การใช้ Metallothionein เป็นตัวบ่งชี้ทางชีวภาพการปนเปื้อนของปรอทในหอยแมลงภู่ *Perna viridis*

นางสาว จินดา ป่าสนธ์

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METALLOTHIONEIN AS A BIOMARKER FOR MERCURY CONTAMINATION IN MUSSEL, *Perna viridis*

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จินดา ป่าสนธ์: การใช้ Metallothionein เป็นตัวบ่งชี้ทางชีวภาพการปนเปื้อนของปรอทในหอยแมลงภู่ *Perna viridis* (METALLOTHIONEIN AS BIOMARKER FOR MERCURY CONTAMINATION IN MUSSEL, *Perna viridis*) อ. ที่ปรึกษา: ผศ. ดร. ศิริชัย ธรรมวานิช, อ. ที่ปรึกษาร่วม: ดร. ณรงค์ศักดิ์ พ่วงลาภ …141…. หน้า. ISBN 974-17-5022-6

ปรอทเป็นโลหะหนักที่มีพิษร้ายแรง เมื่อมีการปนเปื้อนลงสู่สิ่งแวดล้อม แม้ในปริมาณที่ต่ำก็ส่งผลกระทบต่อสิ่งมีชีวิตต่าง ๆได้ ดั้งนั้นจึงจำเป็นต้องมีเครื่องมือที่สามารถวัดปรอทที่ปริมาณต่ำได้ เมทัลโลไธโอนีน เป็นโปรตีนน้ำหนักโมเลกุลต่ำ พบได้ทั่วไปในพวกยูแคริโอต คุณสมบัติของ เมทัลโลไธโอนีน นั้นสามารถจับกับโลหะหนักได้หลายชนิด รวมทั้งปรอทด้วย ในการศึกษาครั้งนี้ จะศึกษาผลกระทบของปรอทที่ความเข้มข้นต่าง ๆ ต่อปริมาณ เมทัลโลไธโอนีน ในหอยแมลงภู่ การทดลองออกแบบเพื่อตรวจหาวิธีวัดปริมาณ เมทัลโลไธโอนีน พบว่า วิธีวัดปริมาณ เมทัลโลไธโอนีน โดยเทคนิค เซมิคอนติเตทีพ อาร์ทีพีซีอาร์ (Semi-Quantitative RT-PCR) มีความเป็นไปได้มากกว่าเทคนิค เจลอิเลคโตรโฟรีซิส และ ซิลเวอร์สเตนนิ่ง (Gel electrophoresis and silver staining) โดยทำการสกัดปริมาณอาร์เอ็นเอรวมจากท่อทางเดินอาหารของหอย ซึ่งเป็นอวัยวะที่มีการสะสมของเมทัลโลไธโอนีน อยู่มาก เพื่อใช้เป็นแม่พิมพ์ในการสังเคราะห์ cDNA ด้วย reverse transcription จากนั้นนำไปเป็นแม่พิมพ์ในปฏิกิริยา PCR ด้วยไพรเมอร์ ที่ออกแบบจาก ยีนเมทัลโลไธโอนีน ได้ผลผลิตขนาด 220 คู่เบส และตรวจสอบความเข้มของแถบดีเอ็นเอโดยเปรียบเทียบกับ ยีนเบต้าแอคติน (β -Actin gene) ซึ่งเป็นตัวอ้างอิง ผลการทดลองพบว่า ปริมาณ เมทัลโลไธโอนีน ของหอยกลุ่มทดลองแตกต่างจากกลุ่มควบคุมอย่างมีนัยสำคัญทางสถิติ ตั้งแต่สัปดาห์ที่ 2 ถึง4 (p<0.05) โดยพบความแตกต่างที่ความเข้มข้นของปรอท 1 ไมโครกรัมต่อลิตร นอกจากนี้ปริมาณ เมทัลโลไธโอนีนยังเพิ่มขึ้นตามความเข้มข้นของปรอทอย่างมีนัยสำคัญทางสถิติ (p<0.05) นอกจากนี้ยังทำการศึกษาการแปรผันของ ยีนเมทัลโลไธโอนีน ด้วยเทคนิค Single Stranded Conformation Polymorphism พบว่าสามารถแยก ไอโซฟอร์มของ เมทัลโลไธโอนีน ได้ทั้งหมด 6 ไอโซฟอร์ม ในการศึกษาครั้งนี้ชี้ให้เห็นว่า ปริมาณ เมทัลโลไธโอนีน จากท่อทางเดินอาหารของหอยแมลงภู่มีการตอบสนองต่อปริมาณของปรอทที่ได้รับ และการตอบสนองนี้สามารถนำไปใช้ตรวจสอบการปนเปื้อนของปรอทในสิ่งแวดล้อมได้

สาขาวิชา สหสาขาวิชาวิทยาศาสตร์สิ่งแวดล้อม ลายมือชื่อนิสิต……..

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KEY WORD: METALLOTHIONEIN/ SEMI-QUANTITATIVE RT-PCR/ MERCURY/ MUSSELS/ BIOMARKER

CHINDA PARSONT: METALLOTHIONEIN AS BIOMARKER FOR MERCURY CONTAMINATION IN MUSSEL, *Perna viridis*. THESIS ADVISOR: ASST. SIRICHAI DHARMVANIJ, Ph.D., THESIS COADVISOR: NARONGSAK PUANGLARP, Ph.D. 141 pp. ISBN 974-17-5022-6

Mercury (Hg) is one of the most toxic heavy metals. Its discharges to the marine environment are of great concern. Very low level of Hg still have chronic effect to many living organisms and sophisticated equipment are needed for the measurement at such low concentration. Metallothioneins (MT) are low molecular weight cytosolic proteins found in eukaryotic species. They are capable of binding a variety of heavy metals, including Hg. The study focused on the effects of the various levels of dissolved mercury to the level of metallothionein in mussels, *Perna viridis*. The experiment was designed to verify the methods, effectiveness for the quantification of metallothioneins in mussel. The results show that semi-quantitative RT-PCR is more promisingtechnique than gel electrophoresis and silver staining in measuring of mussel MT mRNA and eventually this technique can be applied for the assessment of mercury contamination in mussels. The study focused on the digestive tract of the mussels which has been shown to play an important role in tissue distribution of MT. Total RNA from the digestive tract of the mussels exposed to various levels of mercuric chloride was extracted and converted to cDNA by reverse transcription. Oligonucleotide primers were designed to amplify a 220 bp segment of the mussel MT cDNA. The expression of Beta-actin was used as reference. The RT-PCR data showed that there was a significant different between treatments and control in digestive tract MT mRNA in subjects within 2 to 4 weeks of mercury exposure ($p < 0.05$). At concentration of 1 μg/L $HgCl_2$ induced the MT mRNA levels significantly comparing with control. Moreover, this study also investigated the variation of metallothionein forms in mercury-exposed mussels. Six isoforms were identified by single strand conformation polymorphism (SSCP) method. This data suggests that MT mRNA levels of digestive gland from mussel respond to mercury exposure and the response could serve as a more useful assessment of mercury contamination in marine environment.

Field of study Environmental Science Student's signature……..……

Academic year 2003 Advisor's signature……..…..…

Co-Advisor's signature……

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LIST OF ABBREVIATIONS

Abbreviations and symbol

SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
RNA	Ribonucleic acid
DNA	Deoxyribonucleic acid
2-ME	2-mercaptoethanol
PBS	Phosphate buffer saline
BSA	Bovine serum albumin
hr	hour
min	minute
kDa	KiloDalton
v/v	volume by volume
w/v	weight by volume
OD	Optical density
MW	Molecular weight
Kb	Kilobase
bp	base pair
IPTG	Isopropyl-β-D-thiogalactoside
LB	Luria-Bertani medium
µg	microgram
mg	milligram
g	gram
lµ	microlitre
l	litre
ml	millitre
mM	millimolar
M	molar
rpm	revolution per minute
nm	nanometer
SDS	sodium dodecyl sulfate
TE	Tris-EDTA buffer
TEMED	N, N, N', N'-tetraethylenediamine
U	Unit
DW	distilled water
dNTP	deoxynucleotide triphosphate
RT	room temperature
aa	amino acid
CIAA	chloroform: isoamyl alcohol
DEPC-DW	Diethylpyrocarbonate treated distilled water
^{O}C	degree celsius
1x buffer	Working buffer

CHAPTER I

INTRODUCTION

The discharges of heavy metals to the environment are of great concern all over the world. Heavy metals can accumulate in marine organisms and are toxic when present at high concentration. Among them is mercury, the most serious global pollutant. Many of its compounds are highly toxic and readily released into the environment because of their high volatility and mobility. On a molar basis, mercury is far more toxic to marine organisms than any other heavy metals. Low level of mercury still has chronic effects to living organisms.

At very low concentration of mercury, sophisticated method is needed for the measurement. Numerous methods for monitoring mercury contamination based on analytical techniques have been developed. These advances in analytical chemistry provide increasingly accurate measurement on the levels of contamination. However, the main drawback is that it does not evaluate the effects of the contaminant on living organisms or on their health. In the last few decades, recent advances in the biotechnology have allowed us to develop bioassays based on qualitative and quantitative observation of living organisms in their natural environments. This can be considered as potential means of compensating for deficiencies in chemical analysis.

The quantitative measures of changes in the biological system have been defined as biomarker which is used for the measurement of molecular, biochemical, cellular, or physiological parameter. It could provide early warning of effects on biota caused by contaminants present in the marine environment rather than contaminant monitoring. The measurement in term of molecules provides the sensitivity to detect pollutants lower level.

Among three markers biological for exposure assessment in free-living organisms and in environmental pollution assessment, metallothioneins are a potential biomarkers widely used for heavy metal contamination. Metallothioneins are small cystiene-rich metal binding proteins and lack of aromatic amino acids. They are cytosolic proteins found in a variety of eukaryotic and prokaryotic species. They can be induced by a variety of heavy metals, including mercury. Metallothioneins occurs in several isoforms which may differ from each other by only a few amino acid positions and by different isoelectric points and hydrophobicity. The existence of different isoforms with different roles has also been a matter of scientific interest. Their functions involve in the regulation of essential metals and in the detoxification of non-essential metal ions. The induction of metallothionein is, therefore, a potentially powerful biochemical indicator of response to mercury contamination.

Mussels are widely used as species for toxicological studies because of their high accumulation of many heavy metals at high level, long life span, reasonable size for individual analysis, tolerance of wide temperature and salinity ranges, and wide geographical distribution. Mussels are probably the most widespread species of bivalves in seawater of Thailand. For these reasons, they are the most suitable marine organisms for monitoring of contamination levels in coastal water. Therefore, determination of metallothionein level from mussels should be a good monitoring tool for the assessment of the availability of mercury to marine organisms.

The study focused on the effects of the various levels of dissolved mercury to the level of metallothionein in mussels, *Perna viridis*. This provided the information

on the dose-response relationships between the animals and the contaminant for using as early developmental stage in water quality bioassays. The experiments were designed to determine the variation of metallothionein forms in mercury-exposed mussels using single stranded conformation polymorphism (SSCP) method and also to investigate the existence of mercury binding forms using liquid chromatographic techniques. Furthermore, the effectiveness of semi-quantitative RT-PCR for the measurement of expression level of metallothionein gene in mussel was also verified. The use of mussels and monitoring of their metallothionein level may represent an ideal biological endpoint to determine the level of mercury toxicity linked to marine contaminants.

Scope of studies

1. To determine protein level of metallothionein and expression level of metallothionein gene in the tissues of mussel, *Perna viridis*, exposed to various concentration of mercury.
2. To verify the methods for the quantification of metallothioneins in mussel.
3. To investigate the variation of metallothionein forms and the existence of mercury-binding form.

CHAPTER II

LITERATURE REVIEW

2.1 Mercury

Mercury (Hg) is one of the most toxic heavy metals. It is unique in that it is found in the environment in several physical and chemical forms but no clear evidence on essential biochemical function of mercury has been reported (Gochfeld, 2003). In nature, mercury exists in any of three inter-convertible oxidation state: Hg (0), Hg (I) and Hg (II) (Jonasson and Boyle, 1971). Hg (0) (elemental Hg) is a volatile liquid at ambient temperature and surface atmosphere (Lide, 1992). It is rather unreactive but can be oxidazed to HgO by O_2 (Cotton and Wilkinson, 1988). HgO is decomposed to Hg (0) and O_2 on exposure to light (Budavari *et al*., 1989) therefore it can persist for a length of time in contact with air. Hg (I) consists of the diatomic cation Hg^{2+}. It is unstable in most natural environments. It forms unstable aqueous complexes and disproportionates spontaneously to Hg (0) and complexed Hg (II) in the presence of ligands that bind Hg (II) (Fitzgerald, 1995). Thus, the main oxidation states of Hg in nature are Hg(0) and Hg(II).

Hg (II) or Hg^{2+} is the principal form of mercury in aquatic environments. Because of its inert property, mercury does not react readily with O_2 and water (Cotton and Wilkinson, 1988). In natural waters, any dissolved inorganic Hg (II) not bound to organic or sulphide ligands probably consists mainly of hydroxyl and chloride complexes.

2.1.1 Sources

Mercury is released into environment from both natural and anthropogenic sources. Natural sources primarily in the form of elemental mercury include volcanic emissions, degassing from soils, and volatilization from the ocean. Anthropogenic emissions of mercury are from the use of fossil fuel (especially coal) and other extracted, treated, or recycled mineral material as well as from mercury used intentionally in product or processes. Mercury has been used in thousands of products and industrial processes. These include chlorine and acetic soda manufacture, laboratories, electronic lighting (eg. Fluorescent lamps), wiring devices, switches, batteries, thermometer, thermostats, barometer, dental supplies (e.g. dental amalgam filling) and medical equipments (Vostal, 1976; Klaassen, Amdur, and Doull., 1986)

2.1.2 Fate and Transport

Traces of mercury are ubiquitous in soils, natural waters, sediments, organisms, and air (Jonasson and Boyle, 1971). Mercury is released into the atmosphere from anthropogenic emissions as either vapor (elemental or oxidized mercury) or as particles (oxidized compounds). Natural emissions are mainly in elemental mercury form (Vostal, 1976). Mercury is transferred form atmosphere to

the earth's surface via rainfall (Klaassen, Amdur, and Doull, 1986). The majority of mercury in surface soil from terrestrial ecosystem is in the form of oxidized mercury complexes or compounds (Boening, 2000).

In aquatic ecosystems, organic mercury is the most present form and increases from lowest to highest tropic level (Young and Mearns, 1978). Microorganisms are crucial in conversion of inorganic Hg (II) into the readily bioaccumulated toxic organometallic species, methyl mercury (CH_3Hg^+). Methyl mercury undergoes biomagnification up the marine food chain. Therefore, most of the mercury in fish consists of CH_3Hg^+ while inorganic mercury predominantly associates with water and sediments. Elevated CH_3Hg^+ concentrations in marine organisms are found not only in habitats contaminated with mercury but also in unpolluted areas where mercury levels are low. Mercury is adsorbed by living and dead organic matter such as particulate organic carbon, animal membranes, and lipids (Gochfeld, 2003). It entrances into food chain by plankton in water, which can take up mercury in organic form and inorganic form (Mason, Reinfelder and Morel, 1995). In fish and invertebrates, mercuric compounds are accumulated via three major routes of exposure: gills, dermis skin and digestive tract.

Approximately 67% of the studies on organic compounds have claimed to show biomagnification (Duvall and Barron, 2000). Biomagnification is the process where xenobiotic substances are transferred from food to an organism resulting in higher concentrations compared with the source. It is a general phenomenon for marine food webs. However, it has been found only in sea-birds and marine mammals that food intake is the major route and where biomagnification can be clearly shown. (Dietz, Riget and Johansen, 1996). Bioconcentration (uptake from the surrounding water) is the most usual way that organic compounds are accumulated in organisms from invertebrates and fish (Boisson *et al.*, 1998; Leblond and Hontela, 1999).

2.1.3 Toxicity of mercury to marine organism

Mercury as the reactive, free inorganic ion and as various organo-mercury compounds in solution is one of the most toxic metals to marine organism. The toxicity of methyl mercury and related organomercury compounds usually is 10 to 100 times greater than that of dissolved inorganic mercury (Boening, 2000).

Marine phytoplanktons are among the most sensitive marine organisms to inorganic mercury. Concentrations in the range of 0.8 to 1.0 µg/L are capable of inhibiting photosynthesis and reducing growth rate of some species of marine micro algae (Neff, 2002). Developing stage of marine zooplankton is also sensitive to mercury. The reducing rates of embryogenesis and larval attachment by 50% has occurred in *Ciona intestinalis* when exposed to 54 µg.Hg/L and 35 µg.Hg/L, respectively (Bellas *et al.*, 2001). Developing brine shrimp, *artemia* were adversely affected by organic mercury at concentrations less than 0.1 µM (Pandey and MacRae, 1991).

Methyl mercury was accumulated significantly in the brain of Atlantic salmon *Solmo salar* fed 5 or 10 mg Hg/kg body weight. Fish fed with 5 mg/kg methyl mercury had significant increased antioxidant enzyme superoxide dismutase (SOD) in the brain. At dietary level of 10 mg/kg, methyl mercury caused decreasing in antioxidant enzyme activity (SOD) and glutathione preoxidase, pathological damage

and reducing neural enzyme activity and overall post-feeding activity behavior. Fish fed with 100 mgHg/kg inorganic mercury reduced neural MAO activity and pathological changes (astrocyte prolification) in the brain (Berntssen *et al.*, 2003). A severe disorganization of epithelium cell and modification of ciliated olfactory cell was occurred in gill of Arctic charr, *Salvelinus alpinus*, after expose to inorganic mercury 12 hours and 24 hours, respectively (De Oliveira *et al.*, 2002).

In blue mussels *Mytilus edulis*, the effects of sub-lethal concentration of organic and inorganic mercury reduce phosphoarginine and ATP amounts (Aunaas *et al*, 1991) and it was also found to decrease oxygen consumption and ratio of glycogen/protein and glycogen/lipid in marine fouling dreissinid bivalve *Mytilopsis sallei* (Devi, 1996). Inhibition on activity of α-amylase was occurred in digestive gland of green mussel *Perna viridis* that exposed to mercury (0.9mM) (Yan *et al.*, 1996).

2.2 Monitoring of environmental quality

Numerous methods for monitoring environmental health can be divided into 2 distinct categories; the detection of pollutants and their quantification in physical and biological mediums, and the evaluation of the effects of pollution on living organisms, either at the individual level or at the level of populations and/or communities (Amiard, Caquet and Lagadic, 2000).

In the early phase of environmental monitoring of coastal areas, most programs consisted of the measurement of physical and chemical variables and only occasionally were biological variables incorporated. Routine measurements included temperature, salinity, and oxygen concentrations, usually nutrients and some chemical contaminants such as heavy metals, which were perceived to be relatively easy to measure. Sediment monitoring involved sampling the benthos with quantitative grab samplers and measurements included sediment grain size distributions, organic matter content and some contaminants, again usually heavy metals.

For detection and quantification of pollutants, method based on chemical analysis, currently highly improved, is able to measure the extent and level of environmental contamination by using a limited number of samples taken from various matrices (water, soil, sediments, plants, animals etc.). However, apart from the fact that the use of such method is not always possible because of the properties of the substances being studied, no method of analysis is able to quantify all the contaminants present in a sample. Moreover, the analytical techniques do not allow evaluating the effects of the presence of the contaminants on living organisms or the health of ecosystem (Amiard, Caquet and Lagadic, 2000). Bravo-Sanchez *et al.* (2004) developed the method to detect mercury at low level in Sata Catarina Island, Brazil (0.41-1.51 ppb.) by solid phase microextraction-chromatography-inductively couple plasma mass spectrometry. The result was not showing the effect of this low level to marine organism.

2.3 Biomarker as tools for evaluating environmental health

For the biological approach, method based on qualitative and qualitative observations of living organism in their natural environments were soon considered as potential means of compensating for the deficiencies of chemical analysis. At the present, two complementary approaches, base on the study of living organism are used. The study of certain species or groups of species, the presence (or absence) and/or abundance of which provide information about environmental quantity. Another one is the measurement, in individuals from natural environment, of molecular, biochemical, cellular, or physiological parameters. Such indicators were called biomarkers. (Amiard, Caquet and Lagadic, 2000).

Biomarker is becoming an important tool for monitoring programs. There are many definitions of a biomarker. Typically, biomarkers are defined as quantitative measures of changes in the biological system that respond to either (or both) exposure to, and/or doses of, xenobiotic substances that lead to biological effects. Although not explicitly contained in most definitions, the use of the term ‘‘biomarker’’ or ‘‘biomarker response’’ is often restricted to cellular, biochemical, molecular, or physiological changes that are measured in cells, body fluids, tissues, or organs within an organism and are indicative of xenobiotic exposure and/or effect. Changes that occur at the organismic, population and assemblage levels more usually referred to as ‘‘bioindicators’’ hence sometimes the term ‘‘bioindicator organisms’’ is used (Lagadic *et al.*, 2000; Lam and Gray, 2003).

Biomarkers are classified into 3 categories: (1.) biomarkers of exposure, which indicate the presence of one or several pollutants in the organism (2.) biomarkers of effect, which reveal the risks of toxic effects in the long term, and (3.) biomarkers of susceptibility, which indicate the existence of a different sensitivity to a toxin in one part of the population

The use of biomarker presents the advantage of an integrated evaluation in time and space of bioavailable pollutants, not only term of presence, but also in relation to the effects of these products on animal, plant, and microbial populations. Biomarker can also reveal the exposure of individual to compounds like organophosphorous, which are metabolizes rapidly and accumulate very little in organism. Moreover, biomarkers can give an indication of the distribution of contaminants in the environment. Depending on their habitats and their position in the food web, the species that are the source of biomarkers can indicate how pollutants are diluted in the medium or, on the other hand, are concentrated in certain parts of ecosystem (Amiard, Caquet and Lagadic 2000).

There are many biomarkers in organism such as ethoxyresorutin o-diethylase (EROD) which is specific to polyaromatic hydrocarbon (PAH), Polychlorobiphenyl (PCB) and dioxin contamination (Flammarion *et al.*, 2000). Acetylcholinesterase (AchE) which is specific to organophosphorous and cabamet (Galgani and Boequene, 2000) and including metallothionein which is small protein, was induced by heavy metals (Cosson and Amiard, 2000). This protein is becoming an important for monitoring heavy metal contamination. Some biomarkers of pollutants/toxicants are shown in table 2.1

Table 2.1 Biomarkers for pollutants/toxicants in various organisms

Pollutants	**Biomarkers**	**Organism**	**Detection Methods**	**Reference**
1. Pesticide	-cholinesterase (organo-P)	Insects, fish	Chemical analysis, PCR, immunoassay	Porte, Escartin and Borghi (2001)
2. Heavy metal	-metallothionein -δ-minolevulinic acid dehydatase (Pb)	Fish, mollusc, crustacean	Chemical analysis, PCR, immunoassay	Viarango *et al.*(2001) Shakhnazarov, Shakhanazarov andTyushnyallo na (1974)
3. Benzene	-muconic acid	Mollusks, Fish	GC/MS	Bachfold and Handerson (1993)
4. Aromatic hydrocarbon	7,12 dimethylbenz anthracene -cytochrome P450	Fish, mollusks, crustacean	Immunoassay, PCR	Livingstone and Peter(2001)
5. Oxidative damage	-B-hydroxydeoxyguanosine -superoxide dismutase -Glutathione peroxidase	Human, fish, insects, mollusks	Immunoassay, NBT assay, PCR	Downs, Fauth and Woodley (2001)
7. endocrine disruptor	-Vitellogenin -Zona radiata protein	Fish, reptiles, amphibian	PCR, immunoassay, staining	Arukwe *et al.* (2000)
6.environmetal stress (temp, UV, etc.)	-ethoxyresorufin O-diethylase	Fish, mollusks, crustacean	immunoassay, PCR	Romero, Stien and Lafaurie.(2001)

2.4 Metallothionein

Metallothionein (MT) is generally considered as a storage and supply site for essential metals which are utilized in protein synthesis, nucleic acid metabolism and other metabolic processes (Roesijadi, 1994). In addition to this regulatory function, MT may also play a role in metal detoxification. As a general model, MT synthesis is thought to be induced under conditions of elevated metal concentration, providing more binding sites for metal ions and limiting latent damage. The induction of MT is, therefore, a potentially powerful biochemical indicator of response to metal contamination.

In most of the mammalian species studied Zn is thought to be the most potent inducer, with Cd and Hg being less effective, and Cu often a poor inducer. Consequently, Zn usually saturates the binding sites of MT but can be displaced by increasing amounts of Cu, Cd and other metals in high concentrations (Palmiter, 1994). In aquatic invertebrates such as mussels, Cd is, however, the most potent inducer of MT and Zn the least (Longston, Bebianno and Burt, 1998). In view of this diversity in form and function, the application of MT assays in environmental assessment must first be proceeded by validation of responses of the proposed monitoring species. Most native fish populations are considered less suitable candidates as bioindicators of environmental contamination gradients than many invertebrates, because of their mobility. Synthesis of MTmRNA, translation and involvement of this predominantly cytosolic protein in metal-binding, is particularly high in the liver, (George, Todd and Wright, 1996; Olsson, 1996), making it an obvious choice of tissue for study. Evidence is largely based on laboratory exposures (e.g. to Cd, Cu, Hg, Zn), which confirm that, generally, MT levels and associated metals increase as a function of the administered dose (reviewed by George and Olsson, 1994).

2.4.1 Definition of metallothionein

The term metallothionein was introduced to designate the cadmium-, zinc-, copper- containing sulfer-rich protein from equine renal cortex. This protein was characterized as follows; low molecular weight, high metal content, characteristic amino acid composition (high cysteine content, no aromatic amino acid, nor histidine), unique amino acid sequence (characteristic distribution of cysteinyl residues such as cys-x-cys), spectroscopic features characteristic of tetrahedral metal-thiolate (-mercaptile) complexes) and metal thiolate clusters (Kägi *et al.*, 1974; Kojima *et al.*, 1976. Metallothionein are subdivided into 3 classes as follow.

Class I: Polypeptides with locations of cysteine closely related to those in equine renal metallothionein.

Class II: Polypeptides with locations of cystein only related to those in equine renal metallothionein, such as yeast metallothionein. (Winge *et al.*, 1985)

Class III: A typical, non-translationally synthesized metal-thiolate polypeptides, such as cadystin, phytometallothionein, phytochelatin, and homophychelatin (Kojima, 1991).

2.4.2 Nomenclature of metallothionein

The term metallothionein is used for all polypeptides fitting above definition. The metal-free form (apoprotein) may be designated either as apometallothionein or thionein. More specific forms, such as cadmium-metallothionein (or cadmium-thionein) or zinc-metallothionein (or zinc-thionein) are appropriate for metallothioneins that contains only one metal. The molar metal content can be specified by a subscript, i.e. Cd_7 – metallothionein or Zn_7 – metallothionein. When the metallothionein contains more than one metal, for example, 5.3 mol of cadmium and 1.7 mol of zinc, the terms such as Cd, Zn-metallothionein; Cd, Zn-thionein; $Cd_{5.3}$, $Zn_{1.7}$-metallothionein; and $Cd_{5.3}$-$Zn_{1.7}$-thionein; are recommended. In contexts, the term metallothionein of the metal composition is not available or is not specifically interest, the term metallothionein should be used (Kojima, 1991).

The terms for multiple forms of metallothionein or isometallothioneins should be used as broad terms covering all metallothionein occurring naturally in a single species. These terms should apply only to forms of metallothionein arising from genetically determine differences in primary structure and not to forms that differ only in metal composition or are derived by modification of the same primary sequence. Different isometallothioneins should be defined by the term metallothionein followed by Arabic numeral. Where the complex isometallothionein families occur, numbers and the subforms should designate the major groups by letters (metallothionein-1a, metallothionein-1b, metallothionein-1c; metallothionein-2a, metallothionein-2b, etc. (Kojima, 1991).

2.4.3 Structure and isoforms of metallothioneins

Metallothionein is currently classified into 15 families (Binz and Käji, 1999). Other groups of metal binders that are more widespread in plants, including phytochelatins and nonprotein metallothionein have not been included (Robinson *et al*, 1993). Mollusc metallothioneins are single-chain polypeptides of 71-73 amino acid residues. Its member in metallothionein family 2 and subdivided into 4 subfamilies: mo1, mo2, mo and mog. The number and position of the cysteines residues is highly conserve and form C-x-C-x (3) –C-T-G-x (3) –C-x-C-K.

The binding affinity varies between metals (Hg > Ag > Cu > Cd > Zn) with Cu having the greatest stability constant followed by Cd and then Zn. As many as 18 different metals may associate with metallothionein but only Cu^{+}, Cd^{2+}, Pb^{2+}, Ag^{+}, Bi^{2+} and Hg^{2+} can displace Zn. Metallothionein can incorperate up to 7 divalent metal or 12 monovalent Cu atoms per molecule (Cu^{2+} is not bound by metallothionein) (Chen *et al.*, 1996). The wavelength characteristic of metal-cysteine liaisons such as Zn-SH, Cd-SH, Cu-SH and Hg-SH are 230, 250, 270 and 310 nm, respectively (Cosson and Amiard, 2000). Metallothionein has two subunits: the more stable α domain (C-terminal) which incorperates for divalent metal atoms, and the more reactive β domain (N-terminal), which contains only three metal biding sites. The exchangeability depends upon the metal species, and *in vivo* metallothionein exits mainly in Zn form or as mixed-metal protein (Coyle *et al.*, 2002).

