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Qualitative LC-MS-based Protein Profiling of *Helicoverpa armigera* (Hübner, 1808) Larval Midgut under Emamectin Benzoate Stress

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ABSTRACT

Helicoverpa armigera is a major polyphagous pest with an immense potential to develop resistance to conventional insecticides. Emamectin benzoate (EMB), a compound derived from abamectin, is widely used for its efficacy against lepidopteran larvae. Despite several studies revealing its toxic and growth regulatory effects, information regarding its effects on midgut protein composition remains limited. In this study, we examined the midgut protein profile of *H. armigera* larvae following exposure to dietary EMB. Midgut proteins were separated by SDS-PAGE and differentially obtained bands were identified using liquid chromatography-mass spectrometry (LC-MS). The EMB-treated larvae showed a total of 39 differential proteins, among which 35 proteins were differentially deleted while just 4 proteins were differentially detected. The chief differentially absent proteins were found to be associated with metabolism, protein processing, signal transduction and stress response, while the differentially detected proteins were related to glycolysis and signal transduction. The differential absence/presence of these proteins indicated their plausible role in causing the disrupted metabolic and physiological processes in EMB-treated *H. armigera* larvae. This study provides qualitative molecular insights into the midgut-level responses of *H. armigera* to EMB exposure. However, further quantitative estimation of the altered levels of proteins can help formulation of an effective management strategy.

Keywords: differentially detected, differentially absent, Emamectin benzoate, LC-MS, proteins.

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INTRODUCTION

The cotton bollworm, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) is widely spread and highly damaging global agricultural pest, known to impact roughly 300 crops in 68 plant families, including several crops of economic significance like cotton, pigeon pea, tomato, and maize (Haile, Nowatzki, & Storer, 2021; Dagar et al., 2024; Singh et al., 2025). In India, *H. armigera* poses serious threats to the agricultural productivity leading to annual yield loss of ~30% in pigeon pea. In addition, an annual financial loss in cotton and soybean cultivation, exceeding USD 3 billion, has created a grave situation in farming sector (Dhaliwal, Jindal, & Mohindru, 2015; Dagar, Mishra, & Kumar, 2020; Riaz, Johnson, Ahmad, Fitt, & Naiker, 2021). Besides, studies conducted in Himachal Pradesh, India have revealed mean infestation of 32.31% (12.09-41.44%) caused by the pest in the apple orchards. The serious pest status of *H. armigera* posing a significant agricultural challenge can be largely attributed to its adaptive ability, high fecundity and fertility, ability to migrate to long distances, facultative diapause in response to environment stressors, and polyphagy enabling it to multiply in diverse environments and thrive on an extensive range of host plants (Razmjou, Mohammadi, & Hassanpour, 2014). The economic burden and reduced agricultural output caused by this devastating pest, hence, underscore the want for effective and sustainable management strategies.

Effective management of *H. armigera* has always depended on the application of synthetic chemical insecticides because of quick results and high yields (Ishtiaq, Saleem, & Razaq, 2012; Stavrakaki et al., 2024; Alemu, 2025). However, persistence of chemical residues in agricultural produce, surface run-offs carrying pesticides in waterways and adverse effects on farmer's health has always raised concerns. Moreover, rapid development of resistance in *H. armigera* against these chemicals, and adverse effects on non-target and beneficial organisms due to prolonged and indiscriminate use of these chemicals have created a necessity to explore environmentally benign, sustainable, and effective alternatives (Akhtar & Farooq, 2019; Tabashnik & Carrière, 2019).

Hence, the selective insecticides are considered as viable alternatives due to their targeted action, probable safety to non-targets, minimal ecological disruption, and compatibility with integrated pest management frameworks (da Silva et al., 2020). The high control potential of selective insecticides, spinetoram and spinosad, derived from metabolites of the soil bacterium *Saccharopolyspora spinosa,* has been demonstrated against lepidopteran pests with minimal risks to beneficial organisms (El-Sheikh & El-Saleh, 2015; Yao et al., 2021). Similar efficacy of avermectins, a group of macrocyclic lactones, has been evidently shown against various pests, with reduced chances of resistance development (Ahmad, Arif, & Ahmad, 2019).

Emamectin benzoate (EMB) is one of the emerging potent selective toxicants which can be effectively used against *H. armigera* and other lepidopteran pests, imparting both larvicidal and growth-inhibitory properties (Ahmad, Rasool, Ahmad, & Russell, 2019). It disrupts neurological processes in insects by enhancing chloride ion flow through γ-aminobutyric acid (GABA)-gated and glutamate-gated chloride channels resulting in

larval paralysis and eventually death (Ahmad et al., 2003; Dagar & Kumar, 2018). Reports have revealed the toxic impacts of EMB on *H. armigera* at low concentrations, resulting in larval mortality brought about by ingestion from the food as well as cuticular penetration (Parsaeyan, Saber, & Bagheri, 2013; Dagar & Kumar, 2018). The dietary 0.09 µg/mL EMB induced 100% larval mortality in *H. armigera* (Bird, 2015), whereas contact residue method resulted in a comparatively higher LC_{50} value (1.75 µg a.i./mL) (Parsaeyan et al., 2013). Effective application of EMB for cross-disciplinary functions has been promoted by Ahmad et al. (2019) who studied *H. armigera* natural populations for 14 years (2003-2016) to assess the prolonged larval susceptibility status to EMB. Earlier, Prolonged residual activity of EMB leading to >90% growth inhibition in *H. armigera* larvae for 28 days post-application in the fields demonstrated its effective use (Ishaaya, Kontsedalov, & Horowitz, 2002).

