



Distribution, Purification and Biochemical Characterization of an Esterase from Red Palm Weevil *Rhynchophorus ferrugineus* (Olivier) (Coleoptera: Curculionidae) Larvae

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ABSTRACT

Red palm weevil (RPW) *Rhynchophorus ferrugineus* is the most destructive insect pest of numerous palm species in the world and the larval stage (grubs) is the most destructive stage. Great variation in general esterase (GE) activity is recorded among the major tissues and the fat body of the 12th instar larvae has the highest enzyme activity. A major GE isoenzyme is purified to homogeneity to a specific activity 1871.2 U/mg protein. *R. ferrugineus* GE was biochemically characterized to estimate the compounds required for suppressing the enzyme. The enzyme has optimum activity at pH 8.0 and at a temperature of 50°C, irreversibly denatured above 60°C, is induced by Hg²⁺, Ni²⁺, Zn²⁺, Cu²⁺, Co²⁺, completely suppressed by Fe²⁺, appearing to be a Zn²⁺-dependent enzyme. The enzyme hydrolyzed various phosphoric esters as substrates, has high specificity towards PNPA and α -NA with Km values 0.4 and 0.8 mM. *R. ferrugineus* GE is completely inhibited by PMSF, β -ME, DTT, and 49% by EDTA. The enzyme was inhibited competitively by PMSF and noncompetitively by β -ME and DTT with Ki values 1.5, 2.0 and 3.5 mM. The inhibition of GE can increase the susceptibility of the target pest to chemical insecticides and is appropriate for enhancing the insecticidal efficacy of the chemical insecticides. Further studies *in vivo* are in progress to investigate the deleterious effect of EDTA, Fe²⁺, and thiol reducing agent on RPW larval development and the possibility of introducing them as insecticide synergists in IPM programs for developing RPW management strategies.

Keywords: characterization, development, inhibition, esterases, purification.

1. Introduction

Red palm weevil (RPW) *Rhynchophorus ferrugineus* (Coleoptera: Curculionidae) has been proven as the most damaging insect pest of palms in the world, even in all the countries where it has been accidentally introduced. The most destructive stage of RPW is the larval stage (grubs) (Hamadah, 2019; Ghanem *et al.*, 2025). The larvae feed within the apical growing points of the palms, burrow into the trunk and feed on stem tissues, creating extensive damage to palm tissues and weakening the structure of the palm trunk. The resulting damage is often only visible long after infestation, when palms are close to death (Habood *et al.*, 2022; Mohamed *et al.*, 2022). The larvae and adult emergence within the same stem allow successive generations. Numerous application methods are being used for RPW control; the current RPW management strategies are based on manual application of insecticides. The ineffectiveness of viable insecticides for *R. ferrugineus* control is due to the defense system inherent in such pests (Alsanee *et al.*, 2022; Ghanem *et al.*, 2025).

Insecticide breakdown by enzymatic metabolism is the common mechanism that has evolved a dormancy adaptation enabling insects to survive under unfavorable environmental conditions to protect themselves (Srinivas *et al.*, 2006; Nedal and Amin, 2019). Insecticide resistance may arise due to qualitative or quantitative changes of one or more of three classes of enzymes which can bind and metabolize insecticides (Allakwa and Amin, 2022). Esterases have the major importance specifically in the detoxification of organophosphate insecticides (OPs) and can be classified into two groups (Dixon

and Webb, 1979). Esterases (A) that are bind to and detoxify OPs and (B) esterases (including acetylcholinesterase AChE) which are inhibited by OPs.

General esterases, (GEs), the false target enzymes of insecticides, are distributed in the tissues of most organisms (Lopes *et al.*, 2014; Nedal and Amin, 2019). Esterases are involved in several important physiological processes including digestion, reproduction, metabolism of juvenile hormone, molting and play the major role for scavenging and metabolizing synthetic chemical insecticides to less toxic metabolites (Allakwa and Amin, 2022; Hoor *et al.*, 2024).

Insect can develop resistance to an insecticide(s) via several esterase-based mechanisms. A mutant type esterase with an altered substrate specificity which is preferentially metabolizes insecticides (Conyers *et al.*, 1998; Zhou *et al.*, 2004). Increased esterase activities can lead also to resistant development in insects either due to the presence of high amounts of the enzyme protein due to the amplification of the gene esterase or a more catalytically efficient enzyme (Srinivas *et al.*, 2006; Lopes *et al.*, 2014). The involvement of esterases based on elevated activity in the insecticide resistance mechanisms have been documented in various insect species including *Oryzaephilus surinamensis* (Conyers, *et al.*, 1998), *Helicoverpa armigera* (Srinivas *et al.*, 2006), and *Spodoptera frugiperda* (Hoor *et al.*, 2024).

General esterases have been purified and biochemically characterized from different insect species including: *O. surinamensis* (Conyers *et al.*, 1998), *Diabrotica virgifera virgifera* (Zhou *et al.*, 2004), midgut of the silkworm *Bombyx mori* (Murthy and Veerabhadrapa, 1996), rice green leafhopper, *Nephotettix cincticeps* (Chiang and Sun, 1996), insecticides-resistant and susceptible strains of German cockroach, *Blattella germanica* (Prabhakaran and Kamble, 1995). Esterases have also been purified and characterized from insecticide-resistant strains of *H. armigera* (Srinivas *et al.*, 2006) and *Tribolium castaneum* (Haubruge *et al.*, 2002). Esterases have been biochemically characterized, with respect to inhibition kinetics, from the larval hemolymph (Nedal and Amin, 2019) and from cytosol soluble fraction of the 5th instar larvae (Allakwa and Amin, 2022) of *R. ferrugineus*.

General esterase (GE) is one of the enzymes implicated in detoxification of insecticides in addition to its physiological importance. Bakr *et al.* (2013) reported that suppression of GE in an insect could be increases the susceptibility of the insect pests to chemical synthetic insecticides and suitable for enhancing the insecticidal efficacy of the chemical insecticides. Allakwa and Amin (2022) reported that the systematic inhibition of such enzymes has been proven to be an essential biotechnology tool and critical feature for insect management.

The major objective of the present study is to develop RPW control strategy through inhibiting GEs. The present report will investigate the distribution of GEs in different major tissues of *R. ferrugineus*, tracking the variation in the enzyme activity levels during larval development in the tissue of the highest enzyme activity level. In addition, purification, biochemical characterization specifically the susceptibility of the purified major isoenzyme to inhibit by different compounds and the mechanism of inhibition will be conducted. The present study will allow us to speculate on the physiological role of esterases in *R. ferrugineus*. The biochemical characteristics of *R. ferrugineus* GE will be carried out to define the compound(s) that suppress GE activity which can be synergized with the effective chemical insecticide(s) for increasing the efficiency of RPW management. The results of the report will pave the way for *in vivo* investigation for developing RPW management strategy.

2. Materials and Methods

Chemicals, sample preparation, enzyme assay, steps of GE purification and biochemical characterization are described as follows:

2.1. Chemicals

Fast blue RR, α - and β -naphthyl acetate sodium salt (α -NA & β -NA) (99%), *p*-nitrophenol (PNP) (99%), *p*-nitrophenyl acetate (PNPA) (99%), *p*-nitrophenyl phosphate (PNPPh) (99%), *p*-nitrophenyl butyrate (PNPB) (99%), *p*-nitrophenyl palmitate (PNPP) (99%), *p*-nitrophenyl stearate (PNPS) (99%), dithiothreitol (DTT) (99%), acetylthiocholine iodide (ATCI) (99%), β -mercaptoethanol (β -ME) (99%), ethylenediaminetetraacetic acid disodium salt (EDTA), *p*-hydroxymercuribenzoic acid (PHMB) (99%), *p*-methyl sulfonyl fluoride (PMSF), eserine, DEAE-Sepharese for chromatography. All substrates, inhibitors, reagents and resins for polyacrylamide gel electrophoresis (PAGE) were purchased from

Sigma-Aldrich, USA. Sephacryl S-200 was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden) and metals were purchased from BDH Chemicals Ltd Poole England.

2.2. Insect

Different larval instars of *R. ferrugineus* were transported from Central Laboratory for Date Palm Research and Development (CLDPRD), Agricultural Research Center (ARC), Dokki, Giza, Egypt. The larvae were maintained in transparent plastic cups (120 × 60 × 30 cm) with easily removable perforated covers under controlled conditions.

2.3. Samples preparation

Different major tissues, including the hemolymph, whole gut, head, cuticle, and fat body from the 10th instar larvae of *R. ferrugineus* and in fat body during larval development were separated according to Mohamed *et al.* (2022). In brief, the larvae were placed on ice (about 5 min) for immobilization and dissected under microscope according to Alarcon *et al.* (2002). Hemolymphs were collected from the larvae before dissection by puncturing the specimen carefully with a microsurgical knife at the end of the abdomen in Eppendorf tubes. The fat tissues were removed by small brush and collected. The whole guts (foregut, midgut and hindgut) and their contents were separated and collected. For separation of heads, the mouth parts were removed and the brown, hard, outer layers of the heads were peeled off with the aid of sharp surgical blades. The cuticles were separated, washed and cut into small pieces. All fractions were stored at -20°C until used.