All vertebrate examined contains two or more distinct metallothionein isoforms. In mammal, MT-1 and MT-2 are found in all organisms whereas MT-3 expressed mainly in brain and MT-4 is most abundant in certain stratified tissue (Romero-Isart and Vašák, 2002).

Marine mussels *Mytilus spp.* have two major isoforms with molecular weight value of 10 and 20 kDa, define as the MT-10 and MT-20 of MT isoforms, respectively (Carpane *et al*., 1980; Frazier, 1986). Five dimeric and four monomeric have been also identified (Mackay *et al*., 1993).

2.4.4 Function of metallothionein

Metallothionein expression is driven by a number of physiological mediators though several response elements in the metallothionein promoter. Metallothionein acts in zinc trafficking and /or zinc donation to apoproteins. Brady (1982) reviews the reaction of apoenzyme *in vitro*, using Cu-metallothionein and other metallothioneins. Two possible mechanisms have been sown in figure 2.1.

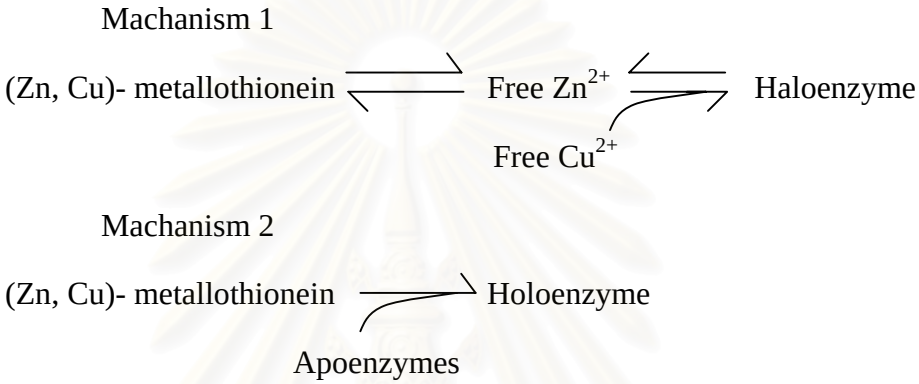

Figure 2.1 Metal donation by metallothionein for transfer of zinc and Cu from metallothionein to apoenzyme (Brady, 1982)

In addition to the functions of essential metal regulation and metal detoxification, it has been reported that metallothionein plays an important role in protection against the toxic effects of anti cancer drugs and oxidative stress The study on the proactive role of metallothionein against toxic effects of dosorubicin (anti cancer drug) in mice indicated that metallothionein reacted against the myocardiotoxicity and hepatoxicity (Kimura *et al*., 2000). Hepatic metallothionein played an important role as an acute phase protein against hose damage, and epidermal metallothionein contribution of epidermis during wound healing in mouse skin (Iwata *et al*., 1999). Metallothionein bound in particular to zinc showed a high capacity antioxidant for protection of DNA damage and lipoprotein damage by radiation and by peroxynitrate, a reactive nitrogen species (Cai and Cherian, 2003; Cai *et al*., 2000). The antioxidant properties of metallothionein in protection of mussel, *Mytilus galloprovincialis*, from oxidative stress that occurred through oxyradical scavenging was detected at both cellular and organism level (Viarengo *et al*., 1999).

2.4.5 Metallothionein induction related to heavy metal toxicity

There have been a number of reports demonstrated that metallotioneins involve that tolerance of heavy metal toxicity in various organisms. In marine diatom, *Phaeodactylum tricomutum*, cadmium reduced the growth rate of the diatom and thereby inducting the synthesis of class III metallothionein. This metallothioneins, composed of long chain polypeptides and bound efficiently to heavy metal, were not generally observed in specimens exposed to higher concentration of metal (Torres *et al*., 1997 and Tessier and Blais, 1995). In Baltic clam, *Macoma baltica*, the levels of metallothioneins were significantly correlated to the concentration of silver, cadmium and mercury (Mouneyrac, *et al*., 2000). The role of metallothionein in cadmium and zinc depuration process was also investigated in bivalve, *Corbicula fluminea*, after in situ exposure on the river Lot in France. It was revealed that cadmium and zinc concentration decreased by 18 and 70 %, respectively. Metallothionein level decreased by 37 % after transferring to unpolluted condition (Baudrimont *et al*., 2003).

An important role of metallothionein in regulation of toxic metals was also demonstrated in a number of organisms and in various tissues. In dogwhalk, *Nucella lapillus*, exposed to cadmium, metallothionein levels in gill, leibleir gland, kidney, digestive gland, and gonad increased gradually relating to the amount of cadmium exposure in sub-lethal biological response (Leung and Furness, 1999). The investigation in the exposure of dietary cadmium in Atlantic salmon, *Salmo solar*, revealed that cadmium entered both the gut and gill where it accumulated in cloride cells and the stimulation of metallothionein expression was detected (Dang *et al*., 2001).

Metal accumulation and metallothionein induction in gills and digestive tracts of *Mytilus galloprovincialis*, *Scapharca inaequivalis* and *Tapes philippinarum*, were investigated. The results confirmed the metal accumulation and metallothionein induction in three species and a higher concentration of metallothionein was detected in digestive tract (Irato *et al*., 2003). In oyster, *Crassostrea gigas*, and in the mussel, *Mytilus edulis*, the impact of metal (silver, cadmium, copper, mercury and zinc) on metallothionein level was investigated. The accumulations of metals were detected from the gill and digestive tract of both mollusks after 4 and 21 day of exposure. During the observation, metallothionein level was correlatively increased (Geret, *et al*., 2000).

The synthesis of metallothionein was found in mussel, *Mytilus edulis* exposed to cadmium and mercury. The observation showed that cadmium and mercury in gill significantly increased metallothionein level (Geret and Cosson, 2000). In addition, the correlation between cadmium, mercury and metallothionein also found in liver and kidney from gray seal, *Halichocrus grypus*, (Teigen *et al.*, 1999). The characters of metallothionein that induced by metals and could be served as biomarker was confirmed in field application. The potential use of metallothionein and stress protein as biomarker of trace metal exposure in crabs *Carcinus maenas* were investigated. The copper and zinc metallothionein concentration measured in gills reflected the copper and zinc exposure gradient. In contrast, stress-70 level in gills did not appear to correlate with the degree of trace metal exposure (Pedersen *et al.*, 1997). Similarity result was found in the monitoring of water quality of St Lawrence River in zebra mussels, *Dreissena polymorpha*, the result indicated that only metallothionein had

positive relationship with copper but no significant correlation was observed between other biomarkers (De Lafontain *et al.*, 2000). In brown trout, *Salmo trutta*, from two rivers with different metal compositions due to mining pollution, cadmium, copper and zinc concentration and metallothionein level of tissues were measured. The result showed that metallothionein level from both sites clearly reflected the presence of these metals. Strong correlation existed between metallothionein-like protein and metal level also found in marine bivalve, *Macoma baltica*, during the period of two and a half years from two locations of Western Scheldt Estuary (Bordin *et al.*, 1997)

2.4.6 Analytical technique for detection and quantification of metallothionein

2.4.6.1. Chromatographic Techniques

Chromatographic techniques permit the separation of MTs from other soluble compounds present in tissue homogenates. Given the absence of specific activity of MTs, the criteria used to characterize isolated fractions rest on the physico-chemical properties of these proteins (Amiard and Cosson, 2000) or on the detection of metals associated with them.

On gel permeation columns, the MTs be eluted behaves compounds of molecular weight from 12 to 15 kDa. MTs are verified by the persistence of these compounds in the denatured homogenates at high heat and that they have a very low absorbency at a wavelength of 280 nm, because MTs do not consist of (or have very little of) aromatic amino acids. On the other hand, these compounds show significant absorbance at a wavelength characteristic of metal-cysteine liaisons (Zn-SH = 230 nm, Cd-SH = 250 nm, Cu-SH = 270 nm, Hg-SH = 310 nm).

If the preparation of samples and their conditions of elution on the column has respected the stability of metal-cystein liaisons (sensitive to oxidation), the peaks containing the MTs must show consistent levels of metals. It is possible from the measurement of metal concentrations in the fraction containing MTs to deduce their concentration, based on stoichiometric rations of metal to apoprotein. The natural MTs are never saturated by single metal. Different metals (silver, cadmium, copper, mercury, zinc) can be linked to proteins, which poses analytical and methodological problems. It is technically possible to measure simultaneously and precisely several metals present in low concentration in a sample, but it requires a high performance and costly apparatus. Moreover, if the stoichiometry of MTs containing zinc or cadmium is well known (7 metallic atoms per molecule of apoprotein), it is not the same for those that contain copper (in principle 12 atoms per apoprotein). The uncertainty is even higher for mercury and silver. The rations not yet entirely clarified with regard to natural MTs, but possibly reaching 18 atoms per apoprotein (Lu *et al*., 1990; Zelazowski and Stillman, 1992). It seems difficult to quantify the concentration of MTs. However, this analytical protocol may provide very useful information on the metallic composition of MTs when metals are analyzed continuously in fractions separated by high performance liquid chromatography (HPLC) (Suzuki, 1980). The applications of HPLC to separation and detection of MTs have been considerable. Presently, the use of anion exchange columns or reversed phase columns allows separation and quantification of different isoforms of MTs, either indirectly by measurement of associated metals (Suzuki *et al.*, 1983), or directly by measurement

of the surface of the corresponding peaks (Richards and Steele, 1987). Despite constant improvements, these techniques remain difficult to implement because they necessitate sophisticated analytical instruments and are relatively time-consuming, which is a serious handicap in analysis of a series of samples.

2.4.6.2 Metal Saturation Assay

The different protocols used are based on the difference in the affinity of metals for apoprotein, which decreases in the following order: Hg > Ag > Cu > Cd > Zn. Once all the sites of MTs are saturated by the metal used (cadmium, mercury, or silver), this metal is quantified (via atomic absorption spectrophotometry or by measurement of the radioactivity of a marked isotope)

The method proposed by Onosaka and Charian (1982) and improved by Eaton and Toal (1982) relies on saturation of binding sites of metals of MTs by radioactive cadmium (^{109}Cd). This method was very frequently used, because the number of Cd ions fixed by a molecule of MT is well established. The process of substitution, on the other hand, does not work perfectly except for MTs that contain only zinc and/or a very small amount of copper. It works even less well for MTs containing mercury of silver.

Piotrowski *et al*. (1973) used mercury (^{203}Hg), but the low specificity of mercury fixation seemed to introduce an overestimation of MT content and the method seemed to lack sensitivity (Webb, 1979; Dieter *et al*., 1987). It appears that these disadvantages can be overcome by the addition of ovalbumin (Dutton *et al.*, 1993) or rat hemoglobin (Couillard *et al*., 1993).

Scheuhammer and Cherian (1986) developed a method very similar to the protocol used for cadmium, but replacing it with silver. The silver fixed by MTs can be quantified by atomic absorption spectrophotometry or using a radioactive isotope (^{110m}Ag). The strong affinity of silver for MTs excludes all possibility of substitution by metals initially present on the MTs (except perhaps mercury).

2.5.6.3 Polarographic Method

The polarographic method relies on the measurement of change in intensity of a current caused by the reduction of hydrogen of sulfhydry1 groups of MT (Olafson and Sim, 1979). The analysis is not influenced by the nature of metals fixed on the protein or by the species (or tissue) analysed (Hogstrand and Haux, 1992). The use of a specific electrolyte, elimination of soluble compounds that are heat-sensitive (or that can be denatured by ethanol), and reduction in the temperature of the analysis allow us to directly quantify MTs in solution without major interference (Thompson and Cosson, 1984). The specificity of this analytic technique was confirmed by chromatographic study (permeation and anion exchange) of polarographic active peaks (Olafson and Olsson, 1991).

2.5.6.4 Immunological Methods (RIA and ELISA)

The quantification of MTs by RIA (radioimmunoassay) or by ELISA methods (enzyme-linked immunosorbent assay) was made possible by the production of polyclonal antibodies (Tohyama and Shaikh, 1978; Van der Malie and Garvey, 1979; Thomas *et al.*, 1986; Roesijadi, Unger and Morris, 1988). These techniques are much more sensitive than the preceeding ones, but their use cannot easily be extended because they remain subjected to the possession of an antibodies produced against the MTs of mammals. However, the antibodies produced against the MTs of mammals may have good reactivity against MTs of other species of mammals, but they react little with MTs of non-mammal species. There are antibodies produced against MTs of different fishes (Kito *et al.*, 1986; Chatterjee and Maiti, 1987; Hogstrand and Haux, 1990; Norey *et al.*, 1990) that can be used in an interspecific study because of strong homology of MTs of fish (George and Olsson, 1994) and anti-MT antibodies of mussel (Roesijadi, Unger and Morris, 1988). The precise quantification of MTs by these highly effective methods demands therefore a study of the appropriate antibody used, particularly with respect to cross-reactions with MTs of the species studied, as well as to different isoforms present.

2.4.6.5 Quantification of mRNA

Regulation of MT synthesis occurs at the transcriptional level, during the production of messenger RNA (mRNA) coding for the apoprotein (Amiard and Cosson, 2000). It is therefore possible to consider measuring and increase in the production of these mRNA in response to a metallic contamination of the environment, rather than seeking to prove an increase in the corresponding protein. For that, it is necessary to use a nuclear probe (simple marked strand) that can be link itself (hybridization by complementarities) with the mRNA and form double strand. This technique, clearly more sensitive than earlier ones, allows us to detect responses at the cellular level. The preparation of specific probes requires cloning of the gene or genes coding for MTs in the species studied. Several cDNA have been cloned in fish (Bonham *et al*., 1987; Chan *et al.*, 1989; Leaver and George, 1989; Kille e*t al*., 1991), in a sea urchin (Nemer e*t al*., 1985), and in on oyster (Roesijadi *et al*., 1991). This method has been used operationally in evaluation of the impact of pollutants in many programs (George and Olsson, 1994), and it has proved to be well adapted to this type of study. At present, PCR techniques have been developed and used widely in this kind of study.

CHAPTER III
MATERIALS AND METHODS

3.1. Chemicals

-Absolute ethanol (BDH, England)
-Acetic Acid (Merck, Germany)
-Acrylamide (Sigma Chemical Co., USA)
-Agarose (FMC Bioproduct, USA)
-Ammonium persulfate (APS) (Sigma Chemical Co., USA)
-Bacto-agar (Oxoid, England)
-Bacto-yeast extract (Oxoid, England)
-100 base-pair DNA ladder (Promega Co., USA)
-Bio-Rad Protein Assay (Bio-Rad, USA)
-Bis-Acrylamide (Promega, Co., USA)
-Boric acid (Merck, Germany)
-Bovine Serum Albumin (BSA) (Promega, Co., USA)
-Bromophenol Blue (Merck, Germany)
-Chloroform (Merck, Germany)
-Peroxidase conjugated rabbit anti-mouse immunoglobulin (DAKO, Denmark)
-Coomassie brilliant blue R250 (Research Organic, USA)
-2.5 mM dATP, dCTP, dGTP, dTTP (Promega Co., USA)
-Diaminobenzidine (DAB) (Sigma Chemical Co., USA)
-Diethyl pyrocarbonate (DEPC) (Sigma Chemical Co., USA)
-Di-sodium hydrogen phosphate (Merck, Germany)
-1,4-Dithiothreitol (DTT) (Fluka, Biochemika, Swizerland)
-Ethidium Bromide (Sigma Chemical Co., USA)
-Ethylene Diamine tretaacetric acid, (EDTA) (Fluka Chemika, Switzerland)
-Formaldehyde (LabScan Asia Co., Thailand)
-Glycine (USB, Amerson Life Science, England)
-Hydrogen peroxide (Siribuncha & CO.LTD, Thailand)
-Improm-II™ Reverse Transciption System (Promega, USA)
-Isopropanol (LabScam Asia, Co., Thailand)
-Methanol (LabScan Asia Co., Thailand)
-Mouse Anti-Metallothionein monoclonal antibody (Stressgen, Canada)
-pGEM® T-easy vector (Promega, Co., USA)
-Potassium chloride (Merck, Germany)
-Potassium di-hydrogen phosphate (Merck, Germany)
-Prep-Gene® DNA Purification Kit (Bio-Rad Laboratories, USA)
-QIAprep® Spin Miniprep Kit (250) (QIAGEN GmbH, Germany.)
-Sephadex G-75 (Amersham Phamacia, Sweden)
-Silver nitrate (Sigma Chemical Co., USA)
-Sodium carbonate (Sigma Chemical Co., USA)
-Sodium chloride (Sigma Chemical Co., USA)
-Sodium dodecyl sulfate (SDS) (Sigma Chemical Co., USA)
-Sodium thiolsulfate (Sigma Chemical Co., USA)
-Spermidine trihydrochloride
-Sucrose (Sigma Chemical Co., USA)
-Treta Methylethylene diamine (TEMED) (Merck, Germany)

-Tri Reagent® (Molecular Research Center, Inc, USA)
-Tris (USR, Amershon Life Science, England)

3.2 Enzymes

-Restriction Enzyme *Eco*RI (Promega, Co., USA)
-DyNAzyme TM II DNA polymerase (Finnzyme, Finland)

3.3 Bacteria

-*Escherichia coli* stain JM 109 (rec A1 supE44 and A1 hsd F17 gyrA 96 rel A1 thiA (lac-pro AB) F (tra D 36 pro AB lac 9 lac ZAM15)

3.4 Equipment

-Automatic micropipette size: P2, P10, P20, P40, P100, P200 and P1000 (Gilson Medical Electrical S.A., France)
-Camera Pentax K1000 (Asahi Opt. Co, Ltd.)
-Chromotography Column (xk 16/70) (Amersham Phamacia, Sweden)
-Fraction Collector and peristaltic Pump. (Acta prime, Amersham Phamacia, Sweden)
-Horizontal gel electrophoresis, Sub-cell GT MINI (Bio-Rad, USA)
-Laminar flow cabinet (Nuaire Class II, NU-440-300E, and USA)
-MegaBACE 1000 (Amersham Phamacia, Sweden)
-PCR thermal cycler: PCR sprint (Hybaid, England)
-Polyacrylamide Electrophoresis, Mini PROTEAN® II Cell (Bio-Rad, USA)
-Polyacrylamide Electrophoresis, PROTEAN® II xi Cell (Bio-Rad, USA)
-Mini trans-Blot electrophoretic transfer cell (Bio-Rad, USA)
-Power supply (Bio-Rad Laboratories, USA)
: Power PAC 300
: Power PAC Junior
: Model 200/0.2
-Refrigerated Centrifuge, 3K18 (Sigma Osterode and Harz, Germany)
-Spectrophotometer (Milton Roy Genesys 5, Germany)
-Water bath, SBS 30 (Stuart Serentific, UK)
-UV transilluminate, M26 (UVP, USA)

3.5 Animals

Mussels, *Perna viridis*, collected from Samutprakarn province and Trad province were maintained in laboratory tanks with aerated seawater, salinity 28 ppt. and temperature 29-30 ºC. The experiment was conducted at the laboratory of Marine Biotechnology Research Unit (MBRU), Department of Marine Science, Chulalongkorn University. The animals were fed daily with artemia. Unwanted sediment was removed and about 10-20 % of seawater was changed every day. Animals were acclimated to laboratory condition for 1 week before the experiment.

3.6 Mercury exposure

The experiment was conducted in five 1000-L tanks. Each tank contained approximately 150-200 mussels with aerated seawater. Mercuric chloride was applied to each tank to get the concentration of 0.0, 0.1, 0.5, 1.0, and 5.0 μg/l , respectively. The concentration of mercuric chloride was maintained for 7 weeks. Specimens from each treatment were collected every week for the first month and twice a week for the second month. During the experiment, the animals were fed and about 10% of same mercuric chloride concentration water was changed daily in each treatment.

3.7 Tissue preparation

After mussels were collected from each treatment, digestive tracts were dissected and homogenized immediately in 3 volumes of Tris sucrose buffer (0.25 M sucrose, 10 mM Tris-HCl pH7.4, 5 mM dithiotheitol) The homogenate was subjected to high speed centrifugation (17,000 g for 1 h at 4ºC). Supernatant was collected and kept at -20ºC.

3.8 Determination of Metallothionein by gradient gel electrophoresis and silver staining

3.8.1 Determination of protein concentration

Protein concentration of sample was determined by the dye assay described by Bradford (1975) using reagents produced by Bio-Rad (Bio-Rad, USA) Bovine serum albumin (Fraction V) was used as protein standard (Appendix C).

3.8.2 Electrophoresis

The method used in this experiment was modified from the method of McCormic and Lin (1991). A vertical slab gel apparatus (Bio-Rad, USA, model Mini PROTEIN® II cell) was used for electrophoresis system. Linear gradient of polyacrylamide gel was conducted using 7.5-30 % native gradient gel (10x7x0.5 cm). Gel preparations was shown in appendix A.

Samples were mixed with an equal volume of loading buffer (appendix A) and loaded into gel lanes. The molecular weight standards included 94, 67, 43, 30 and 14 kDa Electrophoresis was carried out at 180V (20mA) until the bromophenol blue dye left the gel. Gel was then stained for protein (either with Coomassie or silver stain) or blotted.

3.8.3 Staining

Following electrophoresis, gel was removed and placed directly into a solution containing 1.5 mM Coomassie brilliant blue R250, 10 % acetic acid and 40% methanol. Gel was stained for 1 h and de-stained in solution containing 40 % methanol and 10 % acetic acid for at least 2 h with gentle agitation. Gel was further stained (enhanced) with silver staining by fixation in 50 % methanol for 10 min and in

5 % methanol for 10 min. The gel was rinsed 3 times with distilled water before DTT treatment (0.033 μM DTT) for 10 min. The gel was then washed again 3 times with distilled water. The gel was immerged in 0.1 % silver nitrate for 10 min followed by 3 times of distilled water wash. Finally, the gel was developed in developing solution (3% $NaCO_3$) until background turn to yellow the gel was moved to new developing solution until bands appeared.

3.9 Detection of metallothionein by Western blot analysis

The method used in this experiment was adopted from Towbin (1979) with modification.

3.9.1 Blotting

Following electrophoresis as explained in 3.8.2 gel was rinsed briefly with distilled water then soaked with blotting buffer (0.25 M Tris-HCl, 1.92 M glycine) for 30 min. Gel was blotted at 15-25 V for a minimum of 1 h at 4 ºC onto nitrocellulose membrane (Hybound™-C Pure) using mini-trans-blot electrophoretic transfer cell (Bio-Rad.)

3.9.2 Immonochemical staining

Following blotting, membrane was rinsed with PBS (1x PBS, pH 7.3) for 1-2 min and incubated in blocking solution (1% (w/v) BSA in PBS) at room temperature for 1 h. Membrane was then rinsed 3 times (5 min each) with PBS. Membrane was probed with 1:1000 dilution of primary antibody (mouse anti-Metallothionein monoclonal antibody (Stressgen, Canada) in 1%BSA/PBS for at least 1 h. Membrane was rinsed again 3 times (5 min each) with PBS. The membrane was probed with 1:1000 dilution of secondary antibody Peroxidase conjugated rabbit anti-mouse immunoglobulin (DAKO, Denmark) in 1%BSA/PBS for 1 h at room temperature. Unbound secondary antibody was removed by washing 3 times with 0.05 % Tween-20/PBS for 5 min each and 2 times with PBS for 5 min each. Finally, immunoreactive proteins were visualized by soaking with DAB solution (3 mM DAB, 0.03% w/v Hydrogen peroxide in 50 mM Tris-HCl, pH 7.6) until peptide band appeared. The reaction was stopped by washing with distilled water. The membrane was kept in the dark.

3.10 Purification of metallothionein by liquid chromatography

3.10.1 Tissue preparation

Gills and digestive tracts from mercuric chloride treated mussels were prepared as explained in 3.7. Supernatants from each tissue were pooled and subjected to acetonic fractionation.

3.10.2 Mercury Determination

Sample were weighed directly into the nickel boat then, were analysed by Mercury analyzer (AMA254, LEGO, USA) using program Quick Silver (LEGO,

USA). Instrument parameters were set as follow: 60 seconds for drying time, 200 seconds for decomposition time and 45 seconds for waiting time.

3.10.3 Partial purification of metallothionein by acetonic fractionation

Cold acetone (–20ºC) was added to supernatant to a final concentration of 45%. The sample was maintained at 4ºC for 30 min under magnetic stirring and then centrifuged at 14, 500g for 10 min. The pellet was discarded and the acetone concentration of the supernatant was raised to 80%. The preparation was maintained at 4ºC for 40 min under magnetic stirring and centrifuged again at 14,500g for 10 min. The 80% acetonic pellet was resuspended in 6 ml of 20mM Tris-HCl, pH 8.6,containing 0.01% NaN_3 and 10 mM NaCl.

3.10.4 Gel filtration

The method was adopted from the method of Ponzano *et al* (2000). Sephadex G-75 (120 ml) was packed into chromatography column (xk 16/70, Amersham Phamacia, Sweden). The column was then connected to Akta prime liquid chromatography system (Amersham Phamacia, Sweden), calibrated with molecular weight standard proteins (Blue dextan, Bovine serum albumin, and Cytochrom C), and washed extensively with buffer 20mM Tris, 10 mM NaCl, 40 mM EDTA (appendix A). Calibration curve was show in appendix C

Gel filtration was performed at 10ºC on a Sephadex G-75 column equilibrated with buffer20mM Tris, 10 mM NaCl, 40 mM EDTA. Protein concentration of sample was determined as described in 3.8.1 Sample was applied to the column and eluted with buffer 20mM Tris, 10 mM NaCl at the flow rate of 1 ml/min. Sample fraction (1 ml) was collected and analysed for protein by determining the absorbance of fractions at 280 nm. The mercury contents of the fractions were also analysed using Mercury analyzer (AMA254, LEGO, USA). Details as described in 3.10.2

Further purification was carried out on diethylaminoethanol (DEAE)-cellulose ion exchange column (Millipore DEAE MemSep 1010). Expected mercury-containing fractions were pooled and applied directly onto the DEAE column. The column was equilibrated with 20 mM Tris-HCl buffer, pH8.6, containing 0.01% NaN_3 and 1mM DTT. A linear gradient of 150 ml 20-400 mM Tris-HCl, pH 8.6, containing 1 mMDTT and 0.01%NaN_3 was applied to the column, followed by 45 ml 400 mM Tris-HCl buffer, pH 8.6, containing 1 mM DTT and 0.01% NaN_3. Absorbance at 310 nm was measured. Mercury contents from fractions were analysed by Mercury analyzer. Fractions containing mercury were pooled and the proteins were precipitated by adding cold ethanol (-20ºC) /chloroform to final concentration of 87%/1%. The pellet was resuspended in deionized H_20 containing 1 mM DTT and then incubated at 60ºC for 10 min. Protein profile was determined on 10% polyacrylamide gel.

3.11 Expression of metallothionein gene

3.11.1 RNA extraction

Mussels exposed to mercury from each treatment in 3.6 were collected. Gills and digestive tracts were dissected and immediately homogenized in liquid nitrogen. Ground samples were mixed with TRI REAGENT® (Molecular Research Center, Inc, USA) (50-100 mg of tissue per 1 ml of Tri reagent) and maintained for 5

minutes at room temperature to permit the complete dissociation of nucleoprotein complex. The mixture was then centrifuged at 12,000 g for 10 min. The aqueous phase (upper phase) was transferred to a fresh tube and extracted with 0.2 ml of Chloroform per 1 ml of TRI REAGENT®. The mixture was left at room temperature for 2-15 minutes then centrifuged at 12,000 g for 15 minutes at 4°C. The colorless upper aqueous phase containing RNA was transferred to a fresh tube. RNA was then precipitated by the addition of isopropanol (0.5 ml of isopropanol per 1 ml of Tri reagent originally used). The mixture was kept at room temperature for 5 –10 minutes before centrifugation at 12,000 g for 8 min at 4°C. The supernatant was discarded and RNA pellet was washed with 75 % ethanol followed by centrifugation at 7,000 g for 45 min at 4°C. The pellet containing total RNA was air-dried for 3-5 min. and dissolved in DEPC-treated distilled water.

3.11.2 Determination of total RNA concentration

The quantity of total RNA was estimated by the absorbance at the wavelength of 260 nm. The amount of RNA was calculated by the following equation:

At A_{260} = 1, RNA concentration equals 40 µg/ml (Beaven, Holiday and Johnson, 1995; Wilfinger, Mackey and Chomezynski, 1997)

The quality of RNA was also estimated by the ratio of the Absorbance 260 nm / 280 nm. The high purity of RNA should be at the ration of A_{260}/A_{280} between 1.6 – 1.9

3.11.3 Semi-quantitative RT-PCR

Reverse transcription was conducted to make first strand cDNA using total RNA extracted from digestive tracts of mercury-treated mussels in 3.7. The reaction was performed in the final volume of 20µl, at 42°C, for 90 min using Improm II™ reverse transcription kit condition (1 U of Improm II™ reverse transcription, 2µl of 1x Improm II™ reactive buffer, 2.5 mM $MgCl_2$, 0.5 mM dNTP mix, 0.5 µg Oligo dT, and 2.0 U of recombinant RNasin® Ribonocluose Inhibitor). Quantitative PCR was conducted using exact concentration of first-stranded cDNA as template (750 ng). Primers were designed from methallothonien sequence of mussels *Perna viridis* reported in GenBank (AAD02054*).* Forward Primer was *5' ATGCCCAGCCCTT*GTAATTG 3'*(MTI) and Reverse primer* was 5' TTTGCACGAACAACTGGTTA 3'*(MTII) Beta-actin* was used as constitutional expression control. Actin primers were designed from beta-actin sequence *of P. monodon* reported in GenBank (AF11000987). Forward Primer was 5'GGTATCCTCACCCTCAAGTA 3' (Actin1) and reverse primer was 5'AAGAGCGAAACCTTCATAGA 3' (Actin 2). For PCR condition, samples were supplemented with the addition of 1x buffer (10 m M This – HCl, pH 8.8, 50 mM KCl, 0.1% Triton x – 100), 1.5 nM $MgCl_2$, 0.5 µM each of MTI and MTII primer, 0.5 mM dNTP mix, and 1 U Taq DNA polymerase). The PCR reaction for metallothionein gene was performed as follow.

