รายงานวิจัยฉบับสมบูรณ์

การศึกษาคุณลักษณะและการทำลาย (metabolize) ยาฆ่าแมลงโดยเอนไซม์กลูตาไธโอนเอส-ทรานสเฟอเรสที่พบใหม่จากยุงก้นปล่อง (*An.dirus*)

โดย ดร. จีรัง ว่องตระกูล

มีนาคม 2552

สัญญาเลขที่ RMU4880047

รายงานวิจัยฉบับสมบูรณ์

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สนับสนุนโดยสำนักงานคณะกรรมการการอุดมศึกษา
และสำนักงานกองทุนสนับสนุนการวิจัย

กิตติกรรมประกาศ

งานวิจัยนี้ได้รับการสนับสนุนร่วมจากทุนเมธีวิจัย ประจำปี 2548 ของสำนักงานกองทุนสนับสนุนการวิจัย (สกว) และสำนักงานคณะกรรมการการอุดมศึกษา (สกอ) จึงขอขอบพระคุณมาณ.โอกาสนี้ และขอขอบพระคุณผู้อำนวยการสถาบันวิจัยวิทยาศาสตร์สุขภาพที่ได้ให้การสนับสนุนการดำเนินงานโครงการจนสำเร็จลุล่วงตามวัตถุประสงค์ ขอขอบคุณคุณสมศักดิ์ ทะระถา หัวหน้าห้องปฏิบัติการโลหะหนัก บริษัทห้องปฏิบัติการกลาง (ประเทศไทย) จำกัด ที่ได้ให้ความอนุเคราะห์ในการทำ LC-MSD และให้คำแนะนำในเรื่องการแปลผลการทดลอง ขอบพระคุณดร.ละเอียด ประพันธดารา ที่ได้ให้คำแนะนำเกี่ยวกับการพัฒนาการตรวจวิเคราะห์เทมีฟอสด้วยวิธี HPLC ตลอดจนเอื้อเฟื้อสารเคมี คุณสุรางคค์จิตต์ คุตตะเทพ ที่ได้ให้คำแนะนำในการพัฒนาการตรวจวิเคราะห์เพอเมทรินด้วยวิธี HPLC คุณคุณโพธิ์ศรี ลีลาภัทร์ ที่ได้ทำการวิเคราะห์ตัวอย่างด้วยเครื่อง HPLC และคุณวริศรา สุวรรณ ที่ได้ให้คำแนะนำต่างๆ เกี่ยวกับการทำ HPLC ขอบคุณเจ้าหน้าที่ฝ่ายการเงินและฝ่ายนโยบายและแผนสถาบันวิจัยวิทยาศาสตร์สุขภาพที่ได้อำนวยความสะดวกด้านงานเอกสาร

ดร. จีรัง ว่องตระกูล

Abstract

Project Code: RMU4880047

Project Title: Enzyme characterization and insecticide metabolism of new insect GST classes from *An. dirus*

Investigators: Dr. Jeerang Wongtrakul[*], Dr. Saengtong Pongjaroenkit[+] and Assoc. Prof. Albert J. Ketterman[++]

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Project Period: 3 years

Glutathione Transferases (GSTs) (E.C.2.5.1.18) are multifunctional enzymes with major roles in the cellular detoxification of many exogenous and endogenous compounds. This project aimed to characterize and study insecticide metabolism of several new GSTs e.g. epsilon, theta, omega and delta GSTs from *Anopheles cracens*, a major Thai malaria vector. They were studied for their kinetic parameters and their activities toward 11 model substrates. Insecticide assays toward DDT, temephos and pyrethroid insecticides also were performed. It was found that those GSTs exhibited different but overlapping substrate specificities. The AcGSTE3-3 had the greatest ability to conjugate 4-hydroxynonenal. The AcGSTE2-2 had peroxidase activity toward cumene hydroperoxide and t-butyl hydroperoxide which is different from AgGSTE2-2. Both theta class clones had high activity toward p-nitrobenzyl chloride and p-nitrophenethyl bromide which are substrate preferentially of mammalian theta class. The AcGSTO1-1 can utilize CDNB, DCA and HED substrates. DDT metabolites from AcGSTE1-1, AcGSTE4-4, AcGSTE2-2 and R139H which is an allelic variant of the AcGSTE1-1 were detected. AcGSTE2-2 possessed the greatest DDT dehydrochlorinase activity. For temephos metabolism, we found a decrease in the amount of temephos when incubated with AcGSTE1-1, R139H, AcGSTD2-2, AcGSTD3-3 and AcGSTT1-1 enzymes. All the enzymes did not metabolize temephos but they may sequester the insecticide thereby decreasing the amount of temephos in reaction. Permethrin inhibition study found that only AcGSTE1-1, AcGSTE3-3 and AcGSTE4-4 have an interaction with the insecticide. The AcGSTE3-3 interacted with both pyrethroid insecticides with the lowest binding affinity compared to the other Epsilon GSTs. All the Epsilon, theta,

omega and delta class GSTs did not metabolize permethin analyzed by HPLC. AcGSTE1-1, AcGSTE2-2, AcGSTE4-4, AcGSTT1-1, AcGSTD2-2 and AcGSTD3-3 GSTs involve in either insecticide metabolism or sequestering organophosphate insecticide whereas AcGSTE3-3 appeared to have a major role in detoxifying lipid peroxidation products that would confer protection against oxidative damage.

บทคัดย่อ

รหัสโครงการ RMU4880047

ชื่อโครงการ การศึกษาคุณลักษณะและการทำลาย (metabolize) ยาฆ่าแมลงโดยเอนไซม์กลูตาไธโอนเอส-ทรานสเฟอเรสที่พบใหม่จากยุงก้นปล่อง (*An.dirus*)

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ระยะเวลาโครงการ 3 ปี

กลูตาไธโอนเอสทรานสเฟอเรส (GSTs) (E.C.2.5.1.18) เป็นเอนไซม์ที่มีหน้าที่หลายประการโดยหน้าที่หลักคือทำลายสารพิษภายในเซลล์ โครงการนี้มีวัตถุประสงค์คือเพื่อศึกษาคุณลักษณะและศึกษาการทำลายยาฆ่าแมลงของเอนไซม์ GST หลายๆคลาสเช่น เอปซิลอน ทีต้า โอเมกา และเดลตา จากยุง *Anopheles cracens* ซึ่งเป็นพาหะนำโรคมาเลเรียในประเทศไทย ได้ทำการศึกษา kinetic parameter และแอคทิวิตีต่อสับสเตรดต่างจำนวน 11 ชนิด และได้ศึกษาการทำลายยาฆ่าแมลงดีดีที เทมีฟอสและไพรีทรอยด์ เอนไซม์ GST มีค่าจำเพาะต่อสับสเตรดแตกต่างกันและมีการใช้สับสตรดทับซ้อนกัน เอนไซม์ AcGSTE3-3 มีแอคทิวิตีจำเพาะสูงที่สุดต่อ 4-hydroxynonenal เอนไซม์ AcGSTE2-2 มีเพอออกซิเดสแอคทิวิตีต่อสับสเตรดชนิดCumene hydroperoxide และ t-butyl hydroperoxide ซึ่งแตกต่างจากเอนไซม์ AgGSTE2-2 เอนไซม์จากทีต้าคลาสทั้งสองชนิดมีแอคทิวิตีสูงต่อ p-nitrobenzyl chloride และ p-nitrophenethyl bromideซึ่งเป็นสับสเตรดจำเพาะของทีต้าคลาสใน GST ของสัตว์เลี้ยงลูกด้วยนม เอนไซม์ GST ชนิดโอเมกามีค่าแอคทิวิตีจำเพาะต่อสับสเตรด CDNB DCA และ HED ส่วนการศึกษาการทำลายยาฆ่าแมลงพบว่าสามารถตรวจวัด ดีดีทีเมแทบอไลท์จาก AcGSTE1-1, AcGSTE4-4, AcGSTE2-2 และ R139H ซึ่งเป็น allelic variant ของ AcGSTE1-1 โดยเอนไซม์ AcGSTE2-2มีค่าแอคทิวิตีสูงที่สุด ส่วนการทำลายเทมีฟอสพบว่าปริมาณยาฆ่าแมลงลดลงเมื่อทดสอบด้วยเอนไซม์ AcGSTE1-1, R139H, AcGSTD2-2, AcGSTD3-3 และ AcGSTT1-1 ซึ่งเอนไซม์ทุกตัวไม่ได้ทำลายเทมีฟอสแต่อาจมีการจับกับยาฆ่าแมลงทำให้ มีปริมาณลดลงเมื่อทำการตรวจวัด ในการศึกษาการยับยั้งเอนไซม์แอคทิวิตีด้วยยาฆ่าแมลง พบว่า AcGSTE1-1 AcGSTE3-3 และ AcGSTE4-4 สามารถจับกับเพอเมทริน โดยเอนไซม์ AcGSTE3-3 จับได้ทั้งเพอเมทรินและแลมด้าไซฮาโลทริน แต่มีค่าการจับต่ำที่สุดเมื่อเทียบกับตัวอื่น เมื่อศึกษาการทำลายเพอเมทรินด้วย HPLC พบว่า GSTในทุกคลาสที่นำมาทดสอบไม่สามารถทำลายยาฆ่า

แมลงได้ AcGSTE1-1, AcGSTE2-2, AcGSTE4-4, AcGSTT1-1, AcGSTD2-2 and AcGSTD3-3 GSTs มีความเกี่ยวข้องกับการทำลายดีดีทีและหรือจับกับยาฆ่าแมลงกลุ่มออแกโนฟอสเฟตในขณะที่ AcGSTE3-3 มีบทบาทในการทำลายสารพิษที่เกิดจาก ลิพิดเพอออกซิเดชัน ซึ่งเป็นการป้องกันการถูกทำลายจาก 0xidative stress

Executive summary

1. Project title

(Thai language): การศึกษาคุณลักษณะและการทำลาย (metabolize) ยาฆ่าแมลงโดยเอนไซม์กลูตาไธโอนเอส-ทรานสเฟอเรสที่พบใหม่จากยุงกันปล่อง(*An. dirus)*

(English language): Enzyme characterization and insecticide metabolism of new insect GST classes from *An. dirus*

2. Name and address of the Principal investigator:

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3. Field of study

Molecular enzymology

No

5. Project duration

3 years

6. Significance and rationale

Insect glutathione S-transferases are well known for their ability to metabolize insecticides. Previously, they were classified into class I, II and III. However a number of novel insect GSTs have been increasingly identified therefore a unified nomenclature for insects has now been proposed similar to those classes isolated as mammalian GSTs. At present there are six classes of insects GSTs which are Delta, Omega, Sigma, Zeta, Theta and Epsilon from *Anopheles gambiae*, an African malaria vector. Fourteen clones of *An. dirus* GSTs have been obtained using the information from the *An. gambiae* genome project since both species contain orthologous genes. However, there is no information about enzyme characterization e.g. substrate specificity and the roles in the metabolism of insecticides for these newly identified GSTs yet. There is some evidence of insecticide metabolism for isoenzymes from insect delta class and only a few characterization studies of epsilon GSTs.

In Thailand, *An. dirus* is the major malaria vector and was responsible for 128,833 cases of infected people found throughout the country during 1997-1999. *An. dirus* GST isoenzymes have been shown to possess significant amounts of DDT

dehydrochlorinase activity as well as to have interactions with pyrethroid and organophosphate insecticides. Therefore screening using several substrates to determine their role in insecticide resistance for the new GST enzymes obtained from this malaria vector would increase our understanding of the properties of these enzymes and their contributions to insecticide resistance. Different forms of GSTs can be clearly distinguished by their different activities with a given substrate. Some substrates e.g. DCNB and cumene hydroperoxide were found to correlate with insecticide resistance in insects. In addition, characterization of the insecticide resistance will increase our understanding of the mechanism by which the enzymes metabolize the insecticides currently used in our country. Future work would involve obtaining the tertiary structure of the enzyme in complex with an insecticide. This structure will allow us to design either new inhibitors or insecticides with the aim of restoring insecticide susceptibility.

7. Objectives:

1. To study insecticide metabolism in insect Epsilon, Omega, Theta, and the new members of Delta GSTs from *An. dirus* from fourteen clones. For the DDT insecticide, the assay was performed using HPLC to detect the enhanced metabolism of p-p' DDT to p-p' DDE in the presence of reduced GSH.

2. To develop a method for the separation of insecticide permethrin and two of its metabolites m-phenoxybenzoic acid and m-phenoxylbenzyl alcohol using HPLC. Then the insecticide metabolisms in insect Epsilon, Omega, Theta and the new members of Delta GSTs from *An. Dirus* was studied.

3. To study insecticide metabolism in insect Epsilon, Omega, Theta and the new members of Delta GSTs from *An. dirus*. For organophosphate insecticide, the assay was developed using HPLC to detect temephos.

4. To characterize and identify different forms of new insect glutathione S-transferase e.g. Epsilon, Omega, Theta and the new members of Delta GSTs isolated from *An. dirus* which is a major malaria vector in Thailand. A substrate panel that consists of 11 different compounds was used in the study.

8. Methodologies

To achieve aim 1:

To determine DDT-dehydrochlorinase activity, a reaction mixture containing an appropriate amount of GST phosphate buffer and GSH with p,p'-DDT was prepared. The reaction mixtures was incubated at 28°C for 2 hours then dicofol was added before

the samples was extracted three times each with chloroform. The extracts was resuspended in isopropanol and mobile phase before analysis by HPLC. Chromatography was performed on a HPLC system consisting of an LC-20A series (Shimadzu Corporation, Japan) equipped with pump system (LC20-AB), auto-sampler (SIL-20A), photo diode array detector (SPD-M20A), a column oven and software (LC solution).

To achieve aim 2:

Purified GSTs will be incubated with permethrin at 28 °C for 30 minutes then dimethoate was added before the samples was extracted three times each with chloroform. For detection and quantification; the extracts were taken up in mobile phase, 70% methanol, before analysis. HPLC analysis was carried out on a C18 column. The mobile phase was water (adjusted to pH 3.5 using 1N acetic acid): acetonitrile gradient at flow-rate programmed at 1.0 ml/min. The gradient started at 45% acetonitrile, increased to 55% acetonitrile at 5 min, then increased to 95% acetonitrile by 8 min and kept constant for 7 min until 14 min. Then the system returned to 45% acetonitrile at 15 min where it was kept under this condition for 10 min to re-equilibrate. Quantities of permethrin (cis and trans) and metabolites was calculated from standard curves established by HPLC analysis of known concentrations of standards.

To achieve aim 3:

Sample preparation was performed similar to the methods for the DDT and permethrin experiments. Temephos was dissolved in acetronitrile. An HPLC pump with a Rheodyne injector connected to a photodiode array detector will be used with a cartridge column type 8NVC18 (5 micron). The mobile phase was water: acetonitrile gradient at flow-rate programmed at 1.0 ml/min. The gradient started at 70% acetonitrile, decreased to 60% acetonitrile at 5 min, then increased to 65% acetonitrile at 10 min. Then the system returned to 70% acetonitrile at 11 min where it was kept under this condition for 5 min to re-equilibrate. The eluents were monitored by UV detection of wavelength of 250 nm for temephos and pyrene. The chromatographic analysis was performed at 40°C column oven temperature. The Agilent 1100 series, Germany, HPLC system, with DAD detector, coupled with a C18 column and the Agilent LC/MSD SL, USA , a mass selective detector (MSD), were used for analysis of metabolites. Mobile phases consisted of acetonitrile and water (70:30) containing 0.1% (v/v) formic acid. The isocratic mobile phase containing acetonitrile:water (70:30, v/v) had a flow rate of 1.0 ml/min. The MSD was operated as follows: nitrogen gas flow rate, 13 L/min; nebulizer pressure, 50 psi; drying gas temperature 350ºC; capillary voltage positive

4000 V and negative 3500 V. The LC-MS was scanned from 100 to 700 of an m/z unit. GST activity to metabolized temephos was expressed as nmol temephos disappearance/mg enzyme.

To achieve aim 4:

To study the utilization of substrates among different GST classes, a range of chemical compounds was used to detect other GST activities. The screening assays are summarized in a table as follows:

Electrophilic substrate	Substrate concentration (mM)	GSH concentration (mM)	pH	Wavelength (nm)
1-Chloro-2,4-nitrobenzene	1.0	10	6.5	340
1,2-Dichloro-4-nitrobenzene	1.0	10	7.5	345
4-Nitrophenethyl bromide	0.1	10	6.5	310
4-Nitrobenzyl chloride	1.0	10	6.5	310
Ethacrynic acid	0.2	10	6.5	270
Cumene hydroperoxide and t-butyl hydroperoxide	7	10	7.0	340
Dichloroacetic acid	0.5	10	7.4	535
p-Nitrophenyl acetate	0.2	10	7	400
4-hydroxynonenal	0.1	10	6.5	224
Hydroxyethyl disulfide	2	10	7.6	340

9 Results

All the epsilon, theta and omega clones were studied for their kinetic parameters and their activities toward 11 model substrates. Insecticide assays toward DDT, temephos and pyrethroid insecticides also were performed. It was found that a substrate panel can be used to differentiate the recombinant enzymes. The AcGSTE3-3 had the greatest ability to conjugate 4-hydroxynonenal, lipid peroxidation product. The AcGSTE2-2 had peroxidase activity toward cumene hydroperoxide and t-butyl hydroperoxide which is different from AgGSTE2-2. Both theta class clones had high activity toward p-nitrobenzyl chloride and p-nitrophenethyl bromide which are substrate

preferentially of mammalian theta class. The AcGSTO1-1 can utilize CDNB, DCA and HED substrates. DDT metabolites from AcGSTE1-1, AcGSTE4-4, AcGSTE2-2 and R139H which is an allelic variant of the AcGSTE1-1 were detected. AcGSTE2-2 possessed the greatest DDT dehydrochlorinase activity. For temephos metabolism, we found a decrease in the amount of temephos when incubated with AcGSTE1-1, R139H, AcGSTD2-2, AcGSTD3-3 and AcGSTT1-1 enzymes. All the enzymes did not metabolize temephos but they may sequester the insecticide thereby decreasing the amount of temephos in reaction. Permethrin inhibition study found that only AcGSTE1-1, AcGSTE3-3 and AcGSTE4-4 have an interaction with the insecticide. The AcGSTE3-3 interacted with both pyrethroid insecticides with the lowest binding affinity compared to the other Epsilon GSTs.

