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LEAF-DERIVED PROTEOLYTIC ENZYMES AS ECO-FRIENDLY BIOCONTROL AGENTS FOR INSECT PEST MANAGEMENT

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ABSTRACT

The extensive use of synthetic insecticides has raised significant environmental and health concerns, necessitating the development of sustainable and eco-friendly pest management strategies. The present study investigates the isolation, characterization, and insecticidal potential of proteases derived from vegetable leaves against economically important insect pests, including *Sitophilus oryzae*. Plant-derived proteases are integral components of plant defense systems and can adversely affect insect growth and survival by disrupting digestive processes. Fresh vegetable leaves were subjected to protein extraction and purification using ammonium sulfate precipitation. The partially purified proteases were characterized with respect to molecular weight, which showed a protease range of **~40 kDa and ~55 kDa. The** optimum pH was observed at **pH 7;** temperature stability was observed at **37℃**. Insecticidal efficacy was evaluated through laboratory bioassays by incorporating protein extracts into artificial diets and conducting feeding assays. Dose-dependent activity was observed, which showed that as the dose was increased from 0.5 ml, 2 ml, and 5 ml, there was mortality of the pest compared with castor oil as the control. The isolated proteases exhibited significant proteolytic activity, as demonstrated by destaining results, and showed pronounced adverse effects on both target pests, resulting in reduced growth and increased mortality. These findings highlight the potential of vegetable leaf–derived proteases as natural bioactive agents for pest control. The study provides valuable insights into the utilization of plant-based insecticidal proteins for the development of environmentally safe biopesticides, thereby contributing to sustainable agricultural practices and reducing dependence on synthetic chemical insecticides.

KEYWORDS: Plant proteases, insecticidal proteins, biopesticides, *Sitophilus oryzae*, sustainable agriculture, vegetable leaves.

INTRODUCTION

Agricultural crop productivity is significantly affected by insect pests, which are responsible for nearly 20–40% of global crop losses annually, as reported by the Food and Agriculture Organization.[1] The extensive use of synthetic chemical insecticides to manage these pests has resulted in environmental pollution, bioaccumulation, the development of resistant insect populations, and harmful effects on beneficial organisms.[2] Consequently, there is an urgent need to develop eco-friendly and sustainable alternatives such as plant-derived bioinsecticidal proteins.

Plants have evolved complex defense mechanisms against herbivorous insects, including both constitutive and inducible defense responses. Among these, proteinaceous compounds such as protease inhibitors and hydrolytic enzymes like proteases play a crucial role in plant defense.[3,4] Proteases (EC 3.4) are enzymes that catalyze the hydrolysis of peptide bonds in proteins and are broadly classified into serine, cysteine, aspartic, and metallo-proteases based on their catalytic residues.[5] In plant–insect interactions, proteases can interfere with insect digestive physiology by degrading gut proteins, damaging epithelial tissues of the midgut, and disrupting nutrient absorption, ultimately affecting insect growth and survival.[6]

Leafy vegetables are not only nutritionally valuable but also rich in bioactive compounds such as phenolics, flavonoids, latex-associated proteins, and defense-related enzymes. Previous studies have demonstrated that leafy vegetables and latex-producing plants contain cysteine and serine proteases with potential insecticidal properties.[7] These proteases may function either directly by exerting toxic effects on insect gut tissues or indirectly by activating defense signaling pathways within the plant.[8]

Insecticidal proteins from plants have shown promising activity against major agricultural pests such as *Helicoverpa armigera* and *Spodoptera litura*, where disruption of the larval midgut proteolytic balance leads to reduced feeding efficiency, stunted growth, and increased mortality.[9] The ingestion of exogenous plant proteases can result in proteolytic damage to the peritrophic membrane and the induction of apoptosis in midgut epithelial cells, thereby impairing digestive processes.[10]

The isolation and characterization of insecticidal proteases from vegetable leaves involve systematic extraction, ammonium sulfate fractionation, dialysis, and chromatographic purification, followed by biochemical characterization, including molecular weight determination (SDS-PAGE), optimum temperature, optimum pH, and assays. Evaluation of anti-insecticidal activity is typically conducted through in vitro proteolytic assays and in vivo bioassays that measure larval mortality, growth inhibition, and developmental abnormalities.[11]

Exploring proteases from vegetable leaf samples as potential bioinsecticidal agents aligns with the principles of integrated pest management (IPM), emphasizing biodegradable, target-specific, and environmentally safe pest-control strategies. Therefore, the isolation and biochemical characterization of vegetable leaf proteases, along with the assessment of their anti-insecticidal activities, may contribute to the development of novel plant-based biopesticide formulations.

MATERIALS AND METHODS

Plant material collection

Fresh and healthy vegetable leaves were collected from local agricultural fields and thoroughly washed with distilled water to remove dust and contaminants. The samples were shade-dried and stored at 4°C until further use. The leaves were then crushed using a mortar and pestle in the presence of 0.1 M phosphate buffer (pH 7). The leaf extract was transferred into centrifuge tubes. After incubation, the sample was centrifuged at 6000 rpm for 10 minutes at 4°°C. Finally, the supernatant was collected, and the pellet was discarded.