Initial denaturation step:	94°C, 3 min	for 1 cycle
Denaturing step:	94°C, 30 sec	
Annealing step:	50°C, 30 sec	} for 25 cycles
Elongation step:	72°C, 30 sec	
Extension step:	72°C, 7 min	for 1 cycle

For β-Actin reference gene, PCR reaction was performed as follow.

Initial denaturation:	94°C, 3 min	for 1 cycle
Denaturing step:	94°C, 1 min	
Annealing step:	48°C, 1 min	} for 5 cycles
Elongation step:	72°C, 1 min	
Denaturing step:	94°C, 1 min	
Annealing step:	53°C, 1 min	} for 15 cycles
Elongation step:	72°C, 1 min	
Extension step:	72°C, 7 min	for 1 cycle

This method was modified from the method of Marone et al. (2001). The PCR products from each sample were applied to 1.2 % agarose gel electrophoresis. Gel was stained with ethidium bromide. DNA bands were visualized and documented under UV light. The intensity of metallothionein band was detected and compared with that of beta-actin.

3.12 Genetic variation of metallothionein gene detected by single stranded conformation polymorphism (SSCP)

3.12.1 Sample preparation

Samples were prepared and determined as described in 3.11.1 and 3.11.2. First-stranded cDNA from 3.11.3 were used a template and MTI and MTII were used as primers for PCR reaction to amplify metallothionein genes. The PCR condition was as follow.

1x buffer

1.5 nM $MgCl_2$

0.5 μM each of MTI and MTII primers

0.5 mM dNTP mix

1 U Taq DNA polymerase

Initial denaturation step:	94°C, 3 min	for 1 cycle
Denaturing step:	94°C, 30 sec	
Annealing step:	50°C, 30 sec	} for 25 cycles
Elongation step:	72°C, 30 sec	
Extension step:	72°C, 7 min	for 1 cycle

3.12.2 SSCP gel Preparation and running

The method used in this experiment was a method described by Hein *et al.* (2003) using Polyacrylamide Electrophoresis, PROTEAN® II xi Cell (Bio-Rad, USA). Polyacrylamide gel (15% gel, 2.66% cross-link)(16x20x0.4cm) was prepared (Appendix A). The condition used for gel preparation was described as follow.

15% polyacrylamide solution (30 ml) was added 200 µl of fresh 10 % ammonium persulphate and 20 µl of TEMED. The gel was allowed to polymerize for 4 hr and pre-run in the gel box in a 4°C cold room for at least 5 minutes. Load samples were prepared as above and were allowed to run at 200 V, 4°C until the bromophenol blues have reached the bottom.

PCR product (8 µl) from each sample was mixed with 32 µl of loading dye. Sample was heated at 95°C for 5 minutes and immediately transferred to the ice box. Sample was loaded onto polyacrylamide gel for single stranded conformation polymorphism analysis.
The system was running until the dye reached the bottom of the gel.

3.12.3 Silver straining

Gel was removed with on side attached to the glass and placed into the fix-stop solution (10%gracial acetic) for 20 min. After 3 washes with distilled water, the gel was stained with 0.1 % silver nitrate for 30 min. Gel was then washed again with distilled water for 10 s. before placing into the developing solution (30% $NaCO_3$, sodoiumythiosulfate, 0.55%formadehyde). Once the band of DNA started to appear, the gel was transferred into freshly prepared developing solution and shaken until all DNA bands were visualized.

3.12.4 Separation of ssDNA from SSCP gel

Following gel staining, separating bands of ssDNA were analysed. Each ssDNA band was cut out of the gel and washed 3 times (20 min each) with ultrapure water. Each band was added with 20 µl of ultrapure water and incubated at 37^{o}C for 24 h. to allow DNA to diffuse to from gel to the water. This cDNA was used as template for amplification of each variation of metallothionein gene. PCR product was loaded onto 1% agarose gel and the band of interest was cut out and DNA was eluted by Prep-Gene® DNA Purification Kit (QIAGEN GmbH, D-40724 Hilden).

3.12.5 Cloning and sequencing of PCR product of metallothionein genes.

PCR product of metallothionein gene was ligated into pGEM® T-easy vector (Promega, Co., USA). The method was conducted as described by company provided protocol. Briefly, 5 µl of 2x Rapid Ligation Buffer were added to reaction. Then, 0.5 µl ofpGEM- T vector was added and following by 1 µl PCR product. Next, 1 µl T_4 DNA ligase was added and dH_2O was added to 10 µl final volumn.

3.12.6. Transformation of recombinant T-easy vector into *E.coli* JM109

3.12.6.1 preparation of competent cell

The preparation was based on the method of Chung and Miller (1988). *E.coli* strain JM109 was streaked onto LB plate (appendix A) and incubated at 37ºC

overnight. Single colony from the culture plate was sub-cultured into LB media (appendix A) at 37ºC with agitation until the optical density at A_{600} of the culture was between 0.3 and 0.6. The culture was centrifuged at 1000g for 10 min, 4ºC. The cell pellet was resuspended in 1/10 volume of transformation and storage buffer (LB media, containing 10% w/v PEG8000, 5% DMSO, 10mM $MgCl_2$ and 10mM $MgSO_4$) and incubated on ice for 10 min. Competent cells were mixed with glycerol (15% final concentration), aliquots (100µl) and stored at -70ºC

3.12.6.2 Transformation

Aliquot of competent cells (100µl) was thawed, mixed with recombinant vector, and incubated on ice for 30 min prior to heat shock at 42ºC for 60 sec. Cells were immediately moved back on ice for 5 min. SOC medium was added and mixed gently before incubating the mixture at 37ºC for 1 h with agitation. Transformed cells were plated on LB agar plate contained 50 µg/ml of amplicilin, 0.5 mM IPTG, and 40 µg/ml of X-gal. The plate was incubated at 37ºC overnight.

3.12.6.3 Screening for transformant colonies

Blue-white colony technique was applied for screening the transformant colonies. Individual white colonies were selected and subjected to colony PCR as described below.

1x buffer (10 m M This – HCl, pH 8.8 at 25°C, 50 mM KCl, 0.1% Triton x – 100), 1.5 mM $MgCl_2$,
2 µM each of pUC1 and pUC2 primer,
1.0 mM dNTP mix, and
0.25 U Taq DNA polymerase.

PCR condition started with initial denaturation at 94 °C for 3 min and denaturing step at 94 °C for 1 min, 53 °C for 90 sec and 72 °C for 90 sec for 35 cycles and elongation step at 72 °C for 7 min. Alternatively, cells containing DNA target were examined by isolation of plasmid from cell culture and the insert DNA was analyzed by restriction enzyme digestion.

3.12.6.4 Isolation of Plasmid DNA

The method was based on that of Birnboim and Doly (1979). LB or 2xYT broth media (5 ml) containing 10 µg/ml of amplicilin was inoculated with a single recombinant colony and incubated overnight at 37°C with agitation. The cultured cell was centrifuged at 4000xg for 1 min. After the media was discarded completely, the cells were resuspended in 300 µl of GTE buffer (50mM glucose, 25mM Tris-HCl, pH 8.0, and 10mM EDTA). An equal volume of freshly prepared lysis buffer (1% SDS, 0.2 M NaOH) was added and the solution was mixed gently before placing on ice for 5 min. The mixture was neutralized by adding 300 µl of neutralizing buffer (3M potassium acetate pH 4.8). After gently mixing and keeping on ice for 5 min, the mixture was centrifuged at 14,000xg for 10 min. The supernatant containing plasmid DNA was removed and precipitated with equal volume of isopropanol at -20ºC for 30 min. The solution was then centrifuged and washed with 80% ethanol. The pellet was dried and resuspended in 50 µl of water or TE buffer (1

M Tris, 0.5 M EDTA, pH 8.0) prior to the addition of RNase and incubation at 37ºC for 30 min. Plasmid DNA was subjected to restriction enzyme digestion. Further purification of plasmid DNA for sequencing was conducted by phenol/chloroform extraction. The aqueous phase containing plasmid DNA was precipitated by placing at -20ºC for 2 h with the addition of 0.1 volume of 3 M sodium acetate, pH 7.4 and 2 volume of 100% ethanol. After centrifugation, the plasmid pellet was washed with 80% ethanol, dried, and resuspended in distilled water or TE buffer. Alternatively, plasmid DNA was extracted using QIAprep® Spin Miniprep Kit (QIAGEN GmbH, D-40724 Hilden). Briefly, Transfer 1.5 ml of the inoculated culture to a microcentrifuge tube and spin for 5 minutes at13,000 rpm in a microcentrifuge. Pour off supernatant. Repeat this process using the same microcentrifuge tube until the entire culture was transferred. Resuspend pelleted bacterial cells in 250 μl of Buffer P1 and Vortex. Add 250 μl of Buffer P2 and invert tube gently 4-6 times to a mixing. Add 350 μl of Buffer N3 and invert tube immediately but gently 4-6 times. Centrifuge for 10 minutes at maximum speed in tabletop microcentrifuge. During Centrifugation prepare the vacuum manifold and QIAprep columns. Apply the supernatant (about 850 μl) from previous step to the QIAprep column by pipetting. Switch on vacuum source to draw the solution through the QIAprep columns and switch off vacuum source. Wash the QIAprep column by adding 750 μl Buffer PE. Switch on vacuum source to draw wash solution through the column and then switch off vacuum. Transfer the QIAprep columns to a microcentrifuge tube and centrifuge for 1 minute at 13,000 rpm. Place the QIAprep column in a clean 1.5ml microcentrifuge tube. To elute DNA, add 50 μl Buffer EB (10mM Tris-CL, pH 8.5) or water to the center of the column, let stand for 2 minutes and then centrifuge for 1 minute

3.12.6.5 DNA sequencing

Plasmids containing insert DNA of interest were subjected to DNA sequence analysis. The DNA sequencing was conducted on MegaBACE 1000 (Amersham Phamacia, Sweden) at Unit of Shrimp Molecular Biology and Genomic Laboratory at the department of biochemistry, faculty of Science, Chulalongkorn University. The plasmids were also sent to BSU (Bioservice unit) for sequence analysis.

3.12.6.6 Characterization and classification of metallothionein genes

DNA sequences of metallothionein were characterized using BLAST protocol (Altschul *et al*., 1990). Sequence alignment of metallothionein genes was also performed by ClastalX program (Jeanmougin *et al.*, 1998). The classification of metallothionein gene was based on the classification of metallothionein by Binz and Kaji (1997). Phylogenetic analysis was performed by Clustal X program, PHYLIP, Treeview program and Distant model.

CHAPTER IV

RESULTS

4.1 Identification of Metallothionein from mercury-exposed mussels, *Perna viridis*

Metallothioneins in the tissues of mussels, *P. viridis*, exposed to dissolved mercury were determined using gel electrophoresis and the level of metallothionein was detected by silver staining and Immunochemical analysis.

4.1.1 Identification of Metallothionein by gel electrophoresis and silver staining

Mussels, *P.viridis* collected from Samutprakarn province were maintained in seawater at salinity of 28 ppt and temperature 29-30 ºC. After acclimation to laboratory condition for 1 week, the mussels were exposed to mercuric chloride at the concentration of 0.0, 0.1, 0.5, 1.0, and 5.0 μg/l for each treatment, respectively. Samples were extracted from gills and digestive tracts of mussels from each treatment at week 1, 3, 5, and 7, respectively.

Extracted samples from the gills of mercury-exposed mussels were analyzed by native 7.5-30 % gradient polyacrylamide gel electrophoresis and proteins were determined by Coomassie and silver staining. The results are shown in figure 4.1, 4.2, 4.3, and 4.4.

Extracted samples from the digestive tracts of mercury-exposed mussels were analyzed by native 7.5-30 % gradient polyacrylamide gel electrophoresis and proteins were determined by Coomassie and silver staining. The results are shown in figure 4.5, 4.6, 4.7, and 4.8.

As the results revealed that no particular bands of proteins in each sample possessed the high band intensity following silver staining which is a unique characteristic of metallothionein. There were also no clear peptide bands identified as metallothionein by molecular weight comparison with rabbit metallothionein.

In gill, one band was detected at the same molecular weight as standard and the band intensity was high in samples from mussels treated with mercuric chloride at 0.1 and 1.0 ppb. In digestive tract, none particular band was detected as metallothionein in any treatment (see table 4.1-4.2).

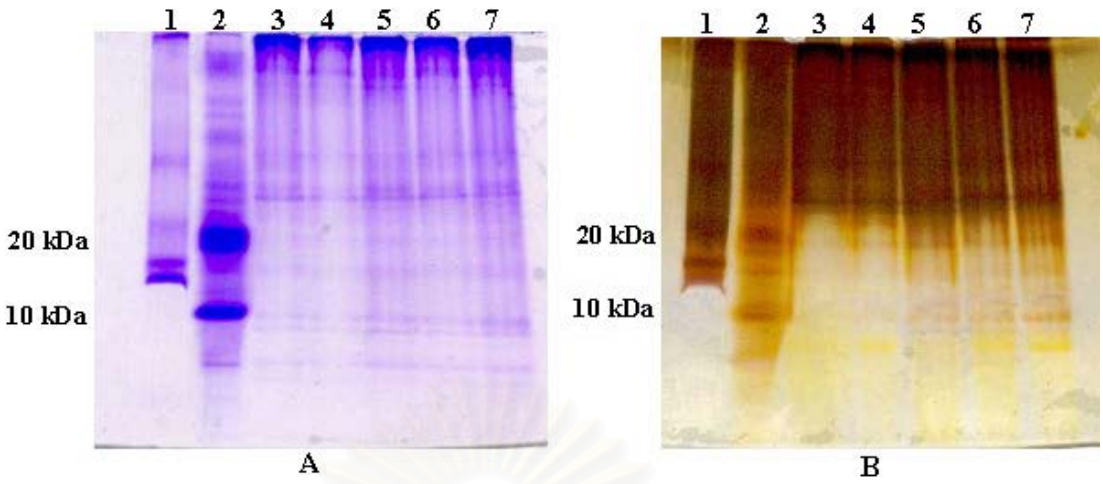

Figure 4.1 Protein profile of gill extracts from mussel treated with mercuric chloride for 1 week. Samples (25 µg each) were loaded onto native 7.5-30% gradient polyacrylamide gels and stained with Coomassie brilliant blue (A) and silver solution (B)

Lane 1 = Low molecular weight standard
Lane 2 = Rabbit Metallothionein (control)
Lane 3 = Gill extract from control mussels
Lane 4 = Gill extracts from mussels treated with 0.1 µg/L $HgCl_2$
Lane 5 = Gill extracts from mussels treated with 0.5 µg/L $HgCl_2$
Lane 6 = Gill extracts from mussels treated with 1.0 µg/L $HgCl_2$
Lane 7 = Gill extracts from mussels treated with 5.0 µg/L $HgCl_2$

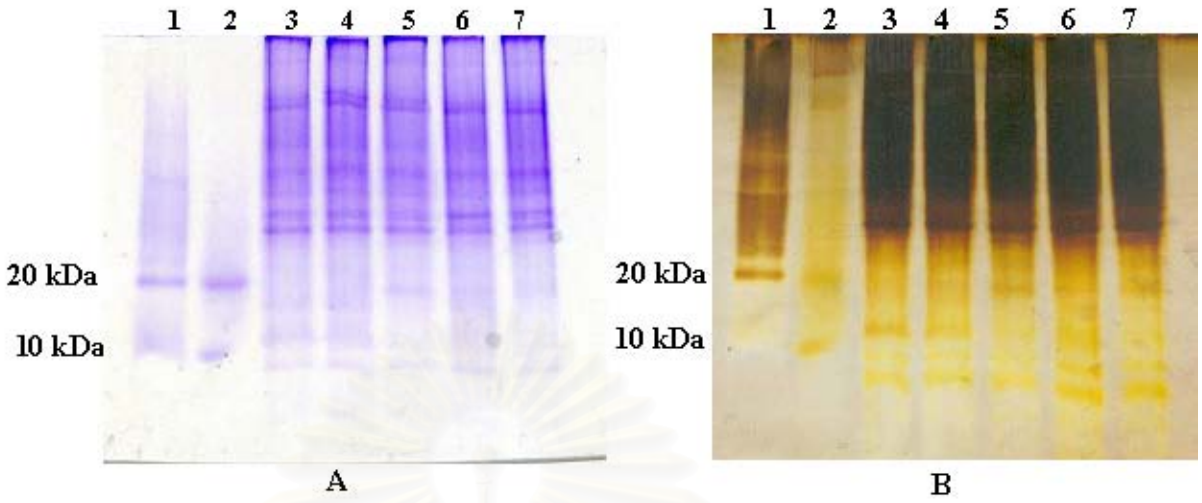

Figure 4.2 Protein profile of gill extracts from mussel treated with mercuric chloride for 3 weeks. Samples (25 µg each) were loaded onto native 7.5-30% gradient polyacrylamide gels and stained with Coomassie brilliant blue (A) and silver solution (B)

Lane 1 = Low molecular weight standard
Lane 2 = Rabbit Metallothionein (control)
Lane 3 = Gill extract from control mussels
Lane 4 = Gill extracts from mussels treated with 0.1 µg/L $HgCl_2$
Lane 5 = Gill extracts from mussels treated with 0.5 µg/L $HgCl_2$
Lane 6 = Gill extracts from mussels treated with 1.0 µg/L $HgCl_2$
Lane 7 = Gill extracts from mussels treated with 5.0 µg/L $HgCl_2$

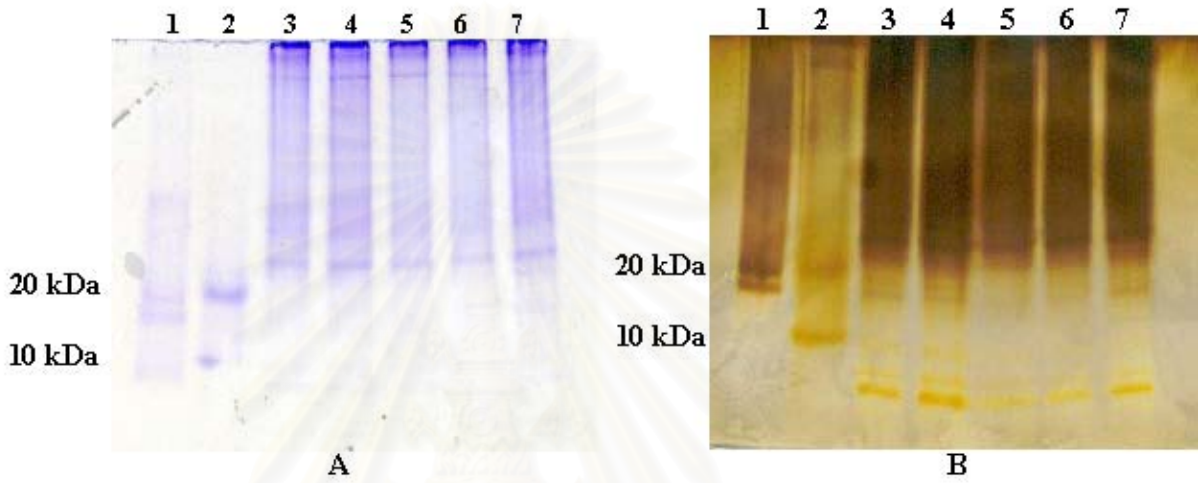

Figure 4.3 Protein profile of gill extracts from mussel treated with mercuric chloride for 5 weeks. Samples (25 µg each) were loaded onto native 7.5-30% gradient polyacrylamide gels and stained with Coomassie brilliant blue (A) and silver solution (B)

Lane 1 = Low molecular weight standard
Lane 2 = Rabbit Metallothionein (control)
Lane 3 = Gill extract from control mussels
Lane 4 = Gill extracts from mussels treated with 0.1 µg/L $HgCl_2$
Lane 5 = Gill extracts from mussels treated with 0.5 µg/L $HgCl_2$
Lane 6 = Gill extracts from mussels treated with 1.0 µg/L $HgCl_2$
Lane 7 = Gill extracts from mussels treated with 5.0 µg/L $HgCl_2$

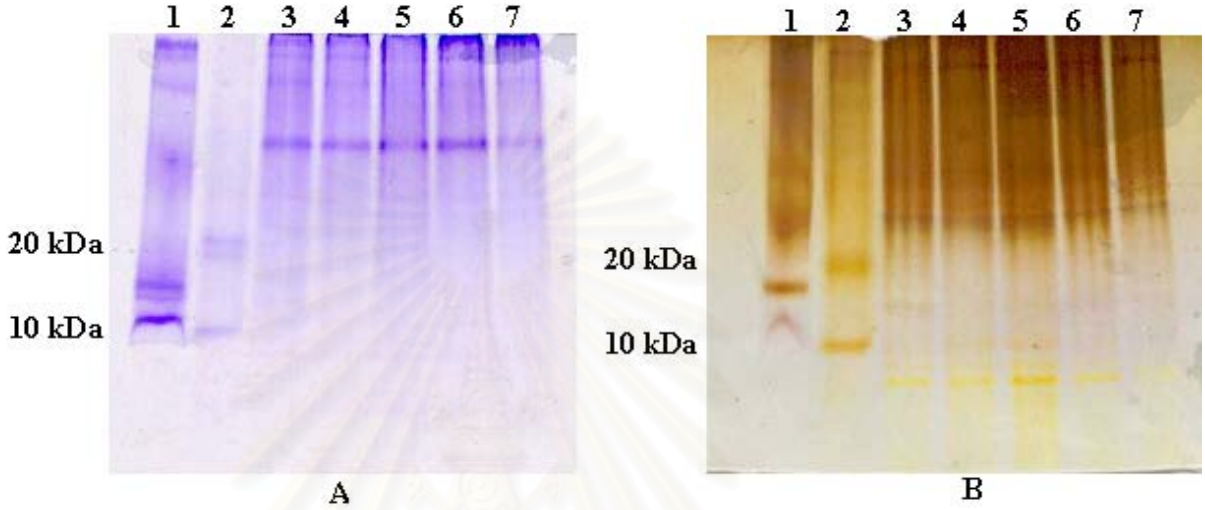

Figure 4.4 Protein profile of gill extracts from mussel treated with mercuric chloride for 7 weeks. Samples (25 µg each) were loaded onto native 7.5-30% gradient polyacrylamide gels and stained with Coomassie brilliant blue (A) and silver solution (B)

Lane 1 = Low molecular weight standard
Lane 2 = Rabbit Metallothionein (control)
Lane 3 = Gill extract from control mussels
Lane 4 = Gill extracts from mussels treated with 0.1 µg/L $HgCl_2$
Lane 5 = Gill extracts from mussels treated with 0.5 µg/L $HgCl_2$
Lane 6 = Gill extracts from mussels treated with 1.0 µg/L $HgCl_2$
Lane 7 = Gill extracts from mussels treated with 5.0 µg/L $HgCl_2$

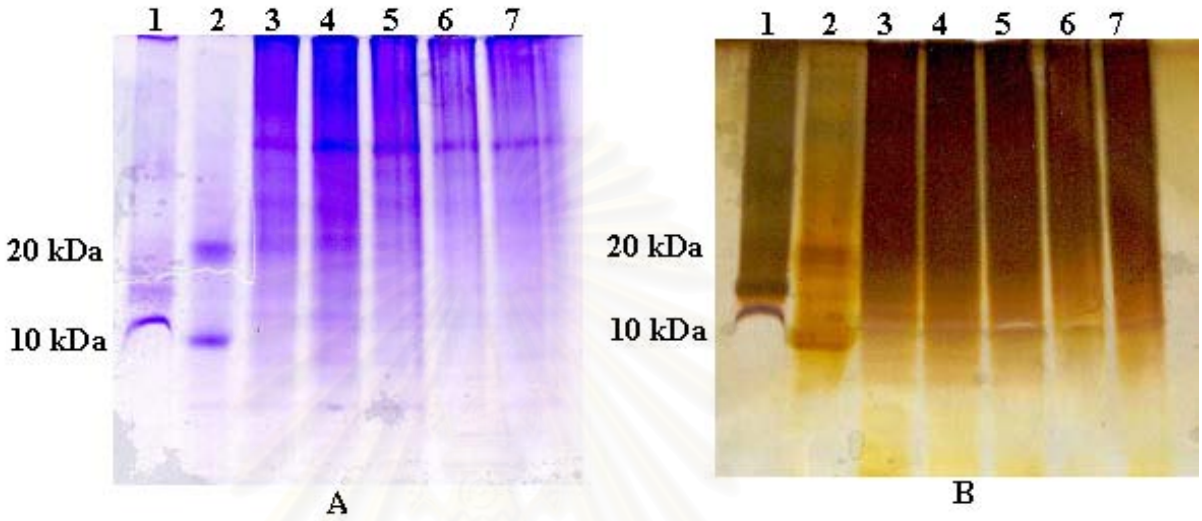

Figure 4.5 Protein profile of digestive tract extracts from mussel treated with mercuric chloride for 1 week. Samples (25 µg each) were loaded onto native 7.5-30% gradient polyacrylamide gels and stained with Coomassie brilliant blue (A) and silver solution (B)

Lane 1 = Low molecular weight standard
Lane 2 = Rabbit Metallothionein (control)
Lane 3 = Digestive tract extracts from control mussels
Lane 4 = Digestive tract extracts from mussels treated with 0.1 µg/L $HgCl_2$
Lane 5 = Digestive tract extracts from mussels treated with 0.5 µg/L $HgCl_2$
Lane 6 = Digestive tract extracts from mussels treated with 1.0 µg/L $HgCl_2$
Lane 7 = Digestive tract extracts from mussels treated with 5.0 µg/L $HgCl_2$

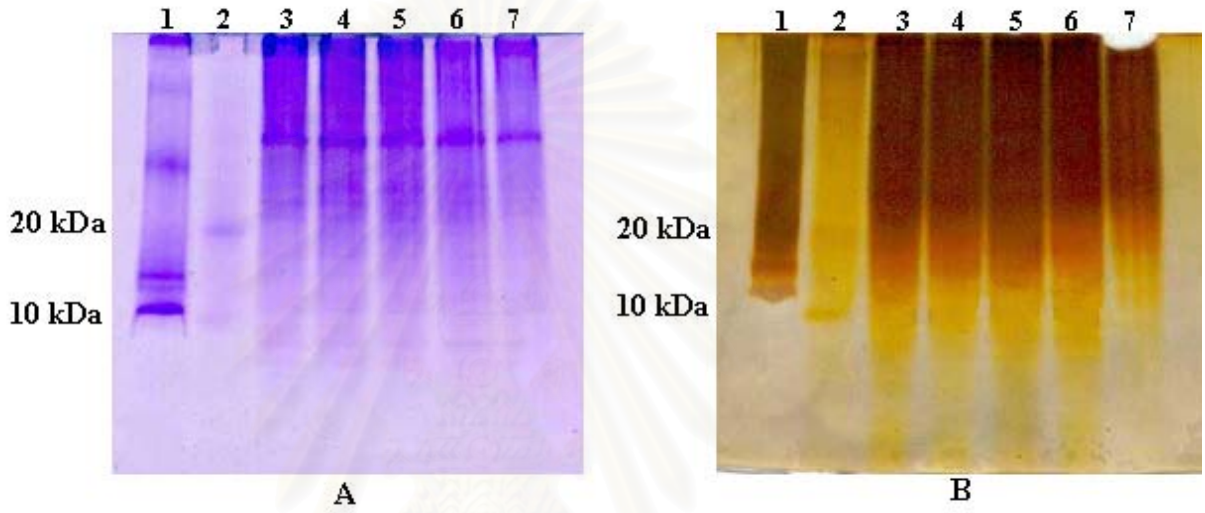

Figure 4.6 Protein profile of digestive tract extracts from mussel treated with mercuric chloride for 3 weeks. Samples (25 µg each) were loaded onto native 7.5-30% gradient polyacrylamide gels and stained with Coomassie brilliant blue (A) and silver solution (B)

Lane 1 = Low molecular weight standard
Lane 2 = Rabbit Metallothionein (control)
Lane 3 = Digestive tract extracts from control mussels
Lane 4 = Digestive tract extracts from mussels treated with 0.1 µg/L $HgCl_2$
Lane 5 = Digestive tract extracts from mussels treated with 0.5 µg/L $HgCl_2$
Lane 6 = Digestive tract extracts from mussels treated with 1.0 µg/L $HgCl_2$
Lane 7 = Digestive tract extracts from mussels treated with 5.0 µg/L $HgCl_2$