2.4. Preparation of enzyme crude extracts

The crude extracts of GE were prepared by homogenizing the tissues individually in pre-cooled pestle (Teflon pestle homogenizer) in 50 mM ice cold sodium phosphate buffer, pH 7.5. The homogenates were centrifuged at 12,000×g for 15 min at 4°C. The precipitates were re-extracted twice. The two supernatants for each extract were pooled individually and designated as enzyme crude extracts, pooled and frozen at -20°C until used for estimating GE activity.

2.5. Enzyme assay

The activity of GE was estimated by Galliard and Dennis (1974) method and modified according to Mohamed *et al.* (2016). The assay reaction mixture contained in 1.0 ml: 80 mM sodium phosphate buffer, pH 8.0, 2.0 mM PNPA as a substrate and incubated for 15 min at 37°C followed by adding enzyme crude extract ranged from 50-100 µl. The increase in absorbance at 407 nm was continuously recorded spectrophotometrically for 3 min in 30 sec intervals for measuring PNP released. One unit of GE activity was defined as the amount of enzyme that is liberated one µmol of PNP per hour under standard assay conditions using a previously calibrated curve of PNP as a standard.

2.6. Protein determination

Protein contents were determined according to Bradford (1976) using bovine serum albumin as a standard.

2.7. Purification of *R. ferrugineus* GE

Unless otherwise stated all steps were performed at 4-7°C.

2.7.1. Preparation of crude extract

R. ferrugineus GE crude extract was prepared from two fat bodies of the 12th instar larvae of *R. ferrugineus* as previously described and concentrated to 1.5 ml by dialysis against pure sucrose.

2.7.2. Sephacryl S-200 chromatography

The concentrated crude extract was applied into a Sephacryl S-200 column (95 x 1.6 cm i.d.) previously equilibrated with 20 mM Tris-HCl buffer, pH 8.0 and developed with the same buffer at a flow rate of 20 ml/ h. The most active fractions were pooled and frozen at -20°C.

2.7.3. DEAE-Sephacryl chromatography

The major pooled active fraction of GE from Sephacryl S-200 column was applied into a DEAE-Sephacryl column (15 x 1.6 cm i.d.) previously equilibrated with 20 mM Tris-HCl buffer, pH 8.0. The adsorbed materials were eluted with a stepwise gradient NaCl ranging from 0.0 to 0.4 M prepared in the same buffer at a flow rate of 60 ml/h. Protein fractions exhibiting GE activities were eluted with 0.1, 0.2 and 0.3 M NaCl and designated as GEI, GEII, and GEIII.

2.8. Optimum pH for activity

The effect of different pH on purified *R. ferrugineus* GE was performed with pH range from 5.0 to 10.5 using sodium acetate, sodium phosphate, and Tris-HCl.

2.9. Optimum temperature for activity and stability

The effect of temperature on activity and stability of purified *R. ferrugineus* GE were investigated over a range of temperatures 10-80°C. For estimating the optimum temperature for activity, the assay reaction mixtures were incubated for 15 min at different temperatures, followed by adding pure enzyme and recording the increase in absorbance. For temperature stability, the enzyme was pre-incubated at different temperatures for 15 min and quickly cooled before estimating the residual activities as previously described.

2.10. Effect of metals

Effect of different metal cations including Co^{2+} , Mg^{2+} , Mn^{2+} , Hg^{2+} , Ni^{2+} , Fe^{2+} , Zn^{2+} , Ca^{2+} , Ba^{2+} , Al^{3+} , Na^{+} in chloride form and Cu^{2+} in sulfate form were examined on *R. ferrugineus* GE activity at 1.0 and 10 mM as final concentrations. The enzyme was pre-incubated in Tris-HCl buffer, pH 8.0 for 15 min at room temperature with the different cations individually prior to substrate addition for estimating the residual activities as previously described using PNPA as substrate.

2.11. Substrate specificity and kinetic parameters

The relative rate for hydrolyzing different aliphatic chains of *p*-nitrophenyl esters, PNPA, PNPB, PNPP and PNPS, as substrates for *R. ferrugineus* GE were determined by the rate of liberation of PNP according to the method of Ljungquist and Augustinsson (1971) and modified by Zera *et al.* (2002). In addition, the relative rate for hydrolysis α -NA and β -NA were also measured by the rate of α -naphthol liberation according to Van Asperen (1962) with some modification by Mohamed *et al.* (2016). The activity of *R. ferrugineus* GE towards various esters including PNPPH at 5.5 (acid phosphatase) and pH 9.0 (alkaline phosphatase) were estimated according to Abbott and Kaplan (1984) and ATCI according to Ellman *et al.* (1961) with some modification by Bilal *et al.* (2018). The kinetic parameters, Michaelis-Menten constant (K_m), maximal velocity (V_{max}) and the catalytic efficiency (V_{max}/K_m) for *R. ferrugineus* GE were estimated using computer analysis of Lineweaver-Burk double reciprocal plots of experimental data according to Lineweaver and Burk (1934) using 6 concentrations of PNPA and α -NA in the range from 0.5-4.0 and 1.0-4.0 mM.

2.12. Inhibition by different compounds

The inhibitory effect of different compounds, including DTT, β -ME, EDTA, PHMB, PMSF and eserine, on the purified of *R. ferrugineus* GE activity was performed. The enzyme was pre-incubated in Tris-HCl buffer, pH 8.0 for 15 min at room temperature with the different compounds individually at 1.0 and 10 mM as final concentrations prior to substrate addition for estimating the residual activities. The median inhibition concentration (IC_{50}) for EDTA, DTT, PMSF, PHMB and β -ME as inhibitors for *R. ferrugineus* GE activity were calculated based on inhibitor (I) concentration versus probit (percentage of inhibition) linear regression according to Zhu and Gao (1998). The bimolecular rate constant (K_i) and the mechanism of inhibition of PMSF, DTT and β -ME are estimated by the double reciprocal plots of initial velocities of *R. ferrugineus* GE versus reciprocal concentrations of PNPA as substrate in absence and presence of three different concentrations of each individually in the range of 2.0 to 6.0 mM.

2.13 Replication and statistical analysis

R. ferrugineus GE activities in different major tissues and in the fat body during larval development were replicated three times. The data are the means of values derived from three separate experiments $\pm$ standard error (SE). One-way ANOVA is used for statistical analysis.

3. Results

Defining RPW tissue and the instar larvae of the highest GE activity

3.1. Distribution of GE activity in different major tissues

The activity of GE was estimated in different major tissues isolated from the 10th instar larvae of *R. ferrugineus* and ranged from 28 ± 0.94 to 491 ± 23.2 U/tissue with specific activity ranged from 89 ± 3.1 to 432 ± 22.4 U/mg protein (Fig.1). Fat body and hemolymph have the highest and the lowest esterase activities. One-way ANOVA showed a significant effect of the tissue type on the total activity ($F=170.9$, $p<0.05$) and specific activity ($F=740.8$, $p<0.05$). The diversity in GE activity has been conducted in the fat body during larval development.

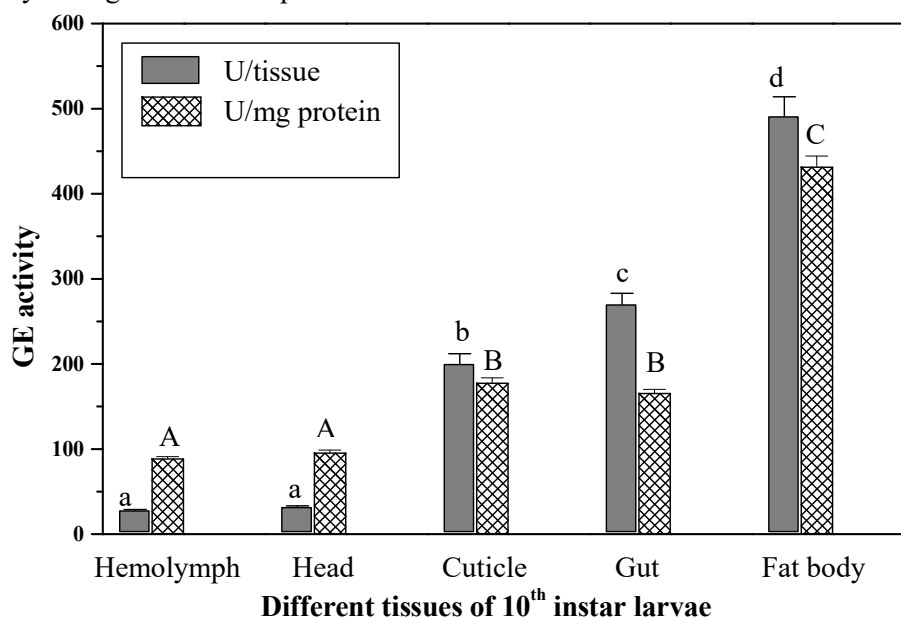


Fig. 1: The activity of GE in different major tissues of the 10th instar larva of *R. ferrugineus*. Each value represents the mean $\pm$ standard error of the mean (SEM). Bars marked with the same letters are similar ($P > 0.05$) whereas those with different one are significantly different ($P < 0.05$). Capital letters refer to statistical analysis for different tissues specific activity.