10 Conclusions:

We attempted to purify, characterized and study insecticide metabolism of epsilon, theta, omega and delta class GSTs from An. cracens which is a major malaria vector in Thailand. Some GST genes e.g. theta and omega also were reported in An. gambiae however the function of these GSTs remained elusive. It was found that epsilon enzymes function in metabolizing, insecticide binding protein and involved in oxidative stress whereas the theta enzymes function as anti-oxidant enzyme and acted as a binding protein with temephos. The Omega GST had a role in oxidative stress response.

11 Output from this project:

The results obtained from this study has been published as indicated below;

1.Poster presentation

1.1 Wongtrakul J., Wongsantichon J., Udomsinprasert R., Vararattanavech A., Leelapat P., Prapanthadara L., Ketterman AJ. Enzyme characterization and insecticide metabolism studies of Anopheles cracens epsilon class Glutathione Transferases. Annual TRF meeting 16-18 October 2008

1.2 Wongtrakul J., Vararattanavech A., Leelapat P., Prapanthadara L., Ketterman AJ. Functional characterization of the epsilon-class GST from An. dirus. Annual TRF meeting 11-13 October 2007

1.3 Wongtrakul J., Leelapat P., Yanola J., Prapanthadara L. and Ketterman AJ. Insecticide resistance study and substrate specificity in insect Glutathione

Transferases. The first annual symposium of the protein society of Thailand. Chulabhorn Research Institute 24-25 October 2006

2.International publication

2.1 Wongtrakul J., Wongsantichon J., Vararattanavech A., Leelapat P., Prapanthadara L. and Ketterman AJ. Molecular cloning and expression of several new Anopheles cracens Epsilon class glutathione transferases. Protein Pept Lett. 2009;16(1):75-81.

2.2 Wongtrakul J., Pongjaroenkit S., Leelapat P., Nachaiwieng W., Udomsinprasert R., and Ketterman AJ. Gene expression and characterization of AcGSTE2-2, AcGSTO1-1 and AcGSTT1-1 manuscript submitted.

3. Detail of project report

3.1 Significance and rationale

Insect glutathione S-transferase are well known for their ability to metabolize insecticides. Previously, they were classified into class I, II and III. However a number of novel insect GSTs have been increasingly identified therefore a unified nomenclature for insects has now been proposed similar to those classes isolated as mammalian GSTs. At present there are six classes of insect GSTs which are Delta, Omega, Sigma, Zeta, Theta and Epsilon from Anopheles gambiae, an African malaria vector. There are 14 new clones of An. dirus GSTs have been obtained using the information from the An. gambiae genome project since both species contain orthologous genes. However, there is no information about enzyme characterization e.g. substrate specificity and the roles in the metabolism of insecticides for these newly identified GSTs yet. There is some evidence of insecticide metabolism for isoenzymes from insect delta class and only a few characterization studies of epsilon GSTs.

In Thailand, An. dirus is the major malaria vector and was responsible for 128,833 cases of infected people found throughout the country during 1997-1999. An. dirus GST isoenzymes have been shown to possess significant amounts of DDT dehydrochlorinase activity as well as to have interactions with pyrethroid and organophosphate insecticides. Therefore screening using several substrates to determine their role in insecticide resistance for the new GST enzymes obtained from this malaria vector would increase our understanding of the properties of these enzymes and their contributions to insecticide resistance. Different forms of GSTs can be clearly distinguished by their different activities with a given substrates. Some substrates e.g. DCNB and cumene hydroperoxide were found to correlate with insecticide resistance in insects. In addition, characterization of the insecticide resistance will increase our understanding of the mechanism by which the enzymes metabolize the insecticides currently used in our country.

3.2 Objectives:

The objectives of this project are as follows:

1. To study insecticide metabolism in insect Epsilon, Omega, Theta and the new members of Delta GSTs from *An. dirus* from 14 recombinant clones. For the DDT insecticide, the assay will be performed using HPLC to detect the enhanced metabolism of p-p' DDT to p-p' DDE in the presence of reduced GSH.

2. To characterize and identify different forms of new insect glutathione S-transferase e.g. Epsilon, Omega, Theta and the new members of Delta GSTs isolated from *An. dirus* which is a major malaria vector in Thailand. A substrate panel that consists of 11 different compounds was used in the study.

3. To develop a method for the separation of insecticide permethrin and two of its metabolites m-phenoxybenzoic acid and m-phenoxylbenzyl alcohol using HPLC. Then the insecticide activity for Epsilon, Omega, Theta and the new members of Delta GSTs from *An. Dirus* was studied.

4. To study insecticide metabolism in insect Epsilon, Omega, Theta and the new members of Delta GSTs from *An. dirus*. For organophosphate insecticide, the assay was developed using HPLC to detect temephos.

5. To express, purify 14 different forms of new insect glutathione S-transferases obtained from the collaborators. All the enzymes were characterized with the developed methods

3.3 Research methodology

3.3.1 Expression of new recombinant clones

E. coli BL21(DE3)pLysS containing a recombinant plasmid was grown in 3 ml of LB broth containing 100 μg/ml ampicillin and 34 μg/ml chloramphenicol at 37°C overnight. The overnight culture was transferred into a new flask containing LB broth and ampicillin to make up 1% of the final concentration. The culture was incubated at 37°C with shaking until the OD at 600 nm was 1.2. An expression was induced by adding IPTG to a final concentration of 0.1 mM for 3 hours. Then the culture was placed on ice for 20 minutes, transferred to a 200 ml-centrifuge bottle and spun at 7000 rpm, 4°C for 10 minutes. The LB broth was decanted and the cell pellet was resuspended in 10 ml of LB, transferred to a new 50 ml-centrifuge tube and spun at 7000 rpm, 4°C for 10 minutes. The pellets were collected and stored at –20°C until used.

3.3.2 Enzyme purification

3.3.2.1 Preparation of Cell lysate

The pellet from 200 ml culture was resuspended with 19.2 ml of phosphate buffer pH 7.3 (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4 pH. 7.3), 800 μl of 100 mg/ml lysozyme and 14.4 μl of 1.4 M β-mercaptoethanol. The mixture was gently vortexed and placed on ice 20 minutes. Then 200 μl of 1M DTT was added. The cell

suspension was lysed by a sonicator and centrifuged at 10,000 rpm, 4°C for 20 minutes. The supernatant was collected and placed on ice for affinity chromatography.

3.3.2.2 Protein purification

Since there are many isoforms of insect GSTs. Therefore, different methods in preparing the enzymes were employed. Therefore different types of column were used in this project.

a. GSTrap column

The soluble recombinant GST, AdGSTD2-2, AdGSTD3-3, AdGSTD4-4, AdGSTD5-5, AdGSTD6-6 and AdGSTE1-1, in the supernatant of total lysate was purified using GSTrap affinity chromatography according to the manufacturer's instructions. The column was equilibrated with 5 bed volumes of the binding buffer, PBS pH 7.3 then the resulting supernatant was subjected to GSTrap affinity column. The non-specific binding proteins were washed out twice with 5 to 7 bed volumes of PBS buffer. The content of the column was eluted with 4 bed volumes of elution buffer (10mM GSH in 1.5 M Tris-HCl pH 8.0, and 10 mM DTT). Only the fractions containing recombinant GST were pooled in the centriprep-10 (Amicon) then centrifuged at 2500 g 4°C. The final volume of the concentrated GST was less than 1.5 ml. Then the GSH was eliminated by using HiTrap desalting column (Amersham Pharmacia Biotech), equilibrated with 5 column volumes of 50 mM phosphate buffer pH 6.5. The concentrated GST was adjusted in volume to 1.5 ml and applied to the column and eluted with 2 ml of the same buffer containing 10 mM DTT. The protein was concentrated until the final volume was less than 1 ml. Sterile glycerol was added to a final concentration of 50%. The purified GSTs were stored at –20°C. All the above steps were performed at 4°C.

b. S-hexyl Glutathione column

For the purification of AdGSTD1-1 and AdGSTE3-3, the S-hexyl glutathione column was used. The column was equilibrated with 5 column volumes of the equilibration buffer (50 mM Tris-HCl pH 7.4, 0.2 M NaCl, 1 mM EDTA pH. 8.0 and 10 mM DTT). The soluble target protein was applied into the column The column was then washed with 10 column volumes of washing buffer, 50 mM Tris–HCl (pH 7.4) containing 1M NaCl and 1 mM EDTA pH 8.0. The GST recombinant protein was eluted from the *S*-hexylglutathione agarose gel with 5 column volumes of elution buffer, 50mM Tris–HCl (pH 7.4), 0.2 M NaCl, 1 mM EDTA, containing 5 mM *S*-hexylglutathione and 10 mM DTT. Only the fractions containing recombinant proteins were pooled in the

centriprep-10 (Amicon) then centrifuged at 2,500 g 4°C. The final volume of the concentrated GST was less than 1.5 ml. Then the S-hexylglutathione was eliminated by using HiTrap desalting column (Amersham Pharmacia Biotech), equilibrated with 5 column volumes of 50 mM phosphate buffer pH 6.5. The concentrated GST was adjusted in volume to 1.5 ml and applied to the column and eluted with 2 ml of the same buffer containing 10 mM DTT. The protein was concentrated until the final volume was less than 1 ml. Sterile glycerol was added to a final concentration of 50%. The purified GSTs were stored at –20°C. All the above steps were performed at 4°C.

c. Ion-exchange chromatography following hydrophobic interaction chromatography

Ion-exchange chromatography is based on the binding of charged sample molecules to oppositely charged groups attached to an insoluble metrix. The proteins are bound to ion exchangers when they carry a net charge opposite to that of the ion exchange. In addition, the hydrophobic interaction chromatography, Phenyl Sepharose High Performance was used in the protein purification, which has phenyl as a hydrophobic ligand. The proteins are separated based on their varying strengths of hydrophobic interaction with hydrophobic ligands immobilized to an uncharged matrix. This technique is usually performed with moderately high concentration of salts in the start buffer since the adsorption is carried out at high salt concentration, the composition of the sample should be adjusted to the pH and ionic strength of the start buffer. Washing and elution are achieved by stepwise decrease in salt concentration.

In this study, the soluble target proteins, 2 recombinant clones of AdGSTT1, AdGSTE4-4 and AdGSTE2-2 were purified using cation exchange chromatography, HiTrap Phenyl Sepharose column (Amersham Biosciences). The SP-XL column was equilibrated with 50 mM phosphate buffer pH 7 and the lysate was applied to the column. For the purification of AdGSTE4-4, the enzyme did not bind to the SP-XL column so flow-through was collected and NaCl added to a final concentration of 3 M before loading to the HiTrap Phenyl Sepharose column pre-equilibrated with five-column volume of 50 mM phosphate buffer pH 7 containing 3 M NaCl at the rate 1 ml/min. Protein was eluted with a linear gradient from 2-0.75 M NaCl in 50 mM phosphate buffer pH 7 containing 10 mM dithiothreitol (DTT). The AdGSTE4-4 was eluted in 50 mM phosphate buffer pH 7 containing 1 M NaCl. For AdGSTT1, the recombinant enzyme was eluted with 50 mM phosphate containing 100 mM NaCl at the rate of 5 ml/min. Then the fraction was applied to HiTrap Phenyl Sepharose column pre-

equilibrated with five-column volume of 50 mM phosphate buffer pH 7 containing 2 M NaCl at the rate 1 ml/min. The enzyme was finally eluted with 50 mM phosphate buffer pH 7.0. For AdGSTE2-2, the enzyme did not bind to the SP-XL column as AdGSTE4-4. So the flow-through was collected and NaCl added to a final concentration of 2 M before loading to the HiTrap Phenyl Sepharose column pre-equilibrated with five-column volume of 50 mM phosphate buffer pH 7 containing 2 M NaCl at the rate 1 ml/min. Protein was eluted with a gradient from 1 M - 0 M NaCl in 50 mM phosphate buffer pH 7 containing 10 mM DTT. The major activity as AdGSTE2-2 was eluted in steriled distilled water containing 10 mM DTT.

For the purification of AdGSTO1-1, HiTrap Q XL column, anion exchanger, was equilibrated with five-column volume of 50 mM Tris pH 8.5. The supernate was applied to the column with the rate of 5 ml/min. Then the column was washed with six-column volume of 50 mM Tris pH 8.5, five-column volume of 50 mM Tris pH 8.5 containing 50 mM NaCl and five-column volume of 50 mM Tris pH 8.5 containing 100 mM NaCl. The fraction containing 100 mM NaCl was collected and NaCl added to 4 M before loading to the HiTrap Phenyl Sepharose column pre-equilibrated with five-column volume of 50 mM Tris pH 8.5 containing 4 M NcCl at the rate 1 ml/min. Protein was eluted with a gradient from 3 M – 1.5 M NaCl in 50 mM Tris pH 8.5

3.3.2.3 Concentration and desalting of purified protein

The target protein was concentrated by Amicon Ultra-4 (Millipore), by centrifuging at 5000X g at 4ºC using Sorvall Centrifuge, SLA-1500 rotor until the final volume reached 1.5 ml. Glutathione that bound to the recombinant GST was eliminated by using HiTrap-desalting column (Amersham Pharmacia Biotech). The column was equilibrated with 5 columns of 50 mM phosphate buffer pH 6.5. Then 1.5 ml of the concentrated protein was applied to the column while the effluents were collected. The purified protein was eluted with 2 ml o the same equilibrating buffer containing 10 mM DTT. Finally, the concentrating step was repeated as previously described, until the final volume of the protein reach 0.5 ml. The purified enzyme was stored at -20°C in 50% glycerol until used.

3.3.2.4 Protein assay

Protein concentration was determined by the method of Bradford using the Bio-Rad protein reagent. The concentrated reagent was diluted 1:5 in distilled water and filtered through Whatman No. 1 filter paper to remove the insoluble dye before use. A protein standard curve was determined at five concentration of bovine serum albumin;

0.1, 0.2, 0.3, 0.4 and 0.5 mg/ml. The assay was started by adding 300 µl of diluted reagent to 10 µl of sample in a microtiter plate. The mixture was incubated at room temperature for 5 minutes. The absorbance at 595 nm was measured and the protein concentration was calculated from the standard curve.

3.3.3 SDS-PAGE Analysis

3.3.3.1 Protein sample preparation

The OD_{600} of the bacterial cell culture was measured. Then the cells corresponding to 0.5 OD were collected by centrifugation at 5,000 rpm for 2 minutes. The cell pellet was resuspended by vortexing in 50 µl sterile distilled water and 20 µl of 4X gel sample buffer (4 mM EDTA, 4% SDS, 40% glycerol, 100 mM DTT, 1.45 mM bromophenol blue, 200 mM Tris-HCl, pH 7.5) and heated to 100°C for 10 minutes in a heating block to denature the proteins. The samples were vortexed, spun at 12000 rpm for 5 minutes and stored in the refrigerator until the SDS-PAGE had been set up.

3.3.3.2 Separation of protein samples

An SDS-PAGE gel was prepared as described in **Table 1**: for stacking gel and separating gel (0.75 mm thickness x 2 gels). After the gel was polymerized for approximately 30 minutes, the well comb was removed. Then the wells were washed with water to remove unpolymerized acrylamide solution. The electrophoresis apparatus was assembled. The samples were loaded, approximately 0.05 OD, including 5 µl of a protein standard marker. The separation was started in descending direction at a constant current of 25 mA until the bromophenol marker was run to the bottom of the gel (approximately 90 minutes). The electrophoresis equipment were disassembled and the gel was stained in Coomassie staining solution for 2 hours at room temperature. Then the gel was destained overnight in destaining solution, dried and made a permanent record by scanning the gel.

Table 1 preparation of SDS-PAGE stacking gel and separating gel (0.75 mm x 2 gels)

Solution	Stacking gel (6%) 3 ml	Separating gel (15%) 10 ml
Acrylamide solution (ml) (30% Acrylamide + 0.3% Bis-crylamide)	0.6	5.0
1.5 M Tris-HCl pH 8.8 (ml)	-	2.5
0.5 M Tris-HCl pH 6.8 (ml)	0.75	-
Distilled water (ml)	1.60	2.29
10% SDS (μl)	30	100
10% (w/v) Ammonium persulphate (μl)	20	100
TEMED (μl)	10	10

3.3.4 Substrate assay panels

For objective 2, a panel of GST substrates consists of 11 substrates ; 1-chloro-2,4-nitrobenzene (CDNB), 1,2-dichlro-4-nitrobenzene (DCNB), 4-nitrophenethyl bromide, 4-nitrobenzyl chloride, ethacrynic acid, cumene hydroperoxide, t-butyl hydroperoxide, dichloro acetic acid (DCA), p-nitrophenyl acetate, 4-hydroxynonenal (4-HNE), hydroxyethyl disulfide (HED). The protocol of the first five substrates are demonstrated in table 2 the protocol for the latter five substrates are as follows:

Table 2 Conditions for spectrophotometric GST assay with various substrates

Substrates	Concentration (mM)	GSH concentration (mM)	pH	Wavelength (nm)	ε (mM^{-1} cm^{-1})
CDNB	1.0	10.0	6.5	340	9.6
DCNB	1.0	10.0	7.5	345	8.5
Ethacrynic acid	0.2	10.0	605	270	5.0
p-nitrophenethyl bromide	0.1	10.0	6.5	310	1.2
P-nitrobenzyl chloride	0.1	10.0	6.5	310	1.14

3.3.4.1 Glutathione peroxidase activity

Glutathione peroxidase activity was determined with cumene hydroperoxide and t-butyl hydroperoxide as substrates. For cumene hydroperoxide each 210 µl coupled-enzyme assay contained 50 mM Tris pH 7.6, 5 mM EDTA, 0.2 mM NADPH, 4 mM GSH, 2 units of glutathione reductase and 1.5 mM cumene hydroperoxide. For the peroxidase assay using t-butyl hydroperoxide as a substrate, the 200 µl reaction consisted of 100 mM Tris pH 8.0, 5 mM EDTA, 0.2 mM NADPH, 2 mM GSH, 1 unit of glutathione reductase and 0.07 mM t-butyl hydroperoxide. Glutathione peroxidase catalyses the oxidation of reduced glutathione (GSH) to oxidized glutathione (GSSG) by hydrogen peroxide. GSSG produced upon reduction of organic peroxide by glutathione peroxidase is recycled to its reduced state by the enzyme glutathione reductase (GR):

$$2GSH + ROOH \xrightarrow{GPX} ROH + H2O + GSSG$$

$$GSSG + NADPH + H^{+} \xrightarrow{GR} 2GSH + NADP^{+}$$

The oxidation of NADPH to $NADP^{+}$ is accompanied by a decrease in absorbance at 340 nm providing a spectrophotometric means for monitoring glutathione peroxidase activity. A spectrophotometer, Spectra MR™, DYNEX Technologies, Inc., was set to measure absorbance at 340 nm at 25°C. The change in A_{340} was recorded for 3 minutes. For calculating the results, the change in absorbance per minute was determined. Then the net rate was obtained by subtracting the value of the sample from the blank. The reaction rate at 340 nm was determined using the NADPH extinction coefficient of 6.22 $mM^{-1}cm^{-1}$. One unit is defined as the amount of enzyme that will cause the oxidation of 1.0 µmol of NADPH to NADP+ per minute at 25ºC. Data were expressed as means of at least three experiments.

GPx activity (µmol/min/mg) = (net rate X 21) / 1000 / 3.732/ protein concentration (mg/ml)

3.3.4.2 Thiolysis reaction

For thiolysis reaction, *p*-nitrophenyl acetate reacts with thiols to produce p-nitrophenol and an acylated thiol. Each of the glutathione transferases that have been tested catalyze this reaction yielding *p*-nitrophenol and acetyl-S GSH. *p*-nitrophenol was quantitated directly by its absorbance at 400 nm. The assay solution included the 0.1 M potassium phosphate buffer pH 7.0 containing 1 mM *p*-nitrophenyl acetate, 4 mM GSH solution and enzyme. The rate of change of the absorbance at 400 nm was recorded.

The extinction coefficient for *p*-nitrophenolate at pH 7 is 8.79 $mM^{-1}cm^{-1}$ at 400 nm. The activity of the enzyme was calculated as follows:

Enzyme activity (μmol/min/mg) = mOD_{400} x dilution factor/1000/5.274/protein concentration (mg/ml)

3.3.4.3 Biotransformation of dichloacetic acid

After DCA was transformed to glyoxylic acid then the latter compound was transformed into glyoxylic acid phenylhydrazone respectively. The formation of glyoxylic acid phenylhydrazone was measured in a mixture of 33 mM acetate buffer pH 5.2, 1 mM GSH, 0.5 mm DCA, 0.115 mM phenyl hydrazine hydrochloride and enzyme. The absorbance at 328 nm was followed. The extinction coefficient of the product is 17.1 mM^{-1} cm^{-1}. The activity of the enzyme was calculated as follows:

Enzyme activity = mOD_{328} x dilution factor/1000/10.26/ protein concentration (mg/ml)

3.3.4.4 Activity toward HNE-substrate

For 4-HNE substrate, GST activities were measured spectrophotometrically in 1-ml quartz cuvettes in a Shimadzu UV-2101PC spectrophotometer. The activity measurements were conducted in 0.1 M sodium phosphate (pH 6.5) at 30°C. 0.1 mM 4-HNE and 0.5 mM GSH were used in the reaction. Enzyme activity with 4-HNE was calculated from the rate of consumption of this substrate (measured by decrease of absorbance at 224 nm; this absorbance is due to the α,β-unsaturated carbonyl structure in 4-HNE) at 30°C. The extinction coefficient for 4-HNE is 13.750 $mM^{-1}cm^{-1}$ at 224 nm. Then the change in A_{224} was recorded for 70 seconds. For calculating the results, the absorbance values as a function of time were plotted to obtain the slope (rate) of the linear portion of the curve. Then the rate of ΔA_{224}/min. for the background or non-enzymatic wells was determined and this rate subtracted from the sample values. The formula below was used to calculate the 4-HNE activity.

4-HNE activity = (ΔA_{224}/min) x 21 /1000/13.750 $mM^{-1}cm^{-1}$ / protein concentration (mg/ml)

3.3.4.5 Thiol transferase activity

Thiol transferase activity was measured by the reduction of the mixed disulfide formed between HED and GSH. The components of the system, NADPH (0.4 mM), GSH (1 mM) and glutathione reductase (6 μg/ml), as well as HED (0.7 mM), were added to a reaction volume of 210 μl in 0.1 M Tris HCl, pH 7.4. A reaction was started by the addition of 10 μl enzyme. The reaction was followed by the decrease in A_{340} for

2 minutes because of the oxidation of NADPH. For the calculation of thiol transferase activity, the principle was similar to peroxidase assay as described in section 3.1.1.

3.3.5 DDTase assay

A 1 ml reaction mixture containing an appropriate amount of GST, in 0.1 M Sodium phosphate buffer pH 6.5 and 10 mM GSH with a final concentration of 0.005 mM p,p'-DDT was prepared. For the RR and RS samples, a 1 ml reaction mixture consisted of 100 µl of supernatant, 0.1 M of Sodium phosphate pH6.5 and 10 mM GSH with 0.005 mM p,p'DDT. The reaction mixtures were incubated at 28°C for 2 hours then 0.005 mM of dicofol was added before the samples were extracted three times each with 1.5 ml of chloroform. The dicofol was used as an internal control to determine the efficiency of extraction. The chloroform extracts (4.5 ml) were pooled and left to air dry overnight then stored at -20°C until used. The extracts were resuspended in 100 µl of isopropanol and 100 µl of mobile phase before analysis. Chromatography was performed on a HPLC system consisting of an LC-20A series (Shimadzu Corporation, Japan).

3.3.6 Pyrethroid metabolized assay

For permethrin-HPLC, the extracts were resuspended in 200 µl of acetonitrile before analysis. Chromatography was performed on a HPLC system as DDT assay. Ten microlitre of mixed standards or sample were injected into a NVC18 (4 micron). The mobile phase was water (adjusted to pH 3.5 using 1N acetic acid): acetonitrile gradient at flow-rate programmed at 1.0 ml/min. The gradient started at 45% acetonitrile, increased to 55% acetonitrile at 5 min, then increased to 95% acetonitrile by 8 min and kept constant for 7 min until 14 min. Then the system returned to 45% acetonitrile at 15 min where it was kept under this condition for 10 min to re-equilibrate. The eluents were monitored by UV detection of wavelength of 230 nm for dimethoate, m-phenoxybenzyl alcohol, m-phenoxybenzoic acid and permethrin.The chromatographic analysis was performed at 35°C column oven temperature.

3.3.7 Temephos metabolized assay

For temephos assay, 200 µl of acetonitrile was added to resuspend the dried extract. HPLC gradient was performed using HPLC system as DDT assay. Ten microlitre of mixed standards or sample were injected into a NVC18 (4 micron). The mobile phase was water: acetonitrile gradient at flow-rate programmed at 1.0 ml/min. The gradient started at 70% acetonitrile, decreased to 60% acetonitrile at 5 min, then increased to 65% acetonitrile at 10 min. Then the system returned to 70% acetonitrile at 11 min

where it was kept under this condition for 5 min to re-equilibrate. The eluents were monitored by UV detection of wavelength of 250 nm for temephos and pyrene. The chromatographic analysis was performed at 40°C column oven temperature. The Agilent 1100 series, Germany, HPLC system, with DAD detector, coupled with a C18 column and the Agilent LC/MSD SL, USA , a mass selective detector (MSD), were used for analysis of metabolites. Mobile phases consisted of acetonitrile and water (70:30) containing 0.1% (v/v) formic acid. The isocratic mobile phase containing acetonitrile:water (70:30, v/v) had a flow rate of 1.0 ml/min. The MSD was operated as follows: nitrogen gas flow rate, 13 L/min; nebulizer pressure, 50 psi; drying gas temperature 350ºC; capillary voltage positive 4000 V and negative 3500 V. The LC-MS was scanned from 100 to 700 of an m/z unit. GST activity to metabolized temephos

4. Results

4.1 DDTase and substrate panel assay of larvae lysate

DDTase activity of *Ae. aegypti* RR and RS strains was determined. It was found that the DDTase activity of RR with new recombinant GSTs. strain was 0.510 ± 0.094 nmol DDE/mg whereas the activity of the RS strain was only 0.066 ± 0.002 nmol DDE/mg. Specific activity towards 11 substrates was performed and the result is shown in Figure 1. Therefore, both assays are successfully developed and will be used

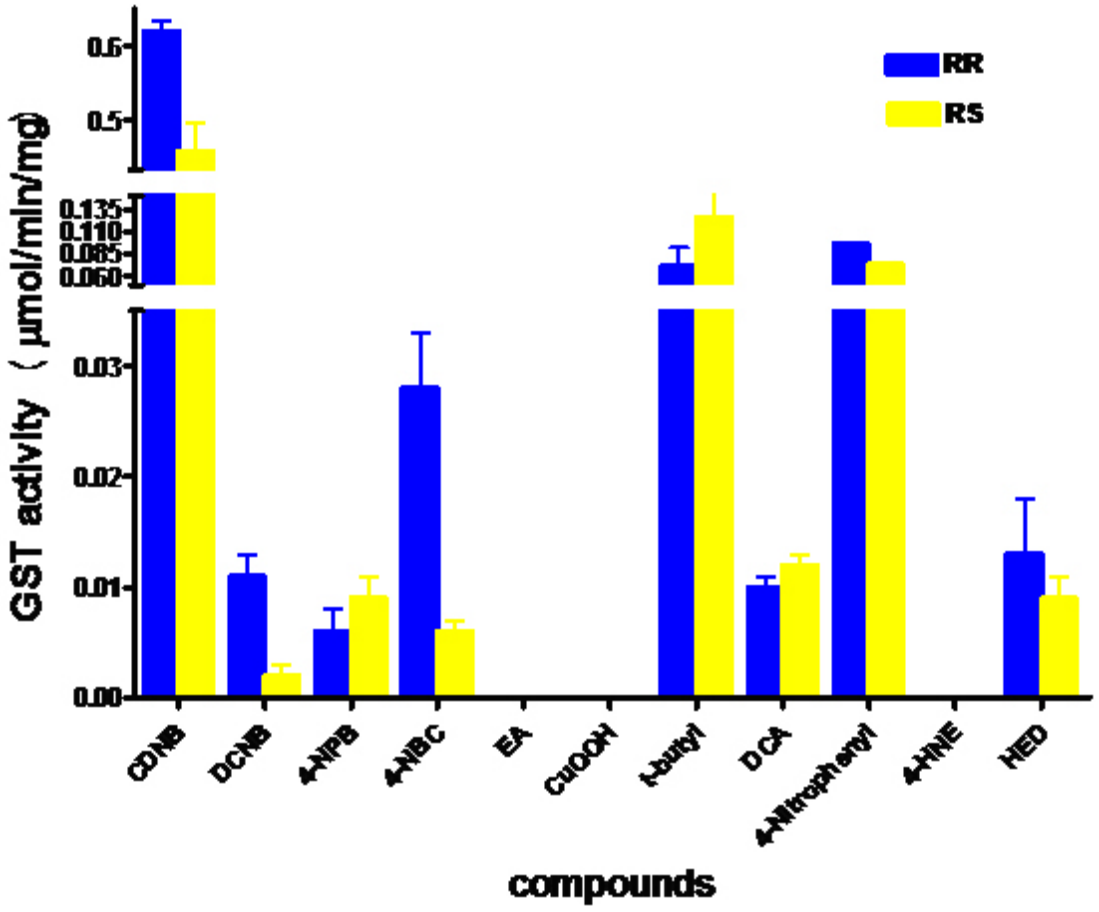


Figure 1: GSTs activity (mean ± SD n= 3) of *Ae. aegypti* larvae RR and RS strains towards 11 different substrates. The units are µmole/min/mg of protein.

4.2 Expression and Purification of Recombinant GSTs in *E.coli*

The expression of the GST in *E.coli* was controlled by the T_7 promoter therefore the plasmids encoding the GST gene were highly expressed in *E.coli* BL21DE3plysS upon IPTG induction. All the recombinant proteins were purified from the lysate by GSTrap affinity column, cation exchange, anion exchange, S-hexyl glutathione and hydrophobic columns. The activities of GSTs were measured in *E.coli* lysate with the standard GST assay and the protein concentration was measured using Bradford reagent. The activity and the yields are shown in Table 3. From all enzyme purifications, approximately 0.6 – 44 % of total GST protein from the bacterial lysate were obtained from the columns. The specific activities of the enzymes were increased approximately 1.3-23-fold greater than unpurified enzymes. All the purified enzymes were stored at -20°C for further characterization.

4.3 Kinetic Parameters

All the kinetic parameters were studied by varying concentrations of both GSH and CDNB substrates. The reactions followed Michaelis-Menten. Kinetic parameters were determined by non-linear regression analysis using Graphpad prism software as shown in Table 4. The maximal velocity or V_{max} of AdGSTE1-1 is 217 µmol/min/mg, 1.2-fold higher than its variant R139H, 175 µmol/min/mg, 6.1-fold and 2.2-fold higher than AdGSTE3-3 and AdGSTE4-4 respectively. The Michaelis constant or K_m, which indicate the binding affinity of the enzyme, is also important in enzyme characterization. Among all the epsilon enzymes, AdGSTE3-3 has the greatest affinity for GSH and CDNB substrates; K_m = 0.05 mM for CDNB and 1.15 mM for GSH, whereas the other enzymes have detectable affinity to CDNB, K_m = 0.08-0.16 mM, but low affinity to GSH, K_m = 4.53-13.7 mM. All the epsilon GSTs has the higher affinity to CDNB than GSH. To determine the catalytic properties, the turn over number for CDNB, kcat and the catalytic efficiency, kcat/Km with respect to CDNB and GSH were calculated. It was found that AdGSTE1-1 demonstrated the highest k_{cat} value, suggesting that AdGSTE1-1 has higher efficiency compared to R139H, AdGSTE3-3 and AdGSTE4-4. For the K_{cat}/k_m values, AdGSTE3-3 possessed the highest specificity toward GSH and R139H possessed the highest specificity toward CDNB substrate.

The kinetic parameters of AcGSTE2-2, AcGSTT1-1 clone2 and AcGSTT1-1 clone3 for GSH and CDNB were determined (Table 5). Comparison of three AcGSTs demonstrated that the AcGSTT1#2 enzyme displayed the highest kinetic parameters e.g. Vmax, kcat, kcat/Km_{GSH} compared to the other AcGSTs, indicating a high rate of

turnover and catalyzing GSH conjugation. The Km values with respect to GSH for AcGSTT1# 2 indicated that this enzyme has similar binding affinities for GSH compared to AcGSTT1#3 while it has higher binding affinities toward GSH compared to AcGSTE2-2 approximately 5.7-fold. For the Km values with respect to CDNB Both AcGSTT1-1 enzymes possessed the relatively high K_mCDNB, approximately 1.9/0.5-fold compared to AcGSTE2-2, thereby significantly decreasing the binding affinity to the substrate. Interestingly, AcGSTE2-2 enzyme also had significant different kinetic parameter compared to *An. gambiae* AgGSTE2-2.

Table 3 Purification of the recombinant GSTs

Enzymes	Step	Column used	Total protein (mg)	Yield (%)	Specific activity (μmol/min/mg)
AdGSTD3-3	Lysate	GSTrap	57	100	12.6
	Purified		25	44	25
AdGSTE1-1	Lysate	GSTrap	83	100	76
	Purified		16	19	173
AdGSTE1-1;R139H	Lysate	GSTrap	42	100	125.2
	Purified		6.3	15	186.8
AdGSTE2-2	Lysate	Cation /	21.8	100	0.9
	Purified	hydrophobic	1.6	7.3	1.2
AdGSTE3-3	Lysate	S-hexyl	40	100	2.2
	Purified	glutathione	0.8	2	47
AdGSTE4-4	Lysate	Cation /	33	100	37
	Purified	hydrophobic	6.5	19.6	69
AdGSTT1 clone 2	Lysate	Cation /	22	100	3.9
	Purified	hydrophobic	0.7	3.2	6.3
AdGSTT1 clone 3	Lysate	Cation /	21.2	100	0.5
	Purified	hydrophobic	0.3	1.4	1.1
AdGSTD2-2	Lysate	GSTrap	41	100	24.5
	Purified		12.8	31	33.6
AdGSTD4-4	Lysate	GSTrap	57.2	100	1.5
	Purified		15.8	27.6	35.2
AdGSTD5-5	Lysate	GSTrap	50.6	100	67.3
	Purified		0.32	0.6	2.7
AdGSTD6-6	Lysate	GSTrap	155.6	100	24.4
	Purified		2.2	1.4	3.2
AdGSTD1-1	Lysate	S-hexyl	15.3	100	0.38
	Purified	glutathione	1.8	11.7	0.86
AdGSTO1-1	Lysate	Anion /	22.5	100	-
	Purified	hydrophobic	0.35	1.6	0.26

Table 4 Kinetic constants for the Epsilon GSTs.

Enzyme	V_{max}	k_{cat}	GSH		CDNB	
			K_m	k_{cat}/K_m	K_m	k_{cat}/K_m
AdGSTE1-1	217 ± 6.0	89.6	8.83 ± 1.02	10.2	0.16 ± 0.03	570
R139H	175 ± 6.9	72.4	13.7 ± 1.11	5.28	0.12 ± 0.01	629
AdGSTE3-3	35.2 ± 0.7	14.6	1.15 ± 0.15	12.7	0.05 ± 0.01	317
AdGSTE4-4*	94.8 ± 5.1	39.3	4.53 ± 1.04	8.7	0.08 ± 0.01	517

The units are: V_{max}: µmole/min/mg, K_m: mM, k_{cat}: s-1, k_{cat}/K_m: mM^{-1} s^{-1}. The data are mean ± standard deviation from at least 3 independent experiments. The data of *An. dirus* AdGSTE4-4 has previously been reported, Charoensilp G. 2006.

Table 5 Kinetic constants for the recombinant GSTE2-2 and GSTT1 from *An. cracens*

Enzyme	V_{max}	k_{cat}	GSH		CDNB	
			K_m	k_{cat}/K_m	K_m	k_{cat}/K_m
AcGSTE2-2	1.4 ± 0.2	0.58	6.80 ± 2.0	0.08	0.05 ± 0.01	68.57
AgGSTE2-2*	13.1 ± 0.4	10.8	6.72 ± 1.70	1.61	0.069 ± 0.01	157.1
AcGSTT1#2	9.4 ± 0.2	4.25	1.27 ± 0.10	3.34	1.94 ± 0.15	2.19
AcGSTT1#3	1.18 ± 0.1	0.53	0.80 ± 0.03	0.67	2.56 ± 0.53	0.21

The units are: V_{max}: µmole/min/mg, K_m: mM, k_{cat}: s-1, k_{cat}/K_m: mM^{-1} s^{-1}. The data are mean ± standard deviation from at least 3 independent experiments. The data of AcGSTE1-1,AcGSTE3-3, AcGSTE4-4 and AgGSTE2-2 has previously been reported, Ortelli F.*

4.4 Substrate Specificity

Specific activities of epsilon GSTs toward 11 substrates were determined and shown in Table 6. The activity was measured with appropriate pH and λ_{max}. CDNB, a general substrate for GSTs, is the substrate that yielded the highest conjugating activity with R139H, the variant of AdGSTE1-1, having the greatest activity and AdGSTE3-3 the lowest activity. The compound 4-nitrobenzyl chloride, 4-nitrophenethyl bromide and ethacrynic acid are rat theta, human theta and pi class substrates respectively. The conjugating activity of AdGSTE1 towards DCNB, the mu class substrate, was similar to R139H variant. In contrast, the activity toward DCNB of AdGSTE3-3 and AdGSTE4-4 is relatively low compared to the AdGSTE1 and R139H approximately 50-fold. AdGSTE1-1 possessed peroxidase activities toward cumene hydroperoxide 1.2-fold, 1.17-fold and 3.8-fold greater than R139H, AdGSTE3-3 and AdGSTE4-4 respectively. In addition, it demonstrated peroxidase activities toward t-butyl hydroperoxide 1.12-fold, 1.5-fold and 1.75-fold greater than R139H, AdGSTE3-3 and AdGSTE4-4 suggesting that they involved in pyrethroid resistance because this peroxidase activity has been shown to be protective against the damage caused by lipid peroxidation products induced by exposure to pyrethroid insecticides. It was found that 4-HNE, the lipid peroxidation product which is specific for alpha and sigma class GSTs, is the substrate that yielded the highest activity with the purified AdGSTE3-3. AdGSTE3-3 and AdGSTE4-4 showed no measurable activity against HED substrate. In contrast, AdGSTE1-1 and R139H showed HEDS-measured TTase activities. The glutathione-dependent oxygenation of DCA to glyoxylic acid is catalyzed by the four epsilon enzymes. However the activity is relatively low. AdGSTE3-3 demonstrated measurable activity towards substrate for the mu class GST p-nitrophenyl acetate where as AdGSTE1-1, R139H and AdGSTE4-4 are highly active toward the substrate.

To characterize the recombinant AcGSTE2-2, AcGSTT1-1 clone2, clone 3 and AcGSTO1-1, GST activity was meausured using CDNB as substrate, Table 7. CDNB is a general substrate for all GST classes. The specific activity of AcGSTT1-1 clone 2 towards CDNB was highest compared to AcGSTE2-2, AcGST1-1 clone3 approximately 3-fold whereas AcGSTO1-1 exhibited lowest GST activity towards CDNB. Unlike CDNB conjugating activity, the specific activity of AcGSTE2-2 toward DCNB can be detected whereas the remaining enzymes did not utilyzed DCNB. For the conjugaing activity toward p-Nitrophenyl bromide, 4-nitrobenzyl chloride, human and rat theta class sustrates, AcGSTT1-1 clone2 had higher specific activity toward both substrates

whereas AcGSTT1-1 clone3 conjugated only a rat-theta class cubstrate. AcGSTE2-2 and AcGSTT1-1 clones can also utilize cumene hydroperoxide and t-butyl hydroperoxide, oxidative stress products suggesting a role in physiological function. However, none of the enzymes are active toward 4-HNE, a lipid peroxidation product. Thioltransferase activity towards HED was determined as a characteristic activity for GSTO 1-1 class. AcGSTO 1-1 exhibited stronger thioltransferase activity than itsown GST CDNB activity. In addition, the enzyme also possessed the activity towardd DCA which is a substrate preference of Zeta class GST. All The recombinant enzymes were further characterized by the thiolysis reaction, it was found that only E2 exhibit specific activity toward p-nitropenyl acetate

Table 6 Specific activity of the Epsilon GST towards 11 different substrates.

Electrophilic substrates	AdGSTE1-1 (µmol/min/mg)	R139H (µmol/min/mg)	AdGSTE3-3 (µmol/min/mg)	AdGSTE4-4 (µmol/min/mg)
1-chloro-2,4-nitrobenzene	207 ± 4.2	219 ± 4.1	33.4 ± 2.78	79.9 ± 0.81
1,2-dichloro-4-nitrobenzene	26.9 ± 0.97	21.5 ± 1.1	0.475 ± 0.008	0.324 ± 0.011
4-nitrophenethyl bromide	0.561 ± 0.036	0.250 ± 0.004	0.114 ± 0.010	0.113 ± 0.009
4-nitrobenzyl chloride	1.653 ± 0.025	2.918 ± 0.048	nd	0.140 ± 0.006
ethacrynic acid	nd	nd	nd	0.034 ± 0.006
cumene hydroperoxide	0.457 ± 0.067	0.376 ± 0.135	0.388 ± 0.037	0.118 ± 0.006
t-butyl hydroperoxide	0.028 ± 0.004	0.025 ± 0.003	0.018 ± 0.004	0.016 ± 0.004
4-hydroxynonenal	0.094 ± 0.010	0.069 ± 0.009	0.169 ± 0.010	0.055 ± 0.013
hydroxyethyl disulfide	0.015 ± 0.005	0.016 ± 0.008	nd	nd
Dichloro acetic acid	0.004 ± 0.0003	0.012 ± 0.0007	0.003 ± 0.0008	0.001 ± 0.0002
p-nitrophenyl acetate	4.24 ± 0.410	4.35 ± 0.069	0.322 ± 0.004	4.753 ± 0.284

The units are µmole/min/mg of protein. The data are mean ± standard deviation from at least 3 independent experiments.

Table 7 Specific activity of the Epsilon, Theta and Omega class GSTs towards 11 different substrates.

Electrophilc substrates	AcGSTE2-2 (µmol/min/mg)	AgGSTE2-2 (µmol/min/mg)	AcGSTT1#2 (µmol/min/mg)	AcGSTT1#3 (µmol/min/mg)	AcGSTO1-1
1-chloro-2,4-nitrobenzene	1.710 ± 0.052	12.5 ± 0.58	6.20 ± 0.28	1.42 ± 0.15	0.04 ± 0.01
1,2-dichloro-4-nitrobenzene	1.50 ± 0.019	5.87 ± 0.24	nd	nd	nd
4-nitrophenethyl bromide	0.116 ± 0.011	-	0.73 ± 0.04	nd	nd
4-nitrobenzyl chloride	0.421 ± 0.018	-	10.8 ± 0.43	1.4 ± 0.08	nd
ethacrynic acid	nd	-	nd	nd	nd
cumene hydroperoxide	0.11 ± 0.002	nd	3.30 ± 0.29	2.18 ± 0.14	nd
t-butyl hydroperoxide	0.04 ± 0.002	-	0.030 ± 0.008	0.030 ± 0.008	nd
4-hydroxynonenal	nd	-	nd	nd	nd
hydroxyethyl disulfide	nd	-	nd	nd	0.262 ± 0.018
Dichloro acetic acid	0.11 ± 0.001	-	0.015 ± 0.001	0.030 ± 0.003	0.022 ± 0.001
p-nitrophenyl acetate	0.178± 0.002	2.37 ± 0.14	nd	nd	nd

The units are µmole/min/mg of protein. The data are mean ± standard deviation from at least 3 independent experiments. A dash indicates data not determined. Nd indicates not detectable.

4.5 Inhibition Kinetics Study

In this assay, permethrin and λ-cyhalothrin, pyrethroid insecticides that are commonly used to control insects, were used as an inhibitor to study the interaction between the insecticide and the GST epsilon enzymes. The binding of permethrin to the four purified GSTs was studied by its inhibitory action against CDNB-conjugating activity. K_m values remained unaltered, while V_{max} values decreased as shown by double-reciprocal plots (Figure 2). It was found that permethrin is a non-competitive inhibitor of all the epsilon enzymes. This type of inhibition means permethrin can bindwith either the enzyme/CDNB complex or the free enzyme. Permethrin inhibition displayed similar inhibition kinetics toward the three epsilon AdGSTE1-1, R139H and AdGSTE3-3 (Table 8). The AdGSTE3-3 possessed the highest K_i value suggesting that the enzyme had the lowest binding affinity toward permethrin. Unlike the permethrin, λ-cyhalothrin inhibition displayed different inhibition kinetics among the four epsilon enzymes. For AdGSTE1-1 and its variant, R139H, the 1/V versus 1/S plots show parallel straight lines as shown in figure 3. The plots indicated that λ-cyhalothrin is an uncompetitive inhibitor for both enzymes. λ-cyhalothrin can only combine with the adGSTE1-1/CDNB complex or the adGSTR139H/CDNB complex but not with the free enzyme hence both K_m and V_{max} of the adGSTE1-1 and R139H reaction are decreased. It was found that AdGSTE3-3 displayed non-competitive inhibition for Lambda-cyhalothrin. The inhibitor constant (Ki) for permethrin and Lambda-cyhalothrin were calculated from the values of the appropriate intercepts of both axes of the double reciprocal plot as shown in Table 8. This data indicated that the binding affinities of AdGSTE1-1 to the permethrin and Lambda-cyhalothrin were similar to R139H while AdGSTE3-3 had 3.9-fold lower binding affinities to permethrin and 3.4-fold lower binding affinities than AdGSTE1-1.

Table 8 Inhibition kinetics of permethrin and λ-cyhalothrin to GST epsilon

Parameters	AdGSTE1-1	R139H	AdGSTE3-3	AdGSTE4-4 *
Ki for Permethrin (μM)	56.5 ± 10.4	55.5 ± 11.2	225.2 ± 75.8	66.3 ± 2.8
Inhibition type	Non-competitive	Non-competitive	Non-competitive	Non-competitive
Ki for λ-cyhalothirn (μM)	47.2 ± 6.2	57.5 ± 10.8	162.1 ± 22.0	115.4 ± 15.2
Inhibition type	Un-competitive	Un-competitive	Non-competitive	Mix-inhibition

The K_i are mean ± standard deviation of at least 3 independent experiments. The data of *An. dirus* AdGSTE4-4 has previously been reported, Charoensilp G. 2006.

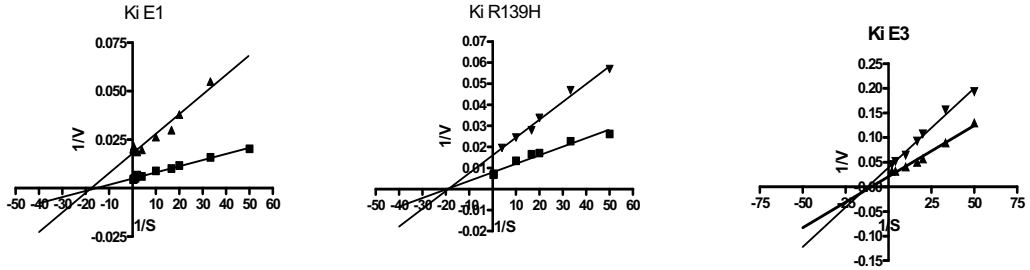


Figure 2 The mechanism of permethrin interaction with epsilon enzymes
The 1/V versus 1/S plot of AdGSTE1, R139H and AdGSTE3-3 in the absence and presence of permethrin indicated that permethrin is a non-competitive inhibitor for all the enzymes. The experiments were performed at least three times.

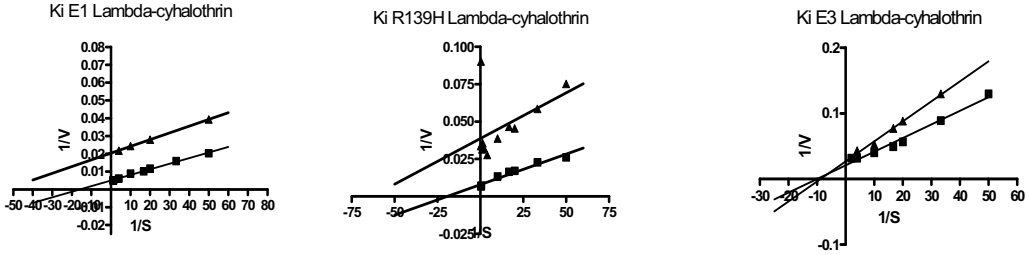


Figure 3 The mechanism of Lambda-cyhalothrin interaction with epsilon enzymes
The 1/V versus 1/S plot of AdGSTE1, R139H and AdGSTE3-3 in the absence and presence of Lambda-cyhalothrin indicated that Lambda-cyhalothrin is a non-competitive inhibitor for AdGSTE3-3 and an un-competitive inhibitor for two enzymes, AdGSTE1-1 and R139H. The experiments were performed at least three times.

4.6 Permethrin metabolized assay

4.6.1 Internal standard

Dimethoate seemed to be the appropriate internal standard since the peak of this chemicat appeared at 1.584 sec after the peaks of solvent front (Figure 4). In contrast, the peak position of malathion and fenthion was 7.191 and 9.150 which is in the transition step of the HPLC system before changing to permethrin condition. This position is not good to separate the internal standard because it will be difficult to reproduce the peak results. For paraooxon, the peak appeared at 2.619 sec (Figure 5-7) which is closed to the PBOH peak. Therefore, from this experiment, dimethoate was chosen as our internal standard. The mixed standard, dimethoate, PBOH, PBCOOH and permethrin was run and shown in Figure 8.

4.6.2 Intraday variability

Intra-day variability was defined as % CV calculated from the values, measured from ten standard bottles containing 100,000 ng/ml PBOH, 10,000 ng/ml PBOOH and 10,000 ng/ml permethrin(Table 9).

Table 9 Intra- day variablility and percent CV measurements

Drug	**Actual concentration (ng/ml)**	**Detected concentration (ng/ml) (mean ± SD)**	**Variablility (%CV)**
PBOH	Intra-day (n=10) 100,000	100,012.08 ± 6.60	0.65
PBCOOH	Intra-day (n=10) 10,000	10,004.903 ± 1.73	1.65
PMT	Intra-day (n=10) 10,000	9,900.863 ± 0.89	0.89

4.6.3 Standard calibration curve

The standard calibration curves of peak area against concentration of PBOOH, permethrin and PBOH are shown in figure 9-11. Linearity of the calibration curves for the first two compounds was achieved at concentrations ranging from 625-20,000 ng/ml while the latter compound ranged from 6,250-200,000 ng/ml. The chromatogram of standards in each level was shown in figure 12.

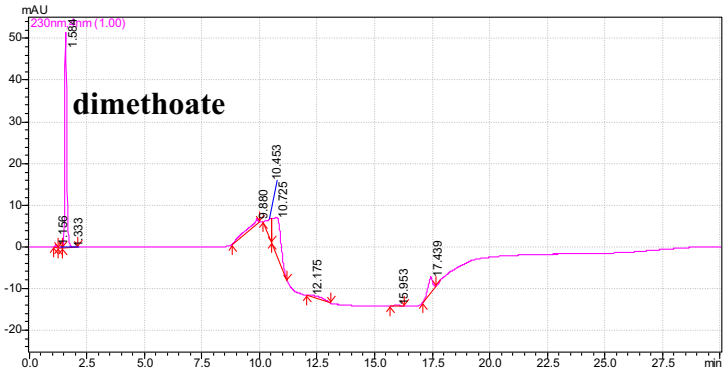


Figure 4 Chromatogram of dimethoate standard

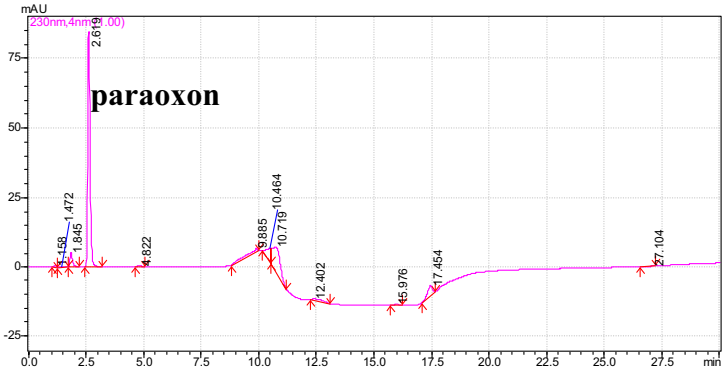


Figure 5 Chromatogram of paraoxon standard

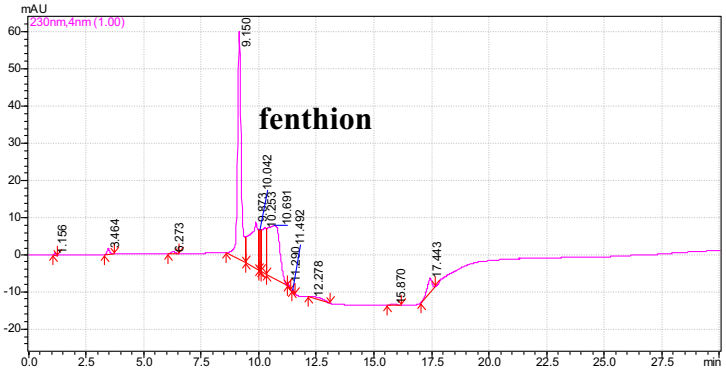


Figure 6 Chromatogram of fenthion standard

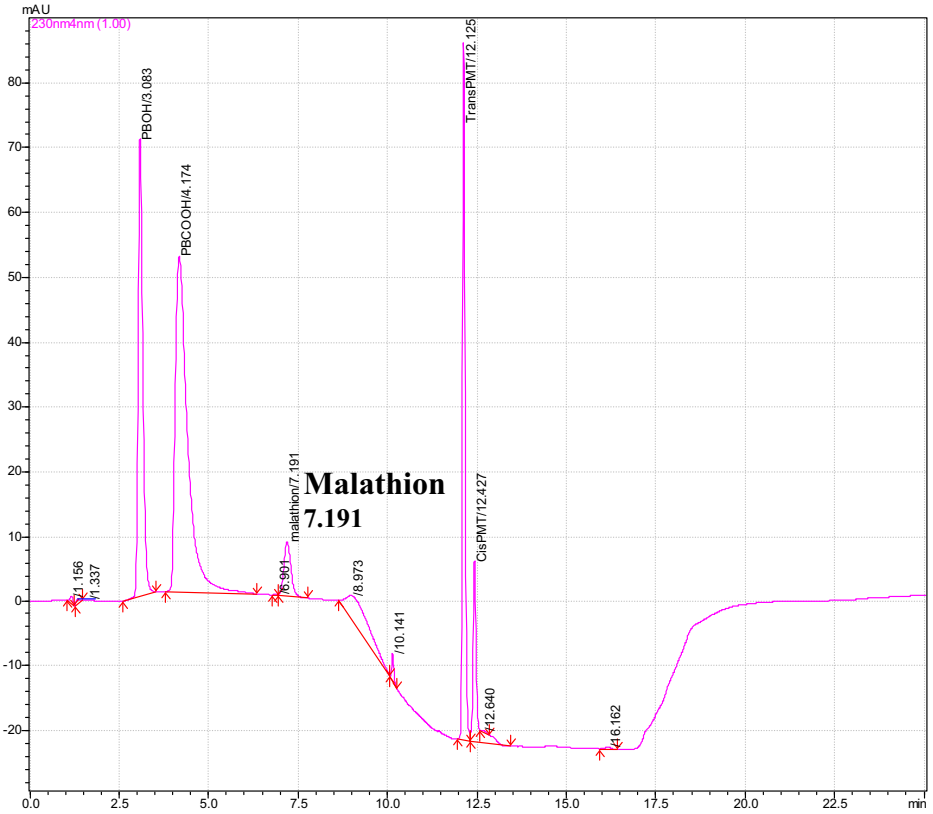


Figure 7 Chromatogram of malathion standard

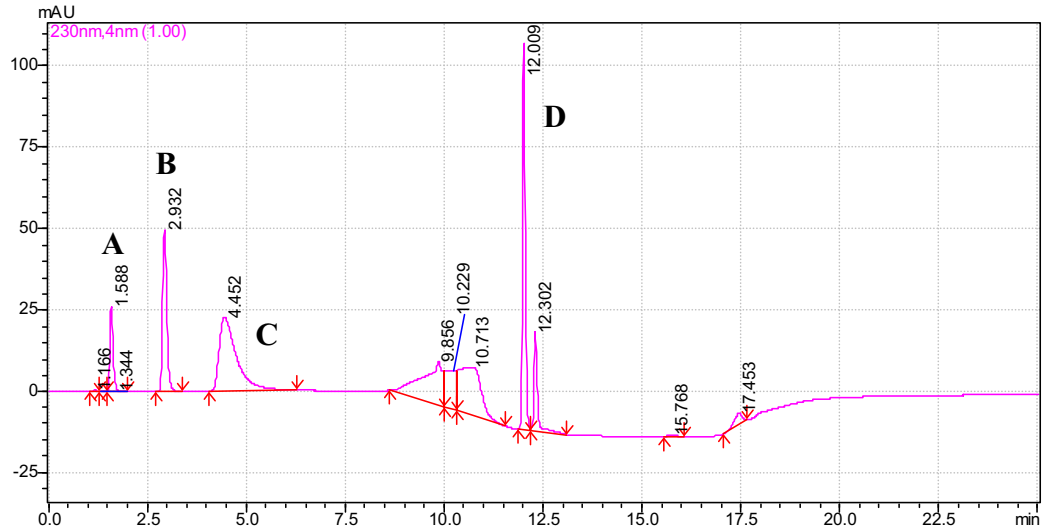


Figure 8 Chromatogram of mixed standard (A) dimethoate (B)PBCOOH (C) PBOH and (D) permethrin

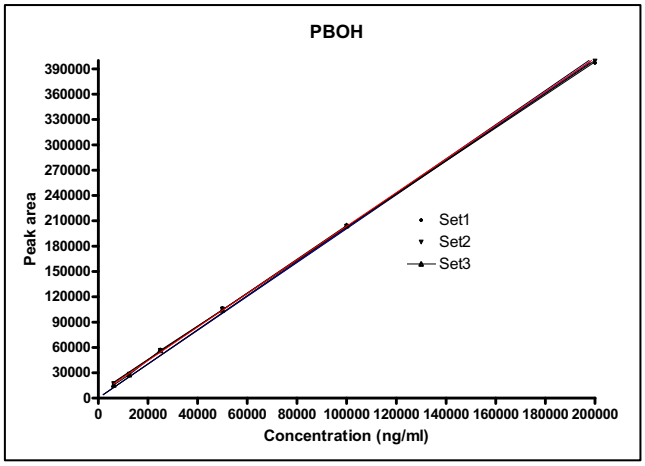


Figure 9 Standard calibration curve of m-phenoxybenzyl alcohol

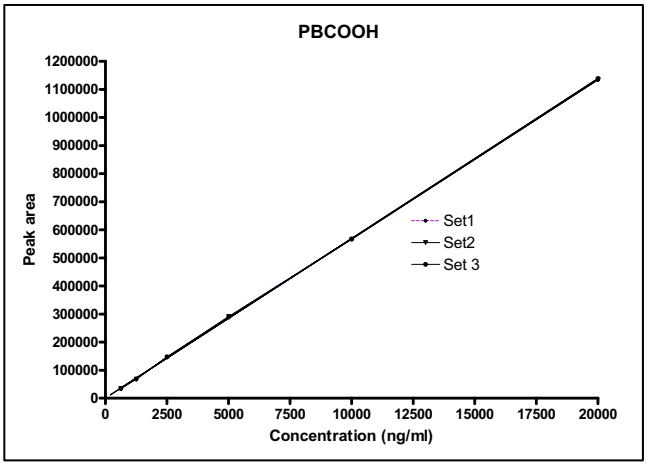


Figure 10 Standard calibration curve of m-phenoxybenzoic acid

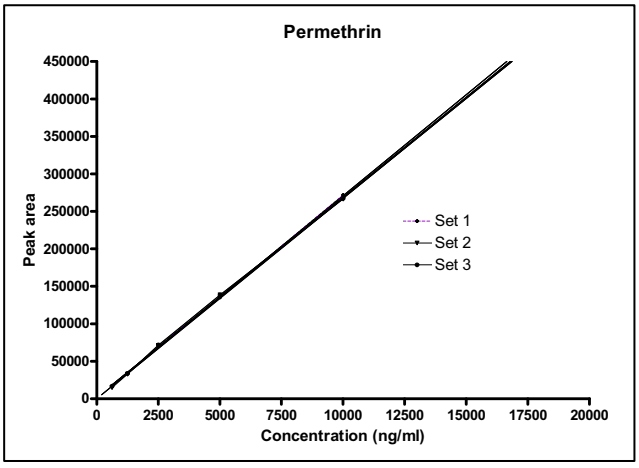


Figure 11 Standard calibration curve of permethrin

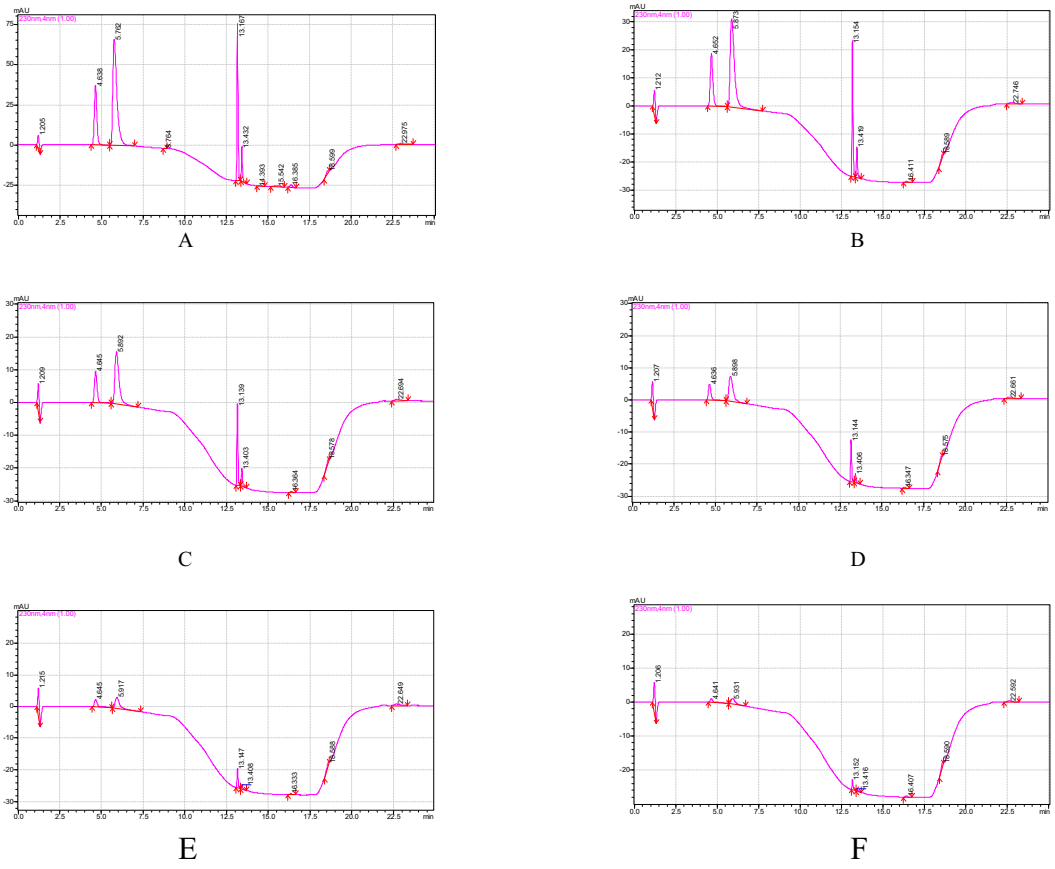


Figure 12 HPLC separations of standards. A, 200,000ng/ml PBOH, 20,000 ng/ml PBCOOH and permethrin, B, 100,000 ng/ml PBOH, 10,000 ng/ml PBCOOH and permethrin, C, 50,000 ng/ml PBOH, 5,000 ng/ml PBCOOH and permethrin D, 25,000 ng/ml PBOH, 2,500 ng/ml PBCOOH and permethrin, E, 12,500 ng/ml PBOH, 1,250 ng/ml PBCOOH and permethrin, F, 6,250 ng/ml PBOH, 625 ng/ml PBCOOH and permethrin

4.6.4 LOD and LOQ

LOD were calculated from a peak signal-to-noise ratio of 3:1. The resulting detection limits ranges were 3,000, 300 and 300 for PBOH, PBCOOH and permethrin respectively. LOQ were determined to be 6,000 ng/ml for PBOH, 600 ng/ml for PBCOOH and 600 ng/ml for permethrin.

4.6.5 Chromatogram

Chromatographic profiles were obtained (figures 13-20) for AdGSTD3-3 in different units after chloroform extraction under HPLC conditions, described above. Retention times of standard were 1.796, 4.646, 6.059 and 13.158 for dimethoate, PBOH, PBCOOH and permethrin respectively. Clean chromatogram of reagent blank and sample blank shows no interference from reagents or the samples. This suggests an efficient sample preparation and clean up method. When the AdGSTD3-3 was incubated with PBCOOH, PBOH and permethrin, no metabolic products were detected in the HPLC chromatogram of the extract of the incubation mixture. The extraction recoveries of dimethoate in AdGSTD3-3 sample ranged from 88.9-92.6%.

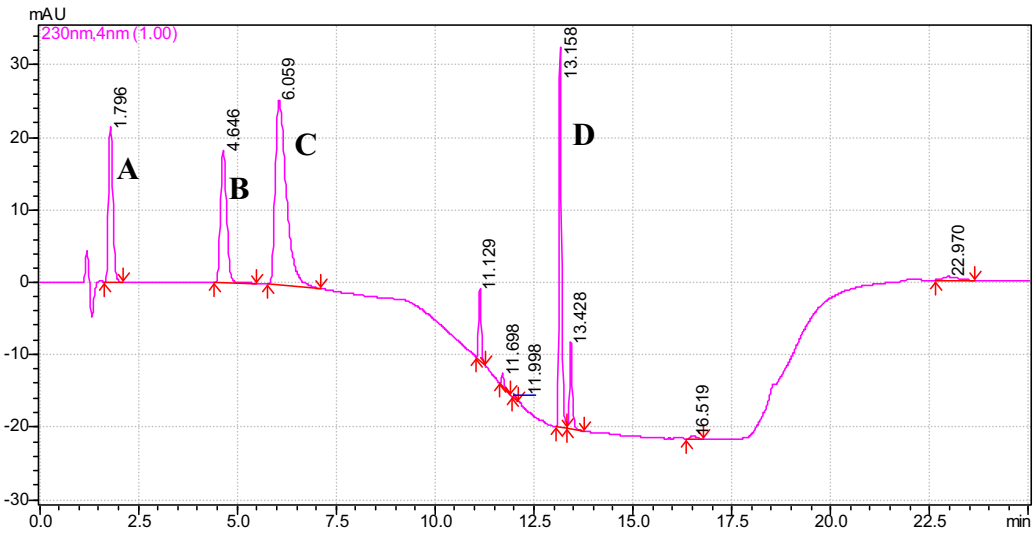


Figure 13 HPLC separations of standards. A, 50,000 ng/ml dimethoate, B, 100,000 ng/ml PBOH, C, 110,000 ng/ml PBCOOH and 110,000 ng/ml permethrin

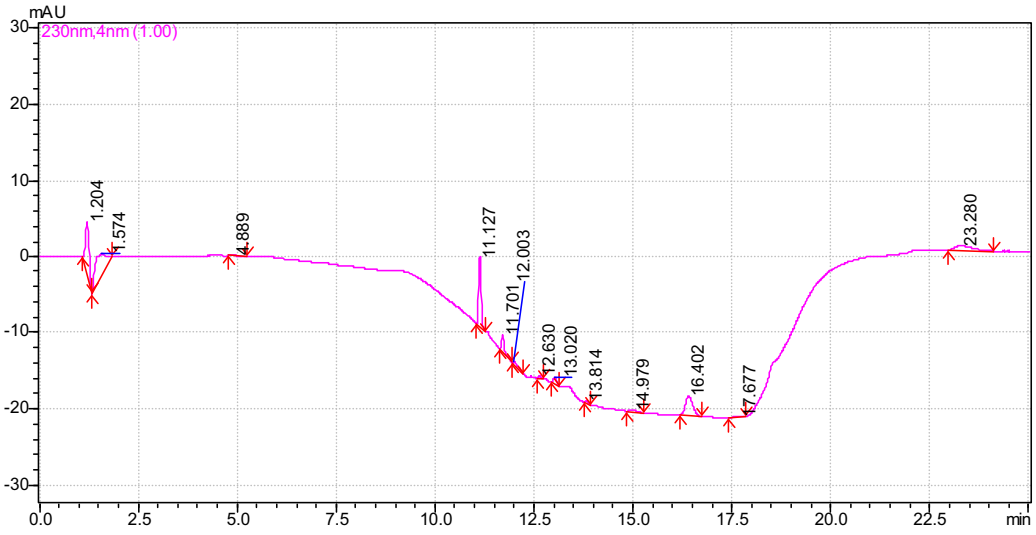


Figure 14 HPLC separations of reagent blank.

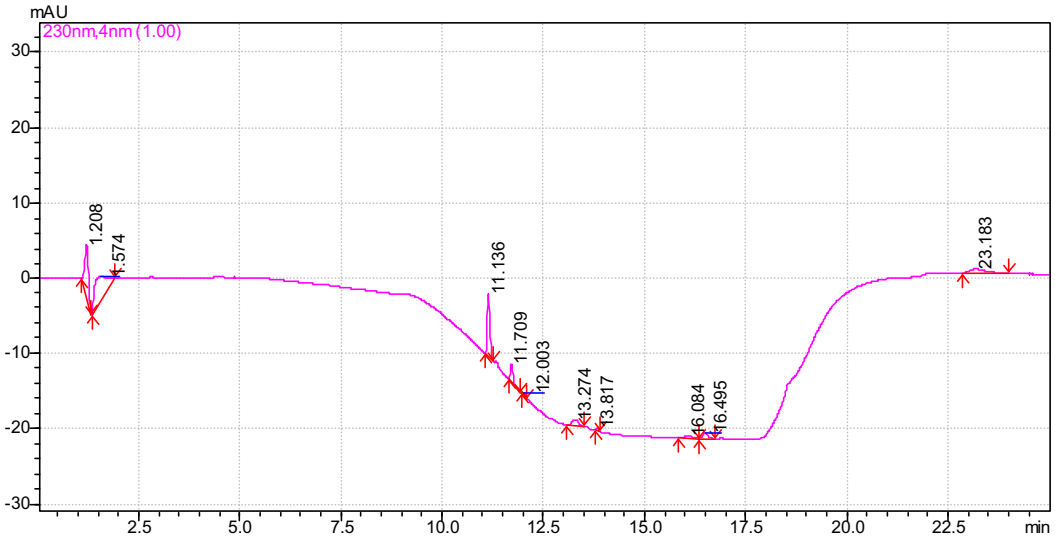


Figure 15 HPLC separations of sample blank containing 2 units of GST AdGSTD3-3

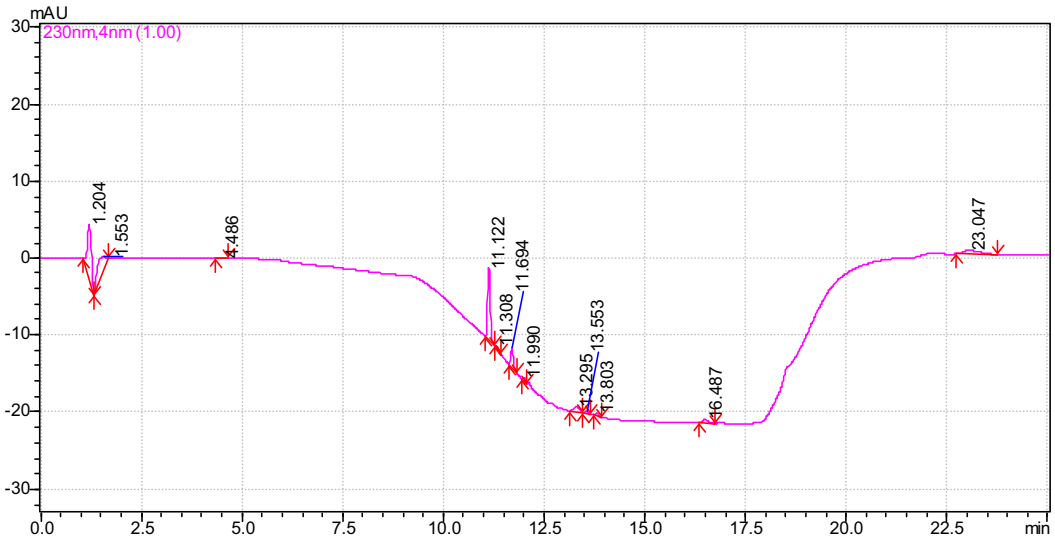


Figure 16 HPLC separations of sample blank containing 4 units of GST AdGSTD3-3

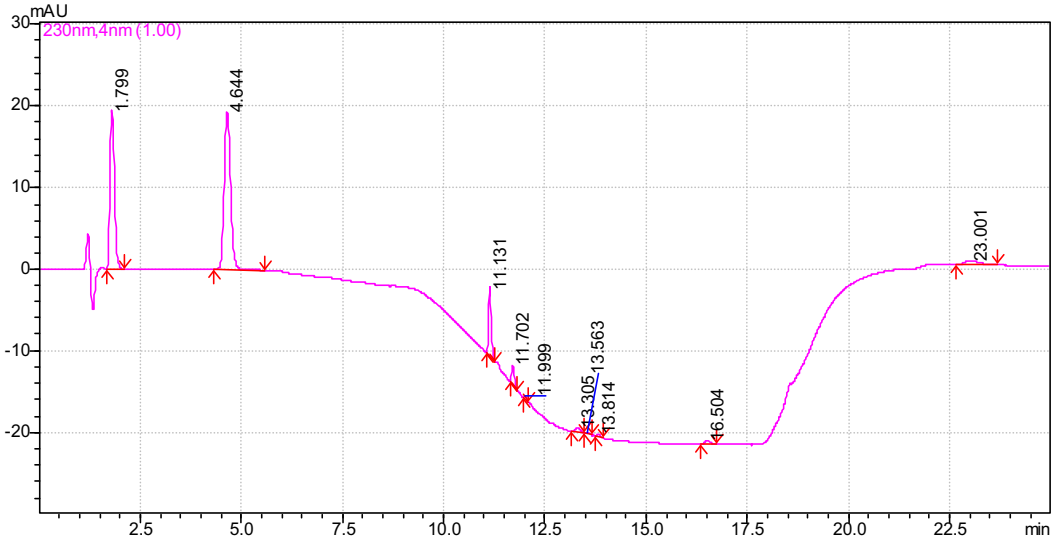


Figure 17 HPLC separations of 2 units of AdGSTD3-3 spiked with internal standard dimethoate 50,000 ng/ml and PBOH 100,000 ng/ml

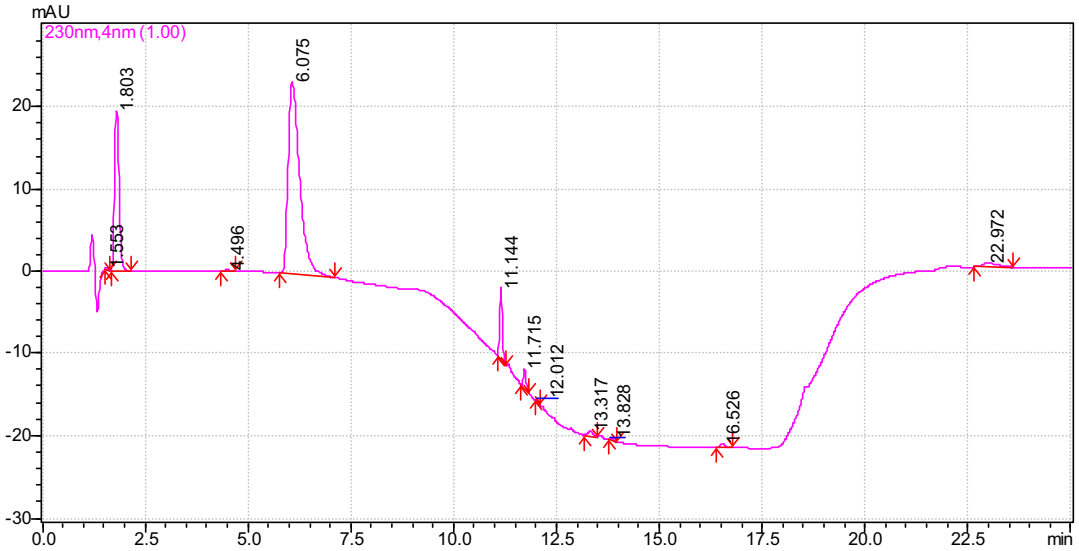


Figure 18 HPLC separations of 2 units of AdGSTD3-3 spiked with internal standard dimethoate 50,000 ng/ml and PBCOOH 110,000 ng/ml

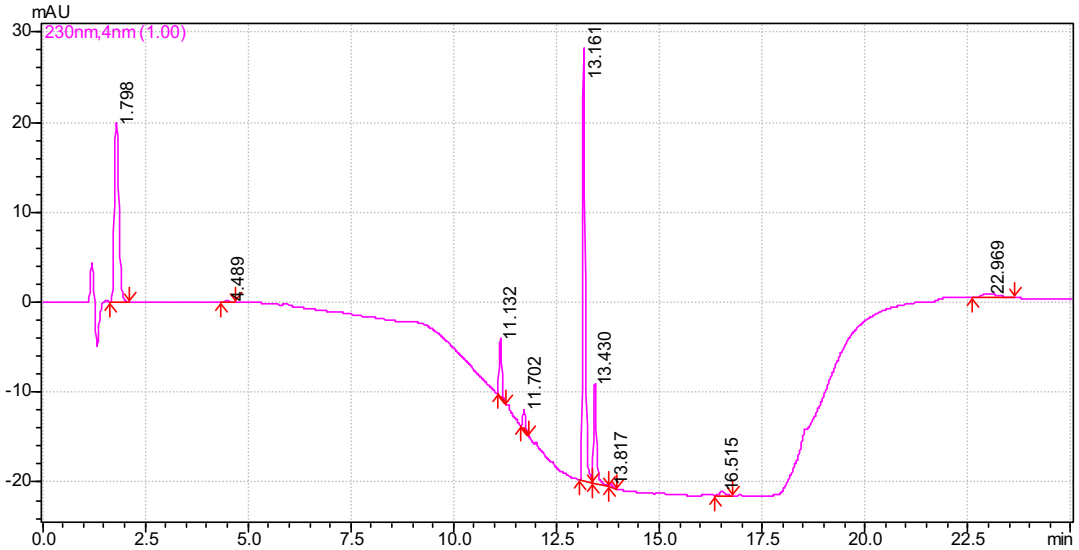


Figure 19 HPLC separations of 2 units of AdGSTD3-3 spiked with internal standard dimethoate 50,000 ng/ml and permethrin 110,000 ng/ml

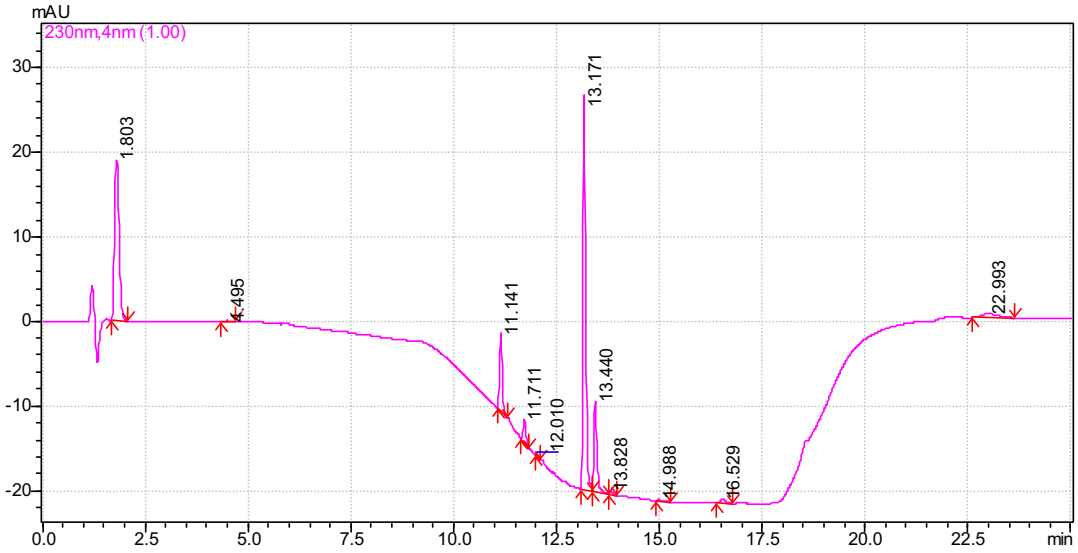


Figure 20 HPLC separations of 4 units of AdGSTD3-3 spiked with internal standard dimethoate 50,000 ng/ml and permethrin 110,000 ng/ml

4.6.4 Permethrin metabolism study

For permethrin metabolized assay, it was found that all delta, epsilon, theta and omega GSTs incubated for 2 hours had no different metabolic profile from sample blank, suggesting that no pyrethroid metabolite product was formed after the incubation (results not shown). Percent inhibitions of AcGSTE2-2, AcGSTT1-1 clone 2 ,clone3 and AcGSTO1-1 with the presence and absence of permethrin revealed less than 10% inhibition (results not shown) suggesting that there is no binding site for the pyrethroid insecticide.

4.7 DDT dehydrochlorinase activity

DDTase activity for epsilon enzymes was determined in triplicate including reagent blank and sample blanks. All the epsilon enzymes possess similar DDT dehydrochlorinase activity (Table10).

Table 10 Comparison of the DDT dehydrochlorinase activities among epsilon GSTs

Enzymes	**DDTase activity (nmolDDE/mg)**
AdGSTE1-1	5.38 ± 0.06
R139H	7.72 ± 0.49
AdGSTE3-3	Not determined
AdGSTE4-4*	2.56 ± 0.32

The DDT dehydrochlorinase activity data were obtained from AdGSTE1-1, R139H, AdGSTE4-4 by conversion of DDT to DDE and detected by high-performance liquid chromatography. Three independent assays were performed. The data of *An. dirus* AdGSTE4-4 has previously been reported, Charoensilp G. 2006.

To study the ability of AcGSTE2-2, AcGSTT1-1 clone 2 ,clone3 and AcGSTO1-1 enzymes to metabolize organochlorine insecticide, DDT dehydrochlorinase activity of all recombinant enzymes measured as nmol of DDE detected by HPLC analysis after incubation of 2 units of enzyme with 5 nmol of DDT. The data is illustrated in Table 11. The recovery of dicofol, an internal standard for chloroform extraction ranged from 85-95%. DDE was undetectable in the reagent blank and sample blank reactions, indicating that no DDE contamination in the system. Only AcGSTE2-2 possessed the DDT dehydrochlorinase activity. The DDTase activity was 2.7-15 fold different among the epsilon AcGSTs. A ratio of DDTase/CDNB activity demonstrated that the AcGSTE2-2 enzyme had a 275-352 fold greater DDT substrate preference compared with the *An. cracens* epsilon GSTs.

Table 11. DDTase activity of the Epsilon, Theta, and Omega GSTs from *An. cracens*

Isoenzyme	**DDTase activity**	**CDNB activity**	**DDTase/CDNB**
AcGSTE2-2	15.074 ± 0.926	1.710	8.81
AcGSTT1#2	ND	6.2	-
AcGSTT1#3	ND	1.4	-
AcGSTO1-1	ND	0.04	-
AcGSTE1-1	5.38 ± 0.06	207	0.025
AcGSTE3-3	ND	33.4	-
AcGSTE4-4	2.56 ± 0.32	79.9	0.032

The DDTase activity is expressed as nmol DDE formation/mg of enzyme protein. The CDNB specific activity is μmol/min/mg protein. ND means that the activity can not be detected

4.8 Temephos HPLC set up method

4.8.1. Testing organophosphate standard

GC-MS was used to identify known standard temephos and temephos sulfoxide. It was found that only temephos standard can be identified correctly with GC-MS whereas temephos sultoxide was failed to identify. Then, temephos, temephos sulfoxide and dicofol was individually run using an HPLC. To check the linearity of standard, the three standards were mixed and prepared in 5 serial concentrations. The mixed standards were analyzed by HPLC in 3 consecutive days. However, the linearity was obtained at some concentration ranges and the sulfoxide can not be used because cannot dissolve properly. In addition, temephos appeared as double peaks at some concentrations. Therefore temephos sulfoxide is discarded from the system. Mixed temephos and dicofol standards were prepared in five different concentrations and tested for linearity for 3 days. It was found that the peak of temephos was separated from dicofol, however, linearity of dicofol was not good and the recovery was low after chloroform extraction. Therefore pyrene was tested by mixing with temephos and gave separate bands (Figure 21). Chloroform extraction of temephos with pyrene gave 73-90% recovery. In conclusion, pyrene can be used as internal standard.