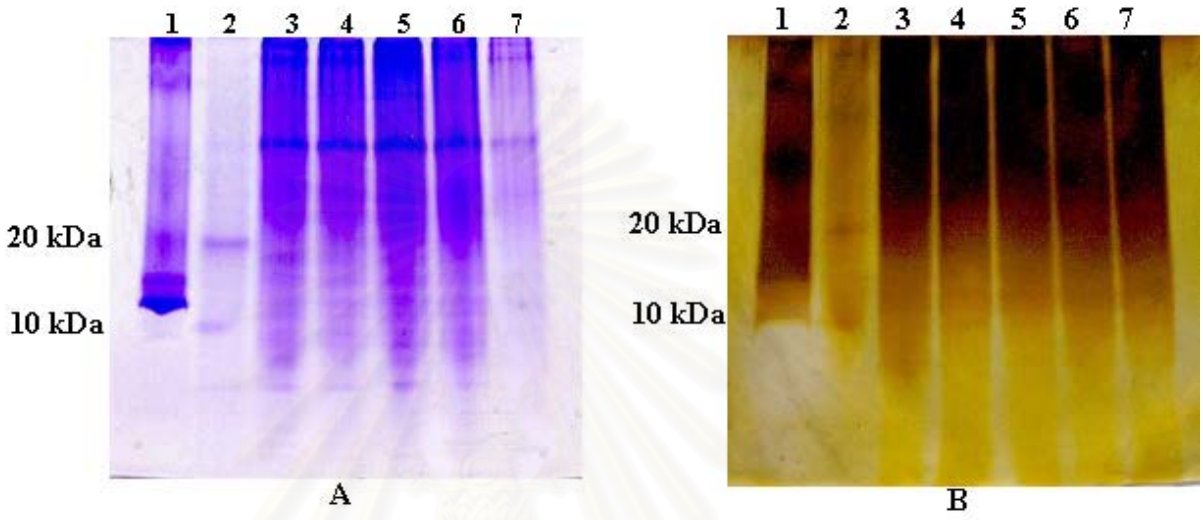

Figure 4.7 Protein profile of digestive tract extracts from mussel treated with mercuric chloride for 5 weeks. Samples (25 µg each) were loaded onto native 7.5-30% gradient polyacrylamide gels and stained with Coomassie brilliant blue (A) and silver solution (B)

Lane 1 = Low molecular weight standard
Lane 2 = Rabbit Metallothionein (control)
Lane 3 = Digestive tract extracts from control mussels
Lane 4 = Digestive tract extracts from mussels treated with 0.1 µg/L $HgCl_2$
Lane 5 = Digestive tract extracts from mussels treated with 0.5 µg/L $HgCl_2$
Lane 6 = Digestive tract extracts from mussels treated with 1.0 µg/L $HgCl_2$
Lane 7 = Digestive tract extracts from mussels treated with 5.0 µg/L $HgCl_2$

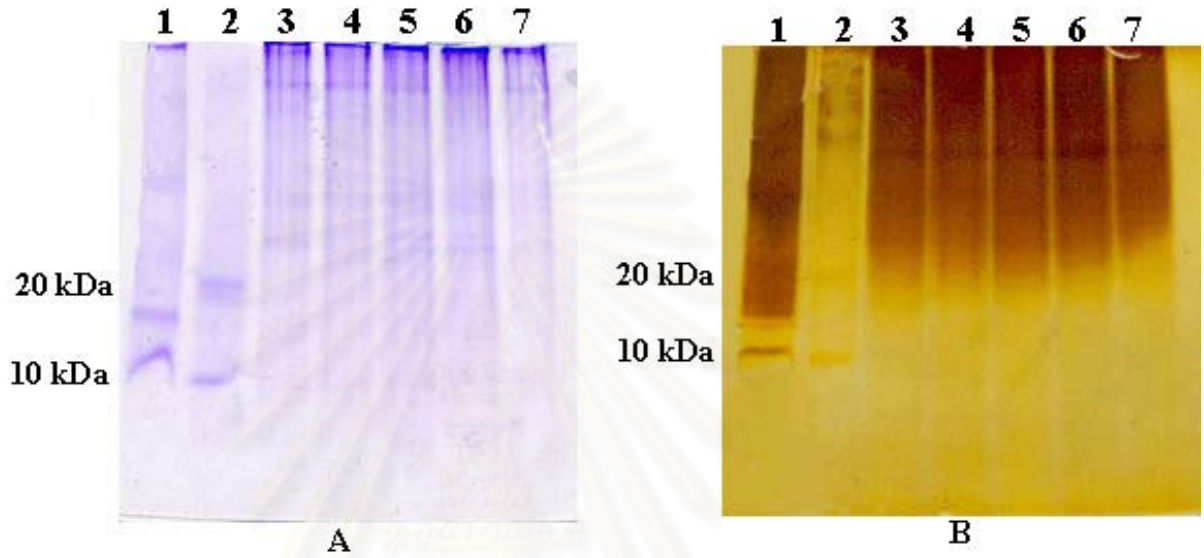

Figure 4.8 Protein profile of digestive tract extracts from mussel treated with mercuric chloride for 7 weeks. Samples (25 μg each) were loaded onto native 7.5-30% gradient polyacrylamide gels and stained with Coomassie brilliant blue (A) and silver solution (B)

Lane 1 = Low molecular weight standard
Lane 2 = Rabbit Metallothionein (control)
Lane 3 = Digestive tract extracts from control mussels
Lane 4 = Digestive tract extracts from mussels treated with 0.1 μg/L $HgCl_2$
Lane 5 = Digestive tract extracts from mussels treated with 0.5 μg/L $HgCl_2$
Lane 6 = Digestive tract extracts from mussels treated with 1.0 μg/L $HgCl_2$
Lane 7 = Digestive tract extracts from mussels treated with 5.0 μg/L $HgCl_2$

Table 4.1 Band intensity from gill extracts comparing with molecular weight of rabbit metallothionein standard, ratios between coomassie brilliant blue staining and silver staining were determined.

Time (week)	$HgCl_2$ (μg/L)	Coomassie brilliant blue		Silver staining		Ratio of Silver/Coomassie	
		10 kDa	20 kDa	10 kDa	20 kDa	10 kDa	20kDa
1 week	Rabbit MT	0.44	0.41	0.35	0.51	0.795	1.24
	C	0	0	-	-	-	-
	0.1	0.01	0.002	-	0.20	-	100
	0.5	0.03	0.03	0.09	0.33	3	11
	1	0.02	0.002	0.12	0.27	6	135
	5	0.04	0.03	0.18	0.35	4.5	11.67
3 week	Rabbit MT	0.05	0.09	0.07	0.14	1.4	1.56
	C	-	-	-	-	-	-
	0.1	-	-	-	-	-	-
	0.5	-	-	-	-	-	-
	1	-	-	-	-	-	-
	5	-	-	-	-	-	-
5 week	Rabbit MT	0.007	0.02	0.01	0.06	1.43	3
	C	-	0.01	-	-	-	-
	0.1	-	-	-	-	-	-
	0.5	-	-	-	-	-	-
	1	-	-	-	-	-	-
	5	-	-	-	-	-	-
7 week	Rabbit MT	0.05	0.06	0.18	0.23	3.6	3.83
	C	-	-	0.06	-	-	-
	0.1	-	-	0.07	-	-	-
	0.5	-	-	0.10	-	-	-
	1	0.01	-	0.06	-	6	-
	5	-	0.02	0.06	0.09	-	4.5

Table 4.2 Band intensity from digestive tract extracts comparing with molecular weight of rabbit metallothionein standard, ratios between coomassie brilliant blue staining and silver staining were determined.

Time (week)	$HgCl_2$ (μg/L)	Coomassie brilliant blue		Silver staining		Ratio of Silver/Coomassie	
		10 kDa	20 kDa	10 kDa	20 kDa	10 kDa	20kDa
1 week	Rabbit MT	0.18	0.18	0.38	0.49	2.1	2.1
	C	-	-	0.26	-	-	-
	0.1	-	-	0.30	-	-	-
	0.5	-	-	0.29	-	-	-
	1	-	0.12	0.260	-	-	2.17
	5	-	0.05	0.26	-	-	-
3 week	Rabbit MT	0.02	0.06	0.11	0.15	1.4	1.56
	C					-	-
	0.1					-	-
	0.5					-	-
	1					-	-
	5					-	-
5 week	Rabbit MT	0.04	0.11	0.14	0.22	3.5	2
	C	0.10	-	-	-	-	-
	0.1	-	-	-	-	-	-
	0.5	-	-	-	-	-	-
	1	-	-	-	-	-	-
	5	0.12	-	-	-	-	-
7 week	Rabbit MT	0.06	0.07	0.12	0.15	2	2.14
	C	0.01	-	-	-	-	-
	0.1	0.01	-	-	-	-	-
	0.5	0.17	-	-	-	-	-
	1	0.02	-	-	-	-	-
	5	0.01	0.01	-	0.06	-	6

4.1.2 Identification of metallothionein by immunochemical analysis

Mouse monoclonal antibody against rabbit metallothionein was used on Western blot analysis. Extracted samples were also analyzed by 7.5-30 % gradient polyacrylamide gel electrophoresis and blotted onto nitrocellulose membranes. After cross reaction between peptide bands on the membrane and antibody, the membranes were stained and photographed. The results were shown in figure 4.9-4.11.

For the experiment on the gill extract of mussels treated with mercuric chloride for 5 weeks (fig 4.9), the results revealed that faint bands of metallothioneins from rabbit (control), Tilapia, and Giant tiger shrimp were detectable. None of the metallothionein bands from normal and mercury-exposed mussels were detected by Western blot analysis. Similar results were obtained from the digestive tract extracts of mussels treated with mercuric chloride for 5 weeks (fig.4.10)

In order to verify that immunochemical analysis can be employed in identification of metallothionein, ten fold of total protein (250 μg each) of digestive tract extracts from mercury-treated mussels, together with the same amount of total protein of the extracts of gill, digestive tract, mantle, gonad, and whole body from the untreated mussels were subjected to the Western blot analysis. The results revealed that metallothionein band from digestive tract from mercury treated mussels was detectable as well as the samples from rabbit (control), giant tiger shrimp, and tilapia (fig.4.11).

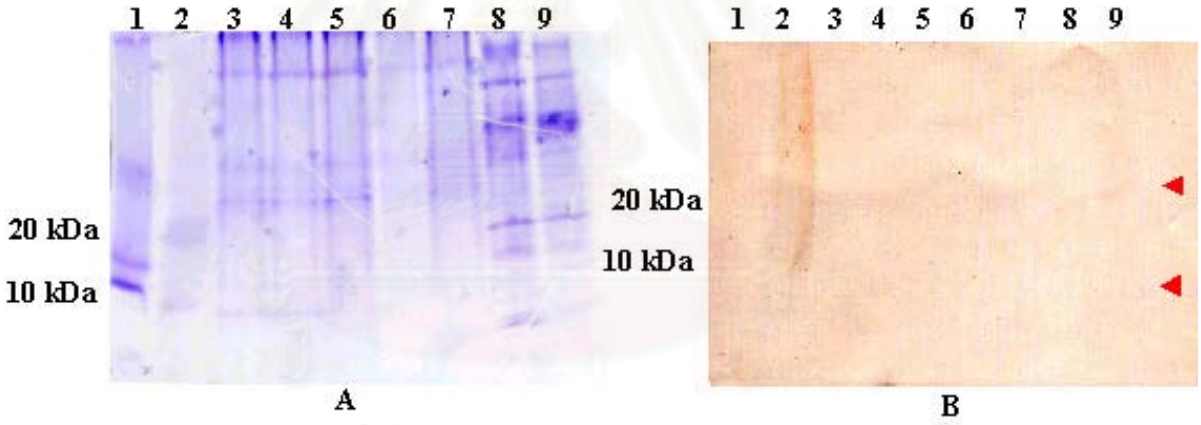

Figure 4.9 Electrophoretic pattern of gill extract from mussels exposed to mercuric chloride for 5 weeks. Each sample (25 μg) was loaded and run on 2 replication of 7.5-30% native gradient polyacrylamid2e gels. A single gel was stained by Coomassie brilliant blue (A) and the other was subjected to Western blotting, stained by immunochemical method using mouse antibody anti- metallothionein monoclonal antibody, and developed by DAB (B)
Lane 1 = Low Molecular weight standard
Lane 2 = Rabbit Metallothionein
Lane 3 = Gill extracts from control mussels
Lane 4 = Gill extracts from mussels treated with 0.1 μg/L $HgCl_2$
Lane 5 = Gill extracts from mussels treated with 0.5 μg/L $HgCl_2$
Lane 6 = Gill extracts from mussels treated with 1.0 μg/L $HgCl_2$
Lane 7 = Gill extracts from mussels treated with 5.0 μg/L $HgCl_2$
Lane 8 = Hepatopancreas extract from giant tiger shrimp, *Penaeus monodon*.
Lane 9 = Liver extract from Tilapia, *Oreochromis nilotica*

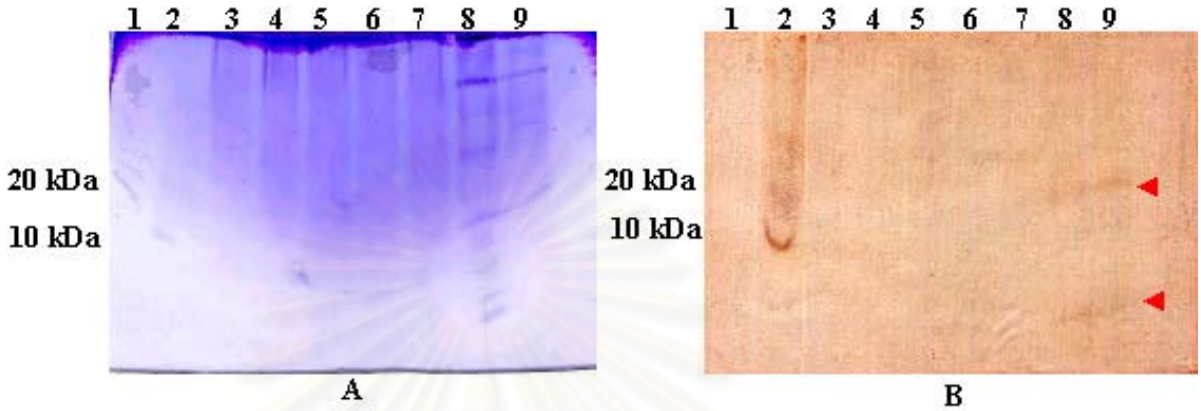

Figure 4.10 Electrophoretic pattern of digestive tract extract from mussels exposed to mercuric chloride for 5 weeks. Each sample (25 µg) was loaded and run on 2 replications of 7.5-30% native gradient polyacrylamide gels. A single gel was stained by Coomassie brilliant blue (A) and the other was subjected to Western blotting, stained by immunochemical method using mouse anti- metallothionein monoclonal antibody, and developed by DAB (B)
Lane 1 = Low Molecular weight standard
Lane 2 = Rabbit Metallothionein
Lane 3 = Digestive tract extracts from control mussels
Lane 4 = Digestive tract extracts from mussels treated with 0.1 µg/L $HgCl_2$
Lane 5 = Digestive tract extracts from mussels treated with 0.5 µg/L $HgCl_2$
Lane 6 = Digestive tract extracts from mussels treated with 1.0 µg/L $HgCl_2$
Lane 7 = Digestive tract extracts from mussels treated with 5.0 µg/L $HgCl_2$
Lane 8 = Hepatopancreas extract from giant tiger shrimp, *Penaeus monodon*.
Lane 9 = Liver extract from Tilapia, *Oreochromis nilotica*

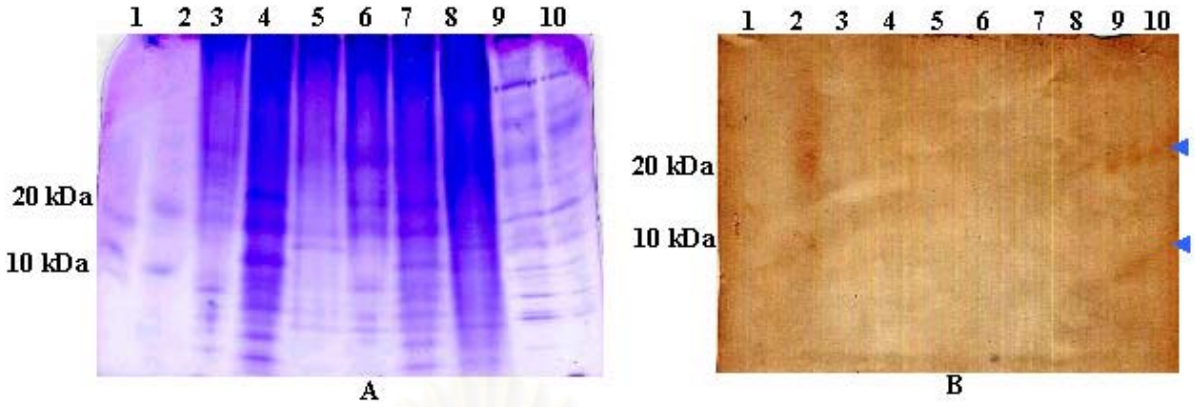

Figure 4.11 Electrophoretic pattern of digestive tract extract from mussels exposed to mercuric chloride for 5 weeks and some tissue extracts from untreated mussels. Each sample (250 µg) was loaded and run on 2 replications of 7.5-30% native gradient polyacrylamide gels. A single gel was stained by Coomassie brilliant blue (A) and the other was subjected to Western blotting, stained by immunochemical method using mouse anti- metallothionein monoclonal antibody, and developed by DAB (B)
Lane 1 = Low Molecular weight standard
Lane 2 = Rabbit Metallothionein
Lane 3 = Gill extracts from untreated mussels
Lane 4 = Digestive tract extracts from untreated mussels
Lane 5 = Mantle extracts from untreated mussels
Lane 6 = Gonad extracts from untreated mussels
Lane 7 = Whole body extracts from untreated mussels
Lane 8 = Digestive tract extracts from mussels treated with 5.0 µg/L $HgCl_2$
Lane 9 = Hepatopancreas extract from giant tiger shrimp, *Penaeus monodon*.
Lane 10 = Liver extract from Tilapia, *Oreochromis nilotica*

4.2 Purification of metallothionein by liquid chromatography

Metallothioneins from gills and digestive tract extracts (171 mg in 9 ml) of mercury treated mussels were partially purified by acetonic fractionation method. Acetonic fractions were combined (12 mg in 6 ml) and loaded (1 mg in 0.5 ml) onto Sephadex G-75 gel filtration column calibrated with molecular weight standard (Blue dextan, Bovine serum albumin, and Cytochrom C). Fractions (120 Fractions, 1 ml each) were collected and the absorbance at 280 nm from each fraction was monitored. The fractions were also subjected to mercury analysis. Protein profiles of gills and digestive tract extracts eluted from Sephadex G-75 column were shown in figure 4.12 and 4.13, respectively. Similar results were detected from the extracts of both gills and digestive tracts. Four peaks, a minor peak (fraction 35-43) at the molecular weight of 2000 kDa., 2 very small peaks (fraction 54-56 and 82-86) at the molecular weight of 121.5 kDa. and 4kDa., and a major peak (fraction 110-120) at the molecular weight of < 4 kDa., were detected.

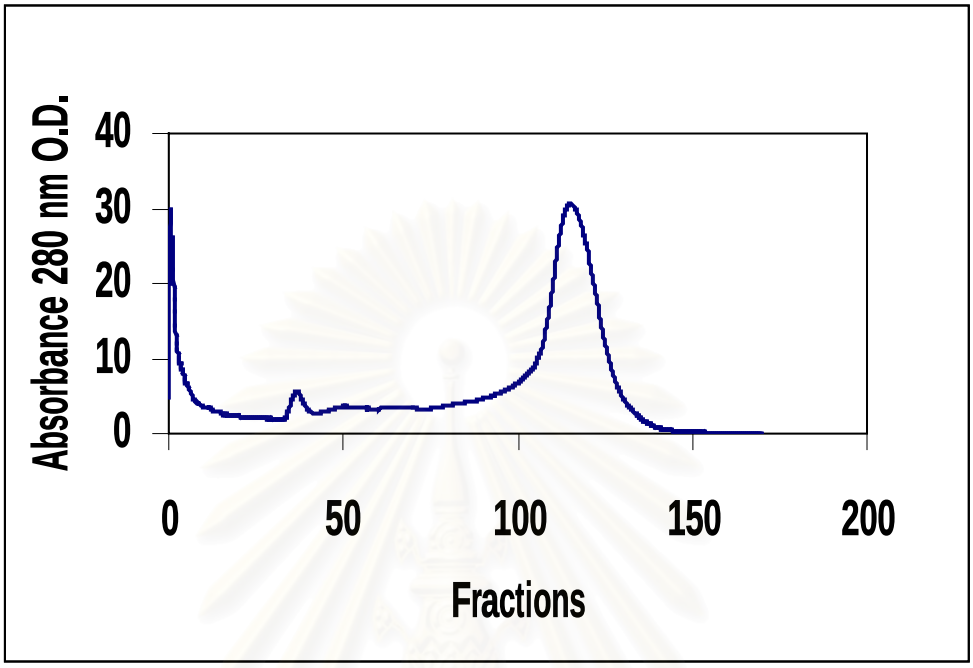

Figure 4.12 Protein profile of acetonic extracts eluted from Sephadex G-75 gel filtration column. Sample was obtained from gill of mercury treated mussels.

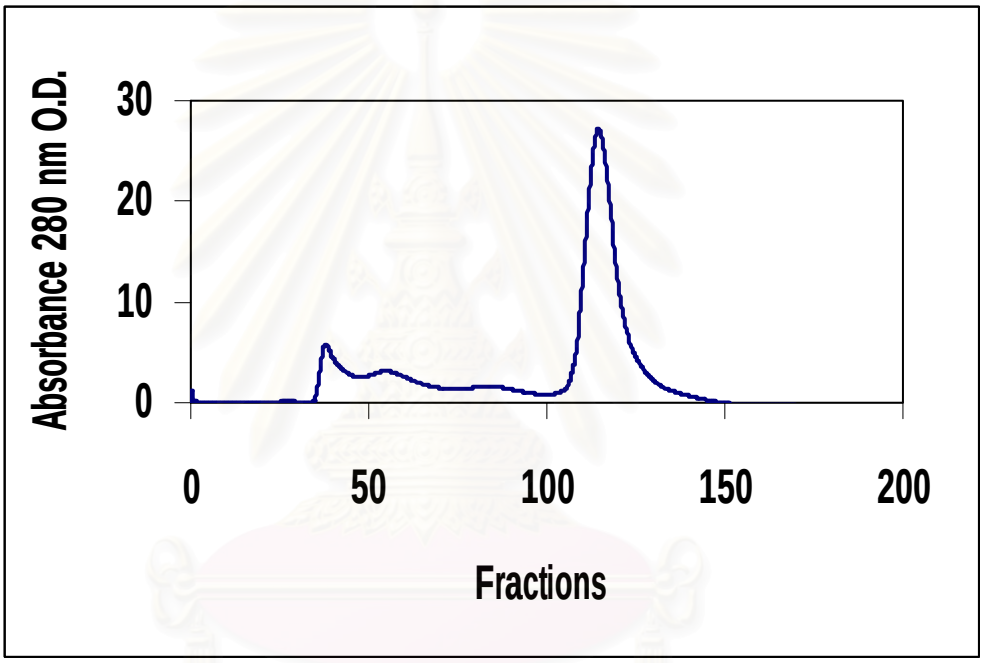

Figure 4.13 Protein profile of acetonic extracts eluted from Sephadex G-75 gel filtration column. Sample was obtained from digestive tracts of mercury treated mussels.

Fractions from each peak were combined and concentrated by lyophilization. Lyophilized samples were dissolved and determined by 15% Native polyacrylamide gel electrophoresis. The gels were stained with Coomassie Brilliant Blue and silver staining. The results were shown in figure 4.14.

High intensity of metallothionein bands stained with silver were detected in the samples from fraction 54-56 (121.5kDa) and fraction 82-86 (4kDa) (see table 4.3). The result of mercury analysis from each fraction revealed no significant difference of mercury level between fractions.(note: mercury content in every sample was lower than detection limite (5 ppb.) of mercury analyser (AMA254).

For further purification on diethylaminoethanol (DEAE)-cellulose ion exchange column, metallothionein was undetectable due to the limited amount of samples.

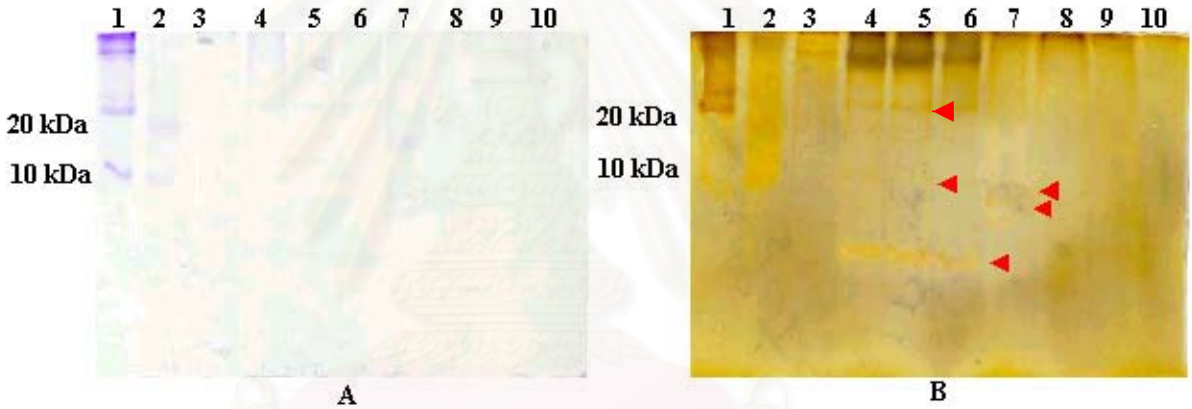

Figure 4.14 Electrophoretic pattern of proteins on 15% native polyacrylamide gel. Samples were obtained from elution fractions of gill extracts separated by Sephadex G-75 column. Gels were stained by Coomassie brilliant blue (A) and silver (B). Red arrows indicate intensified bands of metallothionein stained with silver

Lane 1 = low molecular weight standard
Lane 2 = rabbit metallothionein (5 μg)
Lane 3 = proteins from fraction 38 (74.55 μg)
Lane 4-6 = proteins from fraction 54-56 (72.57, 80.48 and 74.55 μg respectively)
Lane 7-9 = proteins from fraction 82-86 (80.48, 64.66 and 68.62 μg respectively)
Lane 10 = proteins from fraction 115 (74.55 μg)

Table 4.3 Band intensity of fractions obtained from gel filtration**,** which revealed to molecular weight of rabbit metallothionein standard, compared between coomassie brilliant blue staining and silver staining.

Sample	Coomassie brilliant blue				Silver staining			
	20 kDa	10 kDa	~10 kDa	<10 kDa	20 kDa	10 kDa	~10 kDa	<10 kDa
MT Standard	0.03	0.019			0.11	0.09		
Fraction 54				0.016				0.09
Fraction 55				0.019	0.04			0.08
Fraction 56				0.019		0.01		0.04
Fraction 82						0.04	0.04	

4.3 Determination of metallothionein gene expression in digestive tracts of mercury treated mussels

The expression of metallothionein gene in mercury-treated mussel, *P.viridis*, was determined by semi-quantitative RT-PCR using β-actin as reference. First, the sequences of metallothionein and β-actin genes from mussel, *P.viridis*, were retrieved from GenBank (AAD02054 and AF1000987). The DNA sequences were shown in figure 4.15. Primers for amplifying metallothionein gene was then designed at start and stop codons. Primers for β-actin designed from DNA sequence of *P.monodon* was adopted. Details of these primers were shown in table4.4 Appropriate condition for semi-quantitative RT-PCR for metallothionein and actin were conducted by adjusting template concentration, and the number of PCR cycle.

