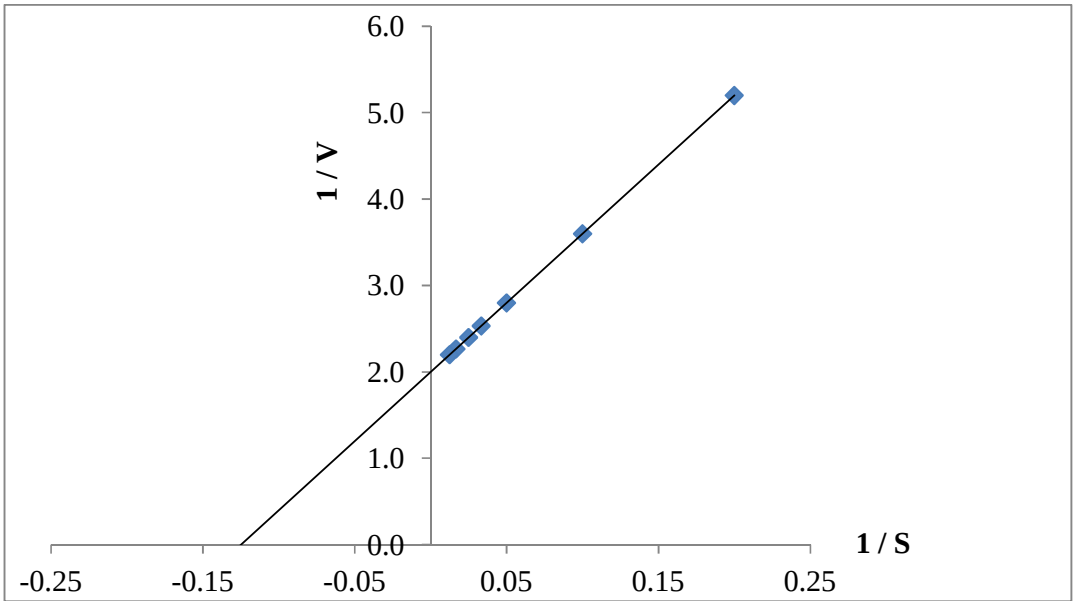

Fig. 3. Line Weaver -Burk plot (Vmax and Km) of lipase extracted from pebrinised fifth instar *Antheraea mylitta drury* larvae

Table 6. Kinetic parameters of lipase enzyme extracted from midgut of 5th instar larvae of healthy and pebrinised *Antheraea mylitta drury* larvae

Type	Enzyme	Vmax (µmol/min/mg protein)	Km (mM)
Healthy Larvae	Lipase	8.2±0.56	26.4±1.45
Pebrinised Larvae	Lipase	0.5±0.05	8.2±1.2

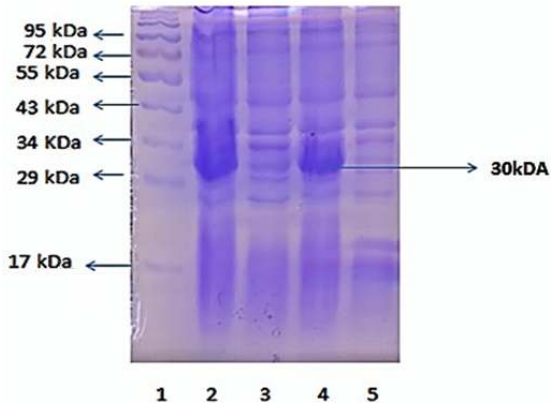

Fig. 4. SDS PAGE

Lane 1: Molecular marker; Lane 2, 4: Pure enzyme with approximately 30 kDa molecular weight. Lane 3,5 : Crude enzyme with other proteins

Impact of Pebrine disease was high on commercial characters of Tasar silkworm through transovarian infection rather than secondary infection [20]. When the digestive enzyme inhibitors are known, enzymes can be inhibited which impairs insect nutrition, retards growth and development of insect and eventually leads to death [21]. Application of suitable enzyme inhibitors on pebrinised larvae reduces the transovarian infection with an increase in mortality rate of pebrinised tasar silkworms as these inhibitors blocks the enzyme activity. Finally, purification and characterization of insect digestive enzymes will help in understanding the enzyme mechanism and helps to design new strategies for control of diseases involved those enzymes.

4. CONCLUSION

In conclusion, recovery percentage, purification fold, yield percentage and also the specific activity of lipase found reduced in pebrinised larvae. Maximum lipase activity was recorded at high temperature and at high pH in both the samples. Various chemicals have various impacts on lipase activity in both the samples.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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