Role of induced glutathione-S-transferase from Helicoverpa armigera (Lepidoptera: Noctuidae) HaGST-8 in detoxification of pesticides

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ABSTRACT

The present study deals with glutathione-S-transferase (GST) based detoxification of pesticides in Helicoverpa armigera and its potential application in eliminating pesticides from the environment. Dietary exposure of a pesticide mixture (organophosphates – chlorpyrifos and dichlorvos, pyrethroid – cypermethrin; 2–15 ppm each) to H. armigera larvae resulted in a dose dependent up-regulation of GST activity and gene expression. A variant GST from H. armigera (HaGST-8) was isolated from larvae fed with 10 ppm pesticide mixture and it was re-combinantly expressed in yeast (Pichia pastoris HaGST-8). HaGST-8 had a molecular mass of 29 kDa and was most active at pH 9 at 30 °C. GC-MS and LC-HRMS analysis validated that HaGST-8 was effective in eliminating organophosphate type of pesticides and partially reduced the cypermethrin content (53%) from aqueous solutions. Unlike the untransformed yeast, P. pastoris HaGST-8 grew efficiently in media supplemented with pesticide mixtures (200 and 400 ppm each pesticide) signifying the detoxification ability of HaGST-8. The amino acid sequence of HaGST-8 and the already reported sequence of HaGST-7 had just 2 mismatches. The studies on molecular interaction strengths revealed that HaGST-8 had stronger binding affinities with organophosphate, pyrethroid, organochloride, carbamate and neonicotinoid type of pesticides. The abilities of recombinant HaGST-8 to eliminate pesticides and P. pastoris HaGST-8 to grow profusely in the presence of high level of pesticide content can be applied for removal of such residues from food, water resources and bioremediation.

1. Introduction

Agriculture relies heavily on array of biocides that protect crop plants from damage caused by insect pest and pathogens. The extensive and persistent use of chemical pesticides, has led to their accumulation in the soil, ground and surface waters. According to a recent report (2015) released by Food Safety and Standards Authority of India (FSSAI) persistence of pesticides was detected in 18.7% food commodities of which 2.5% had pesticide contamination above the maximum residual limits (MRL). This in turn has hazardous effects on non-target organisms in food chains and on human beings (Abhilash and Singh, 2009). In humans and other organisms, pesticide uptake leads to serious health concerns, with multi-generational implications (Wasim et al., 2009). Depending on the chemical nature and class, pesticides have local or systemic reactions in human beings. They generally lead to blurred vision, headaches, dizziness and are also a cause for serious disorders including cancer (Kim et al., 2017; Jeyaratnam et al., 1990).

It is necessary to regulate the use of chemical biocides and design ways to detoxify pesticides present in food and environments for a safe and healthy future.

Organophosphate, carbamate, pyrethroid, organochlorines and neonicotinoids are some commonly used group of pesticides (Uragayala et al., 2015; Bapat et al., 2016). Consumption of contaminated food is one of the important routes by which human beings are exposed to such pesticides (Jiang et al., 2005). Organophosphates and carbamate exposure is associated with an increased risk of Alzheimer’s disease and urinary contraction (Hayden et al., 2010). Pyrethroids can cause neuronal excitation, allergic reactions, dermatitis and asthma (Sharon et al., 2012). Organochlorines can affect central nervous system and cause headaches, nausea, dizziness, vomiting, tremor, lack of co-ordination and mental confusion (Singh et al., 2016).

The polyphagous insect pest Helicoverpa armigera (Lepidoptera: Noctuidae) is responsible for heavy economic losses in a variety of crops including cereals, pulses, cotton, fruit crops and vegetables (Fitt, 1989; Sarate et al., 2012). Pyrethroids, organophosphates, organochlorides, carbamates and plant derived insecticides have been used to control H. armigera infestation (Torres-Vila et al., 2002). However, due to its adaptive and resistance building abilities, several insecticides have been rendered ineffective (Chaturvedi et al., 2007; Nimbalkar et al., 2009).
In general, pesticide resistance in insects involves (i) enhanced detoxification and (ii) modification of target enzyme/receptor sites thereby rendering them insensitive to their presence (Zhu et al., 2016). Metabolic resistance to pesticides in insects is contributed by three major groups of enzymes namely, cytochrome P450, esterases and glutathione S-transferases (GSTs) (Muthusamy et al., 2011; Joulén et al., 2012).