Insect culture

Sitophilus oryzae were obtained from an authorized laboratory or reared under controlled conditions (temperature: 25 ± 2°C; relative humidity: 65–70%; photoperiod: 12:12 h light:dark). The insects were maintained on an artificial diet for experimental purposes.

Protein precipitation and partial purification

The supernatant collected was taken for ammonium sulfate precipitation using a magnetic stirrer. Ammonium sulfate precipitation (60–80% saturation) was carried out to concentrate proteins. The precipitated proteins were collected by centrifugation and dissolved in a minimal volume of phosphate buffer. Dialysis was performed against the same buffer to remove excess salts.

Estimation of Protein Content

Protein concentration in all plant extract samples was determined using Folin's Ciocalteu reagent.[12] The absorbance was measured at 660 nm using a spectrophotometer. The amount of soluble protein was determined as mg of protein per mL of the test sample using a standard curve.[13]

Protease Activity Assay

Protease activity was determined using casein as a substrate

A reaction mixture containing the enzyme extract and casein solution was incubated at 37°C for 30 minutes. The reaction was terminated using trichloroacetic acid (TCA). The liberated amino acids were measured spectrophotometrically at 280 nm. One unit of protease activity was defined as the amount of enzyme required to release 1 µg of tyrosine per minute under the assay conditions.

Characterization of Protease Enzyme

a) Effect of pH

Protease activity was measured at different pH values (4–9) using appropriate buffer systems to determine the optimum pH.

b) Effect of Temperature

The enzyme activity was evaluated at different temperatures (20–60°C) to determine the optimum temperature.

c) Molecular Weight Determination

SDS-PAGE analysis was carried out to determine the molecular weight of the purified protease.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out using the Laemmli method for protein separation. Protein extracts from lettuce leaves were prepared and mixed with 4× Laemmli sample buffer containing Tris-HCl, SDS, glycerol, bromophenol blue, and β-mercaptoethanol in a 3:1 ratio. The mixtures were heated at 95 °C for 5 minutes to denature the proteins. A pre-stained molecular weight marker (10–250 kDa) was included for molecular mass determination. The samples were loaded onto polyacrylamide gels composed of a 4% stacking gel and

a 12% resolving gel, and electrophoresis was performed in Tris–glycine–SDS running buffer.

The gel was run initially at 80 V through the stacking gel and subsequently at 120 V through the resolving gel until the tracking dye reached the bottom. After electrophoresis, gels were immersed in 0.1% Coomassie Brilliant Blue R-250 prepared in methanol and acetic acid, and staining was carried out for about 1 hour. Destaining was performed with repeated changes of methanol–acetic acid solution until the background was clear.

Destaining activity

- The stain removal efficiency and detergent compatibility of partially purified protease from all five lettuce samples were investigated for a wide range of stains, including caffeine, egg yolk, Indian ink, and safranine.
- Detergent solution (Ghari detergent 1%) was prepared. The white muslin cloth pieces (5 cm × 5 cm) were taken and kept in Petri plates.
- Each stain of 0.5 ml was applied to the muslin cloth pieces, which were then air-dried in sunlight for 30 minutes[14].
- The following sets were prepared for each plant source and stains:

T1: 10 ml DW + cloth with stain (control)
T2: 10 ml DW + cloth with stain + 1 ml detergent
T3: 10 ml DW + cloth with stain + 1 ml detergent + 1 ml of enzyme
T4: 10 ml DW + cloth with stain + 1 ml crude leave extract

- For each set, the destaining efficiency of the crude enzyme, with or without detergent, on the stained cloth pieces was visually examined at regular intervals of 5 minutes upto 30minutes.

Insect Bioassay

- To degrade structural proteins of insect cuticles and gut tissues, thereby affecting insect survival and development.[15]
- The anti-insecticidal potential of proteases from five lettuce samples and commercial samples against Sitophilus oryzae (rice weevil) and Tribolium castaneum (red flour beetle), a model insect pest of stored grains, was evaluated by treating insect samples with different concentrations (0.5 ml, 2 ml, and 5 ml) and observing mortality after 24, 48, and 72hours.

Proteases and other bioactive compounds were the most effective in disrupting feeding, growth, survival, and development of insects, highlighting their potential as a natural, eco-friendly alternative to chemical pesticides for controlling insect pests.[16] The following experimental methodology was adopted in:

a) Preparation of Diet

Artificial diet was prepared and supplemented with different concentrations of protein extract.

b) Feeding Assay

- The pests were fed with the treated diet.
- Control group was maintained without protein extract.

c) Observation Parameters

- Larval mortality (%)
- Growth inhibition
- Developmental abnormalities
- Pupation rate

Observations were recorded at regular intervals (24, 48, 72 hours).