4.8.2 Temephos metabolite assay.

Metabolism of temephos was evaluated by measuring the disappearance of parent compound. It was found that disappearance of temephos was linear with respect to AcGSTD3-3 protein concentration from 2-120 units (Figure 22). Therefore the protein concentration equals to 120 unit, approximately 1.5 mg, was chosen and used with all samples. HPLC analysis demonstrated that temephos amount was decreased when it was added to the incubation mixture containing AcGSTE1-1, R139H variant, AcGSTD2-2, AcGSTD3-3 and AcGSTT1-1 GSTs (Figure 23). Of those, AcGSTD2-2 had an ability to decrease temephos with considerably higher rate than the others. To identify the temephos metabolized product, LC-MSD was employed. The UV chromatogram of AcGSTE1-1 and temephos standard had the same retention time at 5.05 minutes and similar UV spectrum that had the highest absorption at 250 nm sugesting similar properties (Figure 24). Based on a scanning results between 100 to 700 of an m/z unit , LC-MSD analysis demonstrated that both standard and AcGSTE1-1 incubating with temephos had identical spectra. The molecular mass of temephos were identified at 467.8, 466.8, 453, 340 and 186.2 m/z (Data not shown). No additional fragment ions, indicating temephos was not metabolized by GSTs, were observed in the

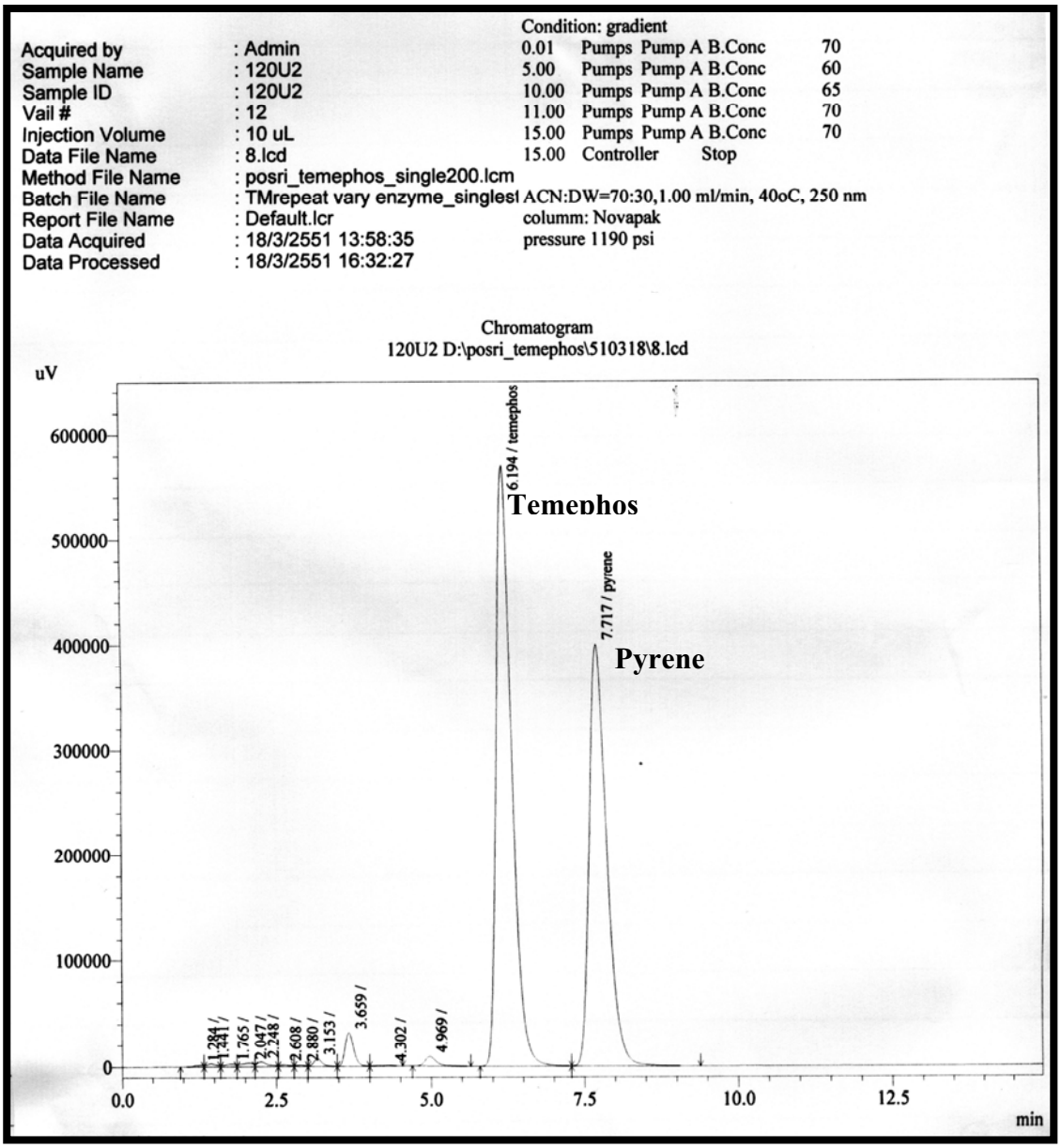


Figure 21 Chromatogram of temephos and pyrene

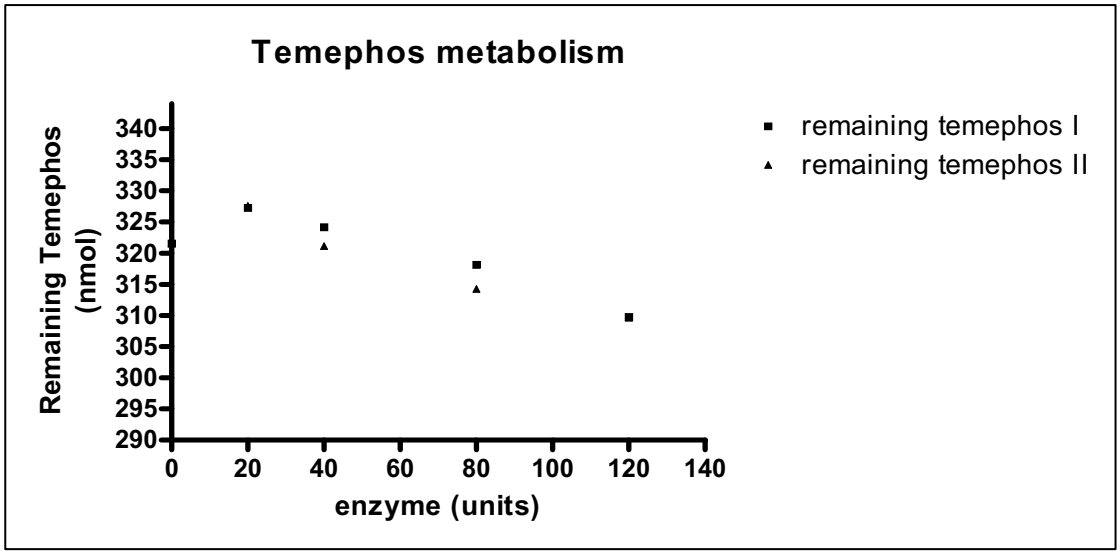
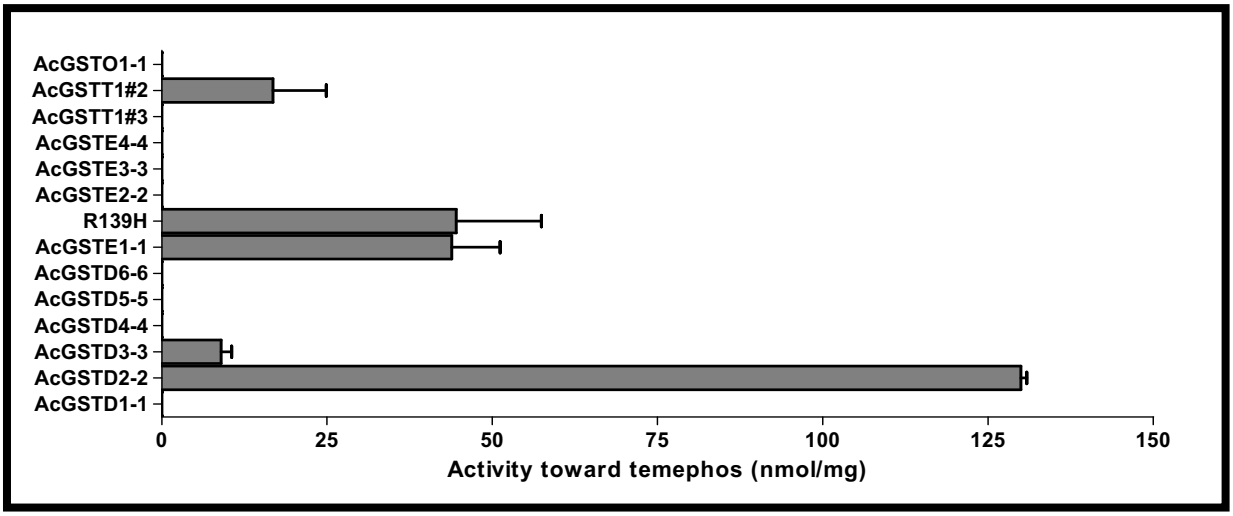


Figure 22: Remaining temephos using diffent amount of GST

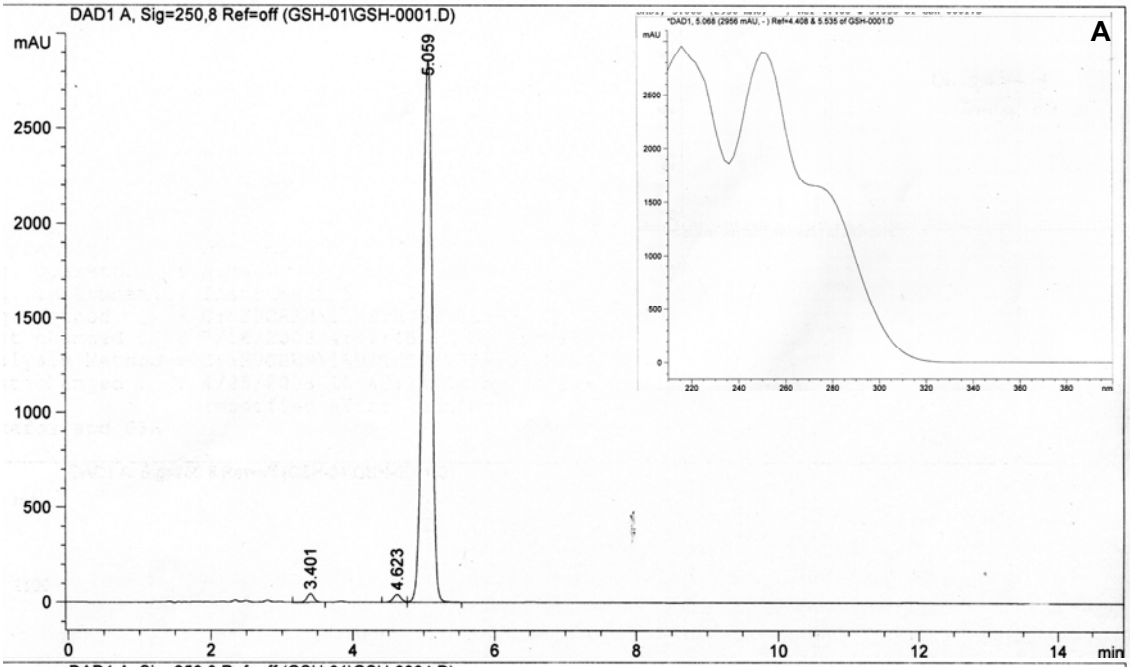
organic solvent. The aques phase was also deteced by LC-MSD and found relatively small peak of temephos suggesting that disapeared temephos was not in this part (Figure 25)

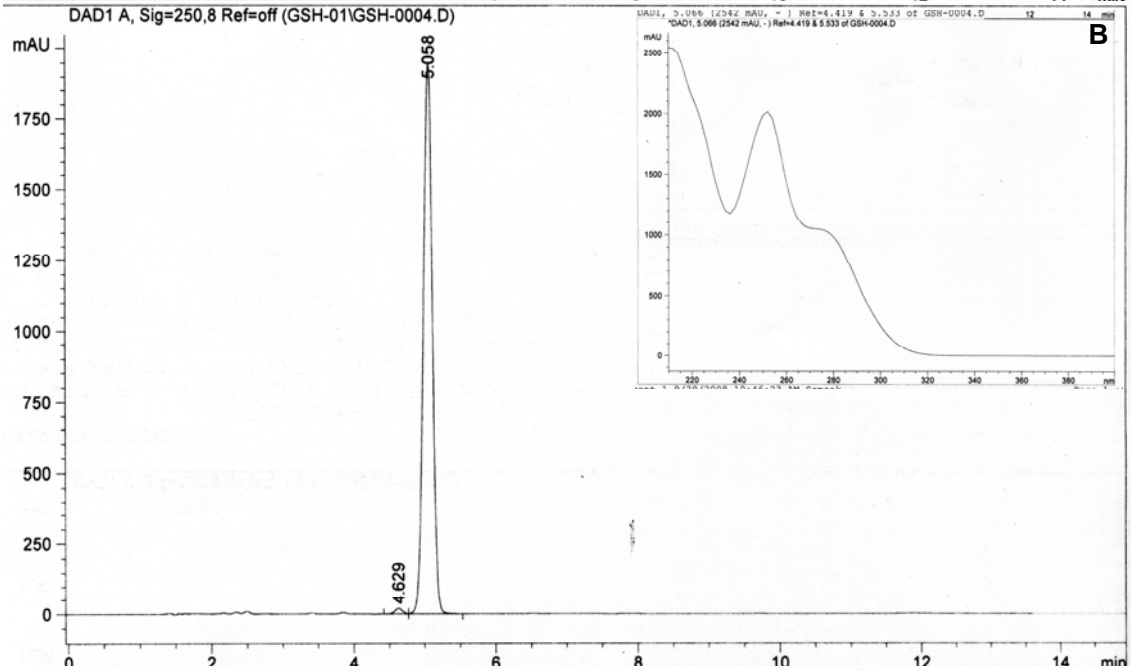
The disappeared insecticide was further studied by LC-MSD using the AcGSTE1-1 sample from the chloroform extracted part compared with the middle part consisting of denatured GST. The white pellet of AcGSTE1-1 in the middle layer was collected and resuspended in acetonitrile, filtered and injected into LC-MSD. In figure 26 ,the HPLC of the chloroform extract (5.232 minutes) had different retention time from the middle part extract (5.171 minutes). It meaned that the latter part had higher polarity compared to the chloroform extract therefore it was eluted eariler than the parent insecticide. Based on the UV spectrum (Figure 26), the suspension from the middle layer had the abilityto absorp UV at 250 nm like the chloroform extract suggesting that temephos may have an interaction with functional group from GST enzyme etc. and form a complex. Although, the MS pattern of the complex consisting of the molecular mass at 483.8 m/z, the overall MS pattern of the complex was different from the chloroform extract suggesting different structure compared to temephos standard (Figure 27). In conclusion GST may act as a binding protein and interacted with temephos and resulting in decreased temephos amount in chlroform extract. The complex had higher polarity than the parent compound. The interaction type between temephos and GSTs may be in hydrophobic and thereby exposing polarity functionl group into the surface of the complex, changing in solubility and ionizing pattern.

Figure 23. Activity of several Epsilon and Delta GSTs from *An. cracens* toward temephos



The activity toward temephos is expressed as nmol of reduced temephos/mg of enzyme protein. The CDNB specific activity is µmol/min/mg protein.


DAD1 A, Sig=250,8 Ref=off (GSH-01\GSH-0001.D)
mAU
5.059
3.401
4.623
min
A


DAD1 A, Sig=250,8 Ref=off (GSH-01\GSH-0004.D)
mAU
5.058
4.629
min
B

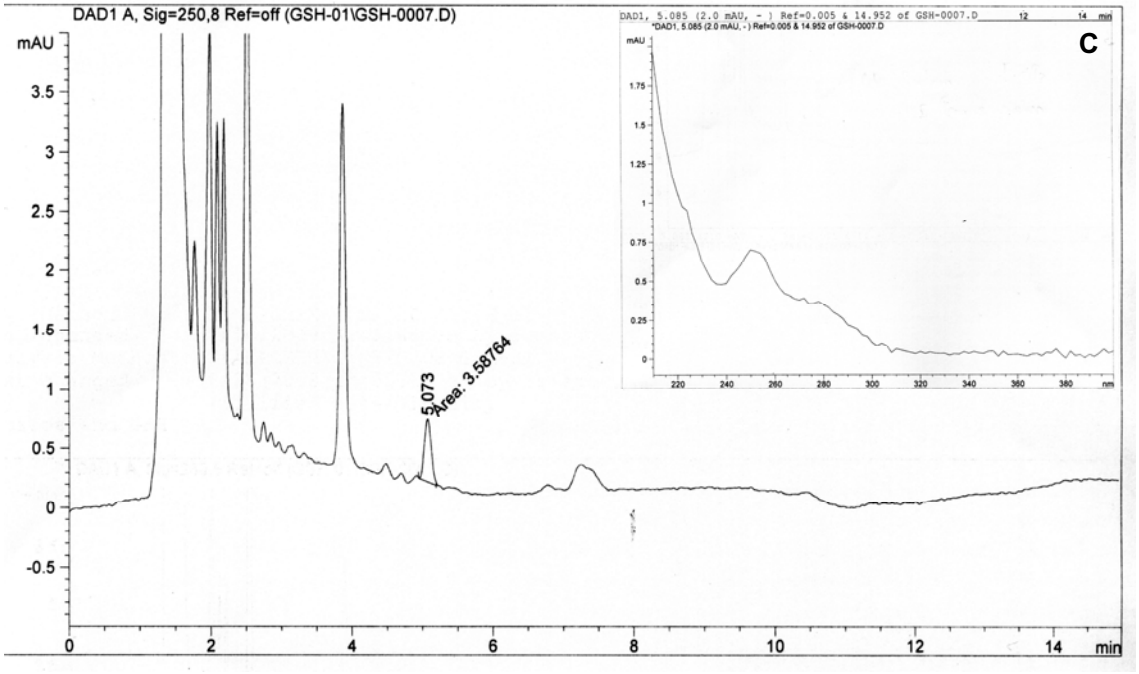
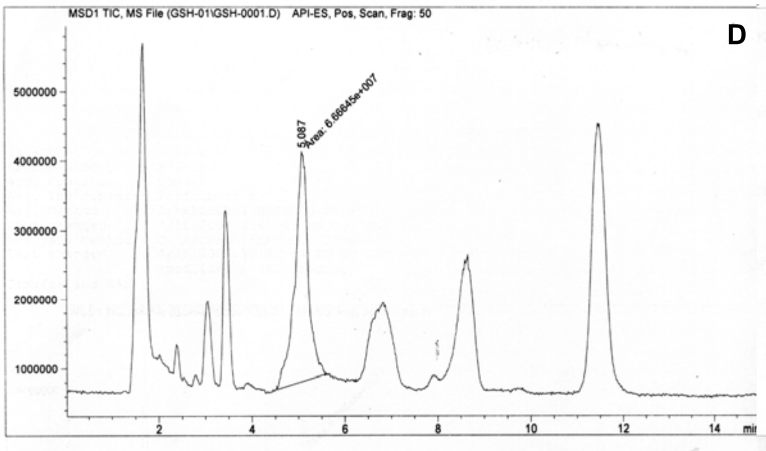
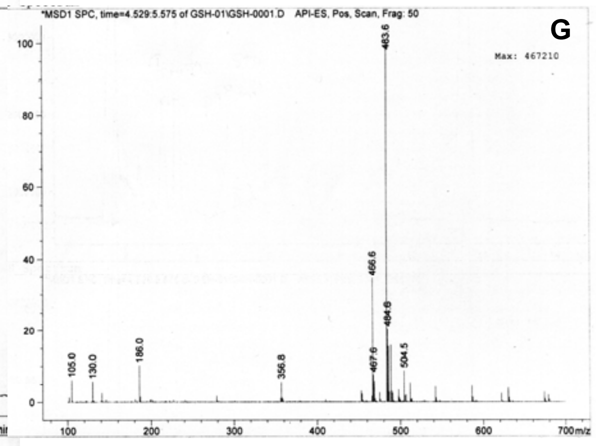
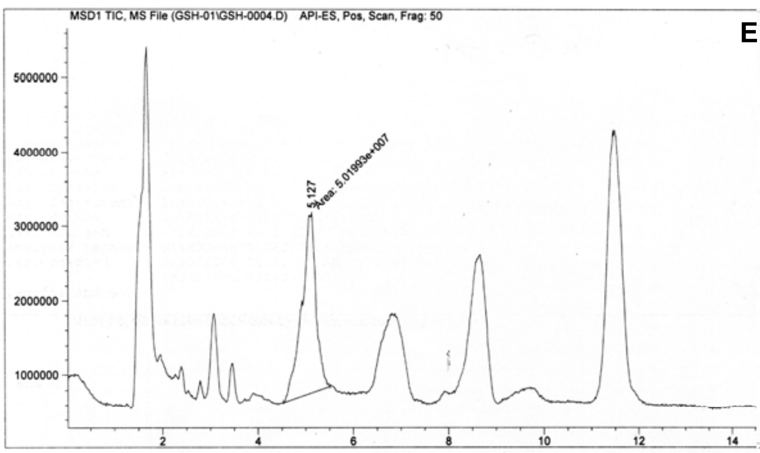
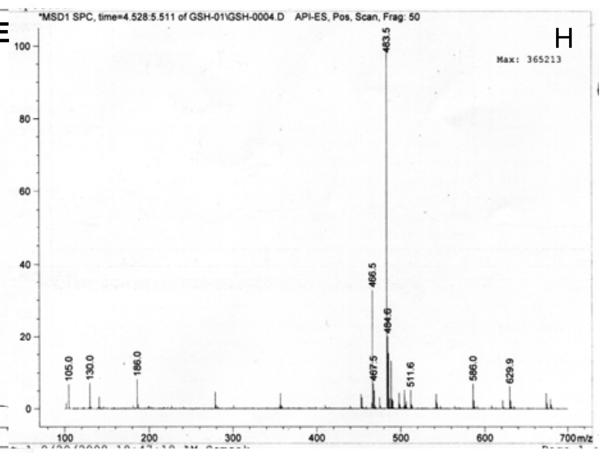