GSTs (EC 2.5.1.18) are a large family of multifunctional enzymes that are involved in phase I detoxification of a wide range of xenobiotic compounds. GSTs play an important role in protecting tissues from oxidative damage and stress (Fournier et al., 1992). According to their cellular location, they fall into three major categories namely: cytosolic, microsomal or mitochondrial GSTs (Enayati et al., 2005). Amongst cytosolic GSTs, only Delta and Epsilon classes are insect specific and the others are present in molluscs, nematodes and mammals (Morgenstern et al., 1983; Goto et al., 2001; Robin et al., 2003; Li et al., 2011). GSTs conjugate reduced glutathione (GSH) to insecticides (Mannervik et al., 1997). In the conjugation reaction, the active site residue of the GST interacts with GSH sulfhydryl group (SH) to generate the catalytically active thiolate anion (GS-). This nucleophilic thiolate anion is then capable of attacking the electrophilic centre of any lipophilic compound to form the corresponding GS-conjugate (Armstrong et al., 1997). The conjugation neutralizes the electrophilic sites of the lipophilic component leading to its detoxification. The product obtained after conjugation is more water soluble and therefore readily excretable from the cells (Enayati et al., 2005) via glutathione S-conjugate export pump and other mechanisms. Attempts to use recombinant GSTs from E. coli (Chen et al., 1997; Cursino et al., 2000) and Bombyx mori (Yamamoto and Yamada, 2016) were successful in detoxifying organophosphate pesticides displaying potential for bioremediation.

We hypothesized that GST expressed in H. armigera exposed to high concentrations of pesticides (2, 4, 7, 10 and 15 ppm of the pesticide mixture) were formulated. H. armigera (3rd instar) larvae were fed with experimental and control diet (without pesticide) for 72 h. Larvae from each set were then flash frozen in liquid nitrogen and stored at −80 °C until further use.

2.2. RNA isolation, cDNA preparation and semi-quantitative RT PCR

Pesticide treated larvae were pulvérised in liquid nitrogen and approximately 50 mg of tissue was used for extraction of total RNA by using the TRI reagent (Sigma, Missouri, USA). Quality of the RNA was checked on 1% agarose gels. The RNA was quantified and equal amounts were used for cdNA synthesis, following manufactures protocols (Promega, Madison, WI, USA). HaGST and 18S primer pairs were used for RT-PCR (Table 1). For 20 μl of reaction, 1 μl of cdNA template, 2 μl of buffer (10X), 0.4 μl of dNTP's (10 mM), 1 μl of forward and reverse primers each (10 μM) and 0.2 μl of Taq polymerase (Sigma-Alrich, Missouri, USA) were used. The reactions were incubated at 95 °C for 5 min for denaturation. This was followed by 30 cycles at 95 °C for 30 s, annealing at 50 °C for 45 s and extension at 68 °C for 90 s. The amplified PCR products were electrophoresed on 1% agarose gels. Initially, the cdNA was normalised with 18S RT-PCR reactions. Normalised quantities of the cdNAs were used for carrying out semi quantitative RT-PCR with HaGST specific primers.

2.3. Cloning and characterization of HaGST gene

HaGST was amplified from H. armigera cdNA (10 ppm pesticide fed) using specific primers designed for the reported HaGST-7 sequence (Table 1) with proofreading polymerase PfX (Invitrogen, Carlsbad, CA, USA). The amplicons were A-tailed and cloned in pGEMT easy vector (Promega, Madison, WI, USA). Plasmids were isolated from the clones, sequenced and analysed using MEGA 6.06 software (http://www.megasoftware.net/). The newly isolated GST gene showed a variation in the amino acid sequence and as such was named as HaGST-8. Plasmids containing HaGST-8 were used for amplification of gene region corresponding to mature peptide of HaGST-8 (Table 1). Amplicons were digested with XhoI and NotI enzymes. Amplicons so obtained were further cloned in yeast expression vector pPICZα; ligated plasmids (HaGST-8 pPICZα) were transformed in E.coli as described earlier (Tamhane et al., 2007). The presence of the plasmid was confirmed by colony PCR using gene specific primers. Sequencing of the HaGST-8 gene in pPICZα was done to ensure the presence of correct reading frame of HaGST-8 in pPICZα.