3.2. GE activity in fat body during larval development

In the fat body (FB), *R. ferrugineus* GE activity sharply increased from the 8th to the 12th instar larvae (Fig. 2). The highest esterase activity was recorded in the 12th instar larvae (934.0 ± 50 U/FB) with the highest specific activity (647 ± 28 U/mg protein). By one-way ANOVA, the larval instar significantly affected both enzyme activity ($F=134.8$, $p<0.05$) and specific activity ($F=98.7$, $p<0.05$). Purification and biochemical characterization of the predominant esterase isoenzyme have been restricted from the FB of the 12th instar larvae of *R. ferrugineus*.

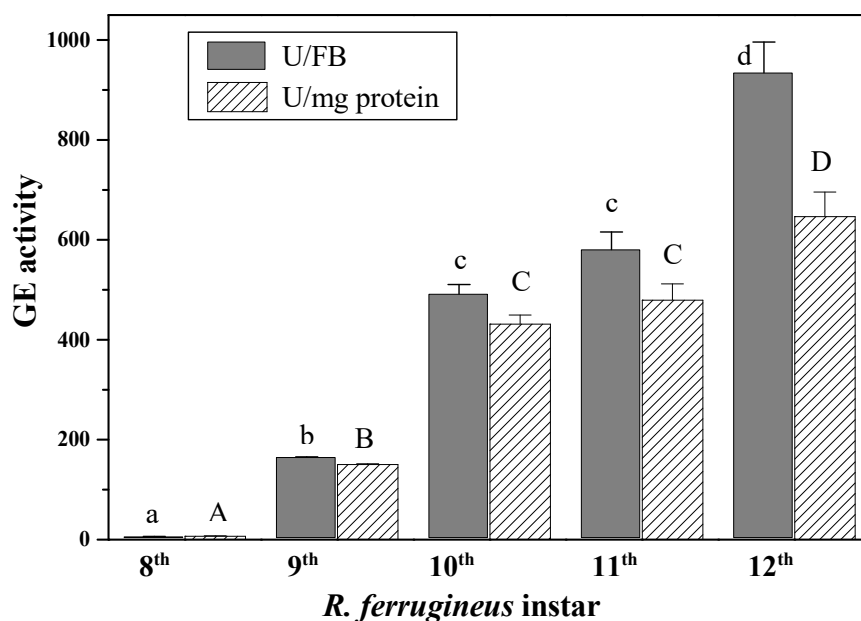


Fig. 2: The activity of GE in the fat body of *R. ferrugineus* during larval development. Each value represents the mean $\pm$ standard error of the mean (SEM). Bars marked with the same letters are similar ($P > 0.05$) whereas those with different one are significantly different ($P < 0.05$). Capital letters refer to statistical analysis for different instars specific activity.

3.3 Purification of *R. ferrugineus* GE

A single GE isoenzyme is purified from fat bodies of the 12th instar larvae of *R. ferrugineus* (Table 1). Chromatography of crude extract on a Sephacryl S-200 column results in a resolution of a one GE peak of activity (Fig. 3). The pooled enzyme has a specific activity of 827.0 U/mg protein with 1.2-fold purification. By applying the concentrated pooled active fractions of Sephacryl S-200 on a DEAE-Sephacryl column, three peaks of GE activities were eluted with 0.1, 0.2 and 0.3 M NaCl and designated as GEI, GEII, and GEIII (Fig. 4). The major purified isoenzyme, *R. ferrugineus* GEII, has a final specific activity 1871.2 U/mg proteins which represents 2.74-fold purification over crude extract.

Table 1: A typical purification scheme for GE from the fat body of *R. ferrugineus*

Purification step	Protein (mg)	Activity (units*)	Specific activity (units/mg protein)	Fold purification	Recovery (%)
Crude extract	1.4	955.5	682.5	--	100
Sephacryl S-200	1.06	876.75	827	1.2	91.8
DEAE-Sephacryl					
GEI	0.19	148.75	782.9	1.15	16
GEII	0.26	486.5	1871.2	2.74	51
GEIII	0.14	105	750	1.10	11

*One unit of GE activity is defined as the amount of enzyme that liberated one μ mol of PNP per hour under standard assay conditions. Each value represents the average of two separate experiments.

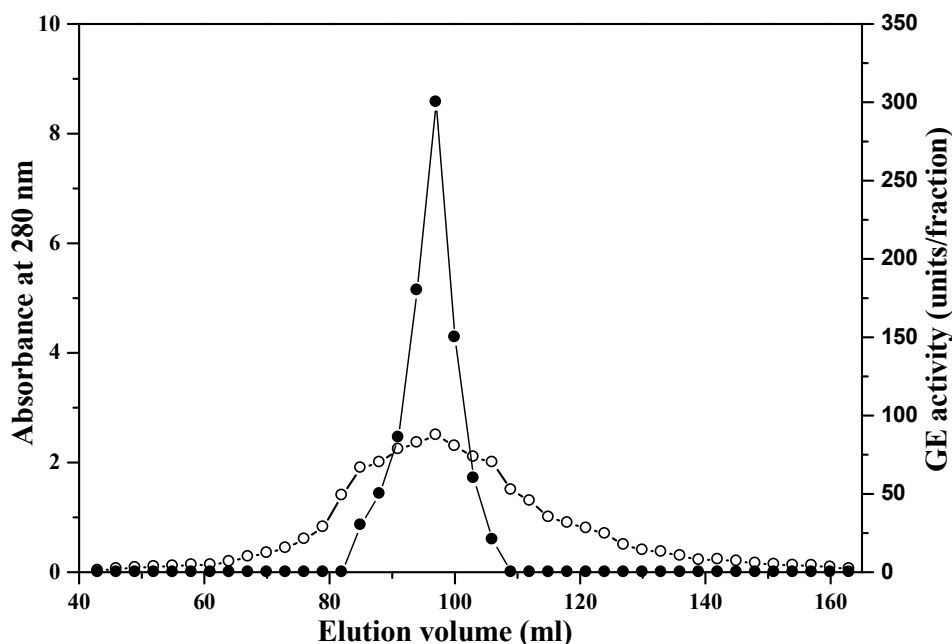


Fig. 3: A typical elution profile for the chromatography of the concentrated crude extract of *R. ferrugineus* GE on a Sephacryl S-200 column (95 x 1.6 cm i.d.) previously equilibrated with 20 mM Tris-HCl buffer, pH 8.0 at a flow rate of 20 ml/h and 3 ml fractions. Absorbance at 280 (o—o) and (•—•) enzyme activity.

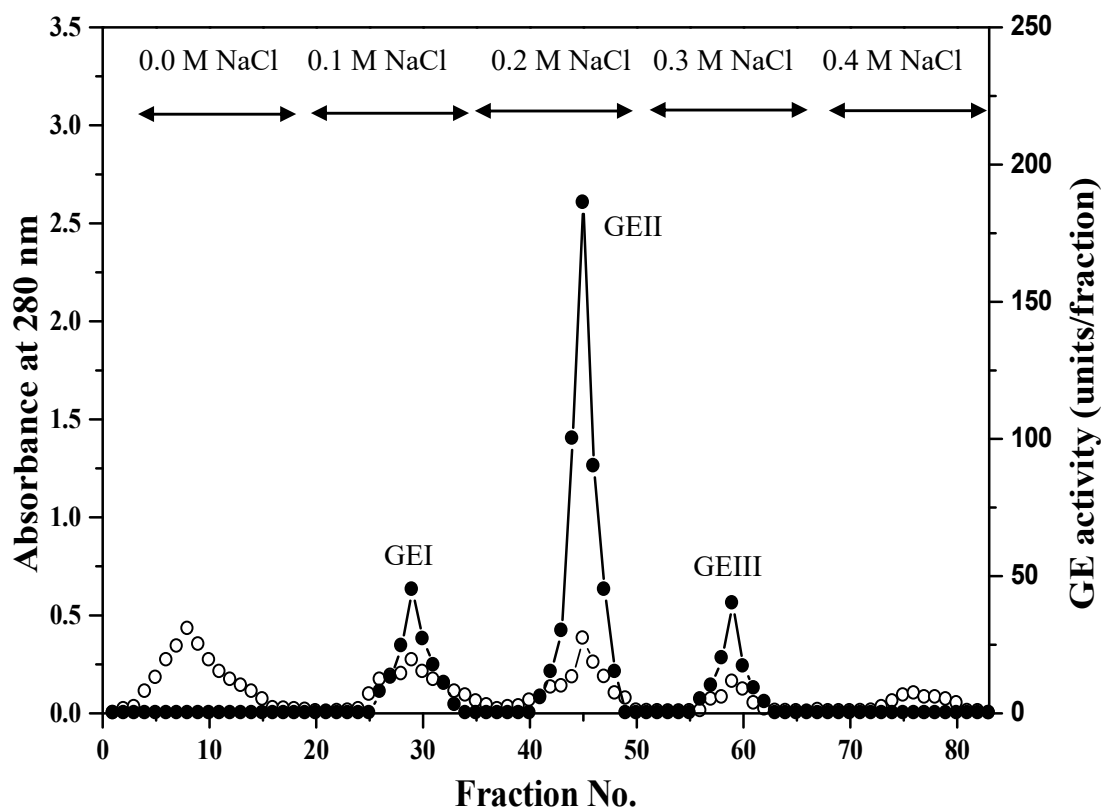


Fig. 4: A typical elution profile for the chromatography of concentrated pooled Sephacryl S-200 active fractions on a DEAE-Sephacryl column (15 x 1.6 cm i.d.) previously equilibrated with 20 mM Tris-HCl buffer, pH 8.0 at a flow rate of 60 ml/h and 5 ml fractions. Absorbance at 280 (o—o) and (•—•) activity enzyme.

