Sains Malaysiana 45(7)(2016): 1057–1062

# Purification of Glutathione Transferases (GSTs) from Identified Rhizospheric Bacteria

(Penulenan Glutation Transferases (GSTs) daripada Bakteria Rizosfera Dikenal Pasti)

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ABSTRACT

*The glutathione S-Transferase (GST) enzyme plays an important role in cellular detoxification. This multifunctional enzyme is involved in Phase II detoxification pathways that protect cellular macromolecules from being attacked by harmful compound. The study is an attempt to isolate glutathione transferase-expressing bacteria from the rhizospheric soil of selected herbal plants. Screening showed nine positive isolates out of twelve bacterial samples from a large microbial population in our soil collection. Crude extract from strain E1 which was isolated from* Piper sarmentosum *(Kadok) showed the highest specific activity against 1-chloro-2, 4-dinitrobenzene substrates ($5.78 \times 10^{-06}$ µmol/min/mg). Based on the carbon utilization of E1 assessed using Biolog system, the strain was identified as* Comamonas testosterone *E1. Glutathione S-transferase purification using GST trap yielded two distinct subunits with molecular weights of 23 and 24 kDa as visualized on 1D SDS-polyacrylamide gel electrophoresis. The purified GST showed reactivity towards 1-chloro-2, 4-dinitrobenzene, 1, 2-dichloro-4-nitrobenzene and ethacrynic acid with specific activity of 0.264* ± 0.038 *nmol/min/mg and 0.056 ± 0.002 nmol/min/mg and 10.500 ± 3.130 nmol/min/mg, respectively. However, no activity was detected against p-Nitrobenzyl chloride, Sulfobromophthalein, trans-4-phenyl-3-butene-2-one, hexa-2, 4- dienal, trans-hepta-2, 4-dienal and trans-oct-2-enal in the study.*

*Keywords:* Comamonas testosterone*; GST-expressing bacteria;* Piper sarmentosum*; rhizospheric soil; xenobiotic*

ABSTRAK

*Enzim glutation S-Transferase (GST) memainkan peranan yang penting dalam detoksifikasi bersel. Enzim pelbagai fungsi ini terlibat dalam Fasa II laluan detoksifikasi yang melindungi sel makromolekul daripada diserang oleh sebatian berbahaya. Kajian ini merupakan cubaan untuk memencilkan bakteria glutation transferase-nyata dari tanah rizosfera beberapa tanaman herba. Daripada keseluruhan dua belas sampel bakteria dari populasi mikroorganisma yang terdapat dalam sampel tanah terkumpul yang diuji, sembilan sampel didapati berpotensi. Ekstrak mentah daripada strain E1 yang telah diasingkan daripada pokok herba* Piper sarmentosum *(Kadok) menunjukkan jumlah aktiviti spesifik yang tinggi terhadap substrat 1-chloro-2, 4-dinitrobenzena ($5.78 \times 10^{-06}$ µmol/min/mg). E1 dikenal pasti sebagai* Comamonas testosterone *berdasarkan penggunaan karbon yang dinilai menggunakan sistem Biolog. Penulenan glutation S-transferase (GST) menggunakan perangkap GST menghasilkan dua subunit berbeza dengan berat molekul masing-masing 23 dan 24 kDa seperti ditunjukkan dalam 1D SDS-poliakrilamida gel elektroforesis. GST tulen menunjukkan reaktiviti terhadap 1-kloro-2, 4-dinitrobenzena (CDNB), 1, 2-dikloro-4-nitrobenzena (DCNB) dan asid etakrinik (EA) iaitu dengan jumlah aktiviti spesifik masing-masing sebanyak 0.264 ± 0.0382, 0.056 ± 0.002 dan 10.500 ± 3.130 nmol/min/mg. Walau bagaimanapun, aktiviti terhadap p-Nitrofenil klorida (NBC), sulfobromoftalein (BSP), trans-4-fenil-3-butena-2-satu (PBO), hexa-2, 4- dienal, trans-hepta-2, 4-dienal dan trans-oct-2-enal tidak dikesan dalam kajian ini.*