2.4. Recombinant expression of HaGST-8 in P. pastoris and enzyme purification

The HaGST-8 pPICZα plasmid was linearized with Pmel restriction enzyme, and was used for transformation of P. pastoris GS115 using Easy Comp Kit (Invitrogen, Carlsbad, CA, USA). HaGST-8 P. pastoris transformants were selected on YPDA plates (Yeast peptone dextrose agar) with Zeocin as a selection marker. Yeast transformants were confirmed by colony PCR using HaGST specific primers. For pre-growth of P. pastoris HaGST-8, YPD medium (yeast: peptone: dextrose) was inoculated with the culture and incubated (48 h, 30 °C, 130 rpm).
2.5. P. pastoris – pesticide assay

Functional analysis of HaGST expressing P. pastoris for pesticide degradation was carried out with the following experiments. P. pastoris HaGST-8 cell suspension with A_{600} = 0.2 was inoculated in 10 ml YPD medium containing Zeocin (25 µg/ml) and kept on a shaker (30 °C, 12 h) for pregrowth. Mixtures of chlorpyrifos, dichlorvos and cypermethrin (200, 400 ppm each) were used for the assays. Methanol was added in the medium containing Zeocin (25 µg/ml) and kept on a shaker (30 °C, 12 h) for pregrowth. Mixtures of chlorpyrifos, dichlorvos and cypermethrin (10,000 ppm each, 4 °C) and the resultant pellet was dissolved in water. The protein was dialysed against water, using 10 kDa cut-off membranes, prior to application on to Centricron tubes with molecular cut off of 3.5 kDa and 10 kDa (Millipore, Darmstadt, Germany). The protein was further applied to a GSH agarose affinity column (Sigma-Aldrich, Missouri, USA) equilibrated with 100 mM potassium phosphate buffer (KPB), pH 7.0. The column was washed with same buffer containing 1 mM NaCl to remove unbound proteins and then with 100 mM KPB (pH 7.0). The protein precipitate was recovered by centrifugation. The precipitate was dissolved in 10X YNB (25 ml), 1 M potassium phosphate buffer pH 6.0, biotin and Zeocin (25 µg/ml) with the addition of 0.5% of methanol at intervals of 24 h for recombinant protein expression. The cell free supernatant (CFS) was collected, filtered through 0.22 µm filter, and subjected to ammonium sulphate precipitation (85% saturation) at 4 °C. The protein precipitate was recovered by centrifugation (10,000 rpm, 20 min, 4 °C) and the resultant pellet was dissolved in water. The protein was dialysed against water using 10 kDa cut-off membranes, prior to application on to Centricron tubes with molecular cut off of 3.5 kDa and 10 kDa (Millipore, Darmstadt, Germany). The protein was further applied to a GSH agarose affinity column (Sigma-Aldrich, Missouri, USA) equilibrated with 100 mM potassium phosphate buffer (KPB), pH 7.0. The column was washed with same buffer containing 1 mM NaCl to remove unbound proteins and then with 100 mM KPB (pH 7.0). The adsorbed enzyme was eluted with 100 mM GSH. The fractions were tested for GST activity using 2, 4-Dinitrochlorobenzene (CDNB) assay. Fractions with high GST activity were pooled and dialyzed against 100 mM KPB (pH 7.0). The affinity purified proteins were quantified, resolved by SDS-PAGE and the gel was stained with Coomassie Brilliant Blue R250 (CBB). Protein concentration and GST activity during all purification steps were determined by Bradford method (Bradford et al., 1976) and CBBN assay, respectively. Fold purification estimates were obtained by taking ratios of specific activity of a fraction at stage by the specific activity of the protein.

2.6. GC-MS and LC-HRMS analysis of pesticides and enzyme activity

For detection of pesticide using GC-MS, 1 ml assay mixture containing (i) 700 ppm pesticide mixture (1 µl) in PBS buffer pH 6.5 (970 µl), (ii) GSH 100 mM (10 µl) (iii) recombinant GST, crude protein from H. armigera extract and protein from empty vector P. pastoris (19 µl, 100 µg protein) was set. A reaction with pesticides but without any of the proteins added, and the volume replaced by water, was considered as a negative control. These reactions were incubated for 1 h at 28 °C, and extracted with equal volume of chloroform. The chloroform phase was separated and used for analysis by GC-MS (Agilent 6540 QTOF MS). The extracted chloroform phase (5 µl) was injected and the column was programmed from 40 °C to 280 °C with a flow rate of 1 ml/min. Pesticides from control samples were detected and identified in the GC-MS data (refer to Supplementary data).

2.7. Phylogenetic analysis of GSTs from H. armigera

FASTA files of full length amino acid sequences of reported GSTs from H. armigera were downloaded from NCBI database. Rattus norvegicus (Mammalia), Crassostrea gigas (Mollusca) and Arabidopsis thaliana (Brassicaceae) GSTs (RnGST, CgGST and AtGST respectively) were also used in the phylogenetic analysis (Table 2). Phylogenetic analysis was carried out using neighbour joining tree method, using MEGA 6.06 software.