Recent advances in molecular biology and proteomics have provided valuable insights into EMB's mechanism of action at biochemical and cellular level. Li, Zuo, Shi, Yang, & Wu, (2023) displayed 358-fold resistance to EMB in transgenic *H. armigera* larvae caused by overexpression of the F116V allele of CYP9A186. Our earlier studies have revealed the ability of EMB to suppress the activity of midgut enzymes critical for larval growth and development in *H. armigera* revealing altered activities of aminotransferase; [alanine (ALT) and aspartate (AST)]; as well as phosphatases [acid (ACP), and alkaline (ALP)] that play pivotal roles in amino acid metabolism, gluconeogenesis, and the breakdown of phosphomonoesters, essential for larval nutrition and development (Dagar et al., 2020; Mishra, Sharma, Dagar, & Kumar, 2020). Complementary *in-silico* docking studies have validated EMB's high-affinity binding interactions with these enzymes, providing a molecular explanation for its inhibitory effects (Dagar et al., 2024).

The EMB's capacity to disrupt the physiological and biochemical processes of *H. armigera*, combined with its selective toxicity and high efficacy, makes it a promising tool for managing pest populations in agro-ecosystems. Hence, the current study is a step further to assess the EMB mode of action on *H. armigera* larvae which investigated the effects of EMB on the protein expression in their midgut and its potential implications for pest control. The research employed a LC-MS-based protein profiling approach to elucidate the molecular impacts of EMB treatment on *H. armigera*, an area that remains largely unexplored as per the literature available. By bridging the gap in understanding the regulatory dynamics of proteins in *H. armigera* larvae under EMB influence, the study sets the stage for novel insights into pest control mechanisms, potentially assisting sustainable agricultural practices.

MATERIAL AND METHODS

Rearing of *Helicoverpa armigera*

The culture of *H. armigera* (NBII-MP-NOC-01) has been maintained at Acharya Narendra Dev College, University of Delhi, India since 2017. The insects are reared in a BOD incubator (M/s Uni-Tech) under regulated temperature (27 ± 1 °C), relative humidity (80 ± 5%), and photoregime (12:12; L:D). The larvae are fed upon artificial

diet, prepared freshly as per the protocol described by Dagar et al. (2020). Post initial mass rearing of the freshly hatched larvae, those attaining third instars, were reared individually to avert cannibalism.

Pupae, surface sterilized with sodium hypochlorite (0.5%), were transferred to the cages (15 ×15 cm) for adult emergence. Adults were provided 10% sucrose solution by placing cotton swabs soaked in the solution. Healthy adult males and females were selected in 3:2 ratios and released in muslin cloth-covered 2 L jars for mating. The eggs laid on the muslin cloths were gathered and transferred to ventilated boxes for hatching (Dagar et al., 2023).

Preparation of experimental diet

Based on our earlier studies, the study was held with the selected dosage of dietary 0.05 µg/mL EMB (Sigma-Aldrich) that imparted sub-lethal effects on *H. armigera* larvae. The experimental diet was prepared by mixing of 1 mL EMB and 100 mL freshly prepared artificial diet followed by solidification by adding agar (Dagar et al., 2020; 2024). Distilled water was used in control diet in place of EMB.

Effect of Emamectin benzoate on midgut protein profile of *Helicoverpa armigera*

The effect of Emamectin benzoate (EMB) was assessed on the midgut protein profile of early fourth instar larvae of *H. armigera* employing a differential proteomic approach.

Experimental Setup and Sample Collection

The experiment was conducted under controlled conditions of temperature, humidity, and photoperiod within a BOD incubator. A total of 50 experimental larvae, taken in 5 replicates of 10 larvae each, were fed upon 0.05 µg/mL EMB-incorporated diet for 24 h, while control larvae were reared concurrently on the untreated diet. Post-feeding, 20 experimental and 20 control larvae were selected randomly and sacrificed by freezing at (-) 20 ºC.

Protein extraction

Protein extraction from larval midgut tissue followed the methodology of Dawkar et al. (2011) with minor modifications. Larval midgut was excised and the tissues were homogenized in liquid nitrogen, using 20 mL of extraction buffer comprising Sucrose (0.7 M), Trisaminomethane (0.5 M), Hydrochloric acid (30mM), Ethylene diamine tetra acetic acid (50 mM), Potassium Chloride (0.1 M), and Mercaptoethanol (2%). The homogenate was vortexed for 10 minutes, followed by centrifugation for 10 min at 14,000 × g at 4 ºC. The supernatant formed was mixed with an equivalent volume of water-saturated phenol. The phenol phase was re-extracted with extraction buffer through vigorous agitation for 10 minutes, followed by centrifugation at 13,000 × g for 10 minutes at 24 ºC. Proteins were precipitated from the re-extracted phenol phase by adding 5 volumes of 0.1 M $NH_4CH_3CO_2$ (ammonium acetate) in methanol and subsequent overnight incubation at (-) 20 ºC. The precipitated proteins were washed thrice with $NH_4CH_3CO_2$ in water and once with acetone. The resultant pellets were dried under air, resuspended in the lysis buffer, centrifuged and stored at (-) 80 ºC.

SDS-PAGE analysis

Proteins were separated using 15% SDS-polyacrylamide gel with 4% stacking gel in a vertical electrophoresis system (Bangalore Genei, India) under a constant electric current of 50 mA. The gels stained with Coomassie Brilliant Blue (CBB), were scanned using a Bio-Rad GS800 high-resolution scanner at 300 dpi. Differentially expressed protein bands of control and EMB-fed larvae were cut, given a distilled water wash, and de-stained with a mixture of 50% acetonitrile solution and 50 mM NH_4HCO_3 (1:1). The dehydration of bands was held with 100% acetonitrile followed by reduction with 10 mM dithiothreitol for 40 min at 50 ºC, alkylation with 50 mM iodoacetamide for 35 min in the dark at room temperature, and overnight digestion with sequencing-grade trypsin (Sigma) at 37 ºC. Peptides were extracted twice using a solution of 50% acetonitrile and 5% trifluoroacetic acid for 20 min, dried and rebuilt in 10 µL of 0.1% formic acid in 5% acetonitrile.

Liquid Chromatography-Mass Spectrometry (LC-MS) analysis

Peptide analysis was conducted using an Agilent 6550 UHD accurate mass QTOF MS coupled with a bridged ethyl hybrid C18 column (50 cm, 3.0 µm particle size). The mobile phase A comprised 80% water+0.1% formic acid, while the mobile phase B had 80% acetonitrile+0.1% formic acid. Samples were pre-concentrated and desalted using a symmetry C18 trapping column. A total of 1.0 µg of digested protein was injected onto a C18 column for separation. Peptides were eluted using a gradient of 0-40% buffer B over 100 min at a flow rate of 300 nL/min and introduced into the mass spectrometer. MS1 spectra were obtained at 70,000 resolutions with dynamic exclusion set to 10 sec, while MS2 spectra were documented at 17,500 resolutions.

Data processing and proteomic analysis

Raw data files generated from the LC-MS analysis were processed using Proteome Discoverer software (v2.2) and searched against the UniProt *H. armigera* reference proteome database. The fragment ion mass tolerances and precursor were set at 0.5 Da and 10 ppm, respectively. Enzyme specificity was defined for trypsin with allowance for a maximum of two missed cleavages. Carbamidomethylation of cysteine was defined as a fixed modification, while oxidation of N-terminal acetylation and methionine was considered as variable modifications. The false discovery rate (FDR) threshold for peptide and protein identifications was set at 0.01. Analysis of independent biological samples was performed in triplicate to ensure reproducibility.

RESULTS

Fig. 1 represents SDS PAGE protein profile of the midgut of *H. armigera* fourth instars reared on control diet and dietary 0.05 µg/mL EMB. First lane (MW) depicts the DNA marker of 10-250 kDa size. Lane 2 (control) shows the midgut protein profile of *H. armigera* larvae reared on control diet, while Lane 3 (0.05 µg/mL EMB) depicts that of the larvae reared on 0.05 µg/mL dietary EMB. The electrophoretic profile revealed

distinct differences in the protein banding patterns of the control and EMB-treated samples with the control lane exhibiting seven prominent protein bands at ~55, 40, 35 and 25 kDa, and lower molecular weight regions while a total of six protein bands, with notable alterations in intensity, were obtained in the EMB-treated larvae.

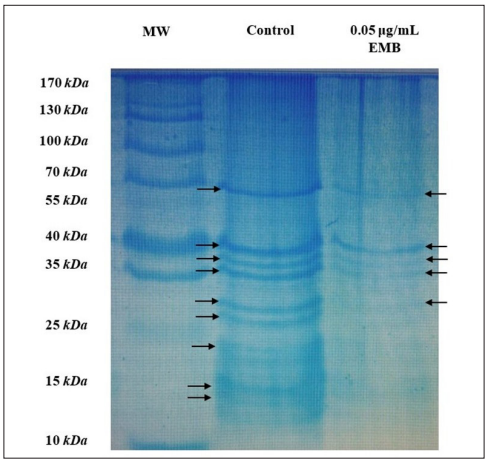

Figure 1. Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) protein profile in *Helicoverpa armigera* fourth instars fed on control and experimental diet incorporated with 0.05 µg/mL Emamectin benzoate.

The LC-MS analysis of the protein bands identified a total of 39 proteins in the experimental larvae including 35 differentially absent and 4 differentially detected proteins in comparison to those found in the control larval midgut (Tables 1, 2). The identified proteins were categorized into - RNA processing, protein processing, stress response, metabolism, structural proteins and signal transduction proteins, based on their functions. A few proteins were hypothetical while functions of a few proteins could not be identified from the database (Tables 1, 2; Fig. 2-4).

Table 1. Characterization of differentially absent proteins in midgut of *Helicoverpa armigera* fourth instars in response to the feeding with 0.05 µg/mL Emamectin benzoate for 24 h.

a) Protein processing

S. No	Experimental peaks	Accession No.	Protein description	Species	Theoretical mass (Da)	Morpheus score	Q-value (%)
1	158	NP_525100.2	Beta subunit of type I geranyl geranyl transferase, isoform A	*Drosophila melanogaster*	1178.597	3.0500	71.5962
2	123	Q9U5K4	Arylphorin subunit	*Spodoptera litura*	914.438	4.0340	71.5962
3	146	Q962T9	Ribosomal protein L14	*Spodoptera frugiperda*	2265.107	2.0174	73.3895

b) RNA processing

S. No.	Experimental peaks	Accession No.	Protein description	Species	Theoretical mass (Da)	Morpheus score	Q-value (%)
1.	229	Q8R0T2	Zinc finger protein 768	*Mus musculus*	1629.733	2.0061	73.9105
2.	174	O18450	Chymotrypsin-like protease precursor	*Helicoverpa armigera*	1219.577	1.0035	82.6409

c) Structural proteins

S. No.	Experimental peaks	Accession No.	Protein Description	Species	Theoretical mass (Da)	Morpheus score	Q-value (%)
1.	134	ACH69160.1	Myosin heavy chain, partial	*Bombyx mori*	1506.698	3.0187	71.5962
2.	229	CAA37309.1	Muscle myosin heavy chain	*Drosophila melanogaster*	1910.879	3.0092	71.5962
3.	117	EEB18387.1	Structural maintenance of chromosome	*Pediculus humanus corporis*	1179.595	3.0750	54.7607

d) Metabolism

S. No.	Experimental peaks	Accession No.	Protein Description	Species	Theoretical mass (Da)	Morpheus score	Q-value (%)
1	232	NP_001040450	H^+ transporting ATP synthase beta subunit isoform 1	*Bombyx mori*	1277.629	3.0062	71.5962
2	265	NP_001041705	H^+ transporting ATP synthase beta subunit isoform 2	*Bombyx mori*	2114.171	2.0114	73.4586
3	360	ABO37162	3-hydroxy-3-methylglutaryl-CoA reductase	*Chrysomela populi*	2040.936	3.0144	71.5962
4	244	Q3S2I9	Phosphoglyceromutase	*Bombyx mori*	1243.707	2.0469	73.3895
5	155	ABI63547	Triosephosphate isomerase	*Blattella germanica*	1506.731	2.0186	73.3895
6	258	ABC86902	Arginine kinase	*Blattella germanica*	1698.807	2.0062	73.9105
7	206	EDW92314	Golgin subfamily A member 4	*Drosophila yakuba*	1376.676	2.0017	75.4739
8	153	NP_001016302	Prenylcysteine oxidase 1 precursor	*Xenopus tropicalis*	1034.507	1.0115	78.6616
9	138	Q0QJJ5	Imaginal disc growth factor-like protein	*Mamestra brassicae*	1094.547	2.0066	73.9105
10	216	Q7Z266	Midgut aminopeptidase N3	*Helicoverpa armigera*	1376.694	1.0081	78.6616
11	138	ABC68516	α-amylase	*Blattella germanica*	894.3832	1.0071	78.7869
12	228	Q964D6	Glutathione-S-transferase-like protein	*Galleria mellonella*	1011.608	1.0033	83.0878
13	275	Q4V831	Adenosine deaminase-like protein A	*Xenopus laevis*	2548.321	1.0013	86.7514
14	175	XP_001663796	Farnesyl pyrophosphate synthase	*Aedes aegypti*	1538.700	0	101.0780
15	174	O18450	HELAM Chymotrypsin like protease precursor	*Helicoverpa armigera*	1219.576	1.0035	99.8578

e) Signal transduction

S. No.	Experimental peaks	Accession No.	Protein description	Species	Theoretical mass (Da)	Morpheus score	Q-value (%)
1	181	EDL96429	ADP-ribosylation factor guanine nucleotide-exchange factor 2	*Rattus norvegicus*	1470.729	2.0314	73.3895
2	115	NP_001036442	Ste20-like kinase, isoform A	*Drosophila melanogaster*	905.4641	2.0068	73.9105
3	81	Q2VIY6	Prophenoloxidase subunit 2	*Helicoverpa armigera*	661.3548	2.0054	73.9105
4	147	EDV98309	GH23041	*Drosophila grimshawi*	1195.539	1.0019	83.8805
5	111	Q9I958	Mitogen-activated protein kinase 14B	*Cyprinus carpio*	1105.580	0	96.4218

e) Stress response

S. No.	Experimental peaks	Accession No.	Protein description	Species	Theoretical mass (Da)	Morpheus score	Q-Value (%)
1	107	EGV94270	Histone H2A type 1	*Cricetulus griseus*	760.419	1.0208	75.4739
2	205	NP_001036985	Heat shock protein hsp21.4	*Bombyx mori*	1224.567	0	98.6300

f) Others

S. No.	Experimental peaks	Accession No.	Protein description	Species	Theoretical mass (Da)	Morpheus score	Q-value (%)
1	117	EEB18387	Structural maintenance of chromosome, putative	*Pediculus humanus corporis*	1179.596	3.0750	71.5962
2	68	NP_001036927	Olfactory receptor 5	*Bombyx mori*	576.3231	1.0676	75.4739

g) Unknown

S. No.	Experimental peaks	Accession No.	Protein description	Species	Theoretical mass (Da)	Morpheus score	Q-value (%)
1	250	EDV43253	Probable serine/threonine-protein kinase MARK-A isoform X1	*Drosophila ananassae*	1565.721	2.021919	73.3895
2	168	O18439	Diverged serine protease precursor	*Helicoverpa armigera*	1219.547	2.0139	73.3895
3	94	ADD17628	Per a 3 allergen	*Periplaneta americana*	685.380	2.0356	73.3895

Differentially absent Proteins

The differentially absent proteins found in the midgut of EMB-fed *H. armigera* larvae comprised 43% metabolic proteins, 14% signal transduction proteins, 8% processing proteins, 8% structural proteins, 6% proteins significant for stress response and RNA processing, and 6% proteins involved in other functions (Table 1; Fig. 2, 4).

Among these, protein processing proteins (3) included arylphorin component, a source of amino acids for protein synthesis; ribosomal protein required for translation, and geranylgeranyl transferase type I subunit beta protein involved in the positive regulation of RAS protein signal transduction which, in turn, helps in protein prenylation, growth and cell proliferation (Table 1a). On the other hand, the RNA processing proteins (2) included zinc finger protein needed in transcriptional regulation; and chymotrypsin-like protease precursor involved in endopeptidase activity (Table 1b).

The differential deletion of three structural proteins in the EMB-fed larvae of *H. armigera*; myosin heavy chain and muscle myosin heavy chain proteins associated with functioning of muscles, and chromosome protein required in cell division and chromosome organization; indicates the effects of EMB causing structural abnormalities, and alterations in the cell multiplication and larval growth (Table 1 c). The maximum effect of dietary EMB was noticed on the proteins (15) associated with carbohydrate metabolism and protein processing. These included proteins associated with ATP production (ATP synthase beta subunit isoform 1 and 2), coenzyme A-catalyzed reactions (3-hydroxy-3-methylglutaryl-coenzyme A reductase), acylation and acyl transfer reactions (3'-phosphoadenosine-(5') diphospho (4') pantetheine), glycolysis (phosphoglycerate mutase) and gluconeogensis (triosephosphate isomerase).

Rest of the metabolic proteins associated with carbohydrate and protein metabolism, found differentially absent in the larvae, comprised imaginal disc growth factor-like protein, arginine kinase, GRIP domain-containing protein, prenylcysteine oxidase 1, geranylgeranyl transferase type I subunit beta protein and midgut aminopeptidase N3. Other metabolic key proteins, like α-amylase and Glutathione-S-transferase-like protein essential in carbohydrate and glutathione metabolism, respectively were also differentially absent in *H. armigera* larvae reared on EMB-incorporated diet (Table 1d).

Apart from these, a few more proteins; such as adenosine deaminase-like protein A), farnesyl pyrophosphate synthase and chymotrypsin-like protease involved in protein processing were found diferentially deleted in the midgut of EMB-fed *H. armigera* larvae.

In addition to these, the experimental larvae of *H. armigera* showed negligible detection of five proteins associated with the signal transduction (Table 1e) consisting of guanine nucleotide-exchange factor 2, ADP-ribosylation factor, Ste20-like kinase, isoform A, Prophenoloxidase subunit 2, GH23041 and Mitogen-activated protein kinase 14B.

As per the database, the ste20-like kinase plays a role in ATP binding and protein phosphorylation; prophenoloxidase subunit 2 is involved in monooxygenase activity; GH23041 is a part of several biological processes like mitotic cell cycle, Hippo signalling, mRNA export from nucleus, negative regulation of synaptic assembly at neuromuscular junction, positive regulation of G1/S transition of mitotic cell cycle and positive regulation of gene expression, etc.; mitogen-activated protein kinase 14B is an vital constituent of the MAP kinase signal transduction pathway; while Mapk14b plays a critical role in the cellular response cascades induced by extracellular stimuli, like cytokines or physical stress causing activation of transcription factors indicating the impact of EMB stress in the larvae.

Interestingly, two stress proteins, histone H2A and heat shock protein (hsp), were also found differentially absent in the EMB-fed larvae. The histone H2A protein is crucial for DNA binding and protein heterodimerization activity while hsp helps in protein maturation, re-folding and degradation. Another protein found was structural maintenance of chromosomes protein which exhibits a specific role in cell division and chromosome organization. Moreover, odorant receptor protein had less expression in the EMB-fed larvae of *H. armigera* that is known to aid transmission of signals based on the olfactory reception (Table 1f).

Three proteins, previously unknown, found differentially absent were isoform A required for positive regulation of the peptidyl serine phosphorylation and growth; diverged serine protease protein needed for serine type endopeptidase activity and Per a 3 allergen with unknown molecular function (Table 1g).

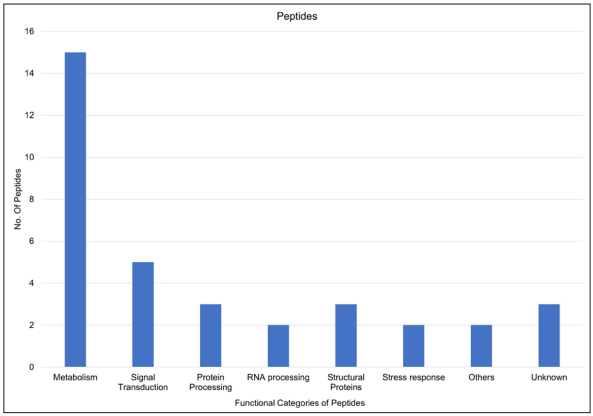

Figure 2. Differentially suppressed proteins in the midgut of *Helicoverpa armigera* fourth instars in response to the feeding with 0.05 µg/mL Emamectin benzoate.

Differentially detected proteins

In contrast to 35 differentially absent proteins, only 4 proteins were detected in EMB-fed larvae of *H. armigera* (Table 2) which had a critical role in metabolism, signal transduction and RNA processing (Figs. 3-4).

Table 2. Characterization of differentially detected proteins in the midgut of *Helicoverpa armigera* fourth instars in response to the feeding with 0.05 µg/mL Emamectin benzoate for 24 h.

S. No.	Experimental peaks	Accession No.	Protein description	Species	Theoretical mass (Da)	Morpheus score	Q-value (%)
Metabolism							
1	256	NP_001091831.1	2-phospho-D-glycerate hydro-lyase	*Bombyx mori*	1519.788	3.0103	54.7607
Signal transduction							
1	250	NP_001036442.1	Ste20-like kinase, isoform C	*Drosophila melanogaster*	16399.774	3.0072	54.7607
2	235	EDL96429.1	ADP-ribosylation factor guanine nucleotide-exchange factor 2 (brefeldin A- inhibited), isoform CRA_a	*Rattus norvegicus*	2247.963	3.0116	54.7600
RNA processing							
1	207	XP_787816.2	Polymerase delta-interacting protein 3	*Strongylocentrotus purpuratus*	996.473	3.0090	54.7600

The metabolic protein (01) expressed in EMB-fed larvae of *H. armigera* was identified as 2-phospho-D-glycerate hydrolyase which catalyses the step 4 of the sub-pathway of glycolysis synthesising pyruvate from D-glyceraldehyde 3-phosphate; the signal transduction proteins (02) were Ste20-like kinase, isoform C protein involved in ATP binding and protein kinase activity; and ADP-ribosylation factor guanine nucleotide-exchange factor 2 (brefeldin A- inhibited), isoform CRA a. The fourth differentially detected protein was polymerase delta-interacting protein 3, which helps in RNA binding.

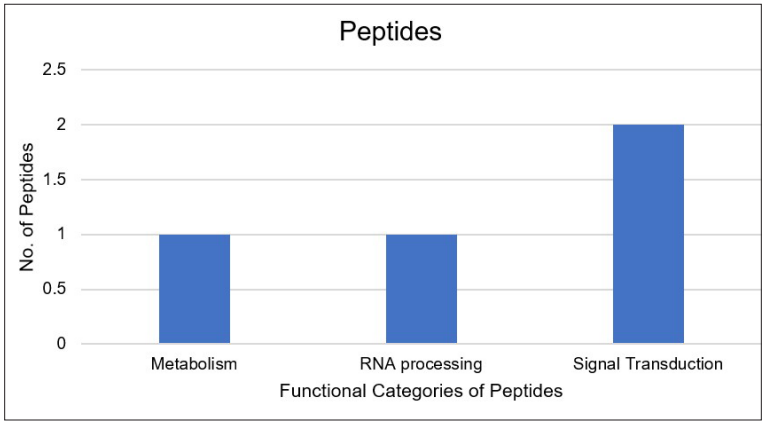

Figure 3. Differentially detected proteins in midgut of *Helicoverpa armigera* fourth instars in response to the feeding with 0.05 µg/mL Emamectin benzoate for 24 h.

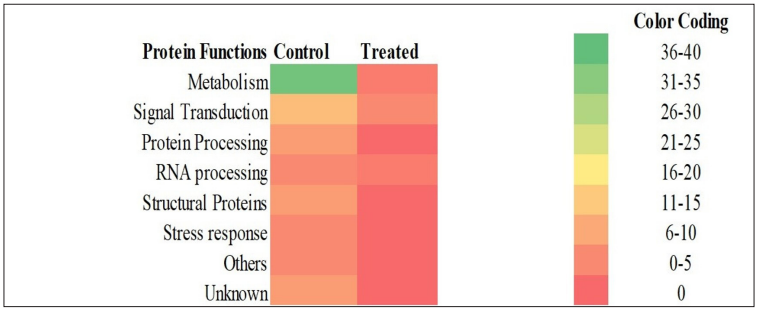

Figure 4. Hierarchical cluster analysis of differentially detected proteins in the midgut of control and 0.05 µg/mL Emamectin benzoate-fed *Helicoverpa armigera* fourth instars

DISCUSSION AND CONCLUSION

H. armigera (Lepidoptera: Noctuidae) is a highly destructive, polyphagous pest. To mitigate *H. armigera* infestations, various synthetic insecticides have been employed (Ishtiaq et al., 2012; Carneiro et al., 2014). Nonetheless, indiscriminate pesticide use has led to the development of resistance mechanisms, primarily through enhanced detoxification enzyme activity and target site insensitivity (Li, Schuler, & Berenbaum, 2007). As a result, alternative control strategies, including selective insecticides with novel modes of action, have gained prominence.

Emamectin benzoate (EMB), a member of the abamectin group, has demonstrated significant efficacy against various lepidopteran pests, including *H. armigera, H. virescens, Pseudoplusia includens, Plutella xylostella, Spodoptera exigua, S. frugiperda, S. littoralis, Tuta absoluta* and *Trichoplusia ni* (Argentine, Jansson, Halliday, Rugg, & Jany, 2002; Taleh et al., 2021) as well as other insects, like thrips (Chen et al., 2023), mosquitoes (Kamran et al., 2022) and hymenopterans among several others (Quyang, Fan, Chen, & Huang, 2023). Despite its proven toxicity, the comprehensive impact of EMB on the biological, physiological, and biochemical parameters of these pests remains insufficiently explored. Since insects have the capability to distinguish different stress signals and respond by adopting numerous tactics to defend the stress, besides metabolic detoxification, insects can regulate their protein expression by reducing the expression of unwanted and deleterious proteins while augmenting the expression of required ones. Hence, analysis of the inclusive protein expression pattern of the EMB-fed larvae could provide significant new insights into the adaptive mechanisms by studying the pattern and level of alteration in the proteins.

In the present studies, gel electrophoresis with enhanced protein solubilization and mass spectrometric technology was used for identifying protein expression in the stress-induced larvae which may provide significant knowledge regarding the variations in physiological processes implicated in stress response (Hancock, Wu, & Shieh, 2002; Rabilloud, Chevallet, Luche, & Lelong, 2002; Beranova-iorgianni, 2003; Wittmann-Liebold, Graack, & Pohl, 2006; Meng, Zhang, & Lei, 2010). The impact of EMB assessed on the midgut protein profile of *H. armigera* larvae using LC-MS technique, revealed a total of 39 affected proteins in *H. armigera* larvae. These included a total of 35

differentially absent and 4 differentially detected proteins as compared to those found in the control group. The analysis of the protein profile showed that most of the undetected proteins were associated with metabolism, hampering their growth and development.

These results are aligned with our previous studies, which showed that EMB-induced dietary stress imposes a significant fitness cost on *H. armigera* (Dagar et al., 2020). The observed physiological impairments, such as delayed larval development, reduced survival rates, and decreased reproductive potential, are consistent with the molecular disruptions identified in the protein profile analysis. The suppression of key metabolic proteins suggests that EMB not only targets the nervous system but also disrupts nutrient assimilation, energy production, and cellular homeostasis, further intensifying its toxic effects (Dagar et al., 2023). Additionally, variations in the enzymatic activity of larval midgut imparted by dietary EMB exposure using biochemical assays and *in-silico* docking studies have provided deeper insights into the mechanism of EMB toxicity. The biochemical analysis demonstrated significantly altered activity of digestive and detoxification enzymes, further corroborating the metabolic disruptions observed at the protein level. The *in-silico* docking studies revealed strong binding affinities of EMB with key metabolic and digestive enzymes, suggesting a direct interaction that may impair their functionality (Dagar et al., 2024).

In a study done by Meng et al. (2010), more than 1200 protein spots were perceived in *H. armigera* adults with 12 proteins more plentiful and 21 proteins abundant. The MS analysis identified 29 differentially expressed proteins in the three-day-old adults of *H. armigera* on UV exposure for 1 h as against 35 differentially unique proteins in control larvae observed in present studies. In accordance with current investigations, they also categorized the identified proteins into cytoskeleton structure, RNA processing, stress response, signal transduction, protein processing and metabolic proteins.