DAD1 A, Sig=250,8 Ref=off (GSH-01\GSH-0007.D)
mAU
5.073
Area: 3.58764
min
C

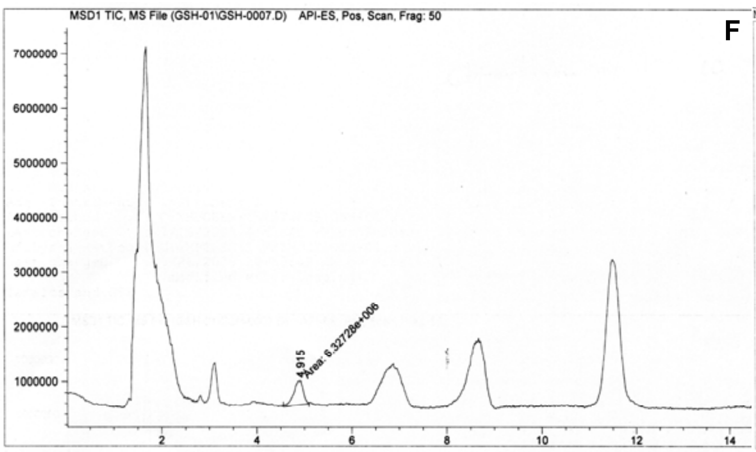
Figure24. LC-DAD chromatogram of standard temehos (A), chloroform extract of AcGSTE1-1 incubated with temephos (B) and aques layer (C) obtained at 250 nm. The isocratic mobile phase containing acetonitrile:water (70:30, v/v) and 0.1% (v/v) formic acid had a flow rate of 1.0 ml/min.


MSD1 TIC, MS File (GSH-01\GSH-0001.D) API-ES, Pos, Scan, Frag: 50
D
5.087
Area: 6.66645e+007
min


*MSD1 SPC, time=4.529:5.575 of GSH-01\GSH-0001.D API-ES, Pos, Scan, Frag: 50
G
Max: 467210
483.6
466.6
484.6
467.6
504.5
105.0
130.0
186.0
356.8
m/z


MSD1 TIC, MS File (GSH-01\GSH-0004.D) API-ES, Pos, Scan, Frag: 50
E
5.127
Area: 5.01993e+007


*MSD1 SPC, time=4.528:5.511 of GSH-01\GSH-0004.D API-ES, Pos, Scan, Frag: 50
H
Max: 365213
483.5
466.5
484.6
467.5
511.6
586.0
629.9
105.0
130.0
186.0
m/z


MSD1 TIC, MS File (GSH-01\GSH-0007.D) API-ES, Pos, Scan, Frag: 50
F
4.915
Area: 6.32728e+006

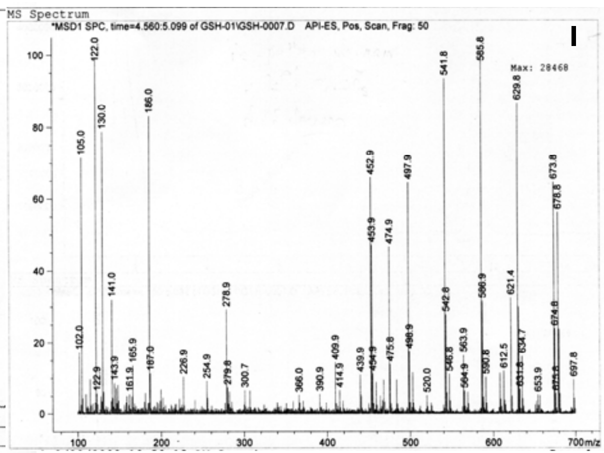

MS Spectrum
*MSD1 SPC, time=4.560:5.099 of GSH-01\GSH-0007.D API-ES, Pos, Scan, Frag: 50
I
Max: 28468
m/z

Figure 25: Identification of temephos (E: t_R 5.127) in chloroform extract of AcGSTE1-1 incubated with temephos compared with aques layer of AcGSTE1-1 incubated with temephos (F) compared with temephos standard (D) showing (D)-(F) total positive ion monitoring (100-700 m/z), (G-I) representatives fragmentation pattern from chloroform extract of AcGSTE1-1 incubated with temephos (H) and aques layer of AcGSTE1-1 incubated with temephos (I) compared with temephos standard (G)

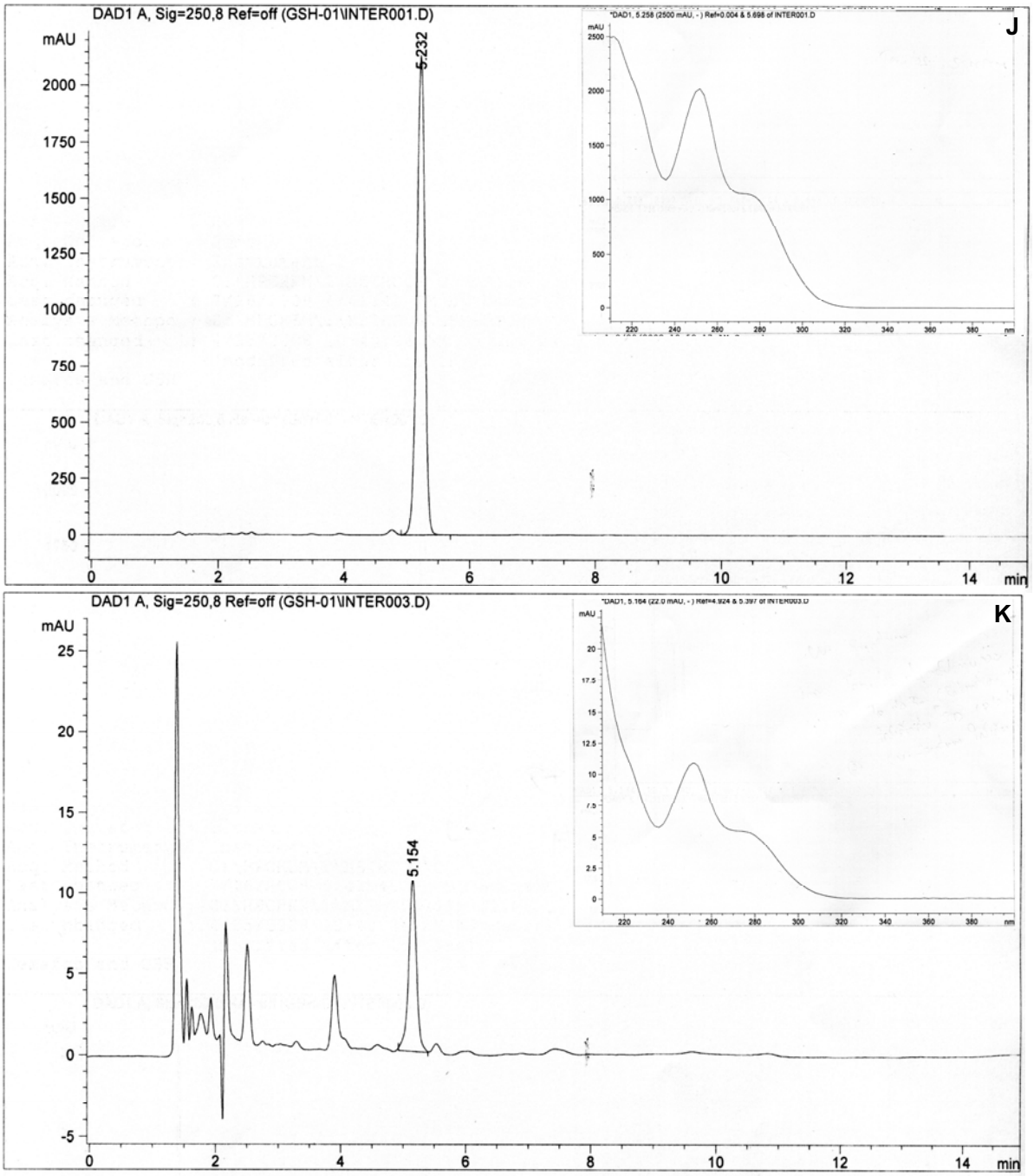

DAD1 A, Sig=250,8 Ref=off (GSH-01\INTER001.D)
mAU
5.232
min
J
DAD1 A, Sig=250,8 Ref=off (GSH-01\INTER003.D)
mAU
5.154
min
K

Figure26: LC–UV chromatograms of AcGSTE1-1 incubated with temephos obtained at 250 nm: (J) chloroform extract (K) after extract the white pellet in the middle layer between aques layer and chloroform with acetonitrile

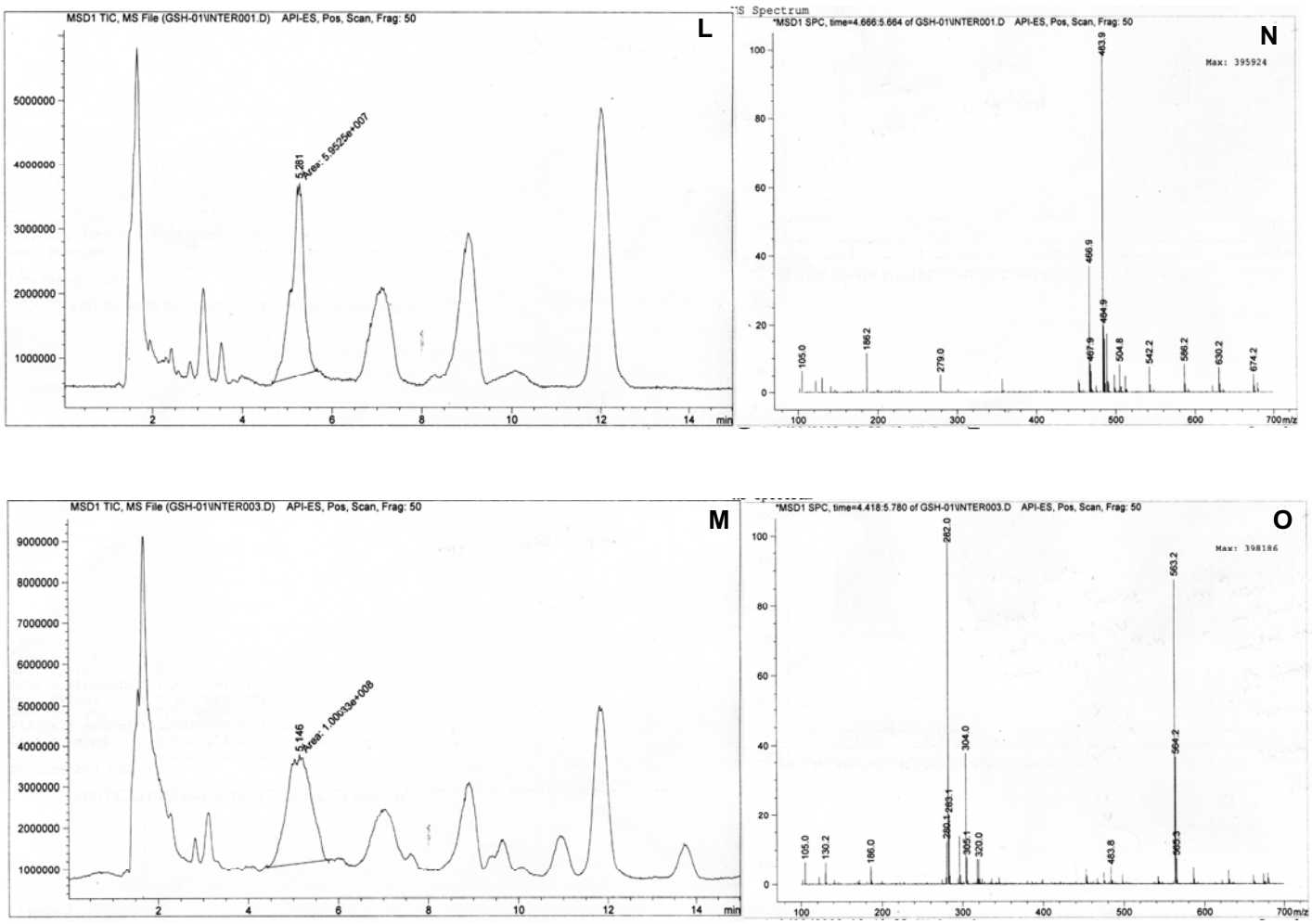


Figure 27: Identification of temephos (E: t_R 5.281) in chloroform extract of AcGSTE1-1 incubated with temephos compared with middle layer of AcGSTE1-1 incubated with temephos (M) showing (L)-(M) total positive ion monitoring (100-700 m/z), (N-O) representatives fragmentation pattern from chloroform extract of AcGSTE1-1 incubated with temephos (N) and middle layer of AcGSTE1-1 incubated with temephos (O).

4.9 Discussion and conclusion

To characterize a role of the enzyme in insecticide resistance including substrate specificity of several new Anopheles cracens, the recombinant AcGSTE1-1, Arg139His variant of AcGSTE1-1, AcGSTE3-3, AcGSTE4-4, AcGSTT1-1 and AcGSTO1-1 from the Thai malaria vector *An. cracens* were expressed and purified. Interestingly, the kinetic parameters of the acGSTE3-3 were different from GST 4a, previously identified by Prapanthadara (1). AcGSTE3-3 had lower binding affinity towards GSH but higher affinity towards CDNB. For the function in insecticide metabolism, AcGSTE3-3 had no DDT dehydrochlorinase activity whereas the GST 4a possessed 4.4 nmol/unit. The data suggests that even after several chromatography steps the GST 4a preparation most likely still consisted of a mixture of different GSTs with acGSTE3-3 possibly being the major one.

As orthologous enzymes acGSTE1-1 from *An. cracens* and agGSTE1-1 from *An. gambiae* share 72% amino acid identity and 84% similarity. However, the sequence changes yielded several substrate specificity differences between the two enzymes. For example, the enzyme from *An. gambiae*, agGSTE1-1, had a 54-fold lower V_{max} value compared to acGSTE1-1. Also unlike the *An. cracens* enzyme, the *An. gambiae* enzyme did not possess detectable DDT dehydrochlorinase activity (2). Interestingly, our acGSTE1-1 and acGSTE4-4 demonstrated 7-fold and 4.8-fold higher CDNB conjugating activity than agGSTE1-1 and agGSTE4-4 of *An. gambiae*. Changing of a positively charged guanidino group at position 139 to a positively charged imidazole group affected the substrate specificity of the enzyme. The mutation increased the enzymatic activity towards 4-nitrobenzyl chloride and dichloroacetic acid. With 4-nitrophenethyl bromide, the specific activity of Arg139His is about half of that observed in the acGSTE1-1 enzyme. acGSTE3-3 may be involved in oxidative stress of the insect because it demonstrated high activity toward 4-HNE. High activity of GST towards 4-HNE was reported in Alpha and Sigma class (3;4). For Epsilon class, the ability to utilize 4-HNE was first reported in DmGSTE1-1 (5). However, there is no information of residues involved in 4-HNE conjugation of GST Epsilon. In drosophila (6) there are two amino acids, Tyr 153 and Tyr 208 that were proposed to function in 4-HNE/GST interaction similar to the mechanism found in mammalian Alpha class (7). Based on the amino acid alignment of acGSTE3-3 and DmGSTS1-1 (8), it is likely that Tyr 153 and Thr 190 residues of acGSTE3-3 perform the same function in drosophila. Both residues are conserved in all Epsilon classes suggesting important functions. Additional evidence