2.8. GST structure prediction, alignment and docking with insecticides

Nucleotide sequence alignment of HaGST-7 and HaGST-8 was performed by using ClustalW. Three dimensional structures of HaGST-7, HaGST-8 were generated using ModWeb (https://modbase.compbio.ucsf.edu/modweb/), an online server based on Modeller algorithm. Predicted structures of GSTs were validated by using PROCHECKv.3.5.4 (http://www.ebi.ac.uk/thornton-srv/software/PROCHECK/download.html) and ProSA (https://prosa.services.came.sbg.ac.at/prosa.php). Validated structures of HaGST-7 and HaGST-8 were used for structure alignment using Chimer-1.10.2 (https://www.cgl.ucsf.edu/chimera/download.html). Structures of different insecticides belonging to pyrethroid, organophosphates, organochlorides, carbamates, and neonicotinoids were downloaded from PubChem (https://pubchem.ncbi.nlm.nih.gov/).
nih.gov/). To study molecular interactions, validated structures of GSTs and insecticides were used for molecular docking using PatchDock online server (http://bioinfo3d.cs.tau.ac.il/PatchDock/) and binding energies were estimated. Heat map of the binding energies of the HaGST insecticide complexes was generated using MS-excel. Gradient ruler with heat map showed the interaction strength as red (strong) to green (weak).

2.9. Assay for glutathione S-transferase activity

The GST activity was estimated by using CDNB (Sigma, Missouri, USA) as the substrate, following the method described earlier (Habig et al., 1974). One unit of GST activity is defined as the amount of enzyme that catalysed the formation of 1 nmol of product per min under the assay conditions. Absorption spectra for the reaction mixtures were acquired on spectrophotometer (Jasco V-530, Tokyo, Japan) operated at a resolution of 1 nm at 340 nm. Reaction mixture (200 µl) contained GSH 20 mM (5 µl), CDNB 20 mM (5 µl), and enzyme 5 µg/ml in glycine NaOH buffer pH 9 (185 µl). Control reactions contained buffer instead of the enzyme. The reactions were carried out in polypropylene 96 well assay plates (Tarson, Kolkata, India) and absorbance was recorded at intervals of 59 s for 15 min at 25 °C at 340 nm. All reactions were done in triplicates with 5 technical replicates each. Average values indicating standard errors were used to generate graphs.

2.10. Biophysical and kinetic properties of HaGST

CDNB based GST activity assays were carried out using 5 µg of affinity purified HaGST enzyme to determine its pH activity range. Enzyme assays were carried out in sodium acetate (pH 5); potassium phosphate (pH 6–7); Tris-HCl (pH 8) or glycine-NaOH (pH 9–10) buffers. The activity of recombinant HaGST-8 at different temperatures ranging from 25 to 45 °C was also determined in 5 replicates.

For the determination of the kinetic parameters of the recombinant HaGST-8, reactions with varying concentrations of CDNB (0.01–10 mM) and 10 mM of GSH were set at 25 °C and Km and Vmax values were determined. The extinction coefficient of CDNB (9.6 mM⁻¹ cm⁻¹) was used for calculations. The enzyme activity was measured spectrophotometrically at 340 nm. The enzyme kinetic parameters were determined on the basis of the equation shown below

\[ V = V_{\max} [S]/[S] + K_m \]

as reported (Enache and Oliveira-Brett, 2014)

2.11. Statistical analysis

Data obtained from the assays for determining pH and temperature optima was analysed by Single factor ANOVA, followed by Tukey’s post hoc Honest Significant Difference (HSD) test. The ANOVA data was considered to be significantly different within the treatments if the F-value obtained was higher than the F critical at the probability of 0.01 (p ≤ 0.01). Critical differences (CDs) between the treatments using Tukey’s HSD were calculated at p ≤ 0.05 and p ≤ 0.01. Letters (a, b, c etc.) in the graph represent the levels of significantly different groups. Values with same letters are not significantly different. For GC-MS and LC-HRMS data represents mean ± S.D. of three independent experiments each conducted in duplicate.

3. Results

3.1. Dietary exposure to pesticide mixture up-regulates gene expression and activity of GST in H. armigera

H. armigera larvae (3rd instar) were fed with diets containing a pesticide mixture of 2, 4, 7, 10 and 15 ppm each for 72 h (Fig. 1A). Subsequently, larval GST gene expression and GST activity was analysed. GST activity of larvae ingesting the pesticide mixture displayed a dose dependant increase from 0.4 to 1.2 units per 5 µl crude enzyme preparation (Fig. 1B). GST gene expressions in H. armigera showed a pesticide dose dependant increase up to 10 ppm (Fig. 1C, black arrow). There was a slight reduction in GST expression in larvae fed with 15 ppm of pesticides.

3.2. Cloning and characterization of H. armigera GST

Work on HaGST genes was initiated by using 3 full length GST sequences reported in the NCBI database, namely HaGST-5, HaGST-7 and HaGST-9 (Table 2). In the present study, cloning and characterization of the gene amplified by using HaGST-7 specific oligonucleotide primers is reported (Table 1).

cDNA prepared from H. armigera larvae fed with 10 ppm pesticide diet, resulting in highest expression of GST and activity (Fig. 1) was used for the gene isolation. Amplicons were obtained, cloned and sequenced. Multiple sequence alignment of cloned GST and HaGST-7 showed 99.5% similarity with only two mismatches, in the amino acid sequences (Fig. 3A, black arrows). Due to this the newly isolated sequence was named as HaGST-8 (Table 2).

Phylogenetic analysis of all reported GSTs from H. armigera and ones from unrelated sources (Table 2) revealed that the GSTs from H. armigera larvae fed on chickpea and cotton, namely HaGST 1, −2, −3, −4 and 5 grouped separately from HaGST-7 and HaGST-8.

HaGST-7, −8, −6 and −9 were closely related. RNst, CgGST groupd away from HaGSTs; while AtGST formed an out-group (Fig. 2).

3D structural models of HaGST-7 and HaGST-8 were validated as over 90% of their residues were present in the most favoured region of Ramachandran plot in PROCHECK analysis and the z-score of both the structures was within the range of scores typically found for native proteins of similar size in ProSA analysis. Validated models of HaGST-7 and HaGST-8 were used for structural alignment (Fig. 3A). Homology modelling showed that HaGST-8 was structurally closely related to HaGST-7 as indicated by root mean square deviation (RMSD) value for the overlap that was found to be 0.151 Å (Fig. 3B).

3.3. Biochemical characterisation of HaGST-8

The mature peptide region of HaGST-8 was cloned in P. pastoris for extracellular secretion of the recombinant protein. GST activity in the broth of recombinant P. pastoris HaGST-8 was found to be more than two fold higher than that in control (Fig. 4A).

After various steps of purification, specific activity of HaGST-8 was found to increase. With affinity purification, a further increase in specific activity of the enzyme was observed (Table 3).

The molecular mass of the purified HaGST-8 was found to be around 29 kDa (Fig. 4B). HaGST-8 showed maximum activity at pH 9 (Fig. 4C). The optimum temperature for HaGST-8 activity was 30 °C (Fig. 4D). At a fixed GSH concentration of 10 mM, HaGST-8 exhibited Michaelis-Menten kinetics in response to changes in CDNB concentration from (0.01 to 10 mM) with Km 0.43 mM and Vmax 0.763 µm/min.

3.4. Growth of recombinant P. pastoris in the presence of high concentrations of pesticide

Functional analysis of the P. pastoris HaGST-8 for pesticide degradation was carried out. Recombinant protein expression in P. pastoris system is driven by an alcohol oxidase promoter, which uses methanol as an inducer. The effect of chlorpyrifos, dichlorvos and cypermethrin on growth of P. pastoris HaGST-8 was determined. The mixture contained equal amount of each pesticide that is either 200 ppm each Fig. 5A and 400 ppm each Fig. 5B.

The recombinant cells were grown in YPD media under the four conditions described in Section 2. After 9 h and 24 h of incubation growth of P. pastoris HaGST-8 in media containing the pesticides (200 and 400 ppm) and methanol as 4 and 5 fold higher (Fig. 5). In media containing the pesticides but without methanol, the growth was
retarded indicating their toxicity. In fact, with higher pesticide concentrations (Fig. 5B) better growth of *P. pastoris* HaGST-8 as compared to culture grown in presence of inducer methanol alone was observed.

### 3.5. Reduction in pesticide content after treatment with rHaGST-8 and crude *H. armigera* GST

A mixture of pesticides (700 ppm each) was treated with HaGST-8 protein and crude enzyme preparation from *H. armigera* tissue. After incubation for 2 h, compounds from the reaction mixture were extracted in chloroform and analysed by GC-MS for obtaining qualitative and quantitative estimates of pesticides. HaGST-8 treatment reduced the contents of pesticides chlorpyrifos, dichlorvos, and cypermethrin by 80%, 60%, and 60%, respectively (Fig. 6A).

