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Biochemical Characterization of the Larval Alpha-amylase of *Leucinodes orbonalis* Guenee (Lepidoptera: Pyralidae) from the Field Collected Population of Uttar Dinajpur, West Bengal, India

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ABSTRACT

Background: Brinjal shoot and fruit borer (*Leucinodes orbonalis* Guenee) significantly harms brinjal crops throughout South Asia. The internal feeding habit of larvae renders pesticides ineffective and contributes to resistance against broad-spectrum insecticides, posing severe management challenges. Targeting insect α-amylase to design enzyme inhibitors presents a promising approach for pest suppression. Therefore, biochemical characterization of larval α-amylase of *L. orbonalis* was carried out.

Methods: Spectrophotometric assays were performed to determine the pH optima and optimal temperature range for α-amylase activity. Effects of various activators and inhibitors on the enzyme were also calculated. Enzyme kinetics study was done to decipher the Km and Vmax of the enzyme-substrate reaction. Enzyme activity was also noted in by zymographic study.

Result: The α-amylase enzyme exhibited a specific activity of 0.294 U/mg of insect protein. It operates optimally at pH 9 and within a temperature range of 45-50°C. Activity of α-amylase was moderately enhanced by Na^+ and K^+ ions, while Mg^{2+} and Ca^{2+} ions significantly increased enzymatic activity. Inhibitory effects were observed with ethylenediaminetetraacetic acid (EDTA), sodium dodecyl sulfate (SDS) and gallic acid (GA) at 5 mM concentrations, reducing activity by 54.62%, 29.07% and 74.04%, respectively. Kinetic analysis of α-amylase revealed that its Km was 0.315% and Vmax was 0.099 mM maltose/min. Two clear zones observed through Native PAGE zymography confirmed the identification of two distinct α-amylase isoforms in the gut.

Key words: α-amylase, Inhibitor, *Leucinodes orbonalis*, Pests, Zymography.

INTRODUCTION

Brinjal or eggplant (*Solanum melongena* L.) is indigenous to the Indian subcontinent and belongs to the family Solanaceae (Nightshade) (Gnanamani and Vijayalakshmi, 2024; Niranjana, 2017; Tsao and Lo, 2006). It is a major vegetable crop in India, cultivated widely across the country except in high-altitude regions. It is often called the “King of Vegetables” because of its versatile use in a wide range of dishes across Indian cuisine (Choudhary and Gour, 2009). India ranks second globally in brinjal production, following China (Nino-Medina *et al.,* 2017), with West Bengal contributing 23.72% of the national output. The major brinjal-producing districts in West Bengal include Murshidabad, Nadia, Malda, Bankura, Jalpaiguri and Uttar Dinajpur (Mandal *et al.,* 2019; Anonymous, 2018).

Brinjal productivity in India is significantly hampered by biotic stresses, particularly insect pests and diseases (Singh, 2019). Among the 26 documented insect pests, the brinjal shoot and fruit borer (BSFB) (*Leucinodes orbonalis* Guenee) (Pyralidae: Lepidoptera) is the most destructive, causing yield losses of 60% to 80% nationally (Kaur *et al.,* 2010; Niranjana *et al.,* 2017) and 32% to 74% fruit damage in West Bengal across seasons (Ghosh *et al.,* 2003). Managing this pest is challenging because the larvae reside inside the fruit or shoot, preventing the pesticides from directly reaching the pest (Ambethgar *et al.,* 2025). Farmers mainly use pesticides to control the pest population to produce

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maximum unblemished brinjal fruits. Moreover, brinjal is typically consumed in its unprocessed form. Therefore, application of uncontrolled conventional insecticides makes it very hazardous to human health, contributes to the emergence of insecticide resistance (Dadmal *et al.,* 2004; Kumar *et al.,* 2025) and increases production costs. Hence, it has become mandatory to search for an effective alternative to conventional chemical control by routine insecticide spray. Pest-resistant crop plant production can be one such alternative.