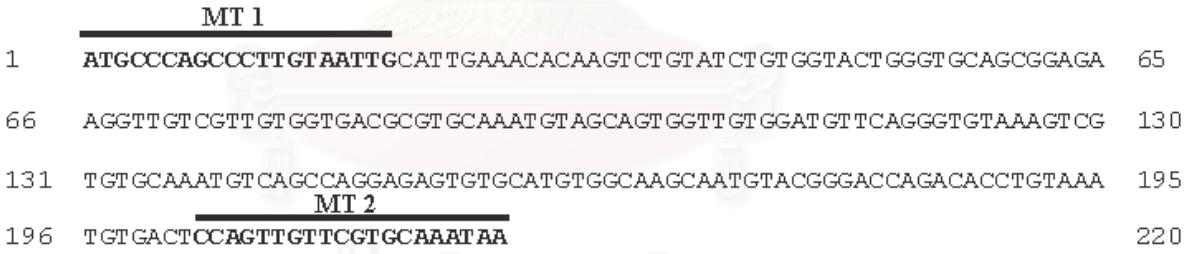

Figure 4.15 Nucleotide sequence of metallothionein gene from mussel *Perna viridis* showing the position of primer MT1 and MT2 at start and stop were shown by bold letter.

```
1    GGGACTGGGGTACTCCTACACTCATAAACCAACGACATCATGTGTGACGACGAGGATCTTACTGC   65
66   CCTTGTGGTTGACAATGGCTCCGGCCTTTGCAAGGCCGGCTTCGCCGGAGACGACGCCCCTCGTG  130
131  CCGTCTTCCCCTCCATCGTCGGCCGTGCCCGTCATCAGGGTGTGATGGTCGGTATGGGTCAGAAG  195
                                                  Actin 1
197  GACGCCTACGTCGGTGATGAGGCCCAGAGCAAACGTGGTATCCTCACCCTCAAGTACCCCATTGA  260
261  ACACGGTATCATCACCAACTGGGATGACATGGAGAAGATCTGGTACCATACTTTCTACAATGAGC  325
326  TCCGTGTTGCCCCCGAGGAGTCCCCCACACTTCTCACTGAGGCTCCCCTCAACCCCAAGGCCAAC  390
391  CGTGAGAAGATGACTCAGATCATGTTCGAGTCCTTCAATGTACCTGCCACTTACATTACCATCCA  455
456  GGCTGTGCTCTCCCTCTACGCCTCTGGTCGTACTACCGGTGAGGTTTGCGACTCTGGTGATGGTG  520
                                  Actin 2
521  TGACTCACTTTGTCCCCGTCTATGAAGGTTTCGCTCTTCCTCATGCTATCCTTCGTCTCGATCTT  585
586  GCTGGTCGTGACCTGACCCACTATCTGATGAAGATCATGACTGAGCGTGGCTACTCCTTCACCAC  650
651  CACCGCTGAACGTGAAATCGTTCGTGACATCAAGGAGAAGCTTTGCTACATTGCCCTTGACTTCG  715
716  AGAGTGAGATGAACGTTGCTGCTGCTTCCTCCTCCTTGGACAAGTCATACGAGCTTCCCGACGGC  780
781  CAGGTCATCACCATTGGTAACGAGCGTTTCCGCTGCGCTGAAGCTCTGTTCCAGCCTTCCTTCCT  845
846  TGGTATGGAATCTGCTGGTATTCAGGAAACCGTCCACAGCTCCATCATGAGGTGTGACATTGACA  910
911  TCAGGAAGGACCTGTTCGCCAATATCGTCATGTCTGGTGGTACCACCATGTACCCTGGTATTGCT  975
976  GACCGCATGCAGAAGGAAATCACTGCTCTTGCTCCTTCCACCATCAAGATCAAGATCATTGCTCC 1040
1041 TCCTGAGCGTAAGTACTCCGTCTGGATCGGTGGTTCCATCCTGTCTTCTCTGTCCACCTTCCAGT 1105
1106 CCATGTGGATCACCAAGGATGAGTACGAAGAGTCTGGTCCCGGCATTGTCCACCGCAAGTGCTTC 1170
1171 TAAATGGAGATTGACAACTTTTACTACAGTTGATAATAAAATTCCGAAACATC              1223
```

Figure 4.16 Nucleotide sequence of Beta-actin gene. The highlight show the position of Actin 1 and Actin 2 primer.

Table 4.4 The details of metallothionein primer and actin primer.

Primer	MT gene				Primer	Actin gene			
	Length	GC%	Tm^{o} C	Ta^{o} C		Length	GC %	Tm^{o} C	Ta ^{o} C
MT1	20	50	60		Actin1	20	50	51.3	
MT2	20	40	54.7		Actin2	20	40	52	
Product	222	49	87.2	52.5	Product	327	52.6	89.7	53.2

4.3.1 Amplification of metallothionein and β-actin genes

Total RNA extracted from gills and digestive tracts of the mussels were subjected to first strand cDNAs production by reverse transcription using oligod(T) primer. Double strand cDNA of metallothionein and β-actin genes were then amplified using first strand DNA as template and MT1-MT2 and actin1-actin2 as primers. PCR products of metallothionein was also cloned and sequenced for approval. The result was shown in figure 4.17. Various concentration of template and the number of PCR cycle used in PCR reaction were adjusted. The PCR products were determined by 1% agarose gel and the intensity of the DNA bands were detected. The appropriate PCR condition for semi-quantitative detection was chosen on the criteria that the PCR product should be on the log phase of amplification. The results (fig. 4.18-4.19) indicated that the condition of using cDNA template at 750 ng with 25 PCR cycles were suitable for the amplification of metallothionein gene while the appropriate condition for β-actin amplification was at the template concentration of 750 ng with 23 cycles.

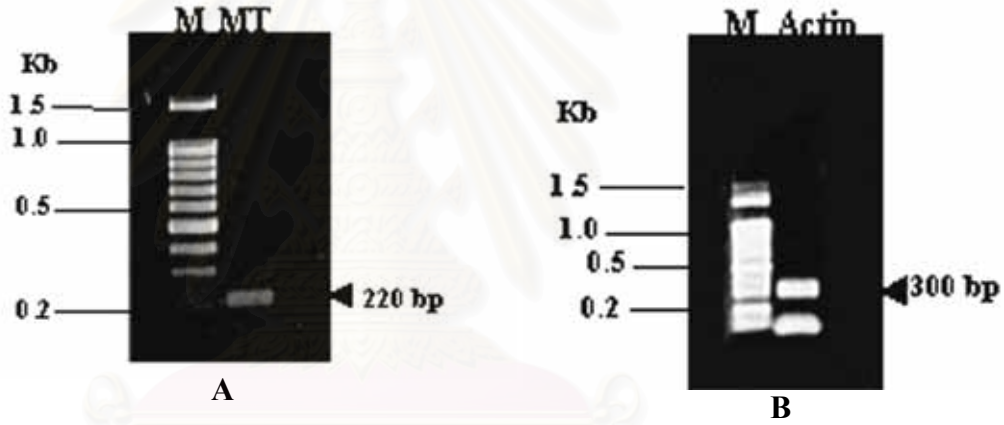

Figure 4.17 Amplification of metallothionein gene (A) and beta-actin gene (B) fragments. Lane M= 100 base pair ladder.

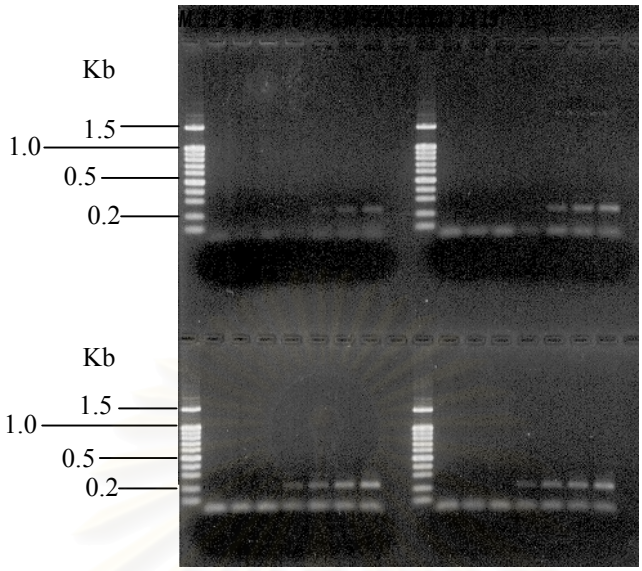

Figure 4.18 PCR products of metallothionein gene amplification determined on 1 % agarose gel and stained with Ethidium bromide. PCR reaction was conducted on 20 (upper lane 1-7), 23 (upper lane 8-14), 25 (lower lane 1-7) and 27 cycles (lower lanes 8-14). The template concentration in each reaction was 10, 50, 100, 250, 500, 750, 1,000 ng, respectively (lane 1-7). A 100 bp DNA standard was shown in lane M.

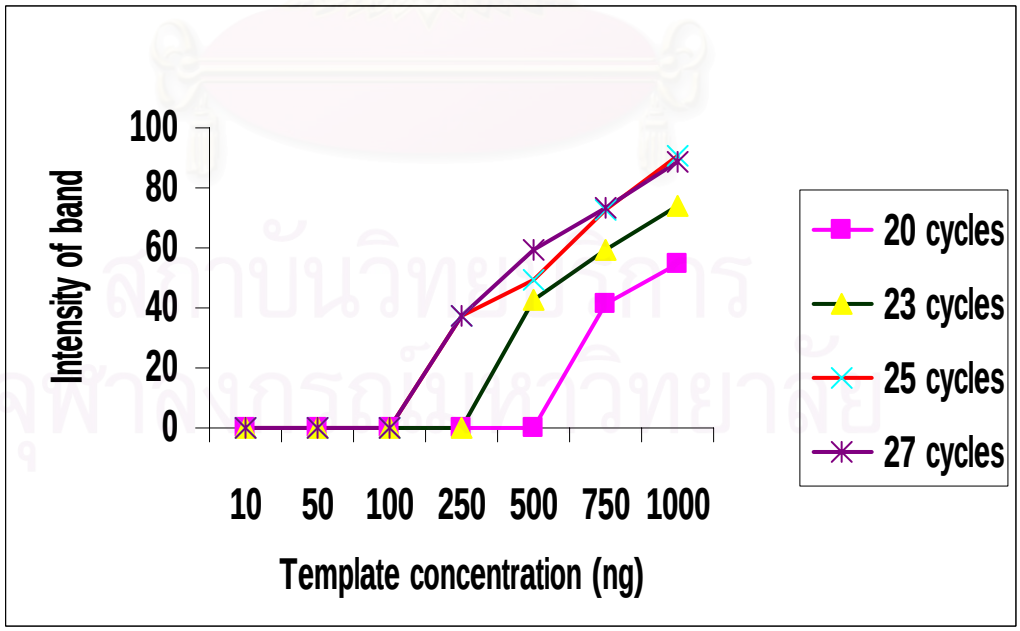

Figure 4.19 Relationship between PCR products of metallothionein gene amplified from digestive tracts of mussels and various amount of DNA template used in PCR reaction.

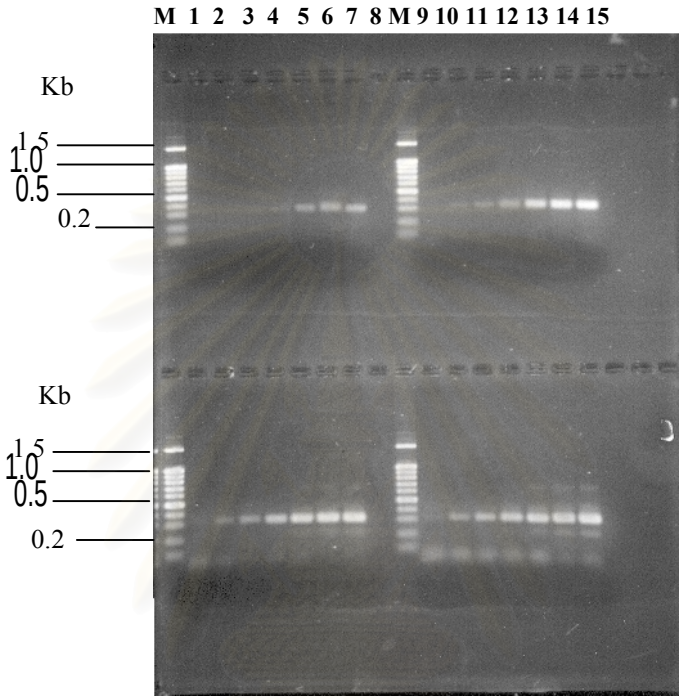

Figure 4.20 PCR products of β-actin gene amplification determined on 1 % agarose gel and stained with Ethidium bromide. PCR reaction was conducted on 20 (upper lane 1-7), 23 (upper lane 8-14), 25 (lower lane 1-7) and 27 cycles (lower lanes 8-14). The template concentration in each reaction was 10, 50, 100, 250, 500, 750, 1,000 ng, respectively (lane 1-7). A 100 bp DNA standard was shown in lane M.

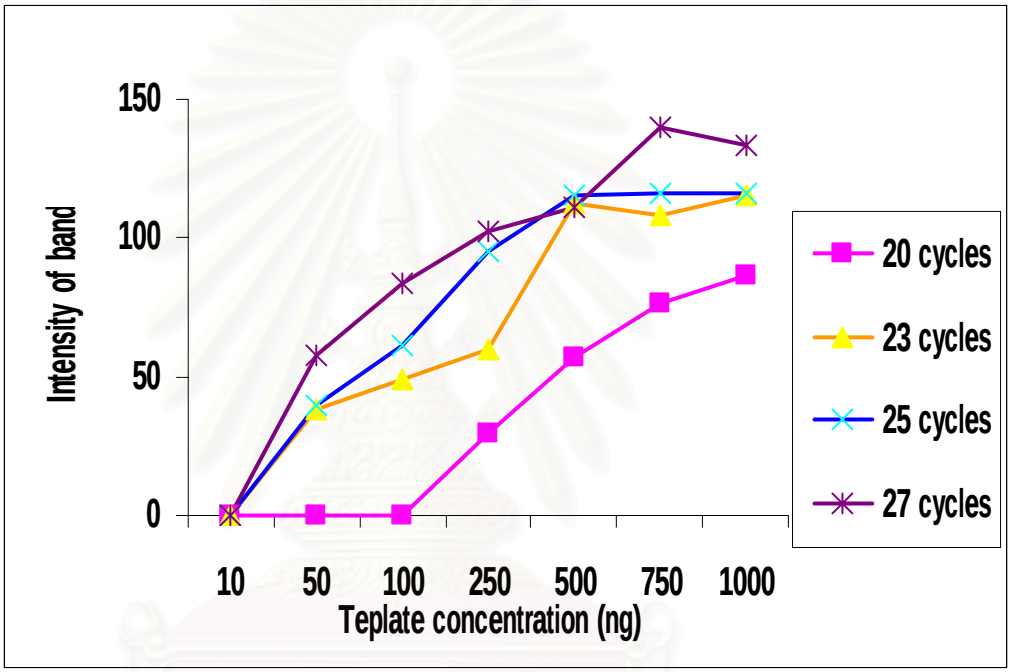

Figure 4.21 Relationship between PCR products of β-actin gene amplified from digestive tracts of mussels and various amount of DNA template used in PCR reaction.

4.3.2 Expression level of metallothionein gene detected by semi-quantitative RT-PCR

Following the adjustment of PCR condition, semi-quantitative RT-PCR was carried out on mercury treated mussels. Mussels collected from 2 locations (Samutprakarn and Trad provinces) were subjected to mercury exposure at the concentration of 0.0, 0.1, 0.5, 1.0, and 5.0 μg/L, respectively. Digestive tracts were dissected from 2 (mussels from Samutprakarn province) and 5 (mussels from Trad province) mussels taken from each treatment every week. Total RNA was then purified from digestive tracts from each sample and reverse transcribed into first strand cDNA using oligod(T) as primer. First strand cDNAs were then used as template for semi-quantitative PCR. β-actin was also amplified and used as transcriptional control. The expression levels of metallothionein gene in gills and digestive tracts of mussels were determined by the ratio between the PCR products of metallothionein and β-actin.

4.3.2.1 Expression level of metallothionein gene in mercury treated mussels from Samutprakarn province.

The results of the experiment on the mussels collected from Samutprakarn were shown in figure 4.22-4.34 and table 4.5-4.11. It was indicated that no significant differences on the expression level of metallothionein gene from mussels treated with every level of mercury were detected ($p > 0.05$). Similar results were obtained from the treatment from week 1 to week 7. However, there were significant correlation between the expression level of metallothionein gene and the increasing level of mercury concentration used in each treatment from week 1 to week 3 ($p< 0.05$). At week 3 to week 7, the expression level of metallothionein gene tended to decrease in mussels treated with high concentration (1.0 and 5.0 μg/L) but there was no statistically significant difference ($p< 0.05$).

Table 4.5 The expression ratio and level of metallothionein and β-actin genes in digestive tracts of mussels treated with various concentrations of mercury at week 1.

Genes	Mercuric chloride concentration (μg/L)				
	0.0	**0.1**	**0.5**	**1.0**	**5.0**
MT	81.83± 0.32	108.23± 3.71	108.61± 70.18	135.67± 45.32	132.69± 20.91
Actin	93.73± 10.72	110.36± 10.5	75.04± 11.59	83.63± 2.07	81.04± 4.75
MT/Actin	0.88±0.10	0.98±0.024	1.54±1.17	1.62±0.50	1.65±0.35

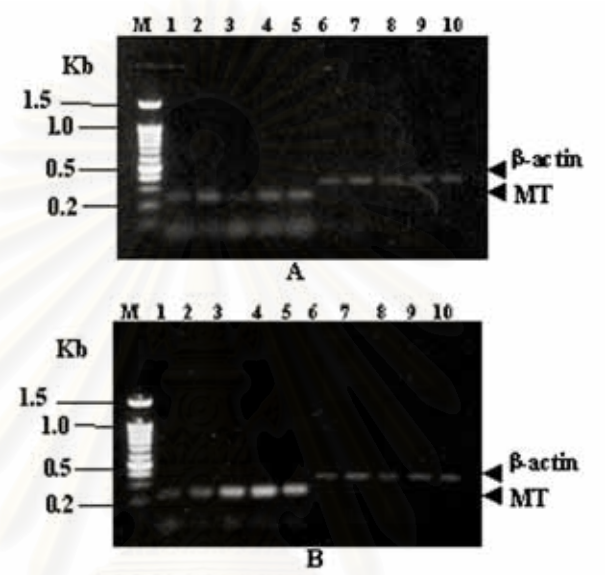

Figure 4.22 The expression levels of metallothionein gene from digestive tracts of *Perna viridis* treated with various concentrations of mercury at week 1 in comparison with β-actin. Samples were obtained from 2 mussels (A and B) and analysed by 1.2% agarose gel electrophoresis.

Lane M= 100 base pairs ladder

Lane 1 = untreated mussel

Lane 2 = expression level of digestive tract from mussel treated with 0.1 μg/L $HgCl_2$

Lane 3 = expression level of digestive tract from mussel treated with 0.5 μg/L $HgCl_2$

Lane 4 = expression level of digestive tract from mussel treated with 1.0 μg/L $HgCl_2$

Lane 5 = expression level of digestive tract from mussel treated with 5.0 μg/L $HgCl_2$

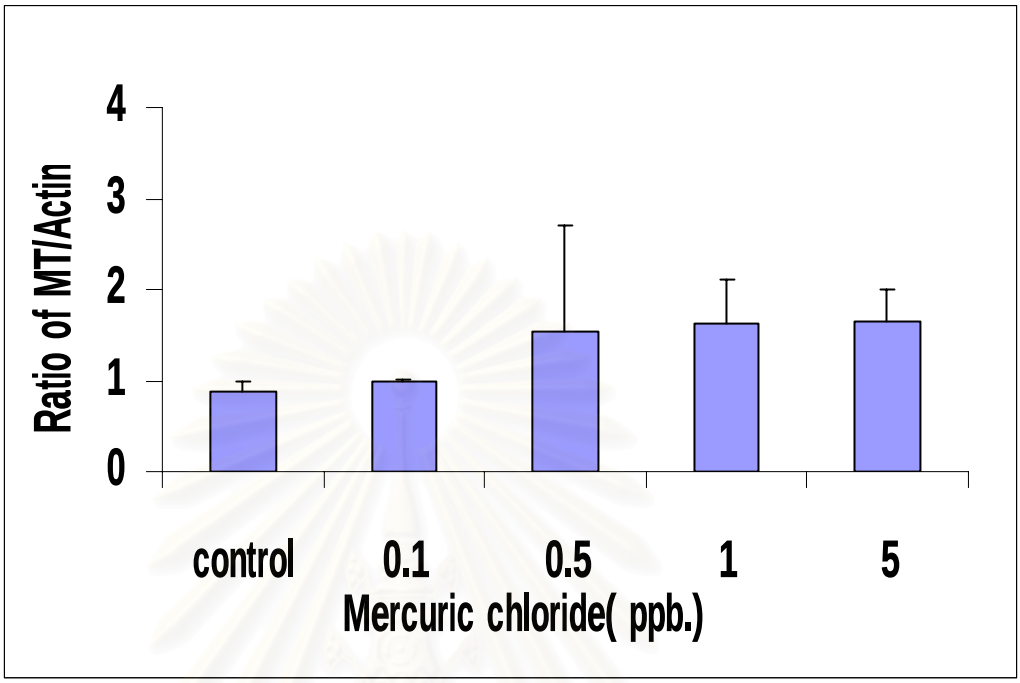

Figure 4.23 The expression ratio of metallothionein and β-actin genes in digestive tracts of mussels treated with various concentrations of mercury at week 1.

Table 4.6 The expression ratio and level of metallothionein gene and β-actin genes in digestive tracts of mussels treated with various concentrations of mercury at week 2.

Genes	Mercuric chloride concentration (μg/L)				
	0.0	**0.1**	**0.5**	**1.0**	**5.0**
MT	92.36± 6.71	97.39± 3.49	106.52± 17.12	120.79± 16.07	113.10± 21.53
Actin	76.06± 23.19	50.87± 1.05	48.60± 0.32	37.72±0.09	57.61± 16.57
MT/Actin	1.26±0.30	1.91±0.03	2.19±0.34	3.20±0.43	2.10±0.98

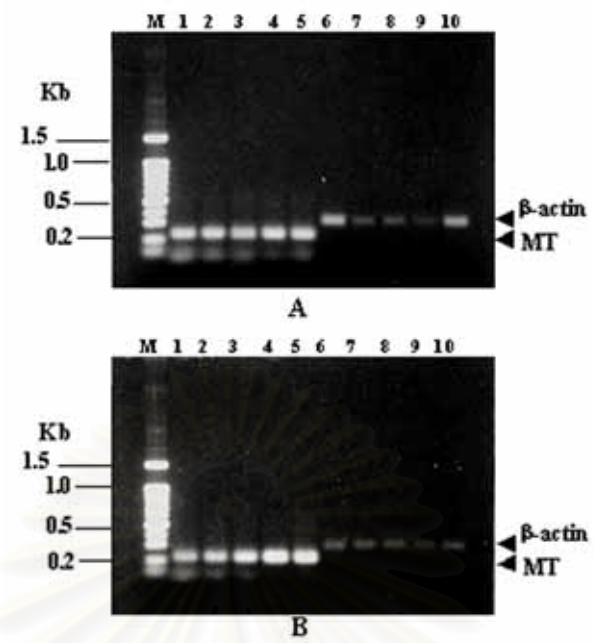

Figure 4.24 The expression levels of metallothionein gene from digestive tracts of *Perna viridis* treated with various concentrations of mercury at week 2 in comparison with β-actin. Samples were obtained from 2 mussels (A and B) and analysed by 1.2% agarose gel electrophoresis.
Lane M = 100 base pair ladder

MT gene

Lane 1 = untreated mussel

Lane 2 = expression level of digestive tract from mussel treated with 0.1 μg/L $HgCl_2$

Lane 3 = expression level of digestive tract from mussel treated with 0.5 μg/L $HgCl_2$

Lane 4 = expression level of digestive tract from mussel treated with 1.0 μg/L $HgCl_2$

Lane 5 = expression level of digestive tract from mussel treated with 5.0 μg/L $HgCl_2$

Beta-actin gene

Lane 6 = untreated mussel

Lane 7 = expression level of digestive tract from mussel treated with 0.1 μg/L $HgCl_2$

Lane 8 = expression level of digestive tract from mussel treated with 0.5 μg/L $HgCl_2$

Lane 9 = expression level of digestive tract from mussel treated with 1.0 μg/L $HgCl_2$

Lane 10= expression level of digestive tract from mussel treated with 5.0 μg/L $HgCl_2$

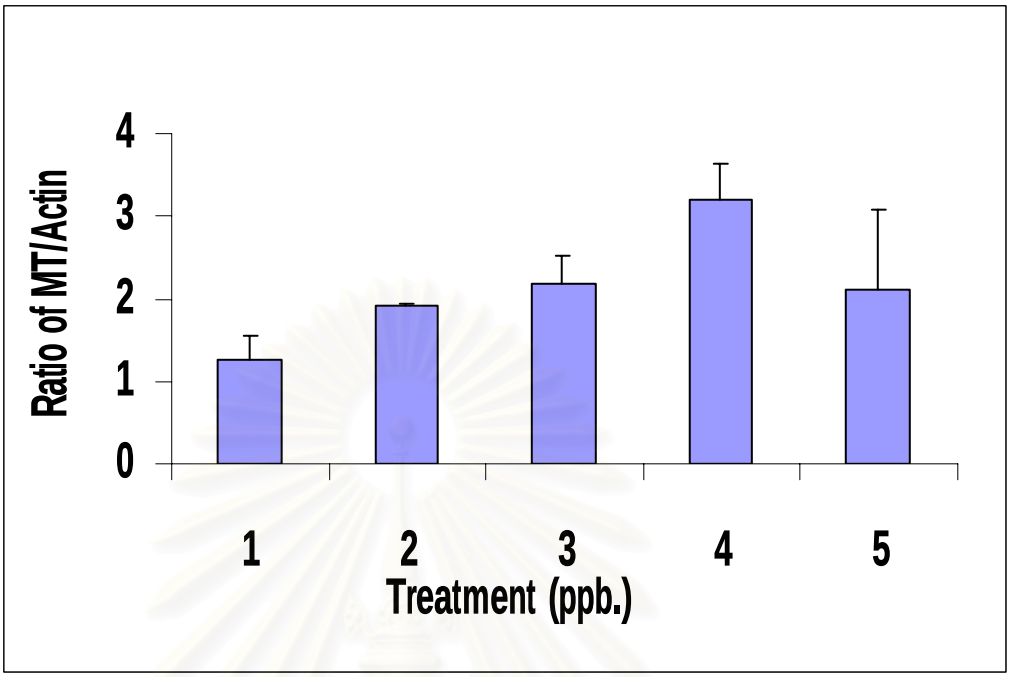

Figure 4.25 The expression ratio of metallothionein and β-actin genes in digestive tracts of mussels treated with various concentrations of mercury at week 2.

Table 4.7 The expression ratio and level of metallothionein gene and β-actin genes in digestive tracts of mussels treated in digestive tracts of mussels treated with various concentrations of mercury at week 3.

Genes	Mercuric chloride concentration (μg/L)				
	0.0	**0.1**	**0.5**	**1.0**	**5.0**
MT	104.62± 11.74	146.14± 1.00	138.82± 1.85	98.75± 43.88	140.92± 4.12
Actin	59.68±8.34	62.26±5.36	59.40± 11.63	42±27.24	71.38± 29.71
MT/Actin	1.76±0.05	2.36±0.19	2.38±0.43	2.55±0.61	2.17±0.96

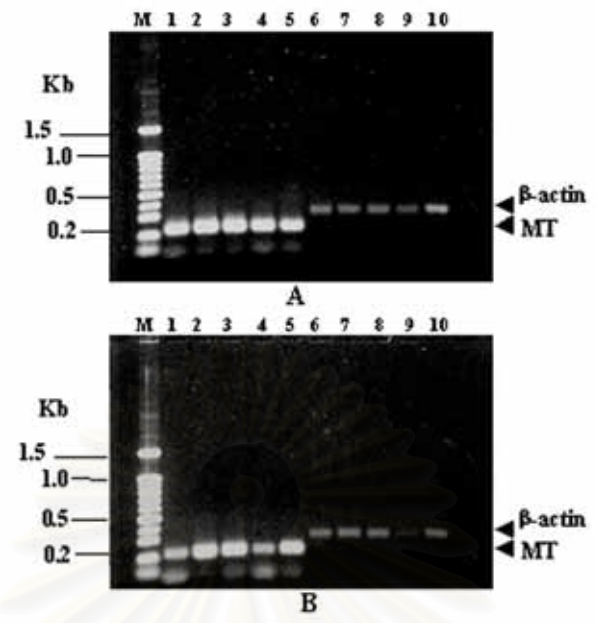

Figure 4.26 The expression levels of metallothionein gene from digestive tracts of *Perna viridis* treated with various concentrations of mercury at week 3 in comparison with β-actin. Samples were obtained from 2 mussels (A and B) and analysed by 1.2% agarose gel electrophoresis.

Lane M = 100 base pair ladder

MT gene

Lane 1 = untreated mussel

Lane 2 = expression level of digestive tract from mussel treated with 0.1 μg/L $HgCl_2$

Lane 3 = expression level of digestive tract from mussel treated with 0.5 μg/L $HgCl_2$

Lane 4 = expression level of digestive tract from mussel treated with 1.0 μg/L $HgCl_2$

Lane 5 = expression level of digestive tract from mussel treated with 5.0 μg/L $HgCl_2$

Beta-actin gene

Lane 6 = untreated mussel

Lane 7 = expression level of digestive tract from mussel treated with 0.1 μg/L $HgCl_2$

Lane 8 = expression level of digestive tract from mussel treated with 0.5 μg/L $HgCl_2$

Lane 9 = expression level of digestive tract from mussel treated with 1.0 μg/L $HgCl_2$

Lane 10= expression level of digestive tract from mussel treated with 5.0 μg/L $HgCl_2$

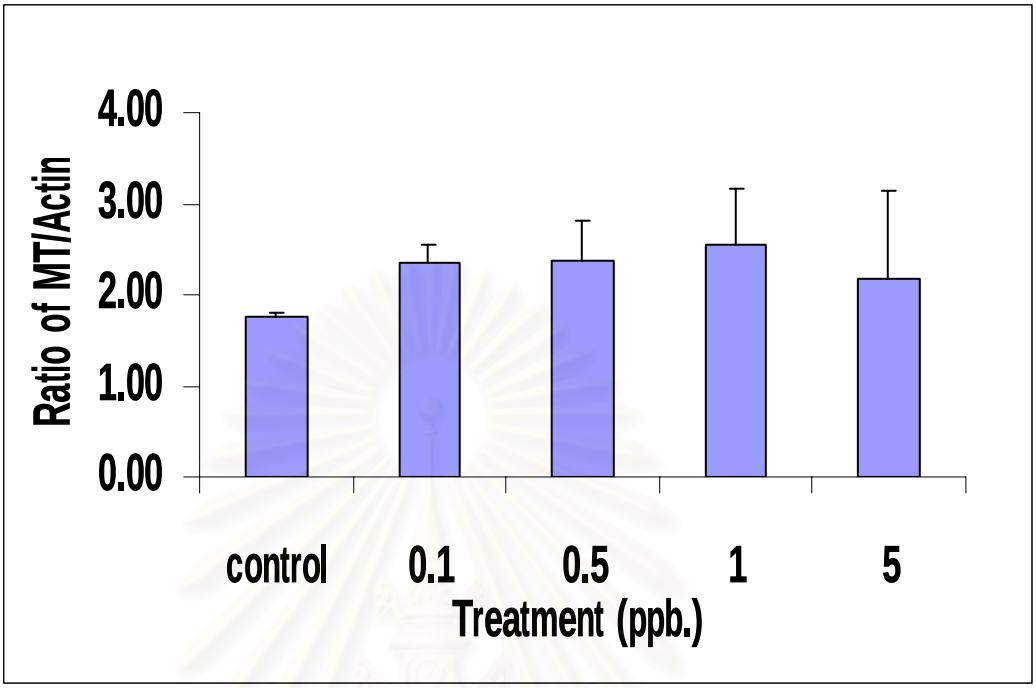

Figure 4.27 The expression ratio of metallothionein and β-actin genes in digestive tracts of mussels treated with various concentrations of mercury at week 3.