A similar reduction in pesticide levels were obtained when crude *H. armigera* enzyme was used (Fig. 6A). Similar tube assays were carried out using with same amount of empty vector (EV) expressed protein to test for the non-specific binding of pesticides to proteins, as a means for their removal from the solutions.

When the pesticides (200 ppm each) were treated with purified HaGST-8 for 4 h at 25 °C and the reaction mixture analysed by LC-HRMS, a complete reduction of chlorpyrifos and dichlorvos and 53% reduction of cypermethrin (Fig. 6B) was noted, as compared to control samples lacking the enzyme.

### 3.6. Effect of amino acid substitutions in HaGST-8 on pesticide binding efficiency

Molecular interaction strength between HaGST-8 and earlier reported HaGST-7, with different insecticides was determined. Among chloropyrifos, cypermethrin and dichlorvos, chloropyrifos and dichlorvos showed strong binding with HaGST-8 whereas Cypermethrin showed strong binding with HaGST-7 (Fig. 7A). Among pyrethroid pesticides, bifenthrin, etofenprox, fenvalerate, phenothrin and resmethrin showed strong binding with HaGST-8 and weak binding with HaGST-7 whereas cypermethrin, permethrin, tralomethrin, tetramethrin showed strong binding with HaGST-7 and weak binding with HaGST-8 (Fig. 7B). Among organophosphates, chlorpyrifos and dichlorovos showed good binding with HaGST-8, than HaGST-7, whereas phorate, phosalone, phoxim, bensulide, tebupirimfos showed strong binding with both HaGST-7 and HaGST-8 (Fig. 7B).

In case of organochlorides and carbamates, chlordane, DDT, endrin, methoxychlor, carbaryl, carbofuran, fenoxycarb showed strong binding with HaGST-8. Amongst neonicotinoids, thiamethoxam showed strong binding with HaGST-8 and HaGST-7, imidacloprid, thiacloprid showed a strong binding with HaGST-8 (Fig. 7B).

### 4. Discussion

#### 4.1. *H. armigera* adapts to the intake of a blend of pesticides by modifying GST expression

On exposure to insecticides, insects exhibit interesting behavioural changes and molecular mechanisms that enable them to adapt to insecticide (Ahmad, 2007). This involves reduced penetration of insecticide through the cuticle, modifications of toxin target-sites or metabolism, up-regulation of detoxification enzymes and development of modified versions of enzymes (Dawkar et al., 2016). In the current study, we have used organophosphate and pyrethroid pesticides in which *H. armigera* displayed tolerance to a mixture of these pesticides. Adaptive and resistance abilities of *H. armigera* have rendered several
Fig. 3. Sequence analysis and homology modelling of HaGST-7 and HaGST-8. A) Sequence analysis of HaGST-7 and HaGST-8 showed Substrate binding site (blue) and GSH binding site (green). Arrow points towards the differences in amino acids in HaGST-7 and HaGST-8. B) Structural alignment of HaGST-7 and HaGST-8 using UCSF Chimera. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

Fig. 4. Purification, expression and biochemical characterization of HaGST-8. A) GST activity of control and recombinant GST expressed in P. pastoris broth assayed by using CDNB as substrate. B) SDS PAGE profiles of GSH affinity purified HaGST-8 stained with CBB (lane 1, black arrow); molecular mass markers (lane 2). C) Effect of pH on activity of HaGST-8. D) Effect of temperature on HaGST-8 activity. Error bars represent mean ± S.D. of three independent experiments each conducted in duplicate. Statistical analysis using Single factor ANOVA and Tukey’s post hoc HSD showed significant difference (p < 0.01) are indicated (a,b,c,d).
insects ineffective (Ahmad et al., 2001), demanding a constant need for new and next generation pesticides. These abilities are due to enhanced metabolic detoxification systems comprising of esterases, cytochrome P450 monoxygenase (MFO) and glutathione enzyme complexes (Fang et al., 2012). These co-ordinate pesticide/toxin modifications for removal from the system.