3.4. Characterization of *R. ferrugineus* GE

The biochemical characterization of the purified GE was carried out with respect to pH, temperature, metal cations, substrate specificity, inhibition by different compounds and mechanism of inhibition.

3.4.1 Effect of pH and temperature

The optimum pH and temperature for estimating GE activity and stability revealed that the maximum activity for *R. ferrugineus* GE is detected at pH 8.0 using Tris-HCl buffer, (Fig. 5) and at a temperature 50°C (Fig. 6a). The enzyme is stable up to temperature 50°C and it irreversibly denatured above such temperature where 37 and 54% of enzyme activities were lost upon incubation for 15 min at 70°C and 80°C. The activation energy (AE) of *R. ferrugineus* GE was calculated from the slope of Arrhenius plot and was found to be 6.7 Kcal/mol (Fig. 6b).

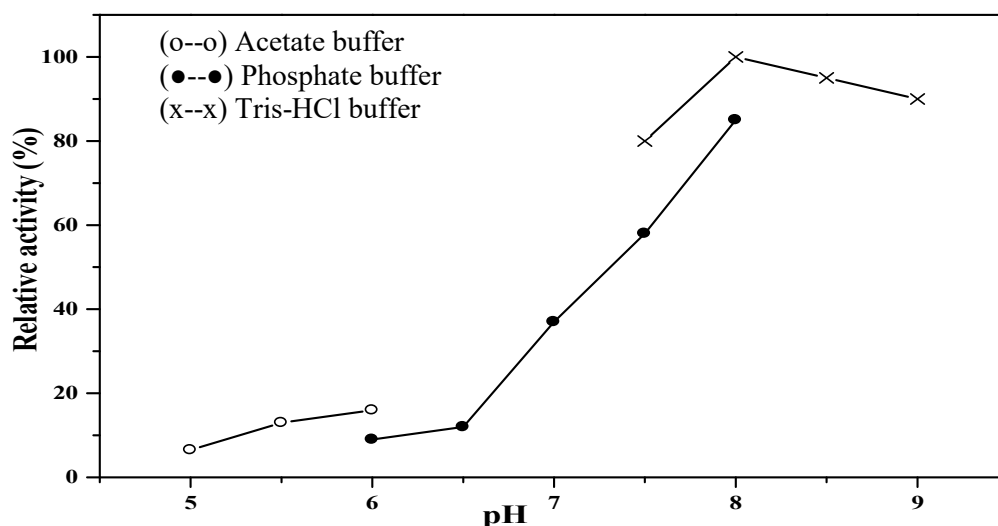


Fig. 5: Effect of different pH on *R. ferrugineus* GE activity. The reaction mixture contained in 1.0 ml: 2mM PNPA and 80 mM of each buffer. Each point represents the average of two separate experiments

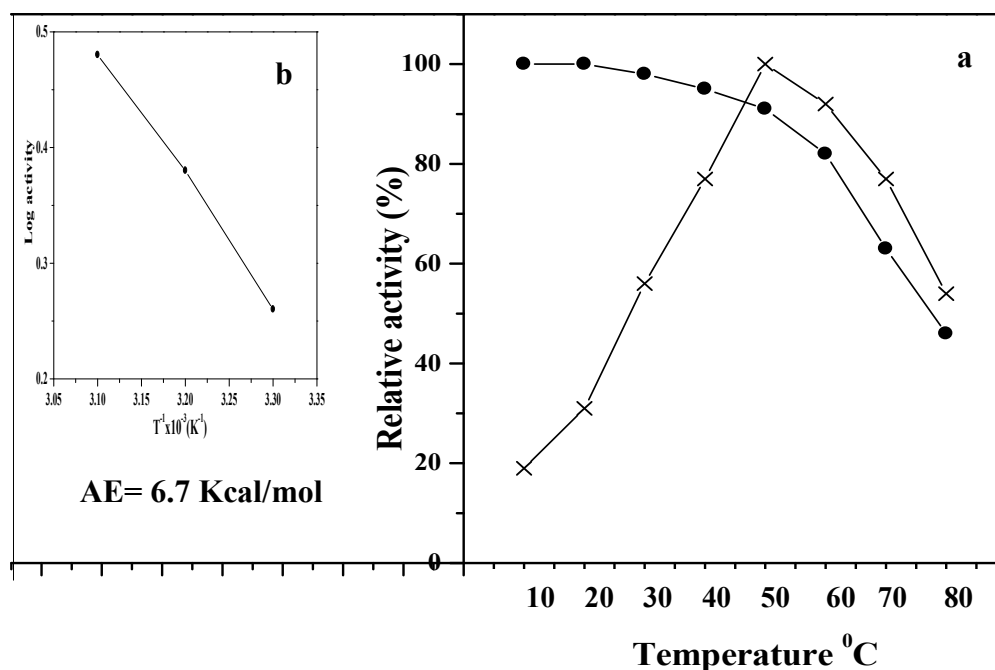


Fig. 6: Effect of temperature on the activity (x--x) and stability (●--●) of *R. ferrugineus* GE (a). Activation energy (AE) was estimated from the replot of log activity versus 1/T (Arrhenius plot) (b). Each point represents the average of two separate experiments.

3.4.2 Effect of metal cations

The effect of different metal cations as inhibitors or activators has been examined. Stimulatory, inhibitory and no effects on *R. ferrugineus* GE activity have been observed using 1.0 and 10 mM as final concentrations of each metal cations examined (Table 2). The enzyme activity is induced by Hg^{2+} , Ni^{2+} , Zn^{2+} , Cu^{2+} and Co^{2+} with induction percent ranged from 13 to 113 % at the two concentration levels 1.0 and 10 mM. Al^{3+} and Fe^{2+} inhibited the enzyme activity by 44.7 and 100 % at concentration 10 mM.

Table 2: Effect of metal cations on *R. ferrugineus* GE activity.

Metal	Relative activity (%)	
	1mM	10mM
Non	100	100
Ni^{2+}	99	160
Ca^{2+}	100	103
Mn^{2+}	100	98
Hg^{2+}	113	155
Na^+	100	100
Mg^{2+}	100	93
Zn^{2+}	166	213
Co^{2+}	133	200
Cu^{2+}	116	180
Fe^{2+}	43	0
Al^{3+}	80	55
Ba^2	100	104

The results are the average of two separate experiments.

3.4.3 Substrate specificity and kinetic parameters

Purified *R. ferrugineus* GE hydrolyzed a number of *p*-nitrophenyl esters (Table 3). The relative activity arranged in a descending order as PNPA > PNPB > PNPP > PNPS with preferability towards PNPA. The enzyme hydrolyzed also α -NA and β -NA with relative rate 100% and 21%. *R. ferrugineus* GE shows Michaelis-Menten behavior for the two substrates PNPA and α -NA in the concentration ranges 0.5 to 2.0 mM and 0.5 to 4.0 mM and exhibited K_m values 0.4 and 0.8 mM with V_{max} 16.7 and 0.75 $\mu\text{mol/ml/h}$. (Fig. 7).

Table 3: Relative rate of *R. ferrugineus* GE for hydrolyzing different substrates

Substrate	Relative activity (%)
PNPA	100
PNPB	60
PNPP	42
PNPS	15
α -NA	100
β -NA	21

The results are the average of two separate experiments.