Information related to the physiology and biochemistry of the insect midgut can play a crucial role in developing novel insecticidal strategies by the incorporation of inhibitor gene in the host plant to create a transgenic plant (Wang *et al.,* 2023). So, it has become essential to understand the biochemical characteristics of gut enzyme α-amylase, a hydrolytic enzyme found in microorganisms (Agüloğlu *et al.,* 2014; Zaferanloo *et al.,* 2014), plants (Amid *et al.,* 2014) and animals (Horii *et al.,* 1987; Grossman *et al.,* 1942). This particular hydrolytic enzyme plays a crucial role in polysaccharide digestion by catalyzing and hydrolyzing α-D-(1,4)-glucan linkages in starch and related polysaccharides (Gupta *et al.,* 2003; Strobl *et al.,* 1998). The function of α-amylase in starch digestion has been extensively studied in various insect species, including Egyptian cotton leaf worm (*Spodoptera littoralis*, Lepidoptera) (Darvishzadeh *et al.,* 2014), olive fruit fly (*Bactrocera oleae*, Diptera) (Delkash-Roudsari *et al.,* 2014), Colorado potato beetle (*Leptinotarsa decemlineata*, Coleoptera) (Hamori *et al.,* 2021), wheat bug (*Eurygaster maura*, Hemiptera) (Ravan *et al.,* 2009) and others.

Understanding the functional dynamics of digestive enzymes on their specific substrates is crucial for formulating advanced insect pest management strategies. Among these, assessing enzyme sensitivity to different inhibitors has proven to be an effective approach for developing transgenic plant-based control programs targeting phytophagous insects (Yezdani *et al.,* 2010). Despite the significant role of α-amylase in carbohydrate metabolism, limited information is available on its properties in the brinjal shoot and fruit borer (*Leucinodes orbonalis* Guenée), a major pest of brinjal. This study quantifies and partially characterizes α-amylase activity in *L. orbonalis* to improve knowledge of its digestive physiology and support innovative pest management approaches.

MATERIALS AND METHODS

Larvae collection

The larvae of different instars of *L. orbonalis* were collected by tearing affected brinjal from different fields of Raiganj Block, Uttar Dinajpur district, West Bengal, India (25.9810° N, 88.0510° E) and its surrounding areas during the years 2023 and 2024. The research work was carried out at the Department of Zoology, Raiganj University.

Chemicals

Bovine serum albumin (BSA), Maltose, Starch, 3,5-Dinitrosalicylic acid (DNS), acrylamide, bis-acrylamide, Tris, Glycine, TEMED (Tetramethylethylenediamine), Ammonium persulphate (APS), Triton X-100 were purchased from SRL, India. Homogenization buffer, solutions of starch (1%) and the staining solutions for gels were prepared fresh before use.

Method of larval digestive tract enzyme extraction

Enzyme samples from the digestive tract of different larval instars were made by the method of Cohen (1993) with slight modifications. The dissected digestive tracts were isolated and homogenized in ice-cold 0.1 M PBS buffer, pH 7.0 (1/4, w/v) and centrifuged at 14,000 rpm for 15 minutes at 4°C. Supernatants were decanted on a separate tube and subsequently centrifuged again in similar conditions (Tierno de Figueroa *et al.,* 2011). The resultant supernatant was collected and stored at -20°C for further use.

Amylase assay

The α-amylase activity was quantified by the dinitrosalicylic acid (DNS) method, employing 1% starch solution as substrate with slight modification of the protocol as described by Champasri *et al.,* 2021. 100 µl of enzyme extract, 5 mL buffer and 500 µl of 1% starch solution (prepared in 0.05M Tris-HCl, pH 8.0) were mixed to prepare the reaction mixture, which was incubated for 30 mins. 1 ml of DNS was added to stop the reaction and subsequently heated in a boiling water bath for 10 minutes. Absorbance of supernatants was measured after cooling to room temperature at 540 nm, using a UV-VIS Spectrophotometer (LABTRONICS, Model No: LT-2201). A blank containing larval extract without the substrate and a control containing the substrate without larval extract were prepared simultaneously. The amount of maltose liberated by the enzyme was measured by using a standard curve of maltose, reacted with DNS. One unit of enzyme activity can be defined as the amount of enzyme needed for the production of 1 mg of maltose in 30 minutes at 35°C. All assays were carried in triplicates.

Determination of optimum temperature and pH for the enzyme activity

To determine the optimal temperature, the enzyme assay mixture was incubated at temperatures ranging from 15°C to 60°C with 5°C increments for 30 minutes. Following incubation, amylase activity was estimated as described above.

To measure the optimum pH required for amylase substrate reaction, the enzyme assays were carried out at different pH values from 4 to 13. The pH values were maintained using buffers like 0.1 M sodium acetate for pH 4-5, 0.1 M sodium phosphate for pH 6-7, 0.1 M Tris-HCl for pH 8-9, 0.1 M glycine-NaOH for pH 10-11 and 0.1 M KCl-NaOH for pH 12-13.

Study of enzyme activators and inhibitors

To measure the efficacy of various ions as activators, enzyme activity was assayed with 20 mM concentration of chloride salts of Na^+, K^+, Ca^{2+} and Mg^{2+}. 5 mM of Ethylene-diaminetetraacetic acid (EDTA), Sodium dodecylsulphate (SDS) and Gallic acid were tested as inhibitors in the reaction mixtures. A reaction mixture containing enzyme, substrate, buffer and none of the above compounds was also used as a control for experimental purpose.

The percentage of α-amylase activation or inhibition was estimated by:

$$\%\ I_{\alpha\text{-amylase}} = 100 \times \frac{(|\Delta A_{540Control} - \Delta A_{540\ Exp.}|)}{\Delta A_{540\ Control}}$$

Total protein concentration (larval gut extract) measurement

The Lowry (1951) method was used to determine protein concentration in larval extract and BSA was used as a standard.

Kinetic studies

The relationship between amylase hydrolytic activity and substrate concentration was estimated. Enzyme assays were conducted across a range of starch concentrations: 0.25%, 0.5%, 0.75%, 1%, 1.25%, 1.5% and 2% (w/v). The same amount of enzyme concentration was used throughout the experiment. In controls, distilled water was used instead of the enzyme at each substrate concentration. Maximum velocity (Vmax) and Michaelis constant (Km) were measured by using a Lineweaver-Burk (double reciprocal) plot.

Electrophoresis and zymograms

The α-amylase found in the crude gut extract was investigated by SDS polyacrylamide gel electrophoresis (PAGE) following the protocols of Lammli (1970) and Andrades *et al.* (2017) with slight modifications. The electrophoresis was carried out on a separating gel (10%, w/v) and a stacking gel (4.5%), both containing SDS (1%). The running buffer was prepared according to Lammli (1970) but excluded SDS. The sample buffer contains Tris-HCl (0.5 M, pH 6.8), SDS (2%), bromophenol blue (without α-mercaptoethanol) (0.01%, w/v) and glycerol (20%) and was not subjected to heating. At 4°C, electrophoresis was performed with a steady voltage of 50 V. Zymogram analysis was performed using the Mini Dual Vertical Electrophoresis Unit (TARSONS). Thorough washing of the gel was done with water and gently rinsed in Triton X-100 (1%, v/v) in PBS (pH 9) for removal of SDS. Subsequently, the gel was incubated for 4 hours in a development buffer (50 mM Tris, pH 9 and 1 mM $CaCl_2$). Staining of the gel was performed with 0.2% KI and 0.1% iodine solution and active enzyme zones were observed as light spots against a dark brownish background.

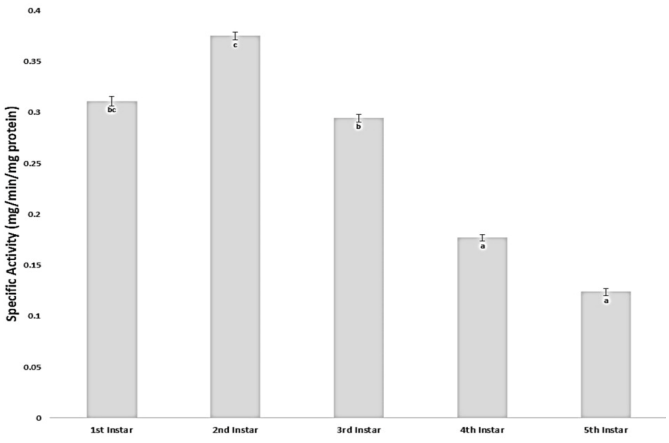
Statistical analysis

One-way analysis of variance (ANOVA) was performed by Microsoft Excel software and Tukey's Test was done at $P \leq 0.05$ significance level for assessment of differences between sample means (n=3) by using IBM SPSS software (Version 18).

RESULTS AND DISCUSSION

α-Amylase activity

Activity of α-amylase was detected in the gut of *L. orbonalis* larvae using starch as a substrate. The average enzyme activity differed significantly across the larval stages (df = 4, F = 741.855, P<0.05). The α-amylase activity increased with larval growth up to the 2nd instar, followed by a gradual decline in subsequent stages (Fig 1). The 2nd instar exhibited the highest enzyme activity (0.374±0.0037 mg/min/mg



The values are the mean ± SEM (n=3). Different letters signify that Tukey's test shows a significant difference (P<0.05) in the activity of enzymes at different instars.

Fig 1: α-amylase activity at different instars of *L. orbonalis*.

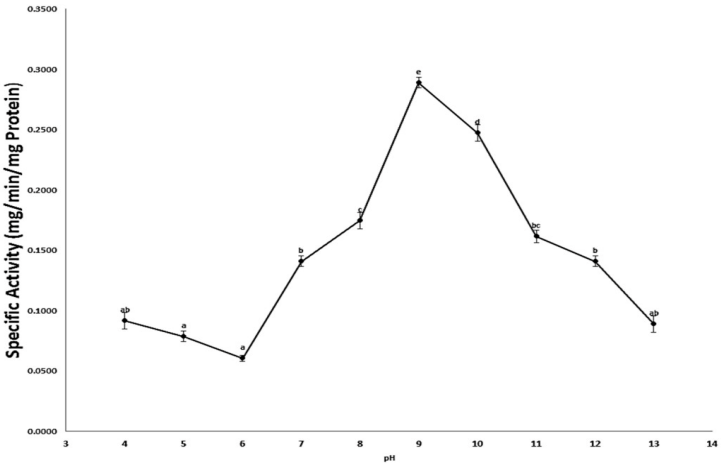
protein or U/mg protein), which was not significantly different from the 1st instar but differed significantly from the other stages. The enzyme activities recorded in the 1st and 3rd instars (0.310±0.0046 and 0.294±.0037 U/mg protein) were comparatively higher, but significant difference was not found, though both differed significantly from the 4th and 5th instars. Low range of enzyme activities were observed in the 5th instar (0.123±0.0033 U/mg protein) and the 4th instar (0.177±0.0032 U/mg protein) and the enzyme activity of both significantly differed from the earlier instars.

Effect of pH and temperature on enzyme activity

The relationship between pH and α-amylase activity on starch in the digestive extract of larval *L. orbonalis* is illustrated in Fig 2. Enzyme activity started to increase from pH 6 and reached the highest activity at pH 9 and then decreased with increasing pH (Fig 2). Amylase activity at optimal pH (pH 9) was 0.2813±0.0045 U/mg protein. The α-amylase activity increased from 25°C, peaked at 40°C and then declined, indicating broad temperature tolerance (Fig 3).

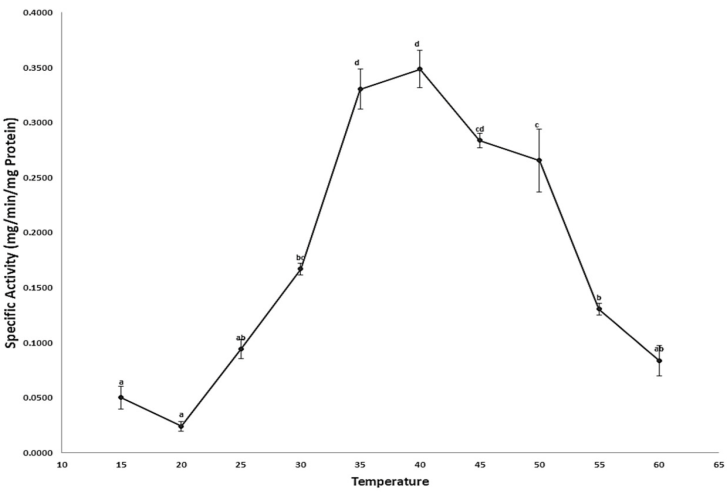
Effect of activators and inhibitors on enzyme activity

Effects of activator and inhibitor on α-amylase activity have been demonstrated in Table 1. The enzyme activity was observed to be increased by the chloride salts of Na^+, K^+, Ca^{2+} and Mg^{2+}. Among these, Ca^{2+} is found to be most effective to increase the activity of the enzyme, while Na^+, K^+



The values are the mean ± SEM (n=3). Different letters signify that Tukey's test shows a significant difference (P<0.05) in the activity of enzymes at different pH.

Fig 2: Effect of pH on the activity of α-amylase from the digestive tract of *L. orbonalis*.



The values are the mean ± SEM (n=3). Different letters signify that Tukey's test shows a significant difference (P<0.05) in the activity of enzymes at different.

Fig 3: Effect of temperature on the activity of α-amylase from the digestive tract of *L. orbonalis*.

and Mg^{+2} ions mildly increase the enzyme activity. Furthermore, EDTA, SDS and Gallic Acid at a concentration of 5 mM were found to inhibit enzyme activity by 51.10%, 31.42% and 73.42%, respectively.

Kinetic studies

Analysis of the Lineweaver-Burk plot derived from reciprocal starch concentration and α-amylase activity revealed a Km of 0.315% starch and a Vmax of 0.099 mM maltose/min for the digestive α-amylase enzyme of *L. orbonalis* (Fig 4 and Fig 5).

Electrophoretic study

Native SDS-PAGE was performed to separate amylase isoforms and their molecular weights were analyzed. Electrophoresis using 10% SDS gels revealed two distinct α-amylase bands (isoforms) when starch was used as the substrate (Fig 6). In the zymographic analysis, clear bands appearing against a dark-stained background indicated the presence of active enzymes capable of hydrolyzing starch. Two amylase isoforms were detected in *L. orbonalis*, with estimated molecular weights of approximately 60 KDa and 180 KDa.

Table 1: Relative activity of α-amylase of *L. orbonalis* towards different compounds.

Compound	Concentration (mmol/lit)	Relative activity (%)
Control	0	100
NaCl	20	115.86
KCl	20	109.45
$MgCl_2$	20	127.66
$CaCl_2$	20	157.19
EDTA	5	48.89
SDS	5	68.57
Gallic acid	5	36.57

The alpha-enzyme activity has been detected in the various larval instars of *L. orbonalis* (Guenee) according to the results of the present study. Active forms of α-amylase have been reported in several other studies with borer pests of Lepidoptera like *Chilo suppressalis* (Zibaee *et al.,* 2008), *Spodoptera littoralis* (Darvishzadeh *et al.,* 2014), or *Pieris brassicae* L. (Sharifloo *et al.,* 2016). Most insect crop pests thrive on a diet rich in complex carbohydrates, such as polysaccharides and therefore their growth and survival depend on the effective utilization of these polysaccharides by α-amylases (Nation, 2008; Chapman, 2012). High content of polysaccharides of brinjal would certainly induce the production of active forms of alpha-amylases in *L. orbonalis*. In the present study, a significant difference in α-amylase activity was noticed among different instars and peak activity was found in the second instar, which may be due to the differentially expressed amylase gene in various larval instars in the *L. orbonalis.* The phenomenon of this differential amylase gene expression within various larval instars has been documented already in studies with other Lepidopteran and Coleopteran insects (Kluh *et al.,* 2005).

The results of the current study have revealed that alpha-amylase of *L. orbonalis* remains active over a wide pH range, with the optimum pH being 9. It has been reported in several other studies (Dow, 1984; Abraham *et al.,* 1992; Zibaee *et al.,* 2008; Kaur *et al.,* 2014) that the optimum pH for lepidopteran alpha-amylase is extremely alkaline (9-11.5), which is in close agreement with our current finding. According to Chapman (1998), the retention of maximum activity of digestive enzymes at high pH values may be an adaptive response to deal with higher tannin content in the insect diet, as tannin precipitates proteins by binding with them at low pH values. Brinjal, the primary host plant of *L. orbonalis*, contains high levels of tannins (Sharma *et al.,* 2019), potentially exerting a selective pressure on the pest. The dependence of alpha-amylase can be due to the

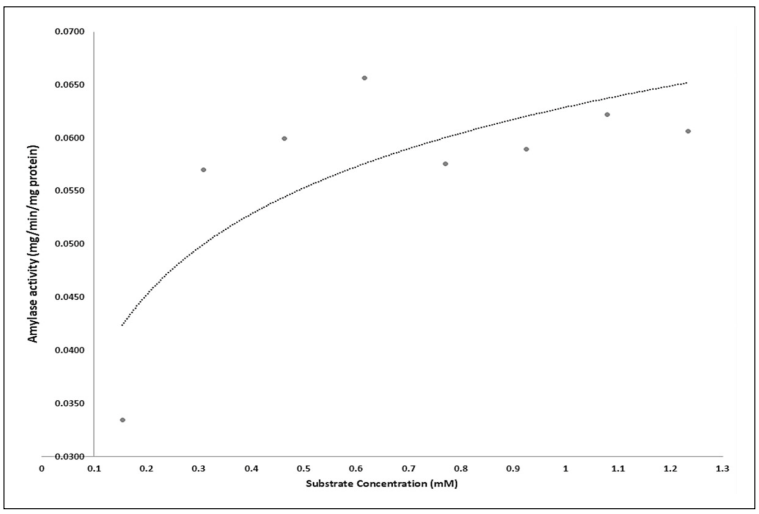


Fig 4: Michaelis-Menten plot of enzyme activity (velocity) versus starch concentration (%) to obtain values for the maximum velocity (Vmax) and Michaelis constant (Km) for α-amylase of *L. orbonalis*.

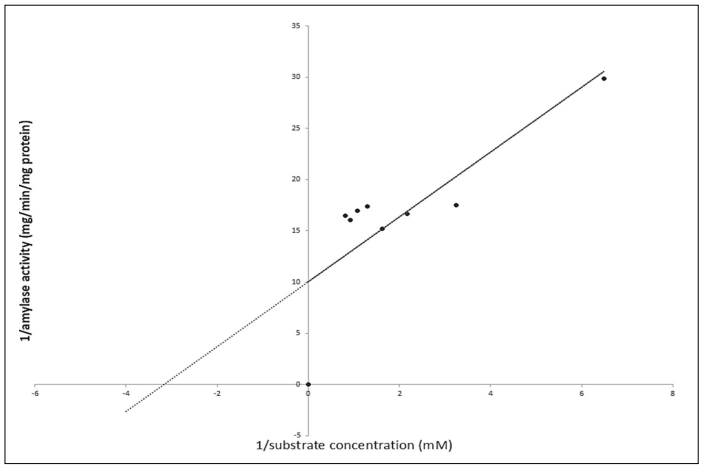


Fig 5: Double reciprocal plot (Lineweaver-Burk plots) of enzyme activity (velocity) versus starch concentration (%) to obtain values for the maximum velocity (Vmax) and Michaelis constant (Km) for α-amylase of *L. orbonalis*.

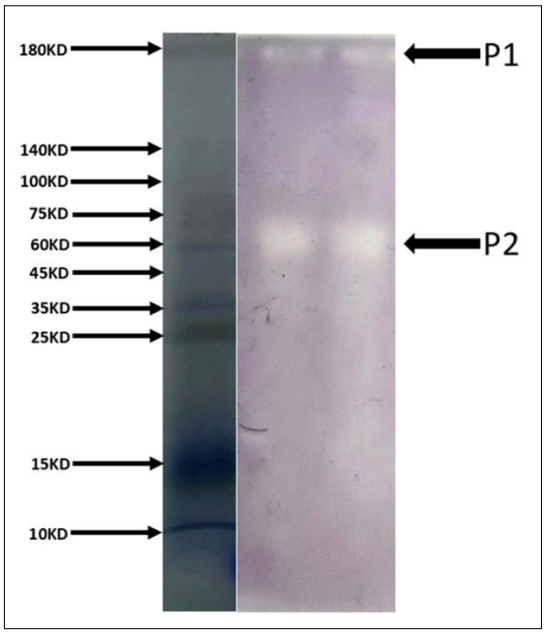


Fig 6: Native gel electrophoresis showing α-amylase bands (P1 and P2) with molecular weights.

selection pressure forcing the evolution of alkaline RNQ-type alpha-amylase in the lepidopteran digestive systems (Terra and Ferreira, 2012).

In the study, the optimal temperature for *L. orbonalis* α-amylase activity was found to be 40°C. The rapid decrease of enzyme activity above 40°C suggests sensitivity of the enzyme towards temperature. The functionality of the enzyme was almost absent beyond 65°C. Our current data on optimal temperature study of α-amylasae of *L. orbonalis* is within the recorded range of 30-60°C (Sharifloo *et al.,* 2016; Kaur *et al.,* 2014; Mendiola-Olaya *et al.,* 2000).

It is very well established by several studies that metal ions are crucial for the proper activity, stability of insect α-amylase (Kaur *et al.,* 2014; Terra and Ferreira, 2012), which is very much in line with our findings of α-amylase enzyme activity. Augmentation of α-amylase activity was noted by addition of sodium, magnesium, or calcium ions. Chloride salts of metal ions were used as the potent activators and the increase of amylolytic enzyme activity can also be brought about by these chloride ions that activate hydrolysis of polysaccharides by the change of optimal pH (Terra and Ferreira, 2012). A significant inhibition of 31-51% of amylolytic activity reduction was observed in our results by the metal-chelating agent EDTA and SDS detergent, which is very much comparable to other studies (Cohen and Hendrix, 1994; Zeng and Cohen, 2000; Sharifloo *et al.,* 2016). Ca^{+2} ion exclusion from the enzyme complex or enzyme denaturation may be the reason behind this inhibition by EDTA or SDS. In the current finding, maximum increase of amylase activity was observed with Ca^{+2} ion, which is strictly in line with other studies of the role imparted by metallic activators on amylolytic activity (Kazzazi *et al.,* 2005; Zibaee *et al.,* 2008; Delkash-Roudsari *et al.,* 2014; and Sorkhabi-Abdolmaleki *et al.,* 2014). Maximum inhibition of amylolytic enzyme activity was seen with gallic acid in the assay mixture. The inhibitory potential of gallic acid or other polyphenolics has been reported in many other works (Ahmed *et al.,* 2013; Lu *et al.,* 2016; Oboh *et al.,* 2019). The conformation of alpha-amylase is altered by the complexation with gallic acid, which is brought about by non-covalent interactions like hydrogen and hydrophobic bonding. The hydroxyl group of the gallic acid structure is responsible for such a kind of hydrogen bond formation by interacting with the active site's amino acids of the enzyme (Long, 2023).

The Km value of α-amylase against starch for *L. orbonalis* was 0.31%. Km value corresponds to the dissociation constant (*Kd*) of the enzyme-substrate reaction; hence low Km indicates a higher affinity of an enzyme towards substrate. Our results of Km of α-amylase are comparable to the Km value obtained in studies with other insects, like 0.42% for click beetle, *Pyrearinus*

termitillumihans (Colepicolo-Neto *et al.,* 1986) and 0.38% in fall army worm, *Spodoptera frugiperda* (Ferreira *et al.,* 1994). A polysaccharide-rich diet may be the reason behind the relatively lower km value observed in the current study.

Electrophoretic zymogram study from the gut extracts of *L. orbonalis* has conspicuously revealed two iso-enzymes of alpha-amylase having distinguishable electrophoretic mobility and molecular weight. A similar isozyme pattern of α-amylase has been identified in other arthropods as well. Two forms of amylolytic enzymes have been recorded in two species of rice weevil, *Sitophilus zeamais* and *S. granarius* (Baker, 1983). In larger grain borer *(Prostephanus truncatus)* and coffee berry borer *(Hypothenemus hampei)* also similar two isozyme patterns have been noted (Mendiola- Olaya *et al.,* 2000*;* Valencia *et al.,* 2000). Multiple isoforms of amylase may increase amylolytic efficiencies over a broad range of pH and temperature (Nadaf *et al.,* 2022). Molecular weight of one of the bands with the highest intensity was found to be 60 KDa, which is well within the recorded reference range of 45-67 KDa (Baker, 1991; Terra and Ferreira, 1994). The second band with a molecular weight of more than 180 KDa may be due to the oligomerization of many monomeric subunits of protein to form an oligomer, which gives a greater apparent molecular weight value during gel visualization.

CONCLUSION

The development of insect-resistant transgenic crops helps reduce dependence on chemical pesticides, lowering environmental contamination and supporting ecological balance. This approach not only enhances crop protection but also promotes human and environmental safety. Biochemical characterization of digestive enzymes, particularly α-amylase, in pests such as *Leucinodes orbonalis*, provides crucial insights into their digestive physiology. Such understanding enables the design of specific enzyme inhibitors that form the basis of sustainable pest management strategies. Future research focusing on purification, structural analysis, and molecular modelling of α-amylase of the target insect will help to design novel inhibitors from plants or other sources.

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Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article. No funding or sponsorship influenced the design of the study, data collection, analysis, decision to publish or preparation of the manuscript.

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