GSTs catalyse the conjugation of GSH to xenobiotic compounds and enable their metabolism out of the cell/body (Shi et al., 2012). Activities of GST enzymes have been detected in mammals, plants and insects where they have been implicated in detoxification of xenobiotics including pesticides (Chasseaud et al., 1973; Yang et al., 1976; Boyland and Chasseaud, 1969). Differential expression of GSTs was observed in housefly Musca domestica displaying varied pesticide tolerance (Wang et al., 1991; Fournier et al., 1992; Syvanen et al., 1994). In the current investigation a dose dependant up-regulation of GST gene expression and enzyme activity were observed when H. armigera larvae were exposed to blend of organophosphate and pyrethroid pesticides (Fig. 1). This up-regulation would be responsible for tolerance and adaptation of the larvae to the pesticide dose as high as 15 ppm, as indicated by larval survival. Higher activities of oxidative and hydrolytic enzymes including GSTs have been shown to be associated with organophosphate and pyrethroid resistance in H. armigera (Vontas et al., 2001, 2002; Srinivas et al., 2006) and Musca domestica (Hayaoka and Dauterman, 1982; Clark et al., 1989). GST overexpression is noted in Spodoptera littoralis after treatment with organochlorines (Lagadic et al., 1993) and in Apis mellifera and Spodoptera frugiperda after interaction with pyrethroids (Yu et al., 1984; Punzo et al., 1993).

### 4.2. Biochemical properties of HaGST-8

HaGST-8 gene was cloned from H. armigera fed with high doses of pesticide mixtures, and it was closely related to HaGST-7, with minor changes in amino acid sequence (Fig. 3). There are reports on the generation of sequence diversity in GSTs which play a key role in survival of in crustacean Calanus finmarchicus (Roncalli et al., 2015).

The biochemical characteristics of HaGST-8 were studied using its recombinant enzyme expression in P. pastoris. Recombinant HaGST-8 had a molecular mass of 29 kDa similar to GST from M. domestica, the plant Zea mays and the fungus Fusarium (Fournier et al., 1992; Izyk and Patrick et al., 1993; Cohen et al., 1986). HaGST-8 exhibited specific activity of 15.14 µmol min⁻¹ mg⁻¹. It is comparable to the values reported for GSTs from fungi Phanerochaete chrysosporium, Mucor circinelloides, Cunninghamella elegans, Fusarium oxysporum and bacteria Thermus thermophile (Dowd et al., 1997; Dowd and Sheehan, 1999; Cha et al., 2001; Cohen et al., 1986; Overbaugh et al., 1988). The Km value (0.043 mM) for HaGST-8 was comparable with earlier reports on GSTs from the snail Biomphalaria alexandrina and insect Tenebrio molitor (Hamed et al., 2009; Kostaropoulos et al., 1996). HaGST-8 was active in pH range 5–10 with maximum activity at pH 9 (Fig. 5). GST purified from Escherichia coli B was stable from pH 5–11 with activity maxima at pH 7 (Iizuka et al., 1989). GST purified from Mucor medeau exhibited maximum activity at pH 8.0, while the activity could not be detected at pH less than 4.5 (Hamed et al., 2005). HaGST-8 due to its high activity at alkaline pH, stability in acidic pH, can be considered further for development of specific bioremedial applications.

### 4.3. Recombinant HaGST-8 efficiently reduces pesticides from aqueous solutions and confers pesticide tolerance in P.pastoris

HaGST-8 was equally effective as the crude enzyme preparation from H. armigera larvae in reducing the pesticide levels from solution. Incubation with recombinant HaGST-8 led to complete reduction of pesticides chlorpyrifos, dichlorovos, and reduced cypermethrin contents from aqueous assay mix (Fig. 6). Earlier reports have indicated that the recombinant strains of GSTs cloned in E. coli showed a great potential for bioremediation (Chen et al., 1997; Cursino et al., 2000). Recombinant GST from Bombyx mori when cloned and expressed was able to detoxify organophosphate insecticides (Yamamoto and Yamada, 2016). Insect-specific epsilon class GSTs are associated with resistance to the organochlorines and pyrethroids and have higher affinities for.

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**Table 3**

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<td>1425.13</td>
<td>11.96</td>
<td>3.6</td>
</tr>
<tr>
<td>GSH Affinity</td>
<td>94.13</td>
<td>1265.85</td>
<td>15.14</td>
<td>4.6</td>
</tr>
</tbody>
</table>

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**Fig. 5.** Growth of P. pastoris HaGST-8 in pesticide enriched media. P. pastoris HaGST-8 was grown in YPD medium supplemented with Set1: only methanol (M+P-); Set 2: no addition (M-P-); Set 3: methanol + pesticides (M+P+); Set 4: only pesticide (M-P+) with A) 200 ppm B) 400 ppm. Methanol was supplemented as a carbon source for the growth of P. pastoris HaGST-8. Single factor ANOVA for the data on different pesticide concentration showed significant difference (at p < 0.01) amongst the various treatments. Tukey’s post hoc HSD comparisons between the data at p < 0.01 indicated by (***) in the figure.
this insecticide (Lumjuan et al., 2011; Wang et al., 2008). When evaluated for pesticide removal potential, recombinant HaGST-8, was found to be efficient in reducing organophosphate levels, while its pyrethroid removal ability was limited. GST catalysed GSH conjugates have been rarely observed in in-vitro reactions. A coordinated action of GSTs and cytochrome P450s on parent molecule leads to the formation of differently substituted derivatives. For example GSH conjugates of chlorpyrifos and chlorpyrifos oxon (CPO) were identified as GSCP and GSCPO (Fujiooka and Casida, 2007; LeBlanc and Sleno, 2011). Though we failed to detect the corresponding pesticide GSH conjugate peak a considerable reduction in parent pesticide peak was observed. The absence of pesticide GSH peak may be due to the subsequent formation

Fig. 6. Pesticide level reduction by recombinant HaGST-8 detected by GC-MS/MS and LC-HRMS. A) Pesticide detoxification by recombinant HaGST-8 and crude H. armigera extract monitored by GC-MS/MS analysis. B) Degradation of pesticides by recombinant HaGST-8 monitored by LC-HRMS. Error bars represent mean ± S.D. of three independent experiments each conducted in duplicate.

Fig. 7. In silico molecular interaction strength of pesticides from various classes with HaGST-7 and HaGST-8. A) Heatmap showed differential binding affinities/ molecular interactions of chlorpyrifos, cypermethrin and dichlorvos with HaGST-7 and HaGST-8. B) Interaction of pesticides belonging to different classes with HaGST-7 and HaGST-8. Gradient ruler at bottom of heatmap shows interaction strength as strong (red) to weak (green). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).
of various derivatives from the GSH pesticide conjugate in the reaction. This needs to be investigated further.

The culture of *P. pastoris* expressing HaGST-8 when exposed to pesticide mixture was able to thrive at concentration of as high as 400 ppm. As anticipated, HaGST-8 *P. pastoris* growth was least in culture not supplemented with methanol but with added pesticide mixture. Methanol acts as a carbon source and inducer for recombinant GST expression driven by the alcohol oxidase promoter. Higher growth of HaGST-8 *P. pastoris* in pesticide enriched media can be attributed to several factors including, GSH availability in HaGST-8 *P. pastoris*, in presence and absence of pesticide, or due to the detoxified products formed from pesticides. There was no effect of glutathione depletion on the growth of *Saccharomyces cerevisiae* as compared to *S. pombe* where glutathione depletion induced growth stasis. For these glutathione metabolism was investigated to understand this delayed growth stasis (Sharma et al., 2000).

4.4. Significance of minor sequence changes on pesticide –enzyme interaction

GST enzyme acts as a monomer and has a site each for binding to GSH and the toxin. It catalyses the conjugation of GSH with the toxin (Deponte et al., 2013). HaGST-7 and HaGST-8 displayed minor variations in amino acid sequence with Arginine (R) to Histidine (H) at interaction 4.4. Significant (Sharma et al., 2000).

metabolism was investigated to understand this delayed growth stasis. Several factors including, GSH availability in HaGST-8, has probably resulted in removal of cypermethrin (Fig. 6B). Minor changes in the amino acid sequence of HaGST-8 as compared to HaGST-7 has substantially weaker binding with cypermethrin and HaGST-8 had substantially weaker binding with cypermethrin than HaGST-7 (Fig. 7A). HaGST-8 was also inefficient in complete removal of cypermethrin (Fig. 6B). Minor change in the amino acid sequence of HaGST-8 as compared to HaGST-7 has probably resulted in modification of its insecticide binding properties. When docking scores of HaGST-7 and HaGST-8 with a range of pesticide belonging to organophosphate, pyrethroid, organochlorides, carbamates and neonicotinoids were compared, HaGST-8 displayed stronger binding/stability with many pesticides. Whether *H. armigera* has a series of variable GST genes that allow it to selectively express the GST best suited for the toxin to which it is exposed to, needs to be further investigated.

5. Conclusion

We have obtained a variant of GST from *H. armigera* after its exposure to high concentrations of organophosphate and pyrethroid pesticides. The gene was cloned in *P. pastoris*, the recombinant protein HaGST-8 was purified and biochemically characterized. Broad range of pH activity, low Km values, binding and interaction with different classes of pesticides and pesticide removal ability makes HaGST-8 and *P. pastoris* expressing HaGST-8 interesting systems for bio remediation of pesticides contaminated environment.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ecoenv.2017.09.028.

References