RESULTS

The specific vegetable leaf samples were collected as shown in the figure.

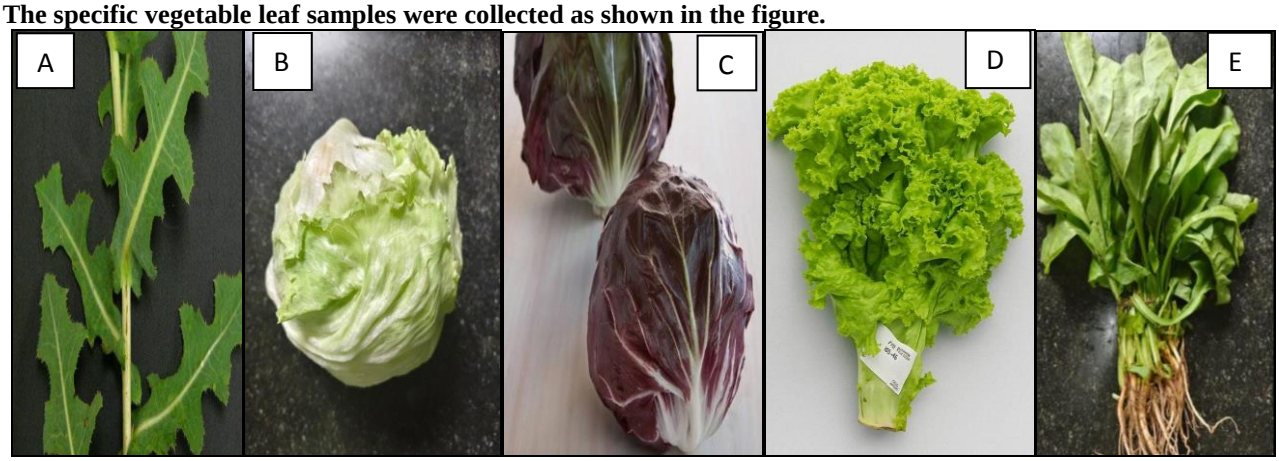

Fig 3.1: Vegetable Leaf Samples Employed in the Study A) WILD LETTUCE (Lactuca *serriata*), ICEBERG LETTUCE (*Lactuca sativa*), C)REDICCHIO LETTUCE (*Cichorium intybus*), D)LEAF LETTUCE (Lactuca *sativa*), E)SPINACH (*Spinacia oleracea*).

The five different leaf sample extracts were collected and stored at 4°°C. Further, the extracts were used for ammonium sulfate precipitation. The precipitate obtained was dissolved in phosphate buffer. Then, partial

purification of the enzyme was performed by dialysis against phosphate buffer at pH 7.

Partial purification of the protease enzyme

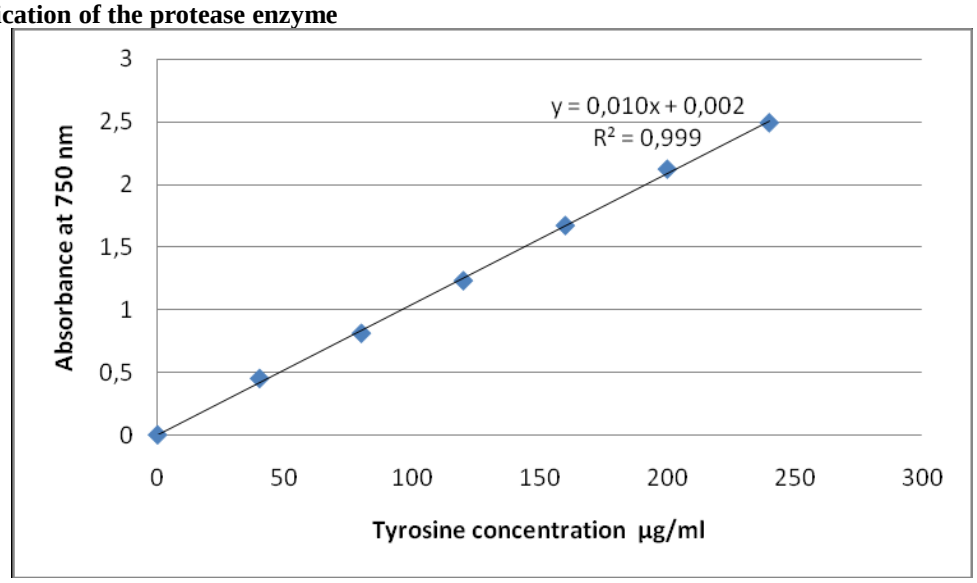

Fig. 3.2: Dialysis of the extract against phosphate buffer.