Table 4.8 The expression ratio and level of metallothionein gene and β-actin genes in digestive tracts of mussels treated in digestive tracts of mussels treated with various concentrations of mercury at week 4.

Genes	Mercuric chloride concentration (μg/L)				
	0.0	**0.1**	**0.5**	**1.0**	**5.0**
MT	99.69± 29.88	106.44± 5.53	116.22± 1.26	132.81± 1.67	130.05± 17.31
Actin	88.17± 12.51	52.46±4.70	56.02± 9.41	71.36± 19.17	78.29± 8.14
MT/Actin	1.17±0.5	2.03±0.08	2.11±0.38	1.93±0.49	1.66± 0.05

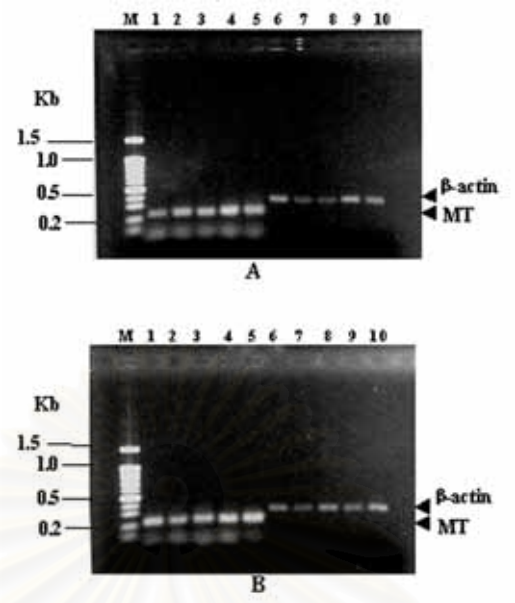

Figure 4.28 The expression levels of metallothionein gene from digestive tracts of *Perna viridis* treated with various concentrations of mercury at week 4 in comparison with β-actin. Samples were obtained from 2 mussels (A and B) and analysed by 1.2% agarose gel electrophoresis.

Lane M = 100 base pair ladder

MT gene

Lane 1 = untreated mussel

Lane 2 = expression level of digestive tract from mussel treated with 0.1 μg/L $HgCl_2$

Lane 3 = expression level of digestive tract from mussel treated with 0.5 μg/L $HgCl_2$

Lane 4 = expression level of digestive tract from mussel treated with 1.0 μg/L $HgCl_2$

Lane 5 = expression level of digestive tract from mussel treated with 5.0 μg/L $HgCl_2$

Beta-actin gene

Lane 6 = untreated mussel

Lane 7 = expression level of digestive tract from mussel treated with 0.1 μg/L $HgCl_2$

Lane 8 = expression level of digestive tract from mussel treated with 0.5 μg/L $HgCl_2$

Lane 9 = expression level of digestive tract from mussel treated with 1.0 μg/L $HgCl_2$

Lane 10= expression level of digestive tract from mussel treated with 5.0 μg/L $HgCl_2$

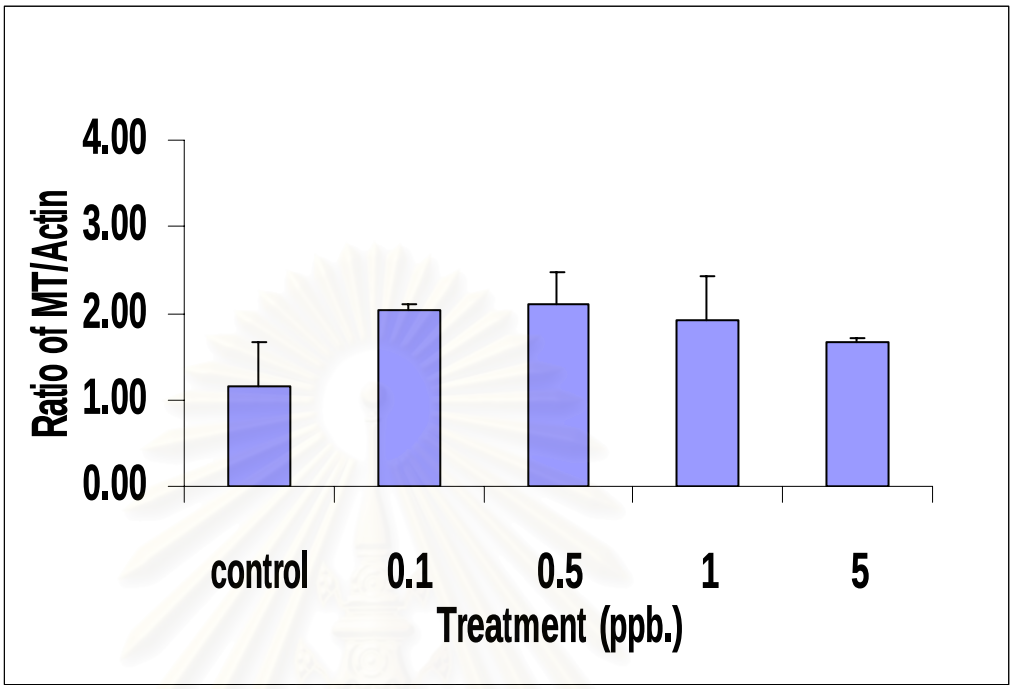

Figure 4.29 The expression ratio of metallothionein and β-actin genes in digestive tracts of mussels treated with various concentrations of mercury at week 4.

Table 4.9 The expression ratio and level of metallothionein gene and β-actin genes in digestive tracts of mussels treated in digestive tracts of mussels treated with various concentrations of mercury at week 5.

Genes	Mercuric chloride concentration (μg/L)				
	0.0	**0.1**	**0.5**	**1.0**	**5.0**
MT	39.79±9.77	80.01± 24.44	116.65± 24.12	136.77± 28.48	146.16± 15.33
Actin	64.82± 20.05	64.14± 24.51	61.18± 20.39	68.00±7.74	84.79± 13.69
MT/Actin	0.62±0.04	1.42±0.93	1.95±0.26	2.00±0.19	1.73±0.10

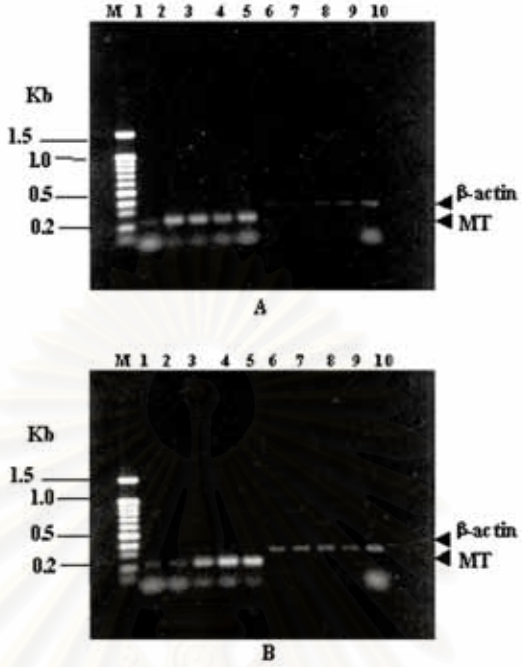

Figure 4.30 The expression levels of metallothionein gene from digestive tracts of *Perna viridis* treated with various concentrations of mercury at week 5 in comparison with β-actin. Samples were obtained from 2 mussels (A and B) and analysed by 1.2% agarose gel electrophoresis.

Lane M = 100 base pair ladder

MT gene

Lane 1 = untreated mussel

Lane 2 = expression level of digestive tract from mussel treated with 0.1 μg/L $HgCl_2$

Lane 3 = expression level of digestive tract from mussel treated with 0.5 μg/L $HgCl_2$

Lane 4 = expression level of digestive tract from mussel treated with 1.0 μg/L $HgCl_2$

Lane 5 = expression level of digestive tract from mussel treated with 5.0 μg/L $HgCl_2$

Beta-actin gene

Lane 6 = untreated mussel

Lane 7 = expression level of digestive tract from mussel treated with 0.1 μg/L $HgCl_2$

Lane 8 = expression level of digestive tract from mussel treated with 0.5 μg/L $HgCl_2$

Lane 9 = expression level of digestive tract from mussel treated with 1.0 μg/L $HgCl_2$

Lane 10= expression level of digestive tract from mussel treated with 5.0 μg/L $HgCl_2$

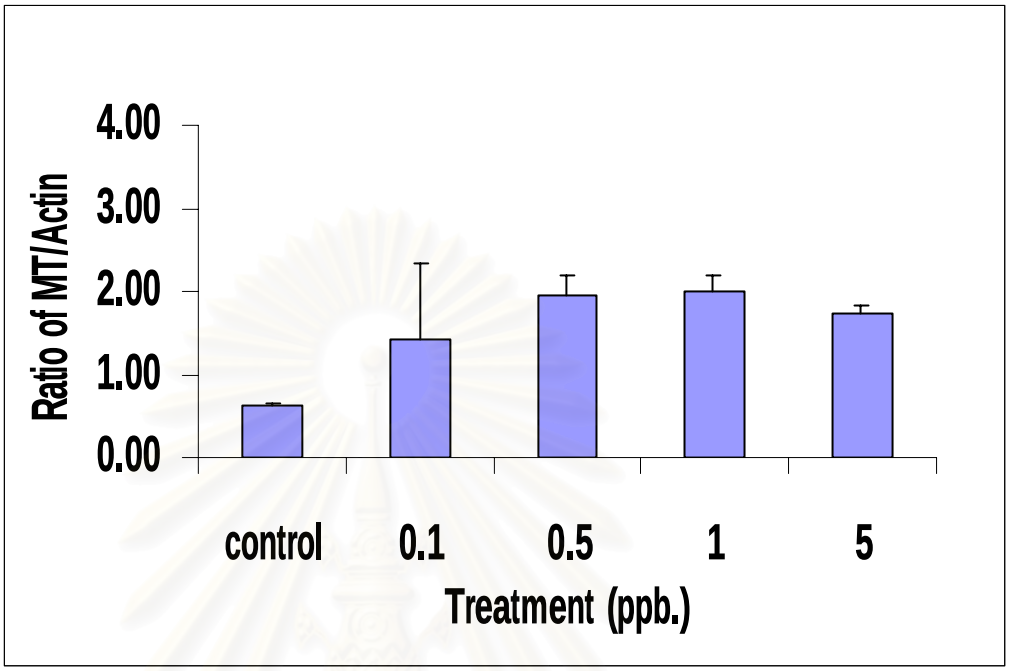

Figure 4.31 The expression ratio of metallothionein and β-actin genes in digestive tracts of mussels treated with various concentrations of mercury at week 5.

Table 4.10 The expression ratio and level of metallothionein gene and β-actin genes in digestive tracts of mussels treated in digestive tracts of mussels treated with various concentrations of mercury at week 7.

Genes	Mercuric chloride concentration (μg/L)				
	0.0	**0.1**	**0.5**	**1.0**	**5.0**
MT	105.12± 2.66	120.14± 4.94	104.17± 6.86	91.14± 10.69	120.56± 15.09
Actin	59.93± 13.45	66.53± 37.05	51.84± 4.39	49.56±3.89	66.87±6.73
MT/Actin	1.80±0.45	2.14±1.14	2.01±0.04	1.85±0.36	1.80±0.04

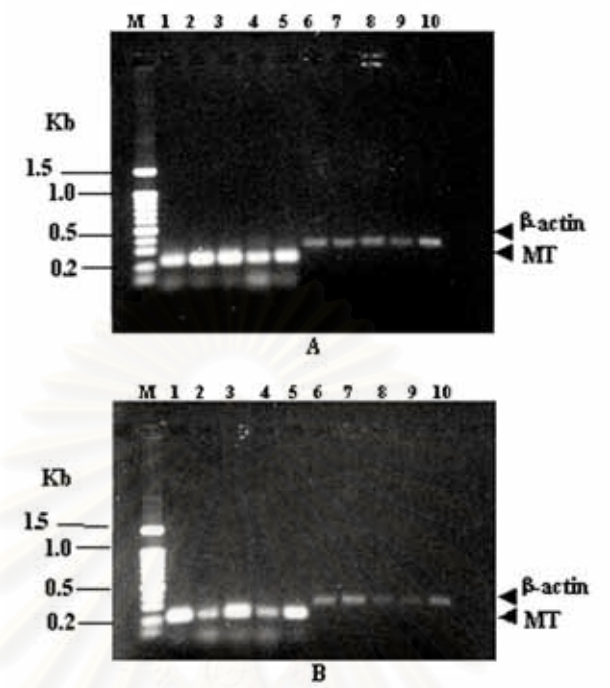

Figure 4.32 The expression levels of metallothionein gene from digestive tracts of *Perna viridis* treated with various concentrations of mercury at week 7 in comparison with β-actin. Samples were obtained from 2 mussels (A and B) and analysed by 1.2% agarose gel electrophoresis.

Lane M = 100 base pair ladder

MT gene

Lane 1 = untreated mussel

Lane 2 = expression level of digestive tract from mussel treated with 0.1 μg/L $HgCl_2$

Lane 3 = expression level of digestive tract from mussel treated with 0.5 μg/L $HgCl_2$

Lane 4 = expression level of digestive tract from mussel treated with 1.0 μg/L $HgCl_2$

Lane 5 = expression level of digestive tract from mussel treated with 5.0 μg/L $HgCl_2$

Beta-actin gene

Lane 6 = untreated mussel

Lane 7 = expression level of digestive tract from mussel treated with 0.1 μg/L $HgCl_2$

Lane 8 = expression level of digestive tract from mussel treated with 0.5 μg/L $HgCl_2$

Lane 9 = expression level of digestive tract from mussel treated with 1.0 μg/L $HgCl_2$

Lane 10= expression level of digestive tract from mussel treated with 5.0 μg/L $HgCl_2$

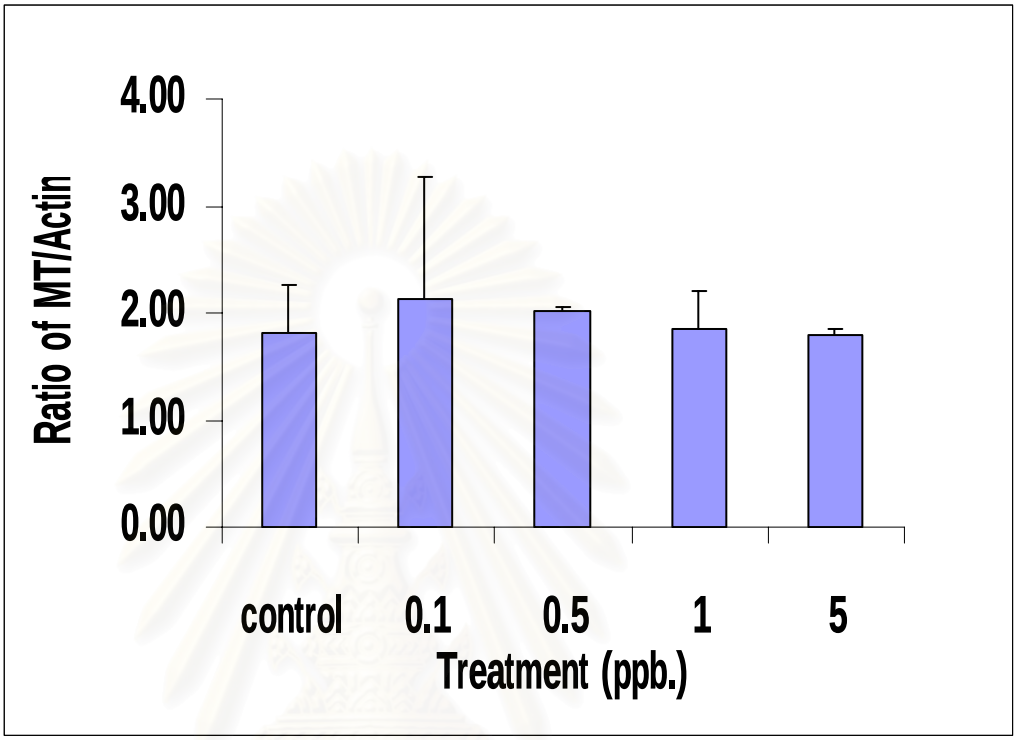

Figure 4.33 The expression ratio of metallothionein and β-actin genes in digestive tracts of mussels treated with various concentrations of mercury at week 7.

4.3.2.2 Expression level of metallothionein gene in mercury-treated mussels from Trad province.

The results of the experiment on the mussels collected from Trad province were shown in figure 4.34-4.41 and table 4.11-4.14. It was indicated that the conditions for semi-quantitative RT-PCR from first experiment were adopted to use in this experiment. For metallothionein gene, template concentration and number of cycle for amplification are 750 ng and 25 cycles. For β-actin gene, template concentration is the same (750 ng) but the cycle for amplification was adjusted to 23 cycles. These conditions were applied to investigate metallothionein gene in digestive tract from mussels were treated with mercuric chloride in different concentration.

When mussels were treated with mercuric chloride for 1 week, ratios of metallothionein gene and β-actin gene were not significantly different between control and treatment ($p > 0.05$) and the expression of metallothionein gene was not corresponded with mercuric chloride concentration (see table 4.11, figure 4.34 and 4.35). After 2 to 4 weeks, the ratio of metallothionein gene and β-actin gene were significantly different between control and treatments ($p < 0.05$). Considering in each week found that 0.5 μg/L, mercuric chloride and 1 μg/L, mercuric chloride concentration could induce higher expression level of metallothionein gene when compared with control groups after exposed for 2 weeks. For 3 weeks exposed, found that 1 μg/L, mercuric chloride and 5 μg/L, mercuric chloride could induce higher expression level of metallothionein gene when compared with control groups. For last week of experiment, the result show 1 μg/L, mercuric chloride induced higher expression level of metallothionein gene when compared with control groups. However, the expression level of metallothionein gene was resistantly induced at 1 μg/L, mercuric chloride. More over the expression level of metallothionein gene tended to increase in corresponding to amount of mercuric chloride up to 1 μg/L and decrease at high concentration 5 μg/L, therefore, the correlation value is significant ($p < 0.05$) (see table 4.12-4.14 and figure4.36-4.41)

Table 4.11 The expression ratio and level of metallothionein gene and β-actin genes in digestive tracts of mussels treated in digestive tracts of mussels treated with various concentrations of mercury at week 1.

Genes	Mercuric chloride concentration (μg/L)				
	0.0	**0.1**	**0.5**	**1.0**	**5.0**
MT	161.69± 40.53	179.61± 56.18	178.58± 54.33	163.00± 53.94	175.83±57.19
Actin	179.2± 47.59	182.65± 50.28	179.99± 43.94	176.29± 45.42	176.0±48.06
MT/Actin	0.88±0.07	0.96±0.08	0.97±0.09	0.89±0.1	0.96±0.12

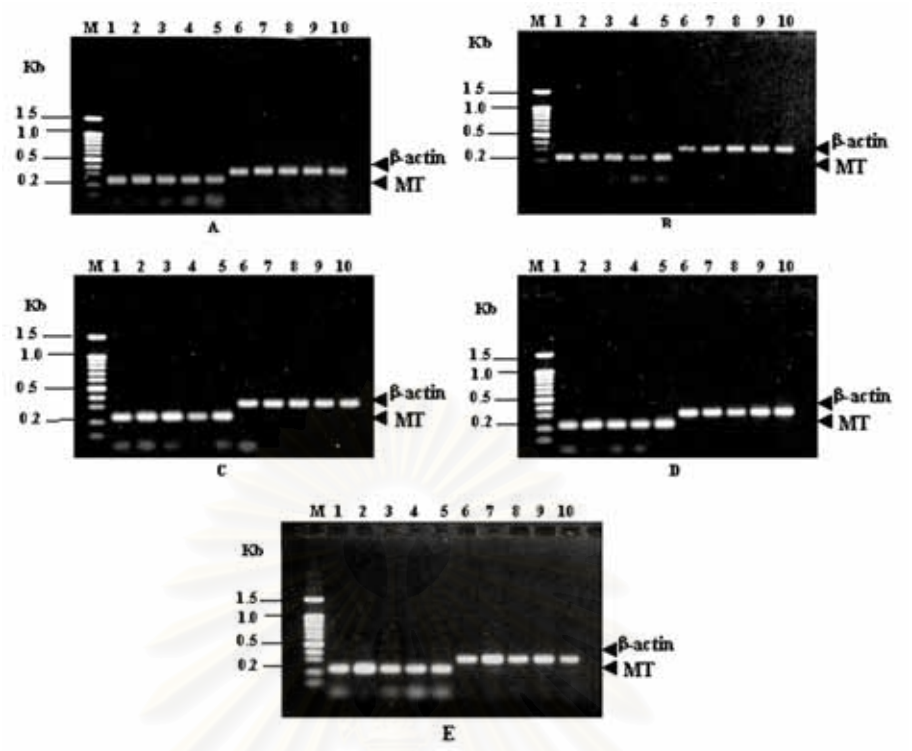

Figure 4.34 The expression levels of metallothionein gene from digestive tracts of *Perna viridis* treated with various concentrations of mercury at week 1 in comparison with β-actin. Samples were obtained from 5 mussels (A, B, C, D and E) and analysed by 1.2% agarose gel electrophoresis.

Lane M = 100 base pair ladder

MT gene

Lane 1 = untreated mussel

Lane 2 = expression level of digestive tract from mussel treated with 0.1 μg/L $HgCl_2$

Lane 3 = expression level of digestive tract from mussel treated with 0.5 μg/L $HgCl_2$

Lane 4 = expression level of digestive tract from mussel treated with 1.0 μg/L $HgCl_2$

Lane 5 = expression level of digestive tract from mussel treated with 5.0 μg/L $HgCl_2$

Beta-actin gene

Lane 6 = untreated mussel

Lane 7 = expression level of digestive tract from mussel treated with 0.1 μg/L $HgCl_2$

Lane 8 = expression level of digestive tract from mussel treated with 0.5 μg/L $HgCl_2$

Lane 9 = expression level of digestive tract from mussel treated with 1.0 μg/L $HgCl_2$

Lane 10= expression level of digestive tract from mussel treated with 5.0 μg/L $HgCl_2$

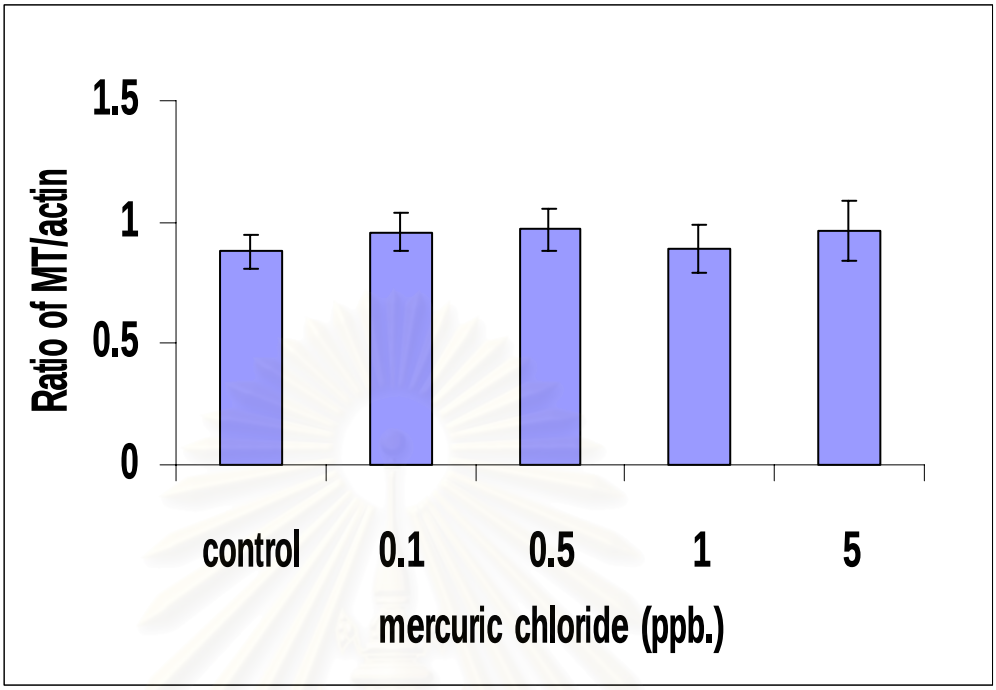

Figure 4.35 The expression ratio of metallothionein and β-actin genes in digestive tracts of mussels treated with various concentrations of mercury at week 1.

Table 4.12 The expression ratio and level of metallothionein gene and β-actin genes in digestive tracts of mussels treated in digestive tracts of mussels treated with various concentrations of mercury at week 2.

Genes	Mercuric chloride concentration (μg/L)				
	0.0	**0.1**	**0.5**	**1.0**	**5.0**
MT	168.47± 49.07	146.08± 44.38	163.20± 42.61	153.58± 56.57	168.78±43.07
Actin	148.56± 40.48	112.91± 41.56	116.96± 19.95	104.14± 39.30	137.88± 45.06
MT/Actin	1.13±0.08	1.36±0.24	1.33±0.12	1.49±0.19	1.28±0.16

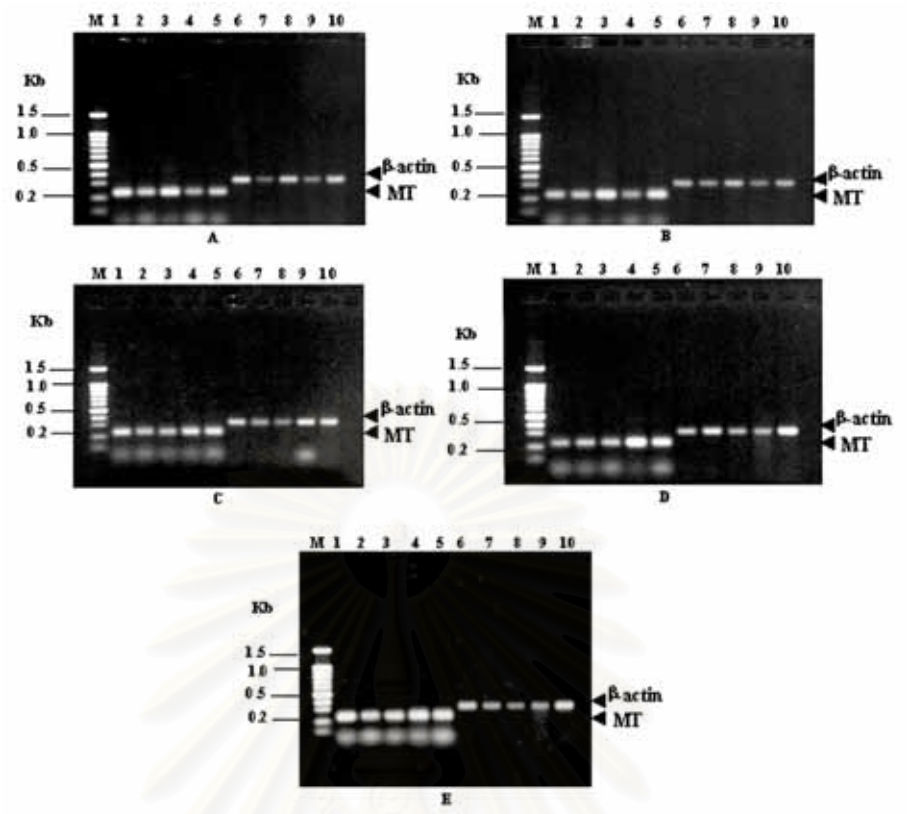

Figure 4.36 The expression levels of metallothionein gene from digestive tracts of *Perna viridis* treated with various concentrations of mercury at week 2 in comparison with β-actin. Samples were obtained from 5 mussels (A, B, C, D and E) and analysed by 1.2% agarose gel electrophoresis.