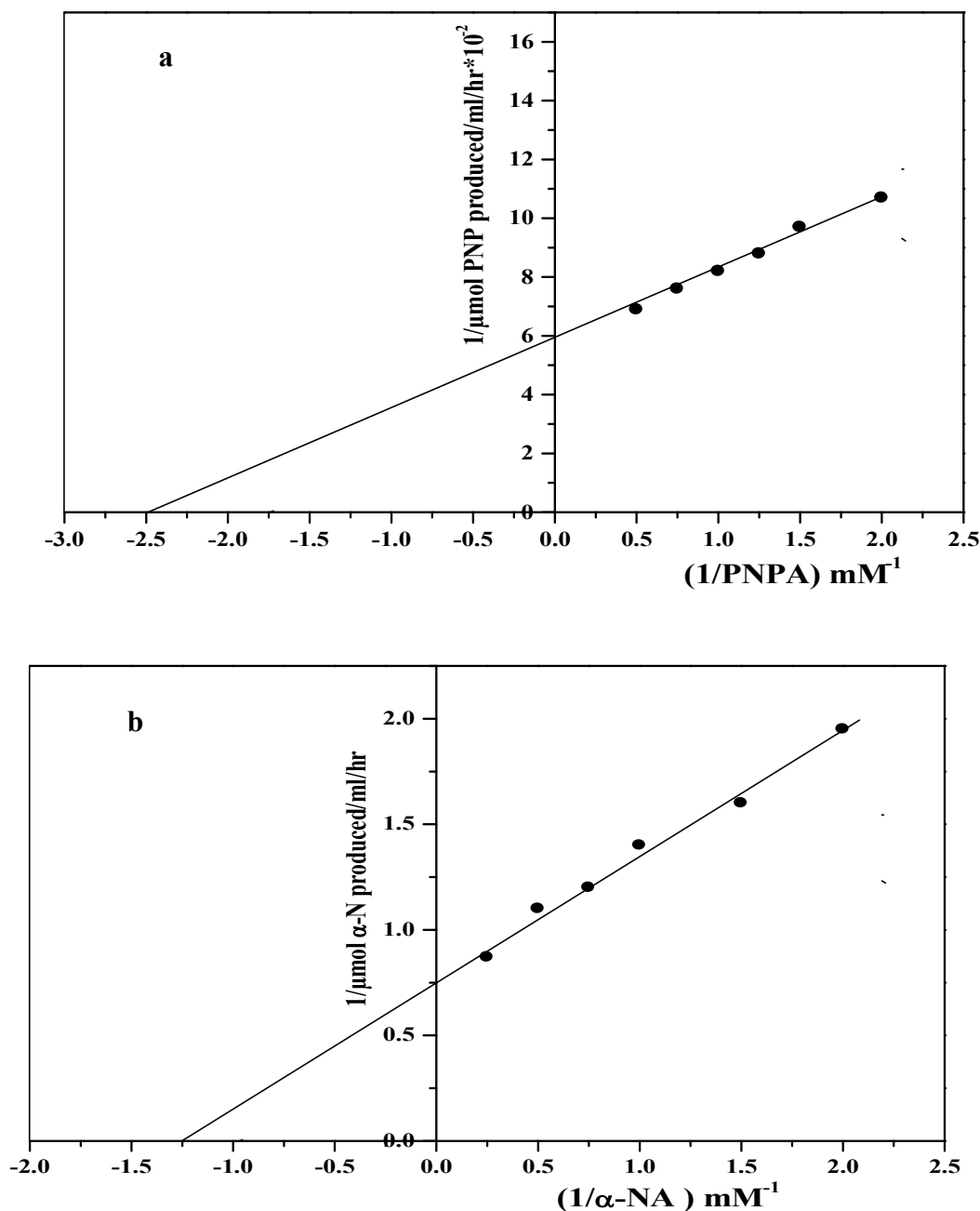


Fig. 7: Lineweaver–Burk plots for estimating K_m and V_{max} for *R. ferrugineus* GE using PNPA (a) and α -NA (b) as substrates. Each point represents the average of two separate experiments.

3.4.4. Inhibition by different compounds

No stimulatory and low inhibitory effect on *R. ferrugineus* GE activity was recorded in presence of IAA and NaF at concentration levels 1.0 and 10 mM. The enzyme moderately inhibited by PHMBA, eserine and EDTA with inhibition percent 45%, 48% and 49% in presence of 10.0 mM of each (Table 4). The enzyme completely inhibited by β -ME, DTT, and PMSF in presence of 10.0 mM of each and the median inhibition constants (IC_{50}) were 3.0, 4.0, and 3.5 mM for (Figs. 8, 9, 10). β -ME and DTT noncompetitively inhibited *R. ferrugineus* GE with K_i values 2.0 and 3.5 mM (Figs. 11, 12). PMSF competitively inhibited the enzyme with K_i values 1.5 mM (Fig.13).

Table 4: Effect of different compounds on *R. ferrugineus* GE.

Compounds	Relative activity (%)	
	1 mM	10 mM
None	100	100
DTT	81.4	0.0
β -ME	75.2	0.0
PMSF	82.8	0.0
Eserin	75.6	52
EDTA	94.0	51
PHMBA	81.8	55
IAA	87.9	75.6
NaF	90.8	79.8

The results are the average of two separate experiments.

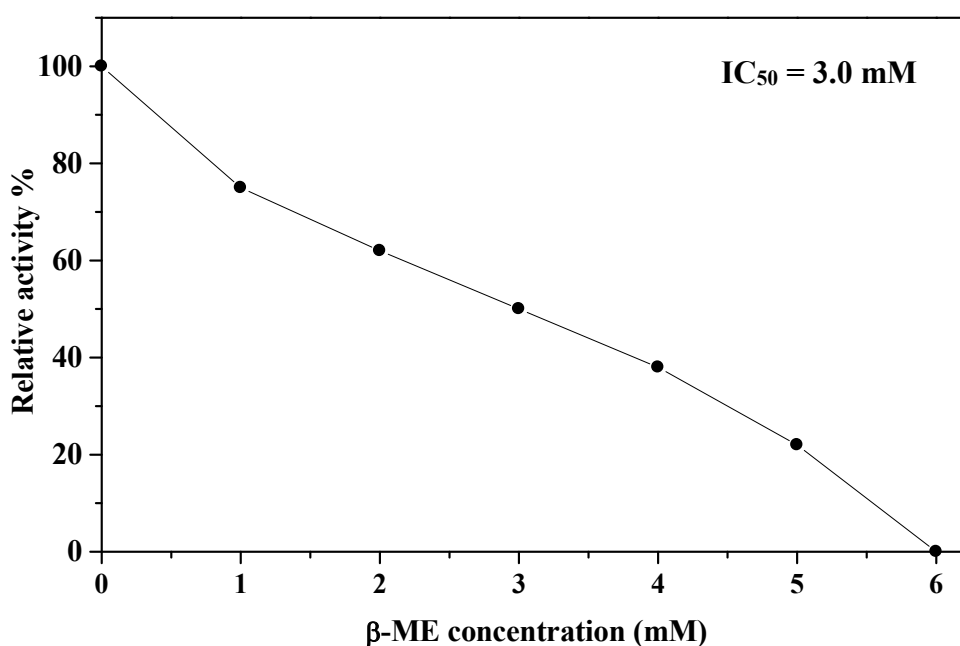


Fig. 8: Inhibition of *R. ferrugineus* GE by β -ME. Purified *R. ferrugineus* GE was incubated for 15 min with different β -ME concentrations (1.0 - 6.0 mM) at room temperature, followed by estimation of the residual activities.

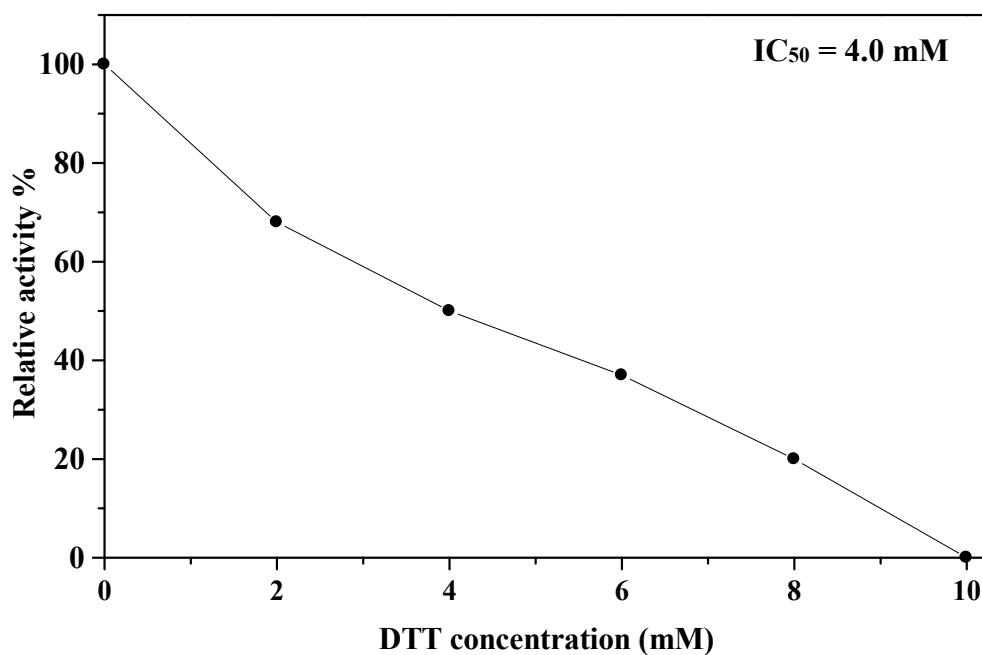


Fig. 9: Inhibition of *R. ferrugineus* GE by DTT. Purified *R. ferrugineus* GE was incubated for 15 min with different DTT concentrations (2.0 - 10.0 mM) at room temperature, followed by estimation of the residual activities.