Enzyme activity estimation using the tyrosine curve

Enzyme activity was determined using casein as the substrate. It is hydrolyzed by casein into smaller peptides, releasing tyrosine residues. The released tyrosine was measured colorimetrically at 660 nm after reaction with the Folin–Ciocalteu reagent.[17] A standard tyrosine curve was prepared (using known concentrations of tyrosine versus absorbance). Unknown absorbance values were compared against the standard curve to calculate the µg of tyrosine liberated.

Enzyme Activity: It is the amount of enzyme that liberates 1 µg of tyrosine per minute under standard assay conditions.

Formula used:

Enzyme Activity = µg tyrosine liberated
Molecular weight × Volume of enzyme taken × Incubation time

Fig. 3.3: Tyrosine standard graph.

Enzyme activity and specific activity of samples

Sample	Crude Activity (U/mL)	Crude Specific Activity (µg)	30% Activity	30% Specific Activity	60% Activity	60% Specific Activity	90% Activity	90% Specific Activity
S1	0.0025	5	0.0069	2.6	0.0025	21	0.0025	18
S2	0.0016	5	0.0047	8	0.0018	21	0.0029	26
S3	0.0025	33	0.0025	13	0.0016	21	0.0016	26
S4	0.0016	47	0.0016	3	0.0007	13	0.0007	21
S5	0.0016	5	0.0012	33	0.0042	21	0.0029	26

The following interpretation can be made

- The highest enzyme activity was observed in the 30% ammonium sulfate fraction of S1 (0.0069 U/mL).
- The lowest enzyme activity was recorded in the 60% and 90% fractions of S4 (0.0007 U/mL).
- The highest specific activity was found in the crude extract of S4 (47 µg).
- The lowest specific activity occurred in the 30% fraction of S1 (2.6 µg).
- Different samples exhibited different optimum precipitation ranges, indicating variability in protease solubility.
- Ammonium sulfate precipitation enhanced enzyme concentration in several fractions while partially purifying the protease by removing non-protein impurities and unwanted proteins.

Enzyme Activity at Different Temperatures

The bar graph illustrates the effect of different temperatures (25°C, 37°C, 60°C, and 100°C) on the enzymatic activity of protease in five different samples (S1, S2, S3, S4, and S5). The data show that the enzyme is active across a range of temperatures, with the highest activity for most samples, particularly S5 (with a concentration value of 0.20), occurring at the optimal temperature of 37°C.

As the temperature increases further to 60°C and 100°C, the enzyme's activity sharply declines for all samples, indicating that the higher temperatures likely cause the enzyme to denature and lose its functional structure.

Table 3.5: Effect of Temperature on Enzyme Activity.

Temperature	S1	S2	S3	S4	S5
25°C	0.07	0.08	0.07	0.08	0.09
37°C	0.18	0.15	0.16	0.15	0.20
60°C	0.05	0.04	0.05	0.06	0.06
100°C	0.02	0.01	0.02	0.03	0.02

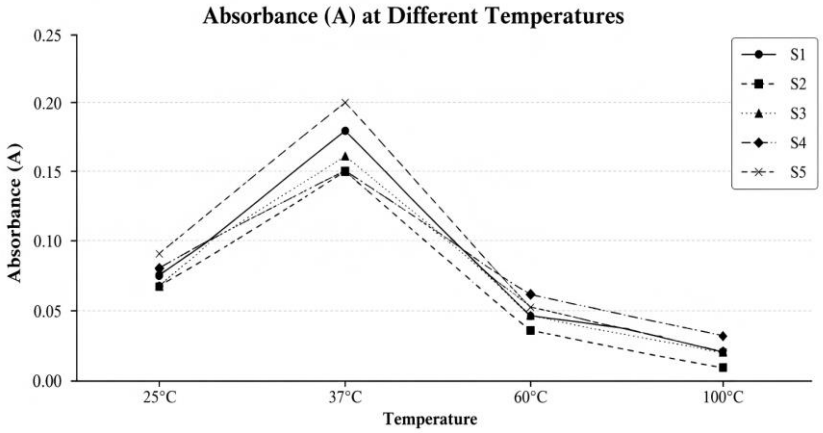

Fig. 3.5: Enzyme Activity at Different Temperatures.

Enzyme Activity at Different pH

The table represents the effect of different pH values on enzyme activity in five samples (S1–S5) using acetate buffer (pH 4, 5) and phosphate buffer (pH 6, 7, 8). The results show that enzyme activity is generally low at acidic pH (4–5) and gradually increases as the pH shifts toward neutral and slightly alkaline conditions.

Maximum activity is observed at pH 7 and pH 8 in most samples, indicating that the enzyme functions optimally under neutral to alkaline conditions, while extreme acidic environments reduce activity significantly. This suggests that pH strongly influences enzyme conformation and catalytic efficiency, with neutral to alkaline conditions being favorable for its stability and function.

Table 3.6: Effect of pH on Enzyme Activity.

BUFFERS	S1	S2	S3	S4	S5
Acetate pH-4	0.14	0.08	0.34	0.13	0.14
Acetate pH-5	0.32	0.15	0.25	0.22	0.18
Phosphate PH -6	0.19	0.20	0.50	0.12	0.14
Phosphate PH -7	0.35	0.30	0.44	0.39	0.34
Phosphate PH -8	0.45	0.26	0.38	0.33	0.29
Phosphate PH -9	0.12	0.09	0.18	0.11	0.1

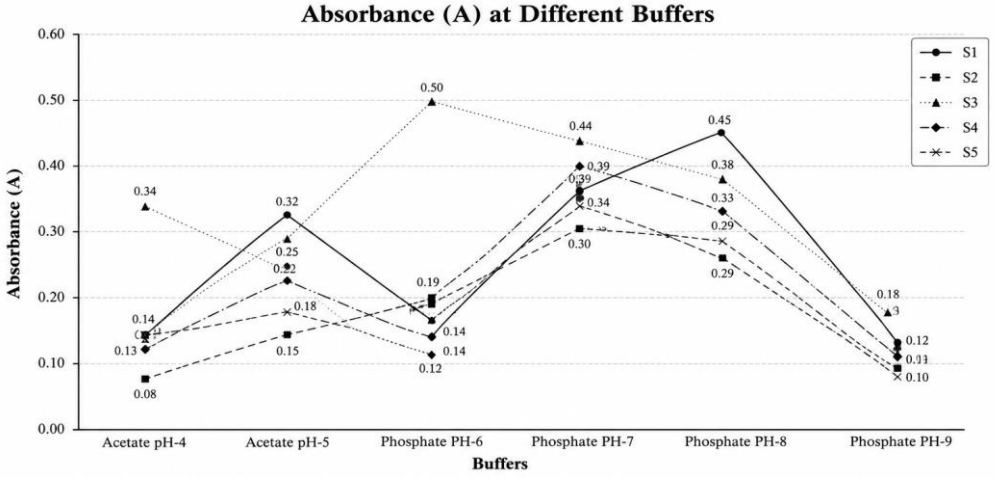

Fig. 3.6: Effects of pH on Enzyme Activity.

SDS-PAGE buffer

The SDS-PAGE (Figure 3.6) shows multiple protein bands in the crude vegetable leaf extracts (lanes 2–6), indicating the expected complexity, with a prominent band at approximately ~40 kDa and ~55 kDa, allowing an approximate molecular mass estimation. In lane 1 a broad molecular-weight marker was used, ranging from ~40 kDa to ~120 kDa.

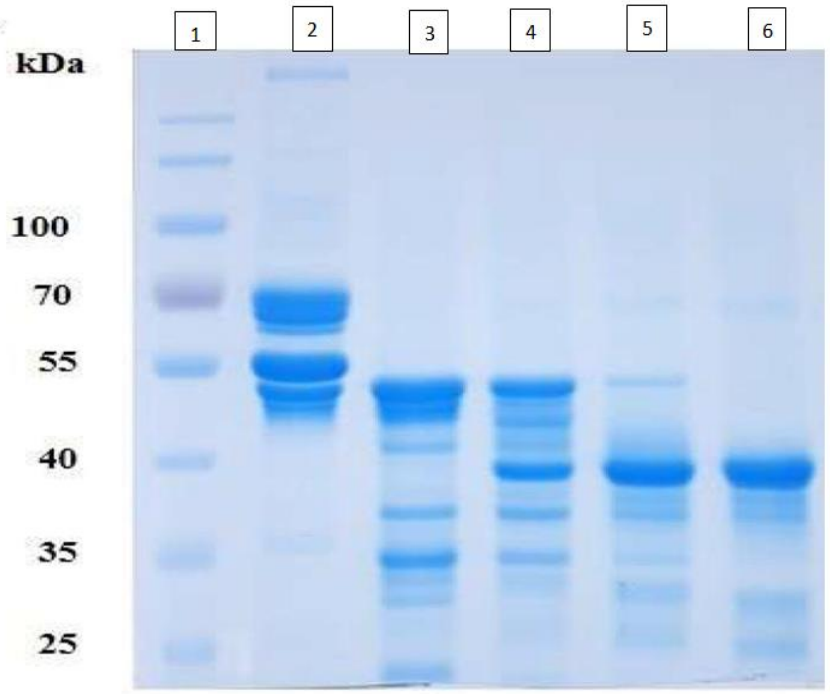

Fig. 3.7: 12% SDS-PAGE profile of enzyme extracts, where lane 1 is a broad-range molecular-weight marker, lanes 2 to 5 are vegetable leaf extracts.

Destaining Activity

Crude acetone-precipitated protease from lettuce leaves was evaluated for its destaining efficiency against protein-based stains (coffee, egg) and ordinary stains (Indian ink, safranin) using a visual grading scale.[18]

Table 5.7: Destaining Activity of Protease Enzyme Samples.

	Control	Detergent	E1	E2	E3	E4	E5
Coffee	+++	++++	++++	+++	++++	++	++++
Egg	++	+++	++	+++	++	++	++
Indian ink	+	++	+++	+++	++	+++	+++
Safranin	+	++	+	+	+	+	+

(+) Very Low
(++) Moderate
(+++) High
(++++) Very High.

The stain-removing efficiency of lettuce enzymes on coffee, egg, Indian ink, and safranin, compared with the control and detergent, was assessed. Wild (E1) and red lettuce (E3) enzymes showed the highest destaining activity, especially against coffee and ink, while iceberg (E2) and leaf lettuce (E4) showed moderate effects. Spinach enzyme (E5) displayed the least activity, indicating variation in stain-removal potential among different lettuce sources.

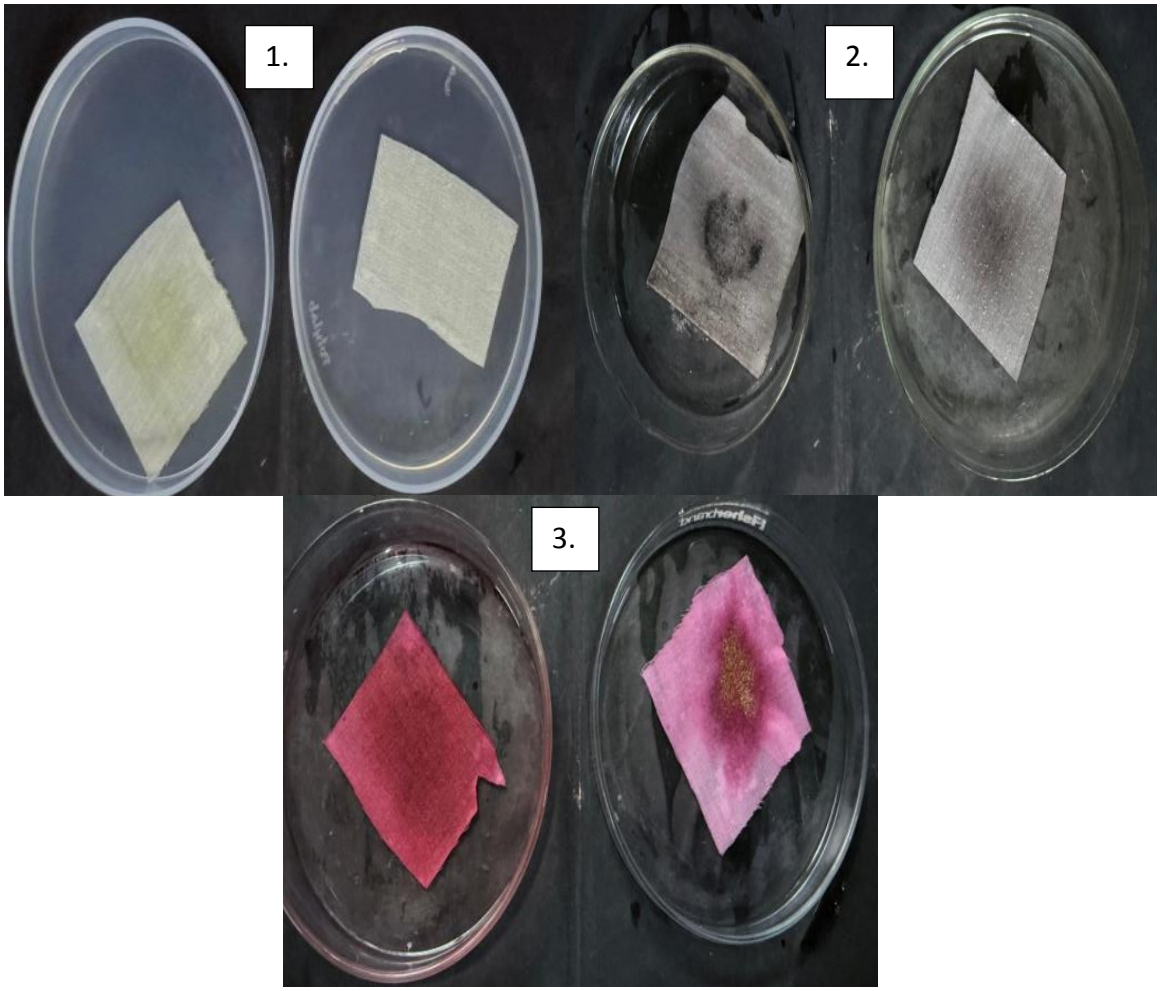

Fig. 3.8: Destaining Activity of Protease Enzyme Samples.

1. 10 ml DW + cloth with a coffee stain + 1 ml detergent + 1 ml of wild lettuce
2.10ml DW + cloth with Indian ink + 1 ml detergent + 1 ml of wild lettuce
3.10ml DW + cloth with safranin + 1 ml detergent + 1 ml of spinach (least activity)

Anti-insecticidal activity

Table 3.9: Bioefficacy of Enzymatic Extracts Against *Sitophilus oryzae*.

Samples	24hrs	24hrs	24hrs	48hrs	48hrs	48hrs	72hrs	72hrs	72hrs
	0.5ml	2ml	5ml	0.5ml	2ml	5ml	0.5ml	2ml	5ml
S1	4	7	8	8	11	12	10	17	20
S2	3	4	7	8	10	12	15	15	20
S3	5	7	10	7	12	14	17	15	18
S4	4	6	11	6	9	15	10	14	19
S5	5	7	10	8	10	12	13	17	18
Castor oil	3	5	8	8	7	15	12	16	20

The given table represents anti-insecticidal activity data measured at three different time intervals—24 hours, 48 hours, and 72 hours—and at three different concentrations—0.5 ml, 2 ml, and 5 ml—for five different samples (S1, S2, S3, S4, and S5), with castor oil used as the control for comparison. A total of 10 insect pairs were used for the experiment, and the initial seed weight was 20 g.

At 24 hours, the activity was generally lower for all samples, showing only early-stage insect mortality or repellency, but the activity gradually increased with higher concentrations; for example, S1 rose from 4 at 0.5 ml to 8 at 5 ml. At 48 hours, a clear increase in insecticidal activity was observed across all samples, showing that prolonged exposure enhanced effectiveness. S3 at 5 mL reached 14 compared to 10 at 24 hours, and S5 also increased to 12 at the same concentration. At this stage, the seed loss weight after 50% insect death was recorded at 19.7 g. By 72 hours, Maximum activity was observed, confirming that the insecticidal effect was both time- and dose-dependent, with S1 at 5 mL reaching 20,

the highest value recorded, and the seed loss weight after 50% insect death reducing further to 19.4 g.

Fig. 3.9: Percent Mortality of *Sitophilus oryzae* Following Protease Treatment.

Overall, the results indicate that higher concentrations and longer exposure times consistently yield stronger insecticidal effects. Among the samples, S1 and S3 demonstrated higher potency at earlier time points, while S4 showed slightly lower activity. The control (castor oil) exhibited a steady increase, but several test samples performed equally well or better, highlighting their potential as effective alternatives for insect control.

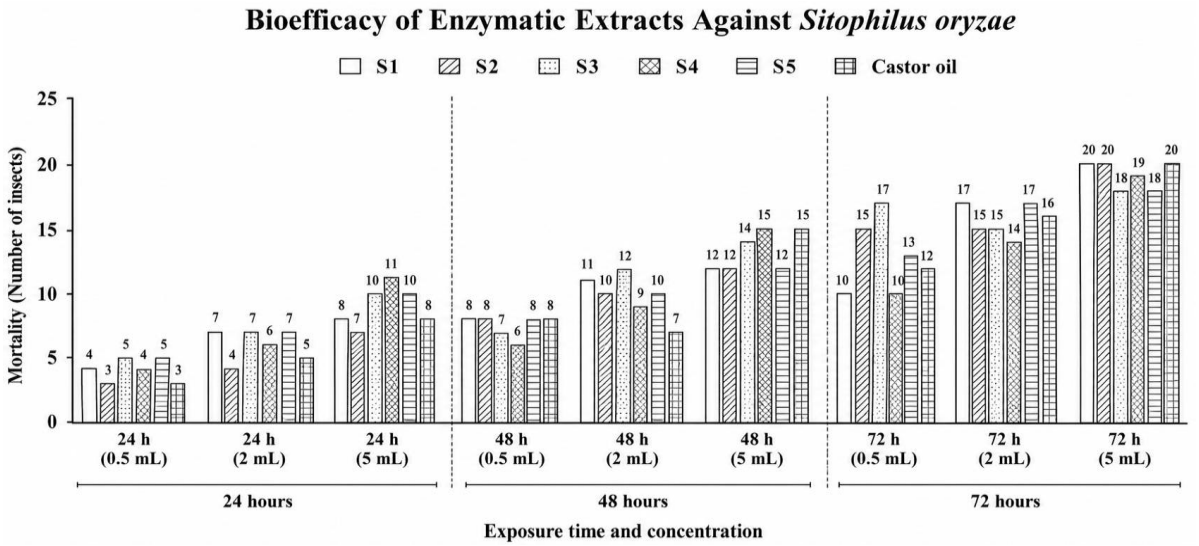

Fig. 3.10: Bioefficacy of Enzymatic Extracts against *Sitophilus oryzae*.