*Kata kunci:* Comamonas testosterone*; bakteria GST-nyata;* Piper sarmentosum*; tanah rizosfera; xenobiosis*

## INTRODUCTION

One of the large protein super-families with a major role in cellular detoxification is the glutathione S-Transferase (GST) family of enzymes. This multifunctional enzyme is involved in Phase II detoxification pathways that protect cellular macromolecules from being attacked by reactive electrophiles (Arakawa et al. 2012). In order to overcome harmful hydrophobic toxic compounds, GST catalyzes the conjugation of tripeptide gluthathione (GSH) into a wide range of electrophilic compounds. The enzyme catalyzes the nucleophilic attack of the sulfur atom of glutathione (-SH) on the electrophilic groups of hydrophobic toxic compounds, thus increasing their solubility and rendering them water soluble. The process promotes their excretion out of the cell (Habig et al. 1974).

GST is widely distributed in nature and can be found present in both eukaryotes and prokaryotes. Living organisms such as mammals, plants, fish, birds and insects were mostly studied to detect the presence of this enzyme (Sherratt & Hayes 2001). Studies on bacterial GSTs were limited but more recent research showed the discovery of GSTs in many kinds of microorganisms including *Escherichia coli*, *Proteus mirabilis* and *Rhizobium*. According to Atkinson and Babbitt (2009), GST enzymes

were divided into different classes (beta, theta, zeta, sigma, pi and other classes) depending on their substrate specificities. Each class of the enzyme displays distinct and broad substrate profiles with some classes exhibiting overlapping substrate recognition. The expression of GST enzymes can be induced by both natural compounds; and compounds from xenobiotic origin were not expected to be present in the system of living organisms such as antibiotic drugs.

Early reports showed the presence of GSTs in higher eukaryotes. GSTs can bind non-catalytically a large number and a diverse range of endogenous and exogenous compounds. Through the binding process, eukaryotic GSTs induce transformation of electrophilic compounds, inactivate their reactivity and later promote excretion and detoxification of both endogenous and exogenous electrophilic substrates (Vuilleumier 1997). The removal of the harmful compounds out of the cell involves formation of corresponding glutathione conjugates.

Compared to eukaryotes, information on prokaryotic GSTs was limited. Allocati et al. (2008) reported the discovery and role of bacterial GSTs in protection against chemical and oxidative stress as well as providing antimicrobial drug resistance. Some GSTs (for example dichloromethane dehalogenase), were involved in metabolic processes associated with detoxification processes. Dehalogenases play a role in the biodegradation of xenobiotic compounds, therefore the presence of these catabolic enzymes allowed the growth of bacteria on recalcitrant chemicals (Copley 1998; Vuilleumier & Pagni 2002). In view of this scenario, GST was expected to be widely spread among organisms that utilize polycyclic aromatic hydrocarbon (PAH) compounds (Lloyd-Jones & Lau 1997).

Evidence has suggested that GST activity towards 1-chloro-2,4-dinitobenzene (CDNB) was relatively higher than that toward other substrates commonly used in GST research. Therefore, CDNB was used here as a standard substrate for screening the enzyme in crude bacterial extracts (Vuilleumier 1997). Zablotowicz et al. (1995) showed the occurrence of GST among rhizospheric bacteria. High levels of GST activity toward CDNB was found in Gram negative bacteria compared to Gram positive bacteria. In the present study, GST was isolated, screened and purified from soil rhizospheric bacteria isolated from Piper sarmentosum. The enzyme isolated from the selected strain was characterized and further analyzed.

## Materials and Methods

### Chemicals

Unless otherwise stipulated, chemicals employed were of the highest grade obtainable. Dithiothreitol and phenylthiourea were obtained from GE Healthcare. Centrifugal concentrators were obtained from Vivascience (Gottingen, Germany). Acrylamide, bis-acrylamide, Biolyte ampholyte solution and concentrated Coomassie Brilliant Blue protein reagent were obtained from BioRad Laboratories, Hercules, CA, USA. For isoelectric focusing, IPG strips were obtained from GE Healthcare. Buffer components were purchased from Sigma Chemical Co., St Louis, USA.

### Sampling

Rhizospheric soil samples of various herbal plants were collected from the botanical garden at the Institute of Biological Sciences, University of Malaya. Soil surrounding the plant roots was sampled at a depth of 10-15 cm from the ground surface (Nasrin & Rahman 2007). The herbal plants used in this study consisted of *Curcuma longa* (Kunyit), *Zingiber officinale* var rubrum (Halia Bara), *Piper sarmentosum* (Kadok), *Phyllanthus niruri* Linn. (Dukung Anak), *Orthosiphon stamineus* (Misai Kucing) and *Zingiber cassumunar* (Bonglai).

### Isolation, Screening and Identification of GST-Expressing Bacteria

One g of soil sample from different herbs was weighed and added into 9 mL of sterile distilled water. Serial dilution was then carried out. The soil cultures were streaked onto tryptic soy agar and incubated for 24 h at 25°C. The colonies were each purified and stock cultures maintained at -20°C in glycerol broth suspensions. All purified isolates were observed and distinguished according to their morphology and primarily identified using Gram staining (Beveridge 2001) and KOH test. Well-separated colonies were purified on the same medium and maintained for screening of GST-expressing bacteria.

Referring to a method by Eklund et al. (2002) monochlorobimane (MCB) was dissolved in acetonitrile at a concentration of 1 mg/mL (40 mM). The solution was stored at –20°C and protected from light to avoid photolytic decomposition of MCB. To screen for GST-expressing bacteria, the MCB solution was sprayed over each agar plate containing the bacterial isolates. Colonies expressing GSTs were fluoresced under UV light (365 nm). Isolates with intense fluorescence were then grown, sediment and lysed. The crude homogenates were tested for GST activity and positive isolate with highest GST conjugating activity was selected. The isolate was identified based on carbon utilization (BIOLOG) using GEN III microplate which was analyzed according to Biology Microbial ID System Database.

### Purification of Glutathione Transferases

A single colony of *Commamonas testosteroni* E1 was picked and grown in tryptic soy broth (TSB) for 48 h and pelleted by centrifugation at 10000 × g for 15 min. This process was followed by washing with eluting buffer (25 mM sodium phosphate buffer, pH7.4). The cells were then re-suspended in buffer solution containing eluting

buffer, protease inhibitor (Sigma), 1.0 mM EDTA, 0.1 mM DTT and lysozyme (0 µg/mL). The suspended cells were disrupted by sonication (Powersonic 603) for 20 min at 4ºC and centrifuged at 100000 × g for 30 min at 4ºC. The supernatant was applied to a GSTrap™ HP (5 mL) column (GE Healthcare) which was connected to an ÄKTA Purifier FPLC equipped with UNICORN software Version 5.1 and a fraction collector (Frac900). The column was pre-equilibrated with eluting buffer and flow rate was set at 0.3 mL/min. The enzymes were eluted with sodium phosphate buffer containing 10 mM of glutathione (GSH). The purified enzyme were used immediately for substrate specificity determination or kept at -20°C until further analysis.

### PROTEIN DETERMINATION

Protein concentration was determined according to Bradford assay using Coomassie Brilliant Blue R-250 and bovine serum albumin as the standard (Kruger 2009).

### MOLECULAR WEIGHT DETERMINATION

Electrophoresis was then run on 12% polyacrylamide gel in the presence of 0.1% SDS at 150 V. Gels were stained with Colloidal Coomassie Blue G-250 (Sedmak & Grossberg 1977). The stained gels were scanned with Image Scanner III (GE Healthcare) and visualized with Image Master Software.

### SUBSTRATE SPECIFICITIES

Enzymatic assays with 1-Chloro-2, 4-dinitrobenzene (CDNB), ethacrynic acid (EA), sulfobromophthalein (BSP), p-nitrobenzyl chloride (NBC) and trans-4-phenyl-3-butene-2-one (PBO) and 3, 4-Dichloronitrobenzene (DCNB) were determined according to Habig et al. (1974). Assays were also done using hexa-2, 4-dienal, trans, trans-2, 4-heptadienal and trans-oct-2-enal according to the method by Brophy et al. (1989).

## RESULTS AND DISCUSSION

### SOIL BACTERIA FROM DIFFERENT HERBAL PLANTS

Twelve different colonies of bacteria were isolated from rhizospheric soil of various herbal plants. The colonies comprised of Gram negative and Gram positive isolates with different morphological characteristics (Table 1). This study focused on Gram negative isolates as CDNB-GST activity has predominantly been detected in Gram negative strains that possess mostly active GSH thiols, either in its oxidized or reduced forms (Zablotowicz et al. 1995).

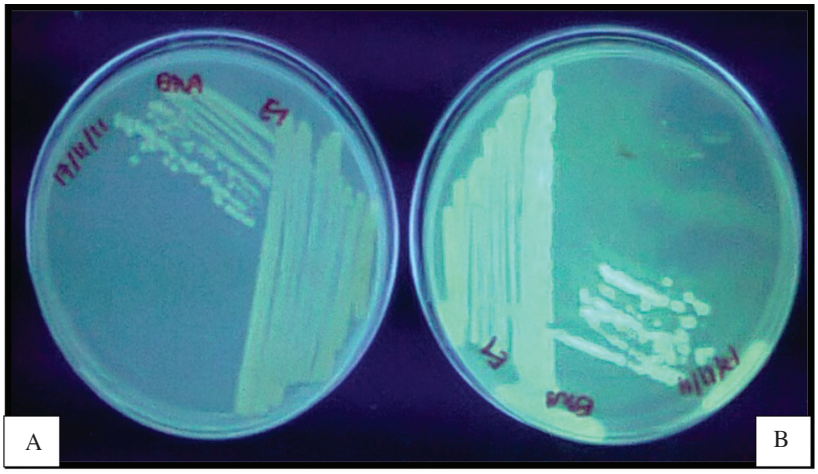
Xenobiotic degradation widely occurred in rhizospheric soil because of the higher population of microbes living in rhizospheres compared to root-free soil. Soil microorganisms were believed to play important roles in detoxification of soil contaminants to support growth of plants. They enhanced soil fertility and increased the uptake of nutrients by plants (Khalvati & Dincer 2013). Considering the role of soil microorganisms in detoxification metabolism, microbial GSTs was expected to occur in rhizospheric bacteria. The microorganisms showed resistance towards toxic compounds and continued to grow due to the presence of intracellular GSTs. It was the enzyme that degrade harmful compounds thus preventing killing of the bacterial community.

### SCREENING AND IDENTIFICATION OF GST-EXPRESSING BACTERIA

All cultured bacteria were tested for the presence of GSTs by spraying monochlorobimane (MCB) solution onto the culture medium (Figure 1). Spraying of the chemical onto the bacterial culture resulted in the production of fluorescence from nine different isolates. However, intensity of fluorescence differed between each isolate. Three isolates; B4, C and D3 were recognized as isolates with non-expressing GSTs because they did not fluoresce upon spraying of MCB solution. Isolates A, B1, B2, and B3 displayed strong fluorescence immediately after being

TABLE 1. Morphological characteristics of microbial isolates

| Rhizosphere | Isolate | Observation | | Bacteria strain |
|---|---|---|---|---|
| | | Gram staining | KOH test | |
| *Orthosiphon stamineus* | A | Pink bacilli | Positive | Negative |
| *Curcuma longa* | B1 | Purple rod | Negative | Positive |
| | B2 | Purple rod | Negative | Positive |
| | B3 | Purple rod | Negative | Positive |
| | B4 | Purple cocci | Negative | Positive |
| *Zingiber officinale* var. *rubrum* | C | Purple rod | Negative | Positive |
| *Phyllanthus niruri* | D1 | Purple rod | Negative | Positive |
| | D2 | Purple rod | Negative | Positive |
| | D3 | Purple rod | Negative | Positive |
| | D4 | Purple hyphae-like | Negative | Positive |
| *Piper sarmentosum* | E1 | Pink bacilli | Positive | Negative |
| | E2 | Purple rod | Negative | Positive |