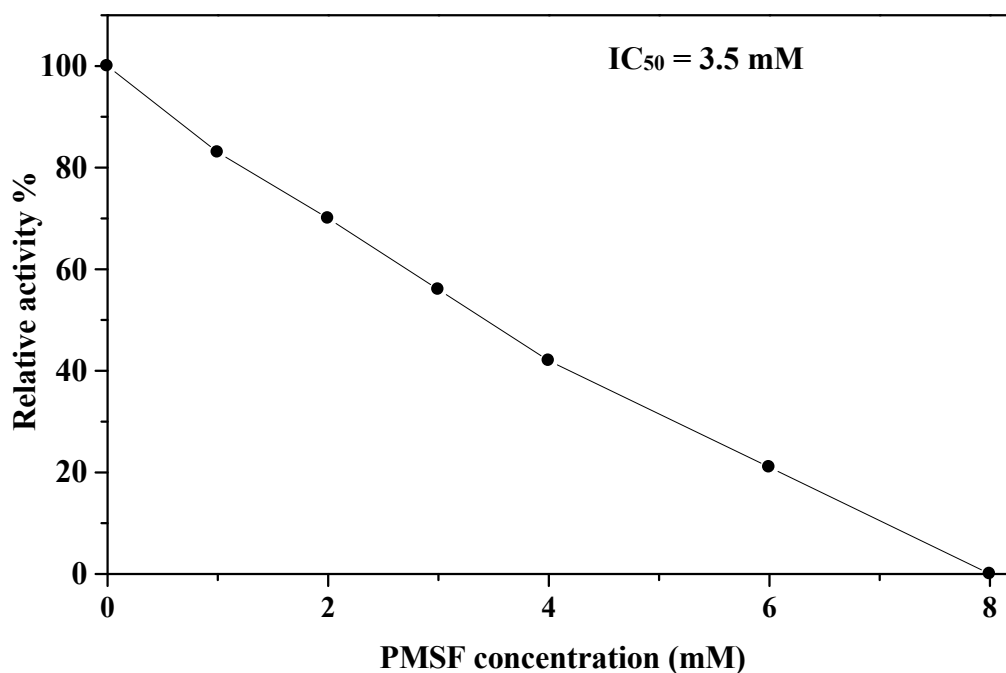


Fig.10: Inhibition of *R. ferrugineus* GE by PMSF. Purified *R. ferrugineus* GE was incubated for 15 min with different PMSF concentrations (1.0 - 8.0 mM) at room temperature, followed by estimation of the residual activities.

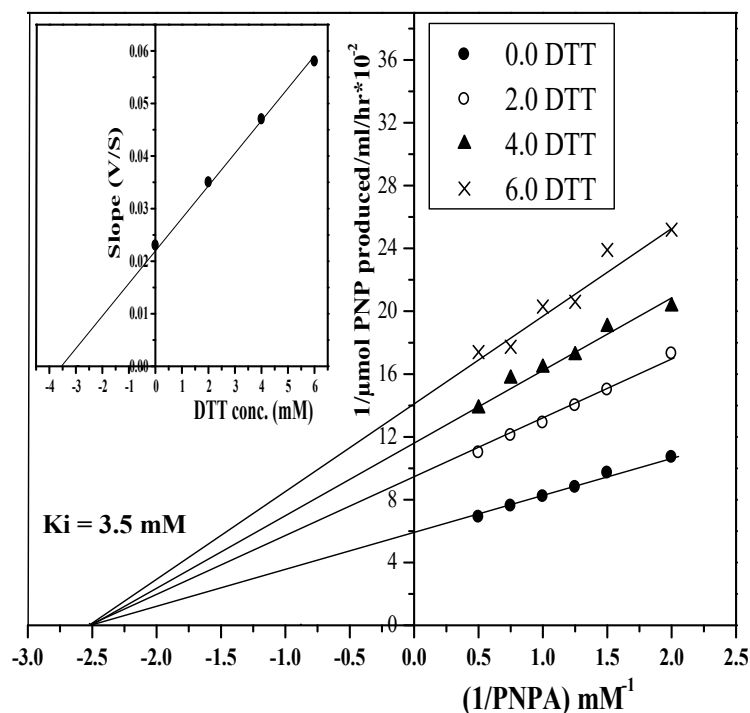


Fig. 11: Reciprocal plots of initial velocities of *R. ferrugineus* GE versus reciprocal concentrations of PNPA in absence and presence of different concentrations of DTT. Inhibition constant (K_i) of the inhibitor as the non-competitor of PNPA was calculated from the replot of V/S against DTT concentrations.

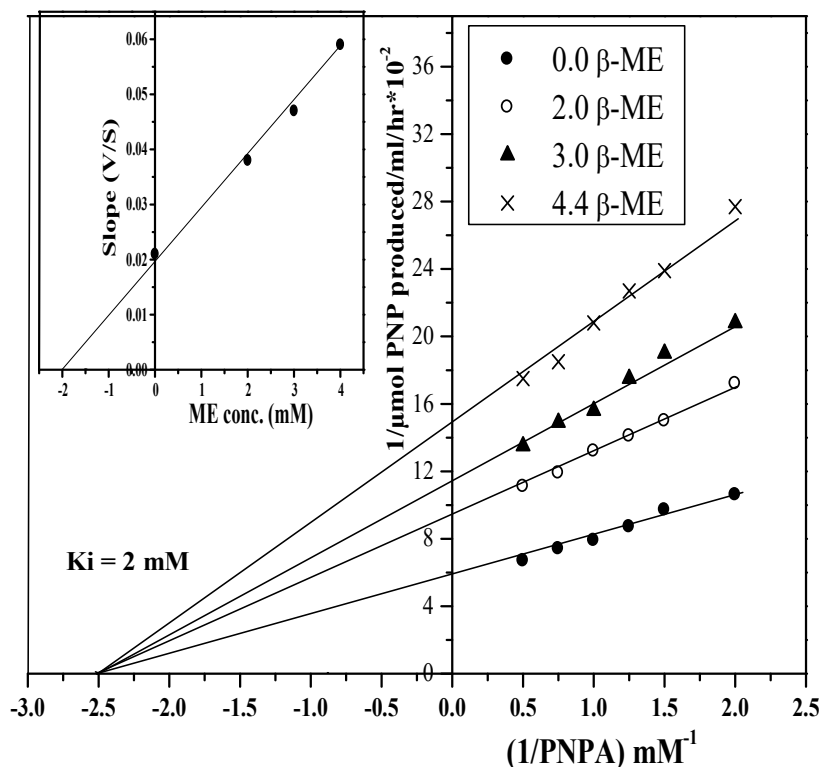


Fig. 12: Reciprocal plots of initial velocities of *R. ferrugineus* GE versus reciprocal concentrations of PNPA in absence and presence of different concentrations of ME. Inhibition constant (K_i) of the inhibitor as the non-competitor of PNPA was calculated from the replot of V/S against β -ME concentrations.

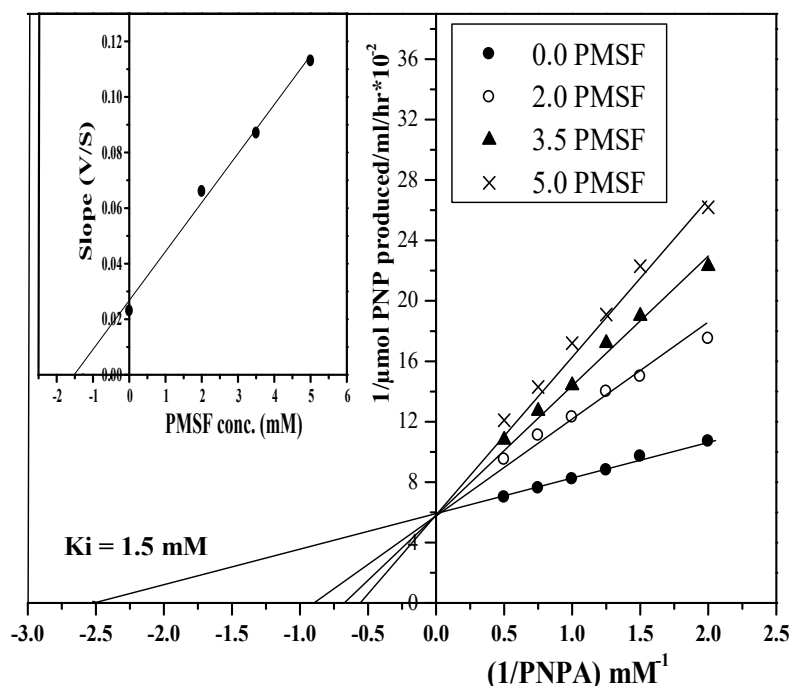


Fig. 13: Reciprocal plots of initial velocities of *R. ferrugineus* GE versus reciprocal concentrations of PNPA in absence and presence of different concentrations of PMSF. Inhibition constant (K_i) of the inhibitor as the competitor of PNPA was calculated from the replot of V/S against PMSF concentrations.

4. Discussion

The management of RPW, *R. ferrugineus* represents a tremendous challenge because of its cryptic life cycle. The intensive use of chemical synthetic insecticides leads to the adaptation and emergence of insecticide(s)-resistant strains (Hamadah, 2019; Mohamed *et al.*, 2021). Resistance that arises from induction of detoxifying enzymes has been investigated in different insect species including RPW (Ghanem *et al.*, 2025). Non-specific esterases or general esterases (GE) catalyze the hydrolysis of a wide range of xenobiotic carboxyl esters and aromatic amides at a high rate and are involved in a major route for insecticide(s)-resistance in various insect species (Ellakwa and Amin, 2022; Hoor *et al.*, 2024).

R. ferrugineus esterases from larval hemolymph (Nedal and Amin, 2019) and from the cytosol soluble fraction of the 5th instar larvae (Ellakwa and Amin, 2022) have been partially characterized with respect to inhibition kinetics and interpreted that usage of inhibitors for suppressing insect metabolic enzymes and the mechanism of inhibition have already proven to be an essential biotechnology method for RPW management. The present study investigates the distribution of GE in different major tissues of the 10th instar larvae of *R. ferrugineus*, in the fat body during larval development as well as purification and biochemical characterization of the major isoenzyme.

The distribution of GE in the 10th instar larvae of the *R. ferrugineus* revealed that the fat body has esterase activity (491 ± 23.2 U/tissue) higher than for whole gut (270 ± 15 U/tissue), cuticle (200 ± 12 U/tissue), head (32 ± 1.1 U/tissue) and hemolymph (28 ± 0.94 U/tissue). Jones and Bancroft (1986) concluded that fat body is the richest source of esterases and showed great variation in activity compared to different tissues including gut, Malpighian tube, testes and ovariole tissues of the adult cotton boll weevil *Anthonomus grandis*. Srinivas *et al.* (2004) suggested that head and muscle of *Helicoverpa armigera* larvae have the highest esterase activities as compared to other tissues examined. Hamed *et al.* (2019) concluded that fat body of an insect is a site of toxins deposition and detoxification. From these results, the authors interpreted that fat body of *R. ferrugineus* has the main role in detoxification of synthetic chemical insecticides and xenobiotic compounds. The variation of GE activity in different major tissues reflects the contribution of each tissue in detoxification and might handle the toxic chemical assault differently.

The alteration in GE activity has been tracked in the fat body during the course of *R. ferrugineus* larval development to elucidate whether larval instar influence the enzyme activity and to select the

larval instar of the highest GE level for purification. The present study demonstrated a significantly sharp increase in GE activity from the 8th instar larvae to 12th instar with the highest activity recorded in the 12th instar of *R. ferrugineus*. Nemec and Zenka (1996) suggested that the young developmental stages are usually more active than the old ones. Such interpretation has been confirmed herein, where the 12th *R. ferrugineus* larvae instar had GE activity (647 ± 48 U/ mg protein) higher than those estimated for the 10th (432 ± 17 U/ mg protein) and 11th (480 ± 32 U/ mg protein) instars larvae. The 6-day old larvae and 4-day old pupae of *Galleria mellonella* have 313.2 and 56.3 U/mg protein, higher GE activity than those recorded for 3-day old larvae (38 U/mg protein) and 1-day old pupae (15.9 U/mg protein) (Nemec and Zenka, 1996).

In insects, GE are involved in several important physiological processes, such as reproduction, digestion, molting, metabolism of juvenile hormone, and detoxification of synthetic chemical insecticides (Ellakwa and Amin, 2022; Hoor *et al.*, 2024). In the vector mosquito *Aedes aegypti*, GE activity increased slightly in pupae than in larvae and such increase may be attributed to the molting of the larvae (Koodalingam *et al.*, 2011). In malathion-resistant and susceptible strains of *Tribolium castaneum*, GE activities in the larval stages have several folds higher than those estimated for nymph and adult (Haubruge *et al.*, 2002). In the light of the above results, it can be hypothesized that the differences in GE activity/synthesis during the course of *R. ferrugineus* larval development are probably due to differences in the regulatory mechanisms acting accordingly with metabolic function requirements of a variable number of processes in which esterases are involved, as interpreted by Lopes *et al.* (2014). Purification and biochemical characterization of a major esterase isoenzyme have been conducted from the fat body of the 12th instar larvae of *R. ferrugineus*.

One major GE isoenzyme has been purified from the fat body of the 12th instar larvae of *R. ferrugineus*. The purified isoenzyme has specific activity of 1871.2 μ mole/h/mg protein. Taken on the account that the activity in terms of μ mole/min/mg protein (31.2), the specific activity herein is consistent to that recorded for E2 isoenzyme of *Nilaparvata lugens* (31.5) (Chen and Sun, 1994), and comparable equal to the major isoenzyme of *Nephotettix cincticeps* (38.3) (Chiang and Sun, 1996). The specific activity of *R. ferrugineus* GE is lower than that of *Diabrotica virgifera virgifera* (42.4) (Zhou *et al.*, 2004) and several folds lower than three isoenzymes from susceptible (467, 528, 615.4), resistant (512, 606, 1666.4) strains of cockroach *Blattella germanica* (Prabhakaran and Kamble, 1995), and two strain of *Drosophila*, *D. mulleri* (1106.7) and *D. arizonae* (4458.3) (Lopes *et al.*, 2014). It is higher than those recorded for GE from resistant (18.3) and susceptible (1.4) strains of *Oryzaephilus surinamensis* (Conyers *et al.*, 1998) and three isoenzymes from *N. cincticeps* (4.5, 13.2, 16.5) (Chiang and Sun, 1996). The fold purification of *R. ferrugineus* GE (2.74) is comparable equal to those estimated for *D. mulleri* (3.37) and *D. arizonae* (3.1) (Lopes *et al.*, 2014), and the E4 isoenzyme of *N. cincticeps* (2.9) (Chiang and Sun, 1996), but it is several folds lower than those recorded for GE from midgut of *S. frugiperda* (5.6) (Hoor *et al.*, 2024), *D. virgifera virgifera* (106.6) (Zhou *et al.*, 2004) and three isoenzymes from *N. lugens* (5.3, 11.0, 24.0) (Chen and Sun, 1994). Such variations in specific activity of the purified *R. ferrugineus* GE and fold purification with those previously documented for different insect species could be attributed to the high or low contents of activity and protein in the crude homogenates of *R. ferrugineus* fat bodies. The biochemical characteristics of *R. ferrugineus* GE are carried out to assure compounds that suppress GE activity in an effective, dependable and safe manner, and subsequently can be synergized with the effective chemical insecticide(s) or introduced in IPM programs for increasing the efficiency of the *R. ferrugineus* management.

The optimal pH for *R. ferrugineus* GE activity was detected at pH 8.0. This result is in agreement with those documented for insecticide-resistant strains of German cockroach *B. germanica* (Prabhakaran and Kamble, 1995), 1- and 4-days old pupae of *G. mellonella* (Nemec and Zenka, 1996), and *D. arizonae* (Lopes *et al.*, 2014). pH optima at 7.5 and 8.5 was observed for *D. mulleri* (Lopes *et al.*, 2014) and midgut of the silkworm, *B. mori* (Murthy and Veerabhadrapa, 1996). An acidic pH optimum at 5.5 was estimated for purified GE from midgut of fall armyworm *S. frugiperda* (Hoor *et al.*, 2024).

The optimum temperature for *R. ferrugineus* GE activity was 50°C and the enzyme is unstable at temperatures above 60°C where 22 and 41% of its activity were lost at 70°C and 80°C. Optimum temperature for activity was detected at 40°C for GE activities from *D. mulleri* and *D. arizonae* (Lopes *et al.*, 2014), insecticide-resistant and -susceptible strains of German cockroach, *B. germanica* (Prabhakaran and Kamble, 1995). At temperature above 50°C, *D. mulleri* and *D. arizonae* esterase

activities were completely vanished (Lopes *et al.*, 2014) and *B. germanica* esterase sharply decreased to 30% of its activity (Prabhakaran and Kamble, 1995). These variations in optimum pH and temperature for GE activities in different insect species could be associated with the different environmental niches occupied by the insect species as recently interpreted by Ghanem *et al.* (2025) and Abd-Elghany *et al.* (2025).

There is a relation between activation energy (AE) and the reaction rate, where the enzymatic reaction rate increases by decreasing the AE (Ellakwa and Amin, 2022). The results herein demonstrated that *R. ferrugineus* GE exhibited AE of 6.7 Kcal/mol for hydrolyzing α -NA. This value is higher than those recorded for cytosol fraction of the 5th instar larvae of *R. ferrugineus* for hydrolysis α -NA (3.57 Kcal/mol) and β -NA (5.95 Kcal/mol) (Ellakwa and Amin, 2022), and for carboxylesterases from the midgut of the silkworm *B. mori* (5.2 Kcal/mol) for hydrolyzing α -naphthyl butyrate (α -NB) (Murthy and Veerabhadrapa, 1996).

Some metal cations can modify GE structure by stimulating or inhibiting enzyme activity (Lopes *et al.*, 2014; Ellakwa and Amin, 2022). Metal cations have various effects on *R. ferrugineus* GE activity including stimulation, inhibition and no effects. Stimulatory effect ranged from 55 to 113% was observed by Hg^{2+} , Ni^{2+} , Zn^{2+} , Cu^{2+} and Co^{2+} . Fe^{2+} completely inhibited *R. ferrugineus* GE activity and Al^{3+} inhibited the enzyme by 45%. GE from insecticide-resistant *H. armigera* was stimulated by Zn^{2+} , inhibited by Mg^{2+} , Mn^{2+} , Ca^{2+} and Cu^{2+} and completely inhibited by Ag^{+} , Cd^{2+} and Hg^{2+} (Srinivas *et al.*, 2006). *D. mulleri* GE activity was stimulated by Na^{+} , Ba^{2+} and Fe^{2+} (Lopes *et al.*, 2014). *D. arizonae* GE activity was stimulated by Ba^{2+} , Na^{+} , Cu^{2+} and Co^{2+} , inhibited by Fe^{2+} , Li^{+} , Mg^{2+} , Ca^{2+} , K^{+} , Mn^{2+} and Zn^{2+} and completely inhibited by Al^{3+} (Lopes *et al.*, 2014). The complete inhibition of *R. ferrugineus* GE by Fe^{2+} herein may be attributed to the binding to the enzyme, promoting negative modulatory effect and preventing it to perform its catalytic function as concluded by Lopes *et al.* (2014). The partially purified GE from the cytosol fraction of the 5th instar larvae of *R. ferrugineus* was stimulated to 110% by Ca^{2+} , and it is significantly inhibited (80%) by Cu^{2+} at concentration level 50 mM (Ellakwa and Amin, 2022). Ding and Wang (2006) estimated the suppression of *D. melanogaster* development, protein synthesis and esterase isoenzymes upon treatment with CuSO_4 . Merheb-Dini *et al.* (2009) suggested that metal cations can bind to amino acids of the enzyme and influence protein structural conformation directly affecting the catalytic performance of an enzyme. It can be hypothesized that Zn^{2+} may be bind to amino acids of *R. ferrugineus* GE and improved the enzyme activity where the enzyme structure becomes better organized as interpreted by Lopes *et al.* (2014).

Esterases exhibiting an elevated activity towards model substrates have been associated with insecticide(s) detoxification and consequently to insecticide(s)-resistance in various insect species (Srinivas *et al.*, 2006; Lopes *et al.*, 2014; Ellakwa and Amin, 2022). Some well-studied esterases may have unsuspected novel functions as proteolytic regulation of cell growth and development (Zera *et al.*, 2002). In the current study, *R. ferrugineus* GE exhibited no detectable AChE, ACP and ALP activities and have specificity towards general esterase substrates as α , β -N- and PNP- esters with prefer ability to hydrolyze α -NA and PNPA, indicating a high specificity towards short chain aliphatic esters. According to such results, *R. ferrugineus* esterase probably can hydrolyze any ester with aliphatic chains as interpreted by Lopes *et al.* (2014). *R. ferrugineus* esterase hydrolyzed α -NA and β -NA with relative rate 100 and 21%. Esterases from silkworm, *B. mori* midgut (Murthy and Veerabhadrapa, 1996), OP-resistant and susceptible strains of *O. surinamensis* (Conyers *et al.*, 1998) also have higher activity towards α -NA than β -NA. Susceptible and field strains of rice green leafhopper, *N. cincticeps* GEs have similar relative rate of activity towards α - and β -NA (Chiang and Sun, 1996).

R. ferrugineus GE showed Michaelis-Menten behavior for the model substrates, PNPA and α -NA with K_m values of 0.4 and 0.8 mM. The affinity of GE to hydrolysis PNPA herein (0.4 mM) is higher than those estimated for silkworm *B. mori* (2.86 mM) (Murthy and Veerabhadrapa, 1996), *D. mulleri* (0.74 mM) (Lopes *et al.*, 2014), and *D. virgifera virgifera* (0.84 mM) (Zhou *et al.*, 2004), but it is lower than that investigated for *D. mulleri* (0.17 mM) (Lopes *et al.*, 2014). The affinity of the enzyme towards α -NA (0.8 mM) is lower than those reported for *B. mori* (0.37) (Murthy and Veerabhadrapa, 1996), resistant (0.064 mM) and susceptible (0.072 mM) strains of *O. surinamensis* (Conyers *et al.*, 1998), cricket *G. assimilis* (0.2 mM) (Zera *et al.*, 2002), and *T. castaneum* (0.014 mM) (Haubruge *et al.*, 2002).

One of the accepted classifications of esterases is by the susceptibility of the enzyme to suppress by different inhibitors (Zhu and Gao; 1998; Lopes *et al.*, 2014; Ellakwa and Amin, 2022). The chemical nature of the catalytic site, i.e., the main residue composition in the enzyme active site is classified

according to Dixon and Webb (1979). In addition, suppression of GEs in insect pests by an inhibitor(s) could be increase the susceptibility of such insect pests to chemical insecticide(s) and appropriate for enhancing the insecticidal efficacy of such chemicals for insect control through introducing as ingredients in IPM programs for improving insect strategy managements (Bakr *et al.*, 2013; Ellakwa and Amin, 2022; Abd-Elghany *et al.*, 2025)

Sulphydryl reagent PHMBA and eserine moderately inhibited *R. ferrugineus* GE suggesting that the sulphydryl groups are not essential in the catalytic action. Based on substrate specificity and the sensitivity to inhibition by PHMBA, the enzyme could be belonging to type B esterases (e.g. carboxylesterase). These results are in consistent with the observation of Murthy and Veerabhadrapa (1996) where midgut esterase of the silkworm, *B. mori* resistant to inhibit by eserine, as a carbamate inhibitor and PHMBA.

The high susceptibility of *R. ferrugineus* GE to inhibit by PMSF at concentration 10mM, indicating that the enzyme belongs to the class of serine esterases and the active site of the enzyme contains a serine and/or cysteine residue. Our finding is corroborated with the reported data for different species of *Drosophila* (Lopes *et al.*, 2014). PMSF irreversibly binds with the hydroxyl side chain of the serine residue, impeding the enzymatic catalysis. Such class of esterases is commonly found in various insect species as *Manduca sexta* (Hinton and Hammock, 2003), silkworm, *B. mori* (Yu *et al.*, 2009). Esterases from *T. castaneum* were not inhibited by PMSF (Gigliolli *et al.*, 2011). On the other hand, *R. ferrugineus* GE is found to be resistant to NaF inhibition. Such result is in agreement with that estimated for cytosol fraction esterase from the 5th instar larvae of *R. ferrugineus* (Allakwa and Amin, 2022).

Considering the results of *R. ferrugineus* GE stimulation to 213 and 200 % by Zn^{+2} and Co^{+2} , the effect of EDTA on the enzyme activity confirmed such results in the current report, where EDTA, is a divalent cation chelating agent, inhibited *R. ferrugineus* GE by 49%. It can be suggested that EDTA can chelate the metal cations present in the active site or those already present in the pure enzyme. Through some types of disorganization in the structural conformation of the enzyme, inhibition is observed and leading to a decrease in enzyme activity as concluded by Lopes *et al.* (2014). The inhibition by EDTA for GEs from *D. mulleri* and *D. arizonae* esterases (Lopes *et al.*, 2014) and from cytosol fraction of the 5th instar larvae of *R. ferrugineus* (Ellakwa and Amin; 2022) suggests that such enzymes were induced in the presence of metal ions. Such interpretation is confirmed herein, where *R. ferrugineus* GE is stimulated by metal cations.

The inhibition of an enzyme by modifying the thiol (SH-) groups at the enzyme's active site can lead to irreversible inactivation, disrupting its catalytic activity by reducing disulfide bonds in proteins, which are crucial for maintaining the enzyme's structure and function (Alliegro, 2000). Our data demonstrated that the thiol-reducing agents β -ME and DTT completely inhibited *R. ferrugineus* GE. The mechanism of action involves the reactivity of the thiol groups which can be attacked by the reagent, resulting in the loss of the enzyme's ability to catalyze its target reaction as interpreted by Alliegro (2000). On the other hand, thiol reagents are known for their metal chelating properties. Thiol reagent may interact with the metals that are already present in the pure *R. ferrugineus* GE, resulting in alteration of the conformational structure of the protein and subsequently in the enzyme activity. The mechanism of β -ME and DTT is noncompetitive inhibition with K_i values 0.2 and 0.4 mM, where the thiol reducing agents are not compete the substrate for the enzyme's active site.

Conclusively, the highest GE activity was observed in the fat body of the 12th instar larvae of *R. ferrugineus* and has the most important role for insecticide(s) detoxification. The biochemical characteristics of the purified *R. ferrugineus* GE isoenzyme as inhibition by EDTA, Fe^{2+} , and thiol reducing agents, can increase the susceptibility of such pest to chemical synthetic insecticides and hence are appropriate for enhancing the insecticidal efficacy of the chemical insecticides, and any impairment on *R. ferrugineus* GE activity will affect RPW development and increase insect mortality. In the future, studies on the deleterious effects of EDTA, Fe^{2+} , and thiol reagents are in progress *in vivo* for different larval instars and to assure the possibility of introducing them as synergists in IPM programs for RPW management.

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