UNIVERSITI PUTRA MALAYSIA

PARTIAL PURIFICATION AND CHARACTERIZATION OF GLUTATHIONE S-TRANSFERASES FROM KEDAH-KELANTAN CATTLE (BOS INDICUS) AND WATER BUFFALO (BUBALUS BUBALIS) LIVER

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By

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Thesis submitted to the School of Graduate Sudies, Universiti Putra Malaysia, in Fulfilment of the Requirement for the Master of Science

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For dearest family and friends

'...man will occasionally stumble over the truth, but usually manages to pick himself up, walk over or around it, and carry on.'

Churchill, Winston S.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Master of Science

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April 2006

Chairman: **Professor Nor Aripin Shamaan, PhD**

Faculty: **Biotechnology and Biomolecular Sciences**

Biotransformation and detoxification process in living organisms consists of two phases, phase I and phase II. Phase I involves in the introduction of functional group into molecule while the phase II involves the conjugation of phase I metabolites. In phase II, glutathione S-transferases (GSTs; EC 2.5.1.18) has aroused much interest because of its involvement in the biotransformation and detoxification of wide spectrum of xenobiotics which can be from pesticides, herbicides and insecticides. The present study was undertaken to purify and characterized cytosolic GSTs from livers of Kedah-Kelantan cattle (*Bos indicus*) and Malaysia water buffalo (*Bubalus bubalis*). The glutathione S-transferases were isolated from two important livestock livers, Kedah-Kelantan cattle (*Bos indicus*) and Malaysian water buffalo (*Bubalus bubalis*) by glutathione affinity chromatography. The affinity-glutathione chromatography successfully purifies the GSTs isoenzymes with 14.73% yield (62.77 purification fold) and 19.71% yield (20.44 purification fold) for KK cattle and water buffalo livers respectively. Initial methods of purification included centrifugation and ultracentrifugation. The affinity elution with

highest activity towards CDNB was estimated for the pI values using isoelectric focusing method via LKB-8100 ampholyte type (LKB Bromna) apparatus. pI values for affinity purified KK cattle liver are 5.7 (C-34), 6.9 (C-38) and 8.8 (C-42). While for the water buffalo liver, the pI values for glutathione affinity purified isoenzymes are 6.85 (B-23) and 7.2 (B-24). The isoenzymes were then tested using SDS-PAGE method for purity and also to estimate the molecular weight estimation. It has been estimated that molecular weight for water buffalo isoenzymes of B-23 was 29.3 ± 0.05 kDa and B-24 was 30.74 ± 0.16 kDa. The KK cattle liver isoenzymes molecular weight was estimated with C-34 was 29.9 ± 0.14; C-38 was 28.3 ± 0.09 and 27.7 ±0.03 for C-42. The study showed that KK cattle liver GSTs exist as isoenzymes (pI 8.8, 6.9 and 5.7), and have high activity towards CDNB, low towards DCNB and no activity towards the ethacrynic acid for the substrate specificities. On the other hand, the water buffalo liver GSTs exist as isoenzymes with pI 6.85 and 7.2. For the substrate specificities, the isoenzymes also have high activity for CDNB, but low for DCNB and could not be detected for the ethacrynic acid.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PENULENAN DAN PENCIRIAN GLUTATHIONE S-TRANSFERASES SEPARA DARI HATI LEMBU KEDAH-KELANTAN (*Bos indicus*) DAN KERBAU (*Bubalus bubalis*)

Oleh

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Proses biotransformasi dan detoksifikasi di dalam organisma hidup merangkumi dua fasa; fasa I dan fasa II. Fasa I melibatkan penambahan kumpulan berfungsi kepada molekul asing manakala fasa II melibatkan konjugasi metabolit fasa I. Dalam fasa II, glutathione S-transferases telah (GSTs;EC 2.5.1.18) telah menarik minat saintis dengan kaitannya dalam biotransformasi dan detoksifikasi bagi sebahagian besar xenobiotik yang biasanya boleh didapati dari racun perosak. Kajian ini dijalankan untuk menulen dan mencirikan GST sitosolik dari hati lembu lembu Kedah-Kelantan (KK) (*Bos indicus*) dan kerbau Malaysia (*Bubalus bubalis*). Glutathion S-transferase telah ditulenkan dari hati dua ternakan penting, lembu Kedah-Kelantan (KK) (*Bos indicus*) dan kerbau Malaysia (*Bubalus bubalis*), dengan menggunakan teknik kromatografi affiniti-glutathione. Kromatografi affiniti-glutathion ini telah berjaya menulenkan isoenzim glutathion S-transferase dengan hasil penulenan sebanyak 14.73% dan 62.7 kali tahap penulenan bagi lembu Kedah-Kelantan, manakala 19.71% hasil penulenan dan 20.44 kali tahap penulenan telah berjaya didapati daripada hati kerbau. Langkah

awal penulenan adalah termasuk teknik pengemparan dan ultrapengemparan. Elusi affiniti yang mempunyai aktiviti enzim yang tertinggi terhadap substrat CDNB telah dianggar bagi nilai pI dengan menggunakan kaedah 'isoelectric focusing' dengan menggunakan alat LKB-8100 jenis 'ampholyte' (LKB Bromna). Nilai pI bagi lembu KK yang ditulenkan adalah 5.7 (C-34), 6.9 (C-38) dan 8.8 (C-42). Manakala bagi hati kerbau, nilai pI bagi isoenzim yang ditulenkan adalah 6.85 (B-23) dan 7.2 (B-24). Isoenzim yang didapati telah diuji dengan menggunakan kaedah SDS-PAGE bagi menganggarkan ketulenan dan berat molekul. Telah dianggarkan bahawa berat molekul bagi isoenzim dari hati kerbau adalah B-23 adalah 29.3 ± 0.05 kDa dan B-24 adalah 30.74 ± 0.16 kDa. Bagi isoenzim hati lembu KK dianggarkan berat molekul; C-34 (29.9 ± 0.14 kDa), C-38 (28.3 ± 0.09 kDa) dan 27.7 ± 0.03 kDa bagi isoezim C-42). Mengikut pemerhatian yang dilakukan GST dari hati lembu KK wujud dalam bentuk isoenzim dengan nilai pI 8.8, 6.9 dan 5.17 dan mempunyai aktiviti enzim yang tinggi terhadap substrat CDNB dan rendah terhadap DCNB dan tiada aktiviti terhadap substrat asid 'ethacrynic' bagi ujian substrat spesifik. Manakala bagi GST yang ditulenkan daripada hati kerbau wujud dalam bentuk isoenzim dengan pI 6.85 (B-23) dan 7.2 (B-24). Bagi penentuan kadar substrat spesifik, isoenzim B-23 dan B-24 tidak menunjukkan sebarang aktiviti terhadap asid 'ethacrynic', rendah terhadap DCNB dan mempunyai aktiviti yang tinggi terhadap substrat CDNB.

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LIST OF ABBREVIATIONS

%	percent
°C	degree Celsius
µg	microgram
µl	microliter
CDNB	1-chloro-2,4-nitrobenzene
DCNB	1,2-dichloro-4-nitrobenzene
EA	ethacrynic acid
EDTA	ethylenediaminetetra acetic acid
g	gram
HCl	hydrochloric acid
KCl	Potassium Chloride
kDa	kiloDalton
NaCl	Sodium chloride
L	liter
M	molar
mA	miliAmpere
mg	milligram
min	minute
mL	milliliter
mM	milimolar
PBS	phosphate buffered saline
pH	- log concentration of H^+ ion (*Puissance hydrogen)*

pI	Isoelectric point
TEMED	N,N,N',N'-Tetramethyl-ethylenediamine
U	units
V	Volts
v/v	volume/volume
W	Watts
w/v	weight/volume
x	times

CHAPTER I

INTRODUCTION

Living organisms are exposed to an increasing number of toxic compounds in the environment, as well as the increasing variety of drugs. The toxic compound, also referred to as xenobiotics, include chemicals in the water, air, food additive or drugs. To get rid of these xenobiotics, the body uses the process of detoxification; a complex series of reaction, to get rid of molecules (toxins) whose prolonged presence may have damaging effects on tissues or lead to undesirable effect.

The detoxification process which is the conversion of non-polar (lipophilic) toxins to polar (hydrophilic) and non-toxic metabolites, occurs in two steps, namely Phase I and Phase II. Most cells are equipped with both of these biotransforming enzymes. Phase I metabolism introduces a functional group into molecule, while phase II metabolism involves conjugation of the phase I metabolites with endogenous substrate such as sulfate, glutathione, glucuronic acid and amino acids (Ionnides *et al.*, 1984). The induction of enzymes involved in detoxification may be caused by substances that selectively unregulated a Phase I enzyme without co-induction of the corresponding Phase II enzyme.