Dawkar, Chikate, Gupta, Slade, & Giri, (2011) studied the effect of host (Chickpea) and non-host (*Cassia tora* - Ct) diets on the growth and molecular responses in *H. armigera* and observed the proteomic differences in their haemolymph, gut and frass by nano-LCMS. They identified a total of 46 proteins with 17 upregulated proteins in the Ct diet-fed larvae. A total of 29 proteins were downregulated in the larvae which included gut proteases and proteins critical for immunity, adaptive stress responses, and detoxification. They also reported that haemolymph proteins of *H. armigera* were involved in defence mechanisms, while frass proteins have their involvement in energy metabolism. They concluded that the Ct diet could alter expression of proteins in *H. armigera* implicated in digestion, nutrient absorption, adaptation, defense, and energy metabolism. These results are in conformity with our study revealing 43% unique metabolism proteins in larvae of *H. armigera* fed on dietary EMB while 14% proteins were involved in signal transduction.

A proteomic approach using both 2DE and tandem MS was used to compare hemolymph expression profiles of a β-cypermethrin-resistant and a β-cypermethrin-susceptible *Blattella germanica* strain (Zhang et al., 2014). They observed 28 differentially expressed haemolymph proteins in the resistant strain comprising 19 upregulated and 9 downregulated proteins in contrast with the susceptible strain. Protein profiling showed the higher expression of nitric oxide synthase, putative cuticular protein, triosephosphate isomerase, ABC transporter, α-amylase, and Per a 3 allergen like those obtained in the

current study. They also reported reduced expression of glycosidase and arginine kinase proposing that this would have plausibly caused alteration in cellular architecture and metabolism associated with resistance to pyrethroids (Zhang et al., 2014).

Similarly, Campbell, Cao, Hines, East, & Gordon, (2008) performed proteome analysis to study the protein profile in the peritrophic matrix (PM) of the *H. armigera* larval gut. The LC-MS/MS analysis identified 41 proteins comparable to those obtained in the current study. They identified the two mucin-like and two chitin deacetylase-like proteins as the most abundantly recognized components in the PM based on the highly intense gel bands and mass spectral signals. Nonetheless they also found diverse hydrolases as the prominent proteins. They correlated the function of these proteins to the major function of PM, serving as an obstacle against parasites and abrasive food particles.

The proteome analysis of the gut tissue of *H. armigera* larvae fed on diet containing chlorpyrifos (CD) and artificial diet (AD) was conducted by Dawkar, Chikate, More, Gupta, & Giri, (2016). They identified 23 proteins that were upregulated in case of CD-fed larvae which had high confidence, 3-28 peptides and 12% to 66% protein coverage. The upregulation of very high-density lipoproteins and elongation factors in CD-fed larvae was documented by >8-fold while that of serine proteases and catalase was 4.4-fold as against AD-fed larvae. Other proteins, *viz*. aminopeptidase, CYP, hsp, carboxyl cholinesterase, arginine kinase, JH esterase, thioredoxin esterase, and glyceraldehyde-3-phosphate dehydrogenase were upregulated by only 2 to 3-fold. The detoxifying enzymes namely CYP, acetyltransferase, thioredoxin peroxidase, and malate dehydrogenase also displayed relatively elevated expression in CD-fed larvae of *H. armigera* indicating larval ability to combat toxin stress.

Similar molecular adaptations in the larval midgut of *H. armigera* were characterized under the pyrethroid-induced stress by Konus et al. (2013) through differential proteome analysis. They compared susceptible strain with two field populations of *H. armigera* for midgut proteins expressions. They concluded that stress primarily increased the expression of proteins associated with the energy metabolism, like ATP synthase and arginine kinase aligned with our study. Additionally, higher expression of NADPH CYP450 reductase could also play a role in detoxification of toxic pyrethroid metabolites (3-phenoxybenzaldehyde).

Emamectin benzoate (EMB) emerges as a promising alternative to conventional insecticides for management of *H. armigera* requiring a deeper understanding of the biochemical and molecular pathways affected by EMB being crucial for optimizing its application and mitigating the risk of resistance development. The present study evaluated the impact of EMB on the gut proteins of *H. armigera* and demonstrated a significantly altered protein profile leading to the differential deletion of 35 proteins while detection of 4 unique ones. These changes suggest a physiological larval response to EMB potentially linked to detoxification mechanisms and metabolic adaptations. Since the present study employed a qualitative protein profiling approach to provide valuable insights into EMB-induced physiological responses, prospective studies using quantitative proteomic platforms will validate protein abundance changes and elucidate regulatory mechanisms in greater detail. Hence, it is recommended that future research elucidating the specific roles of differentially expressed proteins in EMB mechanism of action can help in effective *H. armigera* management.

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Declaration

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Conflict of interest

We declare that there is no conflict of interest.

Authors' Contribution

VSD conceived the idea, conducted the experiments and wrote the manuscript. SK designed and guided the experiments. VSD, and AS analysed the results and SK helped in the analysis. AS edited and SK reviewed the manuscript. All authors were involved in the finalization of the manuscript.

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