Indicators:
A- Colour of colonies before sprayed with MCB
B- Colour of colonies after sprayed with MCB

FIGURE 1. Colour of colonies before and after sprayed with MCB solution

sprayed with MCB while isolates D1, D2, E1 and E2 took about 2-3 min to fluoresce following spraying (Table 2). All isolates were considered positive for having GSTs based on the fluorescence produced when viewed under UV light (wavelength of 365 nm).

Monochlorobimane (MCB) is a substrate commonly used to detect the presence of GST in bacteria. The production of fluorescence was due to formation of glutathione conjugates with fluorogenic MCB substrate which was catalyzed by intracellular GSTs (Eklund et al. 2002). Isolates that displayed fluorescence (A, B1, B2, B3, D1, D2, D4, E1 & E2) indicate high reactivity towards the MCB substrate. The non-fluorescing isolates (B4, C & D3) were colonies which were poorly reactive with monochlorobimane but may show strong reactivity with other substrates.

Crude extracts from the nine potential isolates were further tested for GST activity using CDNB as a substrate. CDNB was widely used as a standard substrate for detection of bacterial GST (Vuilleumier & Pagni 2002). Not all MCB-reactive isolates were found to react with CDNB. Different rates of reaction were observed between crude samples with the highest CDNB-GST activity detected in crude E1 (0.00578 nmol/min/mg). Low CDNB-GST activity was recorded from isolates B1, D4 and E2 displaying 0.00042, 0.00016 and 0.00027 nmol/min/mg of activity, respectively.

Isolate E1 was further identified using semi-automated MicroStation™, a rapid method of identification and characterization based on carbon utilization pattern (Stefanowicz 2006). From the analysis, isolate E1 was

TABLE 2. Intensity of bacterial fluorescence after sprayed using monochlorobimane solution (MCB)

| Rhizosphere | Isolate | Intensity of fluorescence |
|---|---|---|
| *Orthosiphon stamineus* | A | ++++ |
| *Curcuma longa* | B1 | ++++ |
| | B2 | ++++ |
| | B3 | ++++ |
| | B4 | ND |
| *Zingiber officinale* var. *rubrum* | C | ND |
| *Phyllanthus niruri* | D1 | ++ |
| | D2 | ++ |
| | D3 | ND |
| | D4 | ++++ |
| *Piper sarmentosum* | E1 | ++ |
| | E2 | ++ |

Indicators:
++++ Strong fluorescent
++ Modest
ND No detection

not able to metabolize all the added sugars and hexose phosphate. However, the species was able to utilize some amino acids, hexose acids and a variety of carboxylic acids, esters, including fatty acids. This bacterium tolerated pH5-7 range and was able to grow in 1% NaCl. According to Biolog Microbial ID System Database, isolate E1 was identified as *Comamonas testosterone* (Insam & Rangger 1997).

### ENZYME PURIFICATION AND CHARACTERIZATION

The purified intracellular enzyme of *C. testosteroni* was reacted with CDNB, DCNB and EA as substrates. The specific activity towards CDNB, DCNB and EA was recorded as 0.264 ± 0.0382, 0.056 ± 0.0016 and 10.5 ± 3.13 nmol/min/mg, respectively (Table 3).

TABLE 3. Specific activity of purified enzyme towards different substrates

| Substrate | Specific activity ( nmol/min/mg) |
|---|---|
| CDNB | 0.264 ± 0.038 |
| DCNB | 0.056 ± 0.001 |
| EA | 10.50 ± 3.130 |
| NBC | ND |
| PBO | ND |
| BSP | ND |
| Hexa-2,4-dienal | ND |
| trans- hepta-2,4-dienal | ND |
| trans- oct-2-enal | ND |

*Note*: ND – No detection

The reactivity of the GST enzyme towards CDNB indicates its susceptibility to a wide range of diseases and resistance against several antibiotic drugs. Reactivity with CDNB is commonly related to the presence of the beta class GSTs. Beta class GST was found to be able to react with 1-chloro-2, 4- dinitrobenzene (CDNB) substrate which resulted in the formation of glutathione conjugate. To date, homodimeric beta class GSTs have been reported present in *E. coli* and *P. mirabilis* (Allocati et al. 2009). Reactivity with ethacrynic acid (EA) showed the ability of the enzyme to enhance biochemical reaction of alkylating drugs towards drug resistant cells (Iersel et al. 1996). The ability of the enzyme to conjugate EA and DCNB suggested the presence of pi and mu class GSTs (Arakawa et al. 2012) in the bacterial enzyme.

The molecular weight of purified GST that was concentrated using protein concentrator was determined using SDS-PAGE. Figure 2 shows two bands with different molecular weights on the polyacrylamide gel. The purified GST was seemingly a heterodimeric protein based on the formation of two separate bands with molecular weights of 23 and 24 kDa for each subunit. Many reports indicated that subunit molecular weight of GSTs were within the 20-28 kDa range. The formation of two distinct molecular weight bands was considered as protein of interest because it can be induced by stress conditions or action by the hormone system (Chronopoulou & Labrou 2009). Other factors that may contribute to the heterodimeric form include non-specific binding protein during the affinity chromatography that tends to trap unwanted proteins (Skopelitou & Labrou 2010).

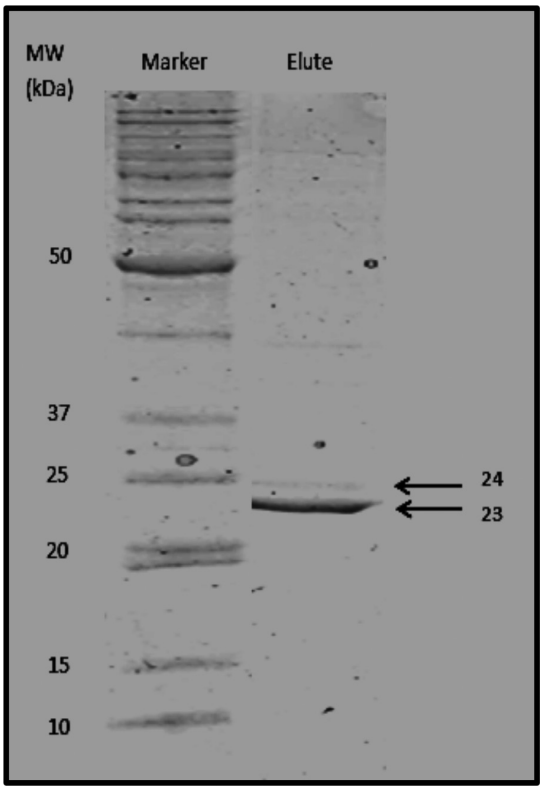



FIGURE 2. SDS-PAGE showed two bands with different molecular weight from purified GST isolated from *Comamonas testosterone*

## CONCLUSION

Rhizospheric bacteria identified as *C. testosteroni* were successfully isolated from *Piper sarmentosum* (Kadok). Using affinity chromatography, the present study showed the presence of beta class GST in *Comamonas* sp. active towards CDNB substrates. The formation of heterodimeric protein is of considerable interest as it is normally induced under stress conditions. Research is in progress to further characterize the purified protein and to study the potential use of the enzyme.

### ACKNOWLEDGEMENTS

This work was funded by the University of Malaya Postgraduate Research Fund (PPP: PV057/2013A), UMRG048 11BIO and the Ministry of Higher Education under the Fundamental Research Grant (FRGS) (FP020/2013A).

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Received: 12 March 2015
Accepted: 27 January 2016