Phase I reactions are catalyzed by a multitude of enzyme activities; the most significant one is the cytochrome P450 (CYP450) supergene family of isoenzymes which has a very broad specificity. The reaction of CYP 450 will generate reactive molecules which often maybe more toxic than the parent compound. The intermediate metabolite is further metabolized by phase II enzymes or otherwise it

may react and cause damage to protein, RNA and DNA within the cell. While in the Phase II metabolism, the biotransformed molecules generated in the Phase I are conjugated by the addition of a water-soluble group to the reactive site; this increase their solubility and thus facilitates excretion in the urine or bile (Grant, 1991). The main types of enzymes catalyzing the Phase II reaction are such as glucuronyl transferase, glutathione S-transferases, amino acid transferases and epoxide hydrolase. Yet, not all xenobiotics go to the same path of metabolism; from Phase I to Phase II route. Instead of going through the Phase I step, they initially undergo the detoxification directly to Phase II.

In the Phase II, glutathione S-transferases (GSTs; EC 2.5.1.18) a phase II enzyme, ubiquitous, inducible, dimeric protein and also the most abundant protein in the cytosolic fraction of the liver (Booth et al., 1961; Wilce and Parker, 1994; Perez-Lopez et al., 1998). The GST have aroused much interest because of its involvement in the biotransformation and detoxification of a wide spectrum of endogenous and xenobiotics compounds. These functions really suit its major role as the phase II detoxification enzyme that conjugates the cellular nucleophile glutathione with a wide range of endogenous or xenobiotic hydrophobic molecules (Mannervik and Danielson 1988; Hayes and Pulford 1995; Armstrong 1997).

The Kedah-Kelantan (KK) cattle (*Bos indicus*) and water buffalo (*Bubalus bubalis)* are two livestocks that are very important to the small holders. The KK cattle and the water buffalo normally are free-range. They are free to roam and feed on vegetation in surrounding areas in rural areas. Thus, they are exposed to the agrochemicals and pesticides which are applied by the villagers and farmers. It is very important to

gather information about biochemical functions especially the patterns of glutathione S-transferases of both animals as it might be a useful tool in environment monitoring.

Objectives of the study

The present study is mainly concerned about the partial purification and characterization of cytosolic glutathione S-transferase (GST) from the livers of Kedah-Kelantan (KK) cattle (*Bos indicus*) and water buffalo (*Bubalus bubalis*). Both species are the most economic importance for the meat market in Malaysia.

Objectives of the study are:

1. To partially purify cytosolic GST from both species using the agarose-glutathione affinity chromatography gel.
2. To partially characterize the partially purified GST by preparative isoelectric focusing, and SDS-PAGE. Characterization of the partially purified and isolated isoforms are carried out using the different substrates; 1-chloro-2,4-dinitrobenzene (CDNB) (broad specificity), 1,2-dichloro-4-nitrobenzene (DCNB) (relatively selective for rat Mu class GST) and ethacrynic acid (EA) (selective for rat Pi class GST) (Habig and Jakoby 1981).

This study hopes to establish the patterns of cytosolic glutathione S-transferase in the KK cattle and water buffalo. This might be useful to further achieve an understanding towards this enzyme in view of using it as a tool in environmental monitoring.

CHAPTER II

LITERATURE REVIEW

Glutathione

Glutathione (GSH) is widely found in all forms of life and plays an essential role in the health of organisms. It is a submajor constituent of all cells and is almost always the major non protein thiol compound present in cells. Glutathione (GSH) is a tripeptide with the sequence of γ-glutamyl-cysteinyl-glycine and with molecular weight of 307.33 daltons. The disulfide derived from GSH by oxidation of the thiol group of the cysteine residue is usually denoted as glutathione disulfide (GSSG).

Glutathione concentration ranges between 0.1 and 10mM in mammalian cells and its sulfhydryl group comprises 10-20% of the non-protein sulfhydryl groups in the cell (Manoharan *et al.*, 1992) representing the major intracellular low molecular weight sulfhydryl compound in animals, plants and in most microorganisms (Sies, 1998). The liver acts as the principal site of glutathione synthesis, the most important chemically active group present in the GSH molecule with respect to its biological and biochemical activity is the thiol group. In healthy tissues, more than 90 percent of the GSH pooled is in the reduced form and less than 10 percent exist in the disulfide form. The enzyme glutathione reductase is the principal enzyme that maintains the GSH in reduced form.

The GSH molecule has two peptide bonds, two carboxylic acid groups, one amino group and one thiol (Figure 1). The high number of hydrophilic functional groups combined with a low molecular weight leads to a high water solubility for GSH.

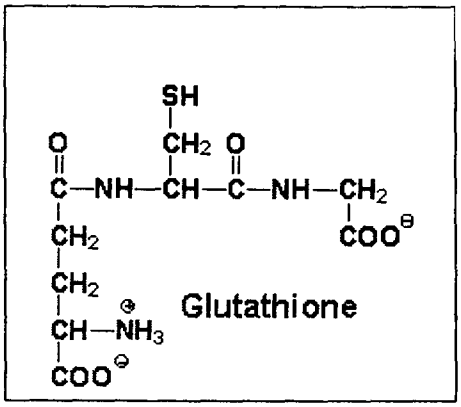

Figure 1: Structure of glutathione (γ-glu-cys-gly).

There are also a number of GSH-dependent enzymes that are part of the cellular protection against endogenous and xenobiotic toxic substances. Glutathione reductase (GR) catalyzes the reduction of GSSG using NADPH as a reductant (Krohne-Ehrich *et al.* 1977). GR is important to keep the high cellular reductive potential. Selenium dependent glutathione peroxidase (GPxs) is another GSH-linked enzyme that catalyzes the reduction of peroxides using GSH as the reducing agent (Krohne-Ehrich *et al.* 1977). Finally, last but not the least, glutathione S-transferases (GSTs) is also a GSH dependent enzymes with many properties among which catalyzing the conjugation of GSH to various electrophilic compounds is one of the most investigated function.

The cystenyl residue of glutathione provides a nucleophilic thiol important for the detoxification of electrophilic metabolites and metabolically produced oxidizing

agents. Its net negative charge and overall hydrophilicity greatly increases the aqueous solubility of the lipophilic moieties with which it becomes conjugated. Its molecular weight ensure that its adducts are preferentially secreted via the biliary system which selects molecules of molecular weight greater than 300 to 500 according to the species (Ketterer *et al.* 1983).

In mammals, GSH conjugates are often further metabolized by hydrolysis and N-acetylcystenyl conjugates known as mercapturic acids, which are excreted in the urine. This is presumed that GSH provides a means whereby the pool of cysteine for detoxification is kept separate from the pool of cysteine for protein synthesis (Ketterer *et al.* 1983).

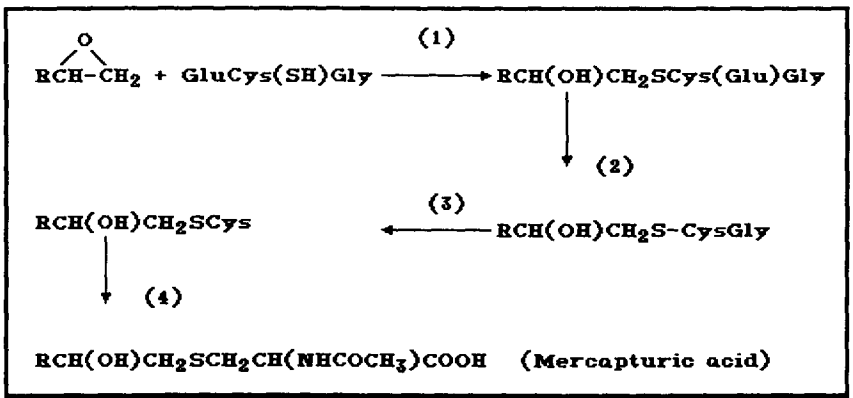

Figure 2: Mercapturic acid biosynthesis pathway.

Taken from www.inchem.org/documents/ ehc/ehc/ehc57.htm

GSH also plays roles in catalysis, metabolism, and signal transduction, gene expression and apoptosis. The most important is that glutathione is a cofactor for glutathione S-transferases (GST), enzymes which are involved in the detoxification of xenobiotics (Meyer *et al.*, 1985).

Though the GSH is undoubtedly a potent (Ioannides *et al.* 1984) antioxidant, indication for its use as supplement are not yet been well established. There is preliminary evidence that it might eventually prove to be useful in management of some cancers, atherosclerosis, and diabetes and also to help prevent or improve various toxicities.

Glutathione S-Transferases

The glutathione S- transferases (also known as GSTs; EC 2.5.1.18) are a family of multifunctional enzymes, found to play an important role in the detoxification of wide variety of xenobiotics (Hunaiti and Owais, 1985; (Habig *et al.* 1974; Prohaska 1980; Hunaiti and Owais 1985; Meyer *et al.* 1985; Ketterer 1986). First found as an enzyme by Booth and co-workers in 1961, GSTs are also proposed to act as carrier protein and were named ligandin (Litwack *et al.*, 1971).Today, despite of research for 40 years, the 'picture' of exact function of the superfamily, is more complex than ever.

GSTs which known as multifunction enzymes are capable of catalyzing a seemingly protean spectrum of reactions; widely distributed and are present at high concentrations in cytosol (Jakoby *et al.* 1984). GSTs can be found mostly in liver,