DISCUSSION

The present investigation demonstrated that vegetable-leaf-derived protease extracts exhibited significant insecticidal activity against *Sitophilus oryzae*, with mortality increasing progressively as both enzyme concentration and exposure duration increased. The highest mortality (18–20 insects) was observed after 72 h at the 5 mL concentration, whereas relatively lower mortality was recorded during the initial 24 h exposure, indicating a clear dose- and time-dependent insecticidal response. Such concentration-dependent toxicity has been widely reported for plant-derived proteases and protease inhibitors, which interfere with digestive physiology and nutrient assimilation in herbivorous insects.[19–21]

Among the evaluated enzyme preparations, S1 exhibited the greatest insecticidal potential by producing complete mortality after 72 h at the highest concentration, while S3 maintained comparatively higher activity even at lower concentrations. The variation in insecticidal efficacy among the extracts is likely associated with differences in protease composition, catalytic efficiency, enzyme stability, or the presence of synergistic phytochemicals such as phenolics and flavonoids. Plant proteases differ considerably in substrate specificity and biochemical properties depending on their botanical source, tissue type, and physiological role, which ultimately influence their biological activity against insect pests.[22]

The gradual increase in mortality with increasing exposure time indicates that these enzymes require continuous ingestion before producing lethal effects. Unlike conventional neurotoxic insecticides that cause rapid paralysis, proteolytic enzymes act by hydrolyzing structural and digestive proteins within the insect alimentary canal. Degradation of the peritrophic membrane and epithelial tissues reduces digestive efficiency, impairs amino acid absorption, disrupts metabolic homeostasis, and ultimately causes starvation and mortality.[23-24] Proteases have therefore been

recognized as promising biological insecticides because they target essential physiological processes rather than the insect nervous system.

The observed insecticidal activity is strongly supported by the biochemical characteristics of the extracted enzymes. The proteases displayed maximum catalytic activity under neutral to slightly alkaline conditions (pH 7–8) and at 37 °C, which closely resemble the digestive environment of many phytophagous insects. Maintenance of enzymatic activity under these physiological conditions is expected to enhance protein hydrolysis following ingestion and consequently increase insect mortality. Furthermore, SDS–PAGE analysis revealed distinct protein bands around 35–40 kDa, suggesting successful enrichment of specific protease fractions that may represent the biologically active molecules responsible for insecticidal activity. These observations agree with previous reports describing plant proteases as stable enzymes capable of maintaining catalytic activity under biologically relevant conditions.[22]

Comparison with the castor oil treatment further demonstrated the effectiveness of the enzyme preparations. Although castor oil achieved complete mortality at 72 h and 5 mL, several enzyme extracts, particularly S1 and S3, produced comparable mortality while exhibiting superior performance during earlier exposure periods or at lower concentrations. These findings indicate that vegetable-derived proteases could serve as viable alternatives to conventional botanical insecticides. Recent reviews on botanical pesticides have similarly concluded that plant-derived bioactive compounds possess multiple modes of action, have lower environmental persistence, have reduced mammalian toxicity, and improved compatibility with integrated pest management (IPM) programmes compared with synthetic insecticides.[25-26]

The insecticidal performance observed in the present study is also consistent with current understanding of plant defence mechanisms. Plants naturally synthesise proteases and protease inhibitors as part of their constitutive and inducible defence systems against herbivorous insects. These proteins inhibit digestive enzymes, reduce protein utilisation, prolong larval development, decrease fecundity, and ultimately increase insect mortality.[21] Consequently, exploitation of naturally occurring proteases represents an environmentally sustainable strategy for protecting stored grains against economically important pests such as *S. oryzae*.

From an applied perspective, the present findings highlight the potential of vegetable leaf-derived proteases as eco-friendly bioinsecticides. Their biodegradable nature, target specificity, and reduced environmental persistence make them attractive alternatives to synthetic chemical insecticides, particularly in stored-grain protection, where concerns regarding pesticide residues and resistance are increasing.[25] Nevertheless, additional investigations are required to purify individual protease isoforms, determine LC_{50} and LT_{50} values, identify active proteins using LC–MS/MS and proteomic approaches, evaluate enzyme stability under storage conditions, and assess toxicity toward non-target organisms before commercial application.

Overall, the present study demonstrates that vegetable leaf-derived proteases possess considerable insecticidal potential against *Sitophilus oryzae*. The observed dose-dependent and time-dependent mortality, together with favorable biochemical characteristics, supports their development as sustainable plant-based bioinsecticides capable of reducing reliance on synthetic pesticides. These findings provide a strong foundation for the future formulation and field evaluation of protease-based bioinsecticides for stored-product pest management.

CONCLUSION

The study demonstrated the isolation and partial characterization of protease enzymes from lettuce and related leafy vegetables, showing optimum activity at 37 °C under neutral to slightly alkaline pH, with enhanced specific activity after ammonium sulfate purification. These enzymes proved effective in practical applications such as removing protein-based and dye stains and exhibited anti-insecticidal activity against storage pests in a dose- and time-dependent manner. Among the tested varieties, wild and red lettuce showed the highest proteolytic potential and application efficiency. Overall, vegetable leaves represent a cost-effective, eco-friendly source of proteases with dual roles in industrial use and natural pest control, while further purification and molecular characterization may improve their stability and broaden their applications in green biotechnology.

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