Lane M = 100 base pair ladder

MT gene

Lane 1 = untreated mussel

Lane 2 = expression level of digestive tract from mussel treated with 0.1 μg/L $HgCl_2$

Lane 3 = expression level of digestive tract from mussel treated with 0.5 μg/L $HgCl_2$

Lane 4 = expression level of digestive tract from mussel treated with 1.0 μg/L $HgCl_2$

Lane 5 = expression level of digestive tract from mussel treated with 5.0 μg/L $HgCl_2$

Beta-actin gene

Lane 6 = untreated mussel

Lane 7 = expression level of digestive tract from mussel treated with 0.1 μg/L $HgCl_2$

Lane 8 = expression level of digestive tract from mussel treated with 0.5 μg/L $HgCl_2$

Lane 9 = expression level of digestive tract from mussel treated with 1.0 μg/L $HgCl_2$

Lane 10= expression level of digestive tract from mussel treated with 5.0 μg/L $HgCl_2$

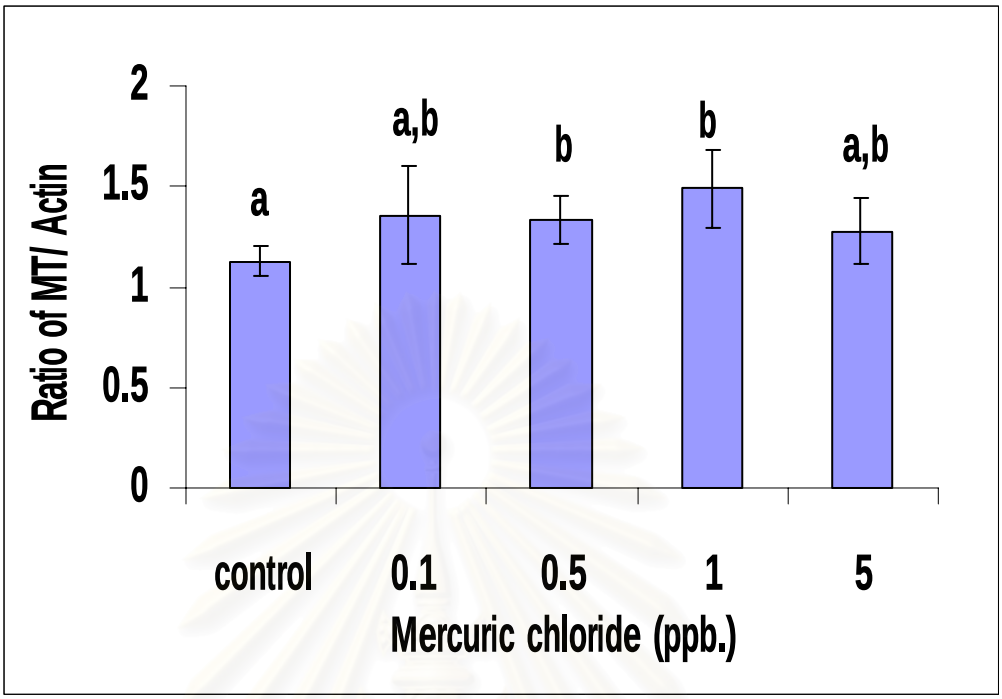

Figure 4.37 The expression ratio of metallothionein and β-actin genes in digestive tracts of mussels treated with various concentrations of mercury at week 2.

Table 4.13 The expression ratio and level of metallothionein gene and β-actin genes in digestive tracts of mussels treated in digestive tracts of mussels treated with various concentrations of mercury at week 3.

Genes	Mercuric chloride concentration (μg/L)				
	0.0	**0.1**	**0.5**	**1.0**	**5.0**
MT	179.33± 13.47	199.73± 20.52	207.85± 8.03	193.42± 14.40	201.86± 16.03
Actin	199.98± 24.04	205.59± 13.57	204.14± 6.01	176.62± 34.40	187.81± 21.62
MT/Actin	0.91±0.11	0.97±0.1	1.02±0.03	1.12±0.15	1.08±0.06

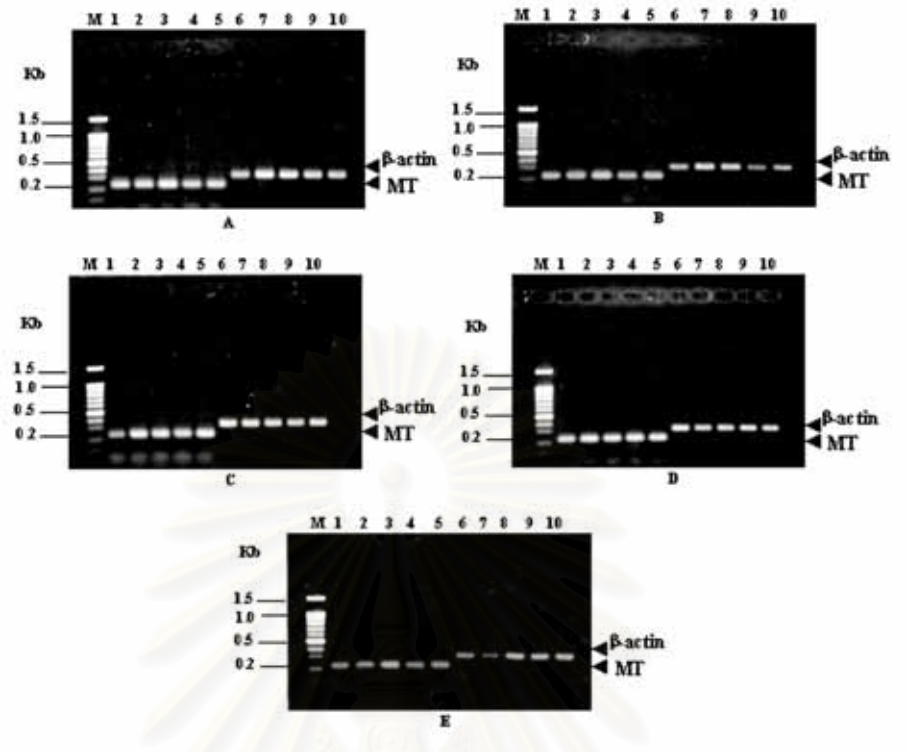

Figure 4.38 The expression levels of metallothionein gene from digestive tracts of *Perna viridis* treated with various concentrations of mercury at week 3 in comparison with β-actin. Samples were obtained from 5 mussels (A, B, C, D and E) and analysed by 1.2% agarose gel electrophoresis.

Lane M = 100 base pair ladder

MT gene

Lane 1 = untreated mussel

Lane 2 = expression level of digestive tract from mussel treated with 0.1 μg/L $HgCl_2$

Lane 3 = expression level of digestive tract from mussel treated with 0.5 μg/L $HgCl_2$

Lane 4 = expression level of digestive tract from mussel treated with 1.0 μg/L $HgCl_2$

Lane 5 = expression level of digestive tract from mussel treated with 5.0 μg/L $HgCl_2$

Beta-actin gene

Lane 6 = untreated mussel

Lane 7 = expression level of digestive tract from mussel treated with 0.1 μg/L $HgCl_2$

Lane 8 = expression level of digestive tract from mussel treated with 0.5 μg/L $HgCl_2$

Lane 9 = expression level of digestive tract from mussel treated with 1.0 μg/L $HgCl_2$

Lane 10= expression level of digestive tract from mussel treated with 5.0 μg/L $HgCl_2$

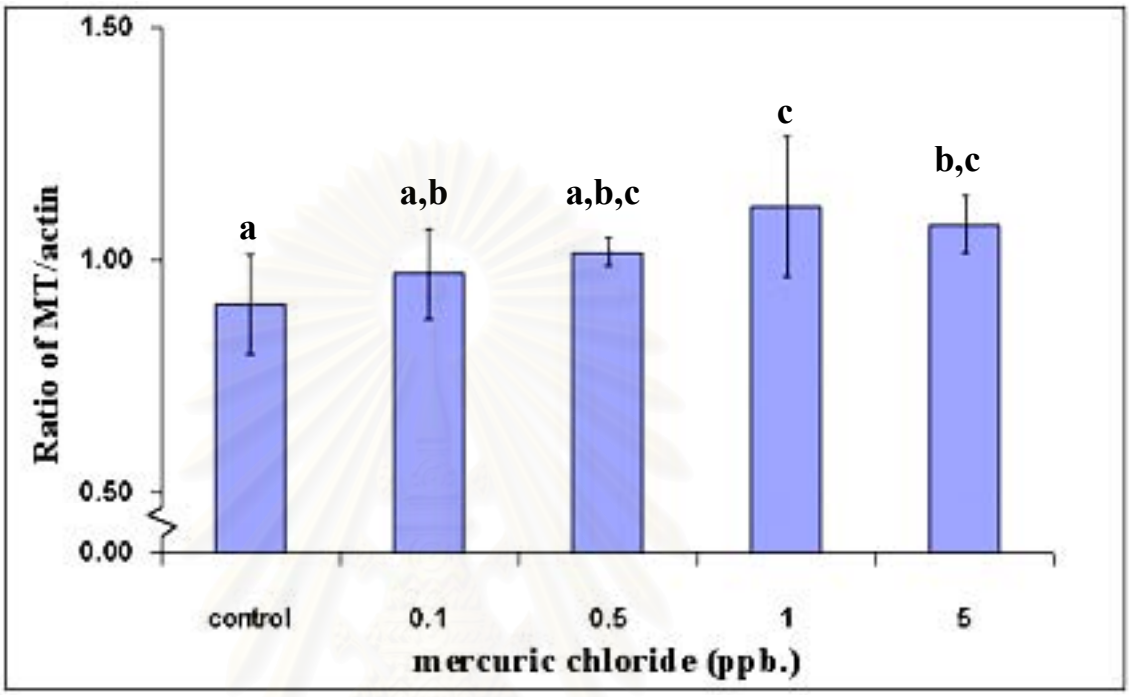

Figure 4.39 The expression ratio of metallothionein and β-actin genes in digestive tracts of mussels treated with various concentrations of mercury at week 3.

Table 4.14 The expression ratio and level of metallothionein gene and β-actin genes in digestive tracts of mussels treated in digestive tracts of mussels treated with various concentrations of mercury at week 4.

Genes	Mercuric chloride concentration (μg/L)				
	0.0	**0.1**	**0.5**	**1.0**	**5.0**
MT	173.62± 33.84	176.25± 44.63	200.90± 22.00	200.47± 26.39	179.89± 49.19
Actin	200.19± 34.14	194.44± 34.87	202.80± 24.25	176.23± 29.18	184.31± 26.91
MT/Actin	0.86±0.06	0.90±0.11	0.99±0.08	1.15±0.19	0.97±0.12

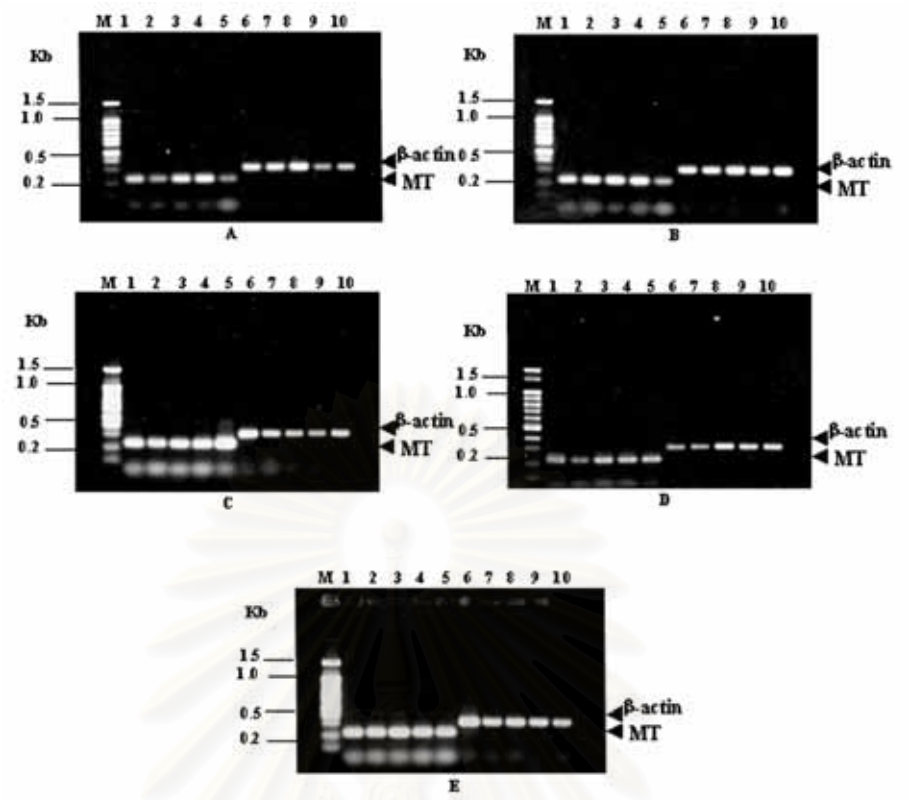

Figure 4.40 The expression levels of metallothionein gene from digestive tracts of *Perna viridis* treated with various concentrations of mercury at week 4 in comparison with β-actin. Samples were obtained from 5 mussels (A, B, C, D and E) and analysed by 1.2% agarose gel electrophoresis.

Lane M = 100 base pair ladder

MT gene

Lane 1 = untreated mussel

Lane 2 = expression level of digestive tract from mussel treated with 0.1 μg/L $HgCl_2$

Lane 3 = expression level of digestive tract from mussel treated with 0.5 μg/L $HgCl_2$

Lane 4 = expression level of digestive tract from mussel treated with 1.0 μg/L $HgCl_2$

Lane 5 = expression level of digestive tract from mussel treated with 5.0 μg/L $HgCl_2$

Beta-actin gene

Lane 6 = untreated mussel

Lane 7 = expression level of digestive tract from mussel treated with 0.1 μg/L $HgCl_2$

Lane 8 = expression level of digestive tract from mussel treated with 0.5 μg/L $HgCl_2$

Lane 9 = expression level of digestive tract from mussel treated with 1.0 μg/L $HgCl_2$

Lane 10= expression level of digestive tract from mussel treated with 5.0 μg/L $HgCl_2$

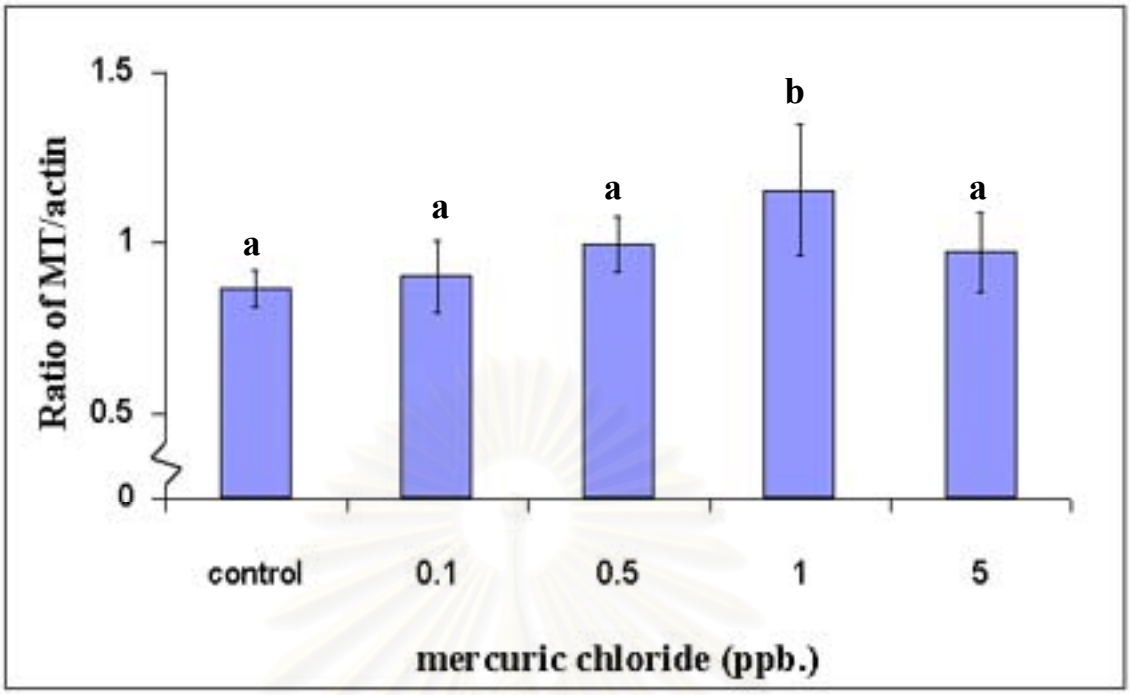

Figure 4.41 The expression ratio of metallothionein and β-actin genes in digestive tracts of mussels treated with various concentrations of mercury at week 4.

4.4 Genetic variation of metallothionein gene detected by single stranded conformation polymorphism (SSCP)

PCR products were amplified from first stand cDNA obtained from gills and digestive tracts of mercury treated mussels using MT1 and MT2 primers. The sizes of DNA products were approximately 220 bp. Variant forms of metallothionein genes from PCR amplification were detected by single stranded conformation polymorphism (SSCP) analysis. The results were shown in figure 4.42-4.44. Twelve different bands of single strand DNA amplified from gills and digestive tracts of mercury treated mussels were detected from SSCP gel. Each band was removed separately from the gel, cloned into T-easy vectors, and subjected to sequencing analysis. The results of the metallothionein sequences were shown in figure 4.45. Deduced protein and DNA sequence alignments of these forms were shown in figure 4.46 and 4.47.

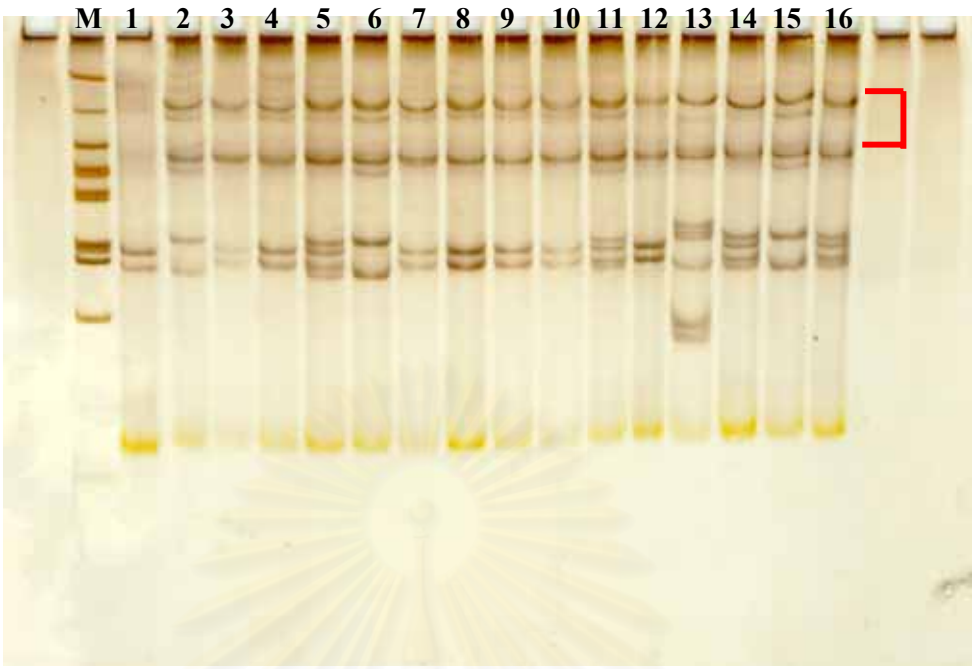

Figure 4.42 PCR- SSCP analysis of metallothionein variants. A 220 bp fragment of the metallothionein gene from gills of mussels treated with different mercuric chloride concentration was determined on 15% polyacrylamide gel.

Lane M = 100 base pairs ladder

Lane 1 = Double stranded cDNA

Lane 2 = Gill extracts from control mussel for 2 weeks

Lane 3 = Gill extracts from mussel treated with 0.1 µg/L $HgCl_2$ for 2 weeks

Lane 4 = Gill extracts from mussel treated with 0.5 µg/L $HgCl_2$ for 2 weeks

Lane 5 = Gill extracts from mussel treated with 1.0 µg/L $HgCl_2$ for 2 weeks

Lane 6 = Gill extracts from mussel treated with 5.0 µg/L $HgCl_2$ for 2 weeks

Lane 7 = Gill extracts from control mussel for 3 weeks

Lane 8 = Gill extracts from mussel treated with 0.1 µg/L $HgCl_2$ for 3 weeks

Lane 9 = Gill extracts from mussel treated with 0.5 µg/L $HgCl_2$ for 3 weeks

Lane 10 = Gill extracts from mussel treated with 1.0 µg/L $HgCl_2$ for 3 weeks

Lane 11 = Gill extracts from mussel treated with 5.0 µg/L $HgCl_2$ for 3 weeks

Lane12 = Gill extracts from control mussel for 7 weeks

Lane 13 = Gill extracts from mussel treated with 0.1 µg/L $HgCl_2$ for 7 weeks

Lane 14 = Gill extracts from mussel treated with 0.5 µg/L $HgCl_2$ for 7 weeks

Lane 15 = Gill extracts from mussel treated with 1.0 µg/L $HgCl_2$ for 7 weeks

Lane 16 = Gill extracts from mussel treated with 5.0 µg/L $HgCl_2$ for 7 weeks

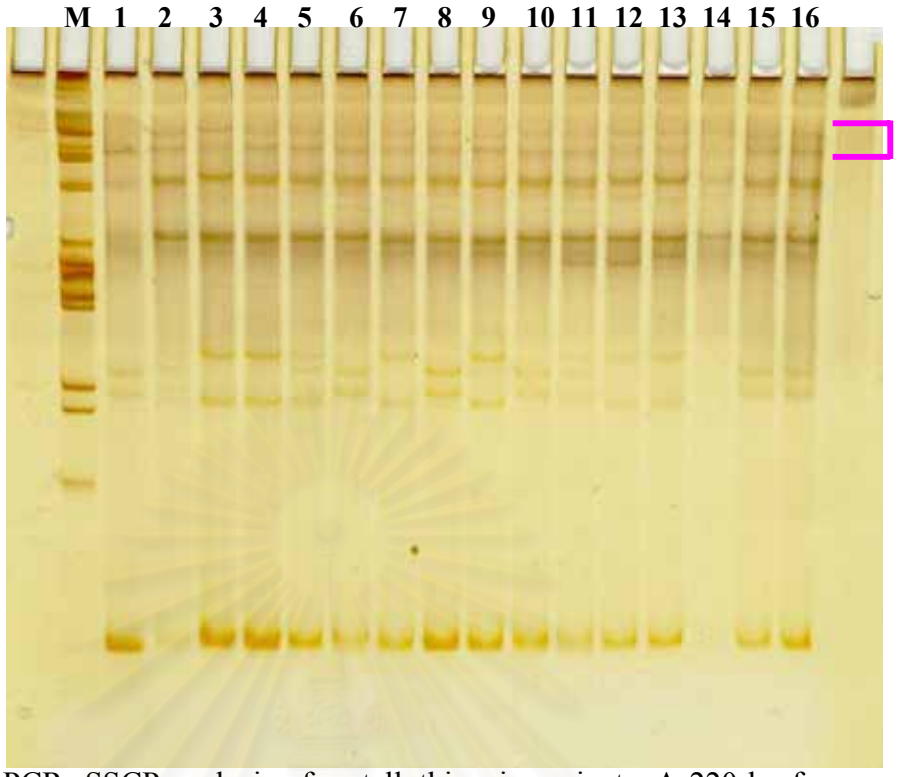

Figure 4.43 PCR- SSCP analysis of metallothionein variants. A 220 bp fragment of the metallothionein gene from digestive tracts of mussels treated with different mercuric chloride concentration was determined on 15% polyacrylamide gel.

Lane M = 100 base pairs ladder

Lane 1 = Double stranded cDNA

Lane 2 = Digestive tract extract from control mussel for 2 weeks

Lane 3 =Digestive tract extract from mussel treated with 0.1 µg/L $HgCl_2$ for 1 weeks

Lane 4 =Digestive tract extract from mussel treated with 0.5 µg/L $HgCl_2$ for 1 weeks

Lane 5 = Digestive tract extract from mussel treated with 1.0 µg/L $HgCl_2$ for 1 weeks

Lane 6 = Digestive tract extract from mussel treated with 5.0 µg/L $HgCl_2$ for 1 weeks

Lane 7 = Digestive tract extract from control for 4 weeks

Lane 8 = Digestive tract extract from mussel treated with 0.1 µg/L $HgCl_2$ for 4 weeks

Lane 9 = Digestive tract extract from mussel treated with 0.5 µg/L $HgCl_2$ for 4 weeks

Lane 10= Digestive tract extract from mussel treated with 1.0 µg/L $HgCl_2$ for 4 weeks

Lane 11= Digestive tract extract from mussel treated with 5.0 µg/L $HgCl_2$ for 4 weeks

Lane 12 = Digestive tract extract from mussel control for 5 weeks

Lane 13= Digestive tract extract from mussel treated with 0.1 µg/L $HgCl_2$ for 5 weeks

Lane 14= Digestive tract extract from mussel treated with 0.5 µg/L $HgCl_2$ for 5 weeks

Lane 15= Digestive tract extract from mussel treated with 1.0 µg/L $HgCl_2$ for 5 weeks

Lane 16= Digestive tract extract from mussel treated with 5.0 µg/L $HgCl_2$ for 5 weeks

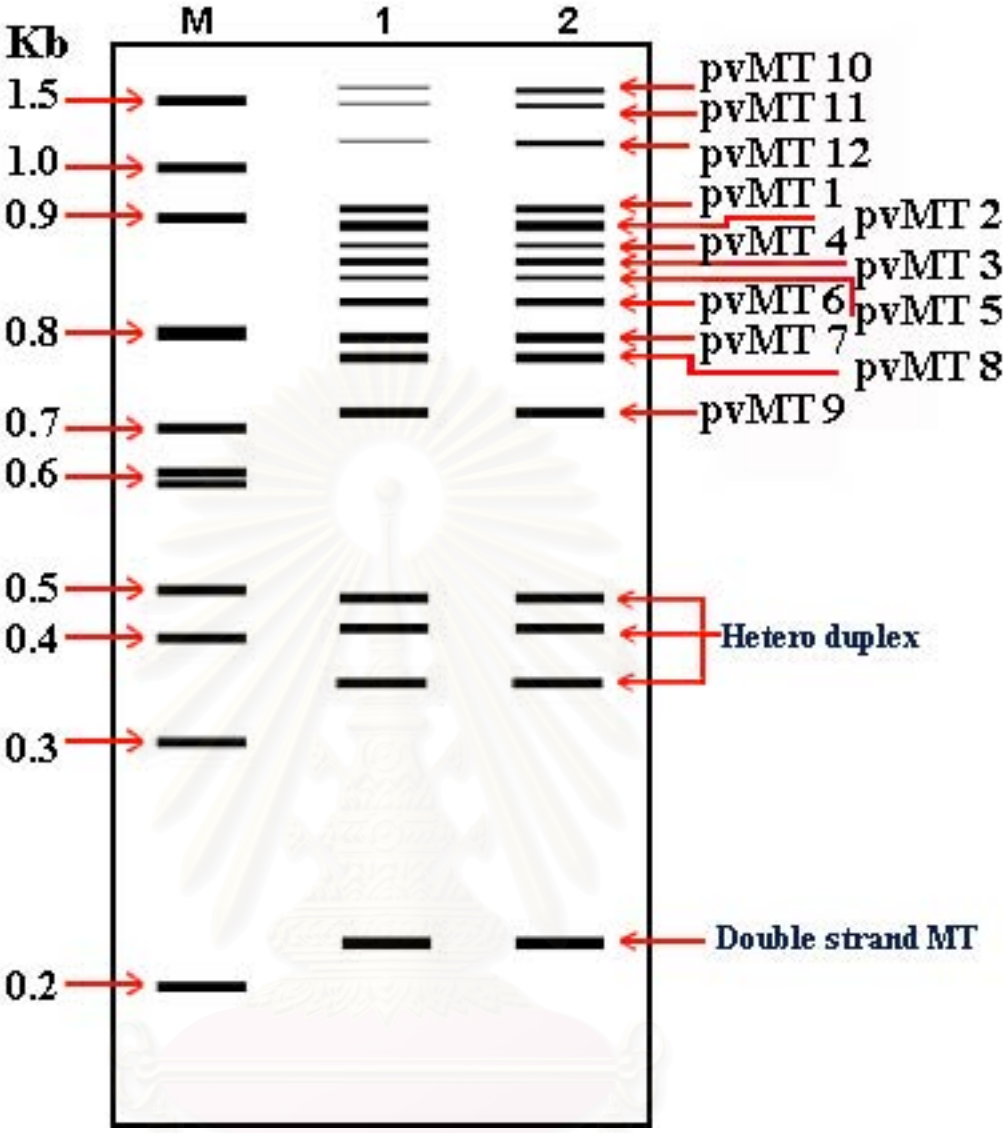

Figure 4.44 Diagram of different visualize metallothionein bands from gills and digestive tracts of mussels, *P. viridis* detected by SSCP analysis Lane M = 100 base pair ladder, lane 1 = gill, lane 2 = digestive tract.

The nucleotide sequences of twelve variant metallothionein genes, designated as pvMT1 to pvMT12, respectively, were translated to amino acid sequence. The classification of metallothionein was based on the classification of metallothionein described by Binze and Käji (1999). SSCP technique was used to obtain the amino acid sequence in variant metallothionein. The sequences found were matched sequence pattern C-x-C-x (3)-C-T-G-x (3)-C-x-C-x (3)-C-x-C-K that belongs to metallothionein family 2 or mollusc metallothioneins (figure 4.48). The other characteristics that matched with this family (table 4.15) were 72-73 amino acids and 21 Cysteins. The molecular weights of these variant metallothionein genes were 7.1-7.3 kDa.

Table 4.15 The characteristic of variant metallothionein from mussels *Perna viridis*

Forms	No. of amino acid	No. of cysteine	Molecular weight	Aromatic amino acid
pvMT1	73	28	7.25	-
pvMT2	73	28	7.31	-
pvMT3	72	28	7.12	-
MT4	72	28	7.12	-
pvMT5	72	28	7.12	-
pvMT6	72	28	7.12	-
pvMT7	72	28	7.19	-
pvMT8	72	28	7.22	-
pvMT9	72	28	7.12	-
pvMT10	73	28	7.25	-
pvMT11	73	28	7.25	-
pvMT12	73	28	7.25	-

The amino acid sequences from variant metallothioneins were aligned with amino acid sequences from other mollusc in Swiss-Prot. The alignments of amino acid sequences showed less than 90% similarity when compared with mo1, mo2, and mog (gastropod metallothionein) indicating that variant metallothionein genes from SSCP technique were members of subfamily mo (other mollusc metallothioneins) (figure 4.51).

Following family identification, all 12 metallothionein sequences were subjected to phylogenetic analysis (figure 4.50). The results indicated that variant metallothionein sequences were classified into 2 main subforms, designated as mop1 and mop2. These 2 subforms were separated by the difference of amino acid sequences at residue 49 to 51 (figure 4.51). Each subforms were also divided into 3 isoforms. Mop1 consisted of mop1-a (pvMT1 and pvMT10), mop1-b (pvMT11 and pvMT12), and mop1-c (pvMT2), respectively and mop2 consisted of mop2-a (pvMT7), mop2-b (pvMT8), and mop2-c (pvMT3, pvMT4, pvMT5, pvMT6, and pvMT9), respectively.

```
                ....|....| ....|....| ....|....| ....|....| ....|....|
                      10         20         30         40         50
pvMT 1          ATGCCCAGCC CTTGTAATTG CATTGAAACA AAAGTCTGTA TCTGTGGTAC
pvMT 2          ATGCCCAGCC CTTGTAATTG CATTGAAACA CAAGTCTGTA TCTGTGGTAC
pvMT 3          ATGCCCAGCC CTTGTAATTG TAATGAAACA CAAGTCTGCA TCTGTGGGAG
pvMT 4          ATGCCCAGCC CTTGTAATTG TAATGAAACA CAAGTCTGCA TCTGTGGGAG
pvMT 5          ATGCCCAGCC CTTGTAATTG TAATGAAACA CAAGTCTGCA TCTGTGGGAG
pvMT 6          ATGCCCAGCC CTTGTAATTG TAATGAAACA CAAGTCTGCA TCTGTGGGAG
pvMT 7          ATGCCCAGCC CTTGTAATTG TAATGAAACA CAAGTCTGCA TCTGTGGGAG
pvMT 8          ATGCCCAGCC CTTGTAATTG TAATGAAACA CAAGTCTGCA TCTGTGGGAG
pvMT 9          ATGCCCAGCC CTTGTAATTG TAATGAAACA CAAGTCTGCA TCTGTGGGAG
pvMT 10         ATGCCCAGCC CTTGTAATTG CATTGAAACA AAAGTCTGTA TCTGTGGTAC
pvMT 11         ATGCCCAGCC CTTGTAATTG CATTGAAACA CAAGTCTGTA TCTGTGGTAC
pvMT 12         ATGCCCAGCC CTTGTAATTG CATTGAAACA CAAGTCTGTA TCTGTGGTAC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                     60         70         80         90        100
pvMT 1          TGGGTGCAGC GGAGAAGGTT GTCGTTGTGG TGACGCGTGC AAATGTAGCA
pvMT 2          TGGGTGCAGC GGAGAAGATT GTCGTTGTGG TGACGCGTGC AAATGTAGCA
pvMT 3          TGGATGCAGC GGGGAAGGTT GTGGCTGCGG TGACGCATGC AAATGTGACA
pvMT 4          TGGGTGCAGC GGGGAAGGTT GTGGCTGCGG TGACGCATGC AAATGTGACA
pvMT 5          TGGGTGCAGC GGGGAAGGTT GTGGCTGCGG TGACGCATGC AAATGTGACA
pvMT 6          TGGGTGCAGC GGGGAAGGTT GTGGCTGCGG TGACGCATGC AAATGTGACA
pvMT 7          TGGGTGCAGA GGGGAAGGTT GTGGCTGCGG TGACGCATGC AAATGTGACA
pvMT 8          TGGGTGCAGA GGGGAAGGTT GTGGCTGCGG TGACGCATGC AAATGTGACA
pvMT 9          TGGATGCAGC GGGGAAGGTT GTGGCTGCGG TGACGCATGC AAATGTGACA
pvMT 10         TGGGTGCAGC GGAGAAGGTT GTCGTTGTGG TGACGCGTGC AAATGTAGCA
pvMT 11         TGGGTGCAGC GGAGAAGGTT GTCGTTGTGG TGACGCGTGC AAATGTAGCA
pvMT 12         TGGGTGCAGC GGAGAAGGTT GTCGTTGTGG TGACGCGTGC AAATGTAGCA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                    110        120        130        140        150
pvMT 1          GTGGTTGTGG ATGTTCAGGG TGTAAAGTCG TGTGCAAATG TCAGCCAGGA
pvMT 2          GTGGTTGTGG ATGTTCAGGG TGTAAAGTCG TGTGCAAATG TCAGCCAGGA
pvMT 3          GTGATTGTGG ATGTTCTGGA TGTAAAATCG TCTGCAAATG TTCAGATGCG
pvMT 4          GTGATTGCGG ATGTTCTGGA TGTAAAATCG TCTGCAAATG TTCAGATGCG
pvMT 5          GTGATTGTGG ATGTTCTGGA TGTAAAATCG TCTGCAAATG TTCAGATGCG
pvMT 6          GTGATTGCGG ATGTTCTGGA TGTAAAATCG TCTGCAAATG TTCAGATGCG
pvMT 7          GTGATTGTGG ATGTTCTGGA TGTAAAATCG TCTGCAAATG TTCAGATGCA
pvMT 8          GTGATTGTGG ATGTTCTGGA TGTAAAATCG TCTGCAAATG TTCAGATGCG
pvMT 9          GTGATTGTGG ATGTTCTGGA TGTAAAATCG TCTGCAAATG TTCAGATGCG
pvMT 10         GTGGTTGTGG ATGTTCAGGG TGTAAAGTCG TGTGCAAATG TCAGCCAGGA
pvMT 11         GTGGTTGTGG ATGTTCAGGG TGTAAAGTCG TGTGCAAATG TCAGCCAGGA
pvMT 12         GTGGTTGTGG ATGTTCAGGG TGTAAAGTCG TGTGCAAATG TCAGCCAGGA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                    160        170        180        190        200
pvMT 1          GAGTGTGCAT GTGGCAAGCA ATGTACGGGA CCAGACACCT GTAAATGTGA
pvMT 2          GAGTGTGCAT GTGGCAAGCA ATGTACGGGA CCAGACACCT GTAAATGTGA
pvMT 3          TGTGCGTGTG GCAAGCAATG TACGGGACCA AAGACCTGTA AATGTGACTC
pvMT 4          TGTGCGTGTG GCAAGCAATG TACGGGACCA AAGACCTGTA AATGTGACTC
pvMT 5          TGTGCGTGTG GCAAGCAATG TACGGGACCA AAGACCTGTA AATGTGACTC
pvMT 6          TGTGCGTGTG GCAAGCAATG TACGGGACCA AAGACCTGTA AATGTGACTC
pvMT 7          TGTGCGTGTG GCAAGCAATG TACGGGACCA AAGACCTGTA AATGTGACTC
pvMT 8          TGTGCGTGTG GCAAGCAATG TATGGGACCA AAGACCTGTA AATGTGACTC
pvMT 9          TGTGCGTGTG GCAAGCAATG TACGGGACCA AAGACCTGTA AATGTGACTC
pvMT 10         GAGTGTGCAT GTGGCAAGCA ATGTACGGGA CCAGACACCT GTAAATGTGA
pvMT 11         GAGTGTGCAT GTGGCAAGCA ATGTACGGGA CCAGACACCT GTAAATGTGA
pvMT 12         GAGTGTGCAT GTGGCAAGCA ATGTACGGGA CCAGACACCT GTAAATGTGA

                ....|....| ....|....| ..
                    210        220
pvMT 1          CTCCAGTTGT TCGTGCAAAT AA
pvMT 2          CTCCAGTTGT TCGTGCAAAT AA
pvMT 3          CAGTTGTTCG TGCAAATAA. ..
pvMT 4          CAGTTGTTCG TGCAAATAA. ..
pvMT 5          CAGTTGTTCG TGCAAATAA. ..
pvMT 6          CAGTTGTTCG TGCAAATAA. ..
pvMT 7          CAGTTGTTCG TGCAAATAA. ..
pvMT 8          CAGTTGTTCG TGCAAATAA. ..
pvMT 9          CAGTTGTTCG TGCAAATAA. ..
pvMT 10         CTCCAGTTGT TCGTGCAAAT AA
pvMT 11         CTCCAGTTGT TCGTGCAAAT AA
pvMT 12         CTCCAGTTGT TCGTGCAAAT AA
```

Figure 4.45 The nucleotide sequence of metallothionein genes detected from gills and digestive tracts of mussels.

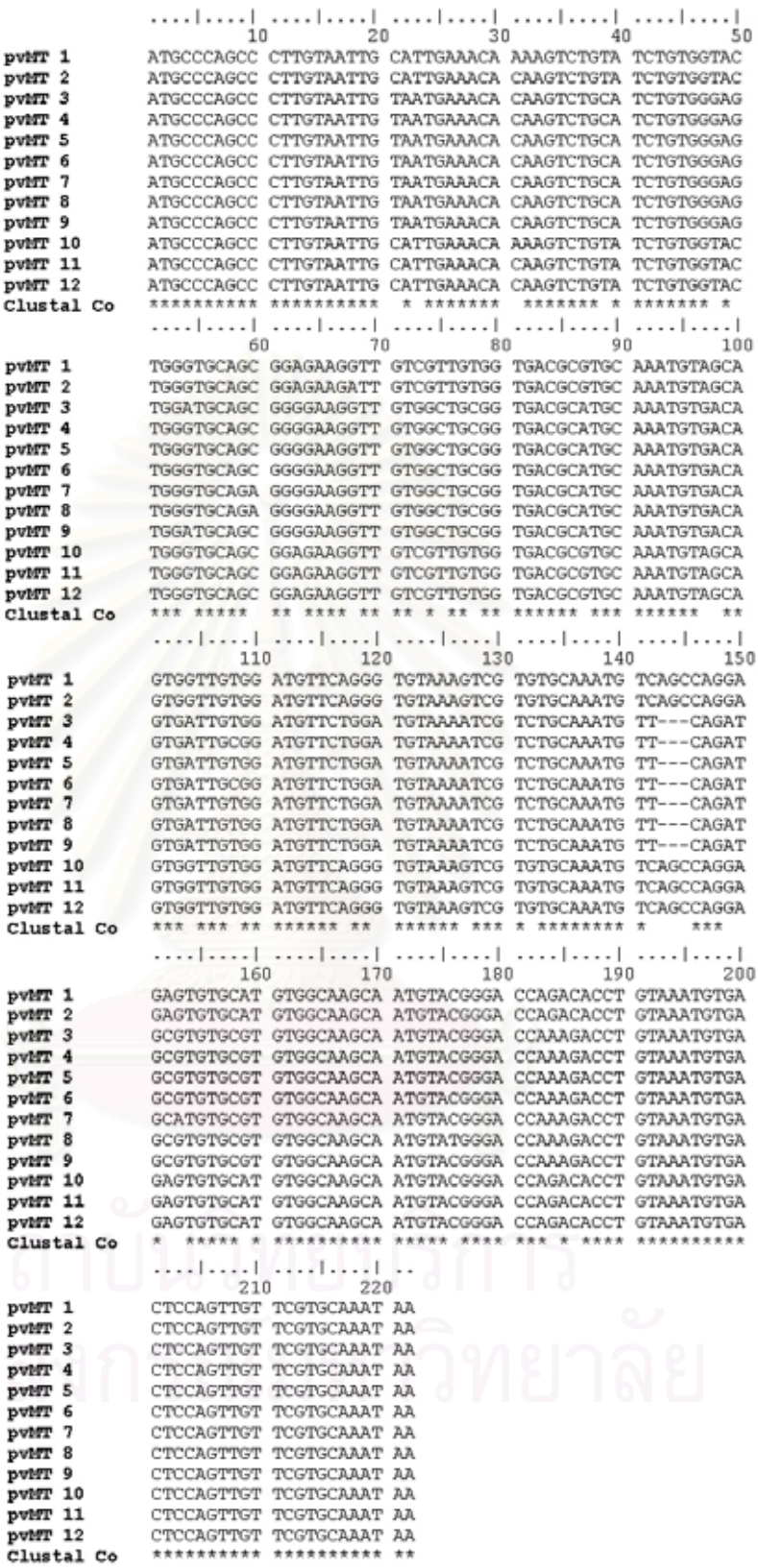

Figure 4.46 The nucleotide sequence alignment of metallothionein genes detected from gills and digestive tracts of mussels.

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                ....|....| ....|....| ....|....| ....|....| ....|....|
                        10         20         30         40         50
pvMT 1          MPSPCNCIET KVCICGTGCS GEGCRCGDAC KCSSGCGCSG CKVVCKCQPG
pvMT 2          MPSPCNCIET QVCICGTGCS GEDCRCGDAC KCSSGCGCSG CKVVCKCQPG
pvMT 3          MPSPCNCNET QVCICGSGCS GEGCGCGDAC KCDSDCGCSG CKIVCKCS-D
pvMT 4          MPSPCNCNET QVCICGSGCS GEGCGCGDAC KCDSDCGCSG CKIVCKCS-D
pvMT 5          MPSPCNCNET QVCICGSGCS GEGCGCGDAC KCDSDCGCSG CKIVCKCS-D
pvMT 6          MPSPCNCNET QVCICGSGCS GEGCGCGDAC KCDSDCGCSG CKIVCKCS-D
pvMT 7          MPSPCNCNET QVCICGSGCR GEGCGCGDAC KCDSDCGCSG CKIVCKCS-D
pvMT 8          MPSPCNCNET QVCICGSGCR GEGCGCGDAC KCDSDCGCSG CKIVCKCS-D
pvMT 9          MPSPCNCNET QVCICGSGCS GEGCGCGDAC KCDSDCGCSG CKIVCKCS-D
pvMT 10         MPSPCNCIET KVCICGTGCS GEGCRCGDAC KCSSGCGCSG CKVVCKCQPG
pvMT 11         MPSPCNCIET QVCICGTGCS GEGCRCGDAC KCSSGCGCSG CKVVCKCQPG
pvMT 12         MPSPCNCIET QVCICGTGCS GEGCRCGDAC KCSSGCGCSG CKVVCKCQPG
Clustal Co      ******* ** :*****:**  **.* ***** **.*.***** **:****. .

                ....|....| ....|....| ...
                        60         70
pvMT 1          ECACGKQCTG PDTCKCDSSC SCK
pvMT 2          ECACGKQCTG PDTCKCDSSC SCK
pvMT 3          ACACGKQCTG PKTCKCDSSC SCK
pvMT 4          ACACGKQCTG PKTCKCDSSC SCK
pvMT 5          ACACGKQCTG PKTCKCDSSC SCK
pvMT 6          ACACGKQCTG PKTCKCDSSC SCK
pvMT 7          ACACGKQCTG PKTCKCDSSC SCK
pvMT 8          ACACGKQCMG PKTCKCDSSC SCK
pvMT 9          ACACGKQCTG PKTCKCDSSC SCK
pvMT 10         ECACGKQCTG PDTCKCDSSC SCK
pvMT 11         ECACGKQCTG PDTCKCDSSC SCK
pvMT 12         ECACGKQCTG PDTCKCDSSC SCK
Clustal Co       ******* * *.******** ***
```

Figure 4.47 The deduced amino acid sequences of metallothionein genes detected from gills and digestive tracts of mussels.

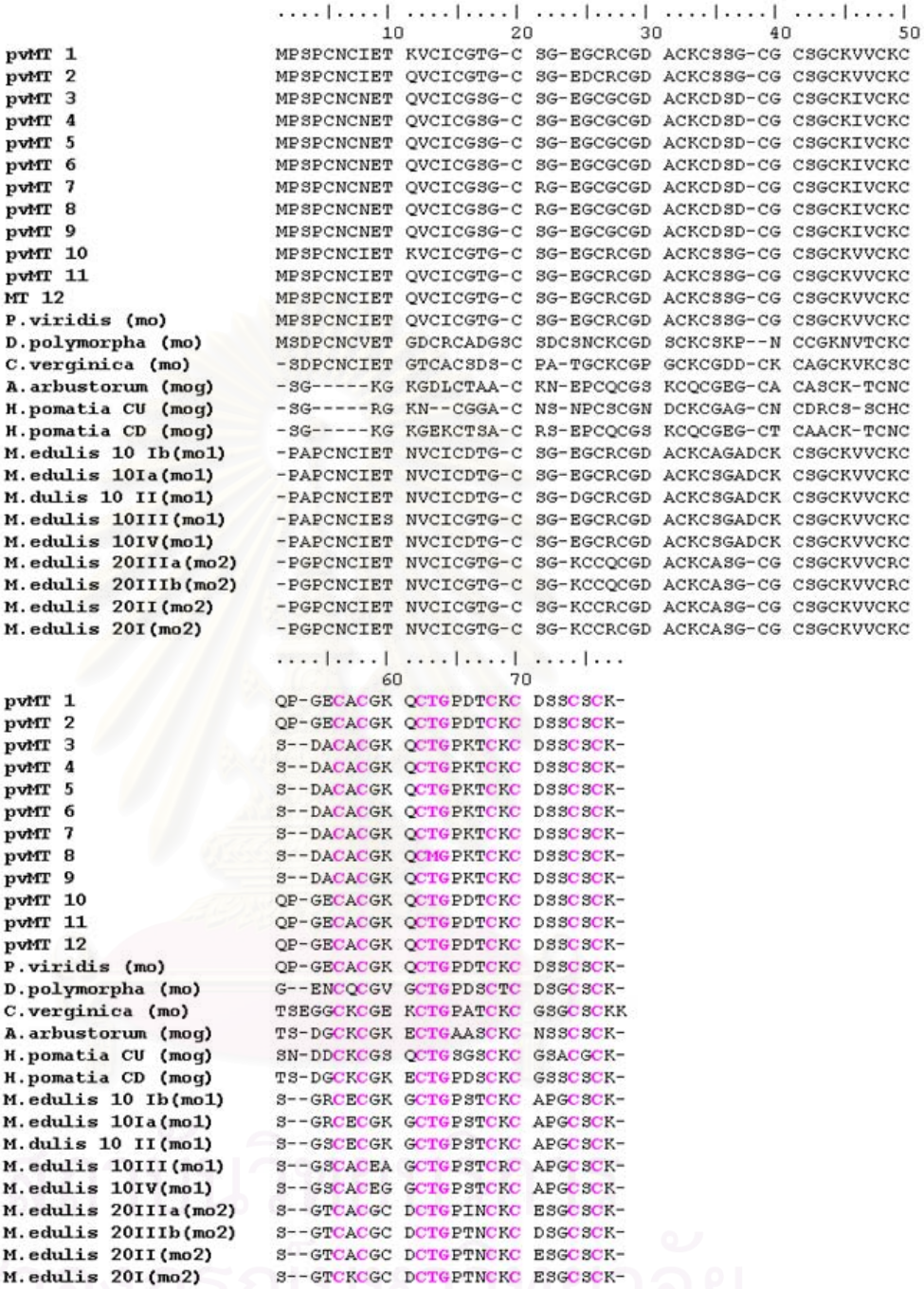

Figure 4.48 Deduced amino acid sequence alignment of metallothionein genes detected from gills and digestive tracts of mussels. Identical residues were highlighted. Alignments were made to reflect the conservation of cysteines that matched with sequence pattern of metallothionein family 2 (molluscan metallothioneins).

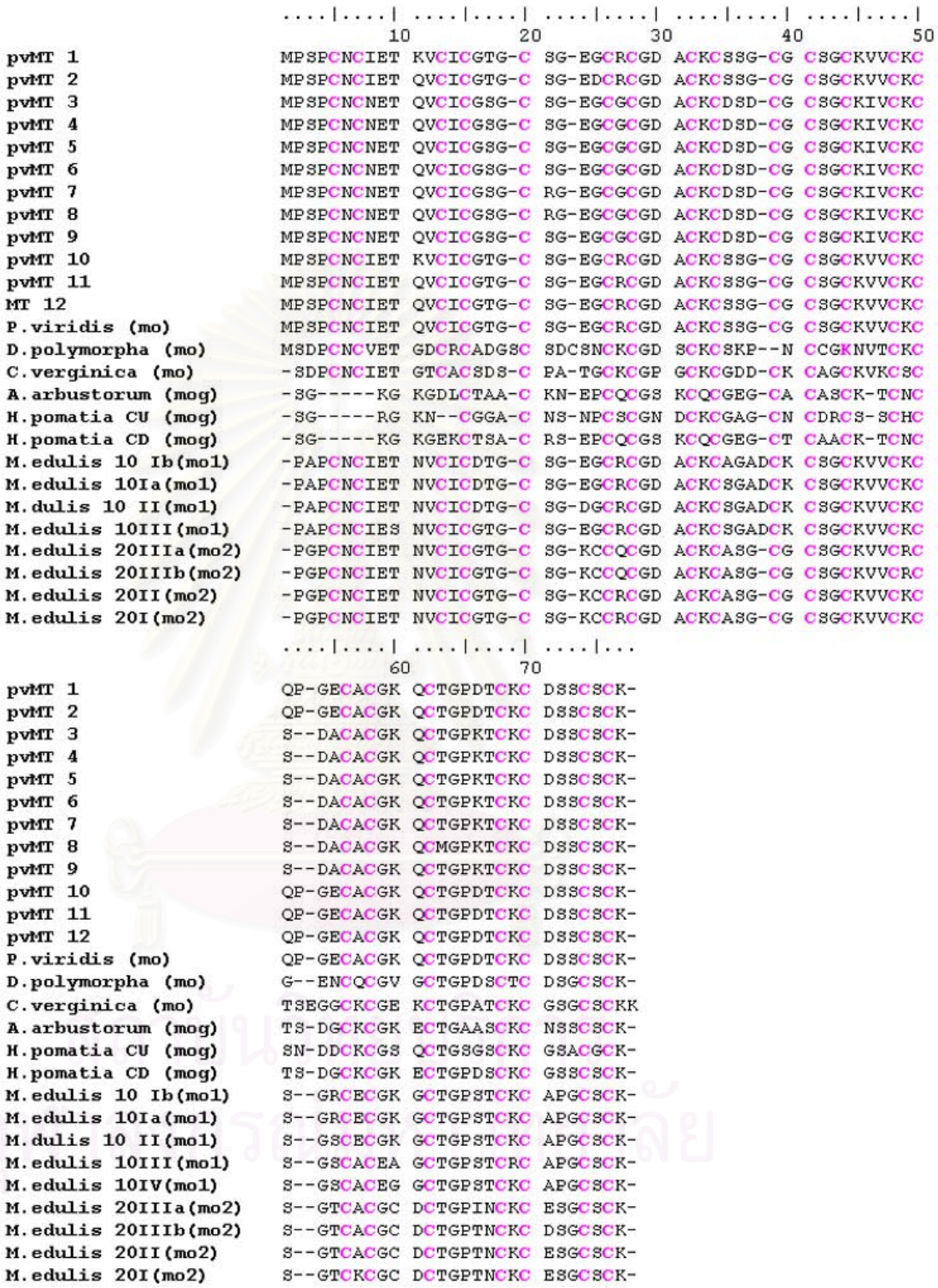

Figure 4.49 Comparison of deduced amino acid sequences of metallothioneins from *P.viridis* and other molluscan metallothioneins. Similarity amino acids were highlight.

Figure 4.50 Phylogenetic tree analysis of amino acid sequence of variant metallothionein gene from mussel *Perna viridis*

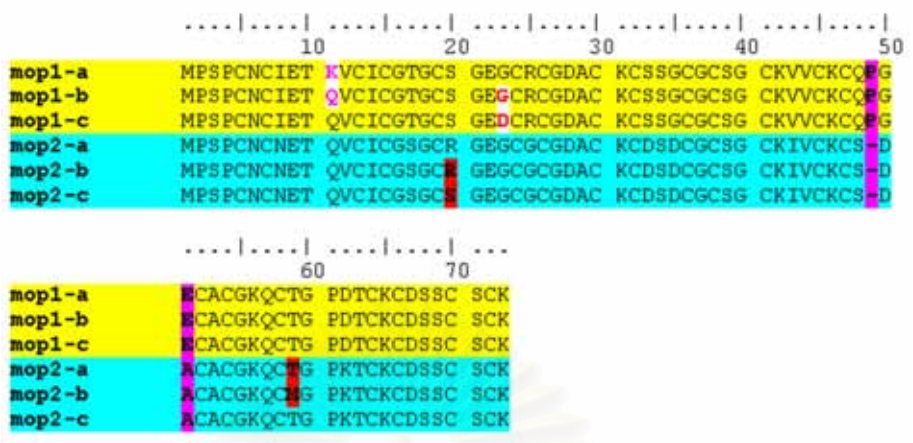

Figure 4.51 The alignment of amino acid of metallothionein isoforms. The highlight shows the different of amino acid residues position.

CHAPTER V

DISCUSSIONS

5.1 Determination of metallothionein

The experiment was designed to identify metallothionein level in the tissues of mussels, *P. viridis*, exposed to a very low concentrations of dissolved mercury using gel electrophoresis. The protein level of metallothionein was assayed by Coomassie staining, silver staining, and immunochemical analysis.

The protocol used in the experiment was modified from Gel electrophoresis and silver staining (McCormick and Lin, 1991). This method was based on the very high affinity of metals to metallothionein, a unique characteristic of metallothionein. The electrophoretic gels containing peptide bands were firstly stained by coomassie Brilliant blue, and followed by silver staining. The metallothionein content was quantified by the ratio of the peptide band intensity from silver and Coomassie staining. As revealed by the results (see section 4.1.1), the method of detecting metallothionein by silver staining was unable to detect the normal or the different level of metallothionein in mussels treated with mercuric chloride. Even high purity metallothionein (rabbit metallothionein) used as control did not give the reliable result. When higher amount of protein sample (250 μg) (see section 4.1.2) was loaded to the gel, metallothionein bands were detectable. Unfortunately, the result is not consistency. This is probably because of the difficulty in controlling the appropriate condition of the gradient gel and the very low level of metallothionein in mercury-exposed mussels. Gel electrophoresis and silver staining has been improved for a number of times (Lin and McCormick, 1986; McCormick and Lin, 1991) and was frequently employed for metallothionein detection in a number of organisms (Lin and McCormick, 1986; McCormick and Lin, 1991, Geret and Cosson, 2002). However, most of them were successfully used in higher animals and with very high level of metal contamination (Lin and McCormick, 1986; McCormick and Lin, 1991). A number of studies have been reported in using this method successfully but the amount of samples has to be quite high (Lin and McCormick, 1986; McCormick and Lin, 1991). Therefore, it can be concluded that the increasing level of metallothionein in mussels exposed to very low level of dissolved mercury can not be detected by gel electrophoresis and silver staining, indicating the low sensitivity of this method for the detection of metallothionein in mussels, *P.viridis*.

By detecting mussel metallothionein using antibody specific to rabbit metallothionein, the results revealed that faint positive bands were detected while the metallothioneins from rabbit, fish and shrimp were clearly detected by this antibody. Positive bands from mussels appeared clearer by increasing the amount of samples by 10 fold. This can be explained by the low level of metallothionein in mussels beyond the minimum sensitivity of the method. Also there was less cross reactivity between mussel metallothionein and the antibody. Either ways, it can be concluded that the metallothionein level in mussels is not detectable by Western blot analysis using antibody specific to metallothionein from other species, in this case, from mouse. The quantification of metallothioneins by immunochemical methods (Western blot and

ELISA, enzyme-linked immunosorbent assay) are much more sensitive than gel electrophoresis and silver staining, but their use cannot easily be extended because almost all of antibodies are produced against the MTs of mammals which have good reactivity against metallothioneins from other species of mammals, but they react little with metallothioneins of non-mammal species (Roesijadi, Unger and Morris, 1988).

By molecular weight comparison, peptide bands from both gills and digestive tracts of mussels with the same molecular weight as rabbit metallothionein were detectable. However, the content of metallothionein bands was not consistent with the level of mercury. Since Western blot analysis failed to identify metallothionein bands, the bands, therefore, can not be confirmed as metallothioneins.

5.2 Purification of metallothionein

After partially purification by Sephadex G-75 gel filtration chromatography, four peaks at the molecular weight of 2000 kDa, 121.5 kDa, 4 kDa, and less than 4 kDa, were detected from both gill and digestive extracts. The elution profiles were correspondent with a number of reports using similar protocols (Frazier *et al.*, 1985; Schlenk and Brouwer, 1991; Ponzano *et al.*, 2000). Peptide bands given high intensity in silver staining was detected in Peak 2 and 3. Cross reaction of these bands and anti-metallothionein antibody confirmed that these bands were metallothioneins. The productions of metallothionein were approximately 1.75 % of total protein concentration from gill extracts and approximately 2.72 % of total protein concentration from digestive extracts (table 5.1). The result was in correspondent with the result of metallothionein purification in *Mytilus edulis* (Roesijadi, 1986; Geret and Cosson, 2002). At low level, the amount of metallothionein tends to be underestimated because its lack of aromatic amino acid causing the low absorbency at wavelength of 280 nm (Frazier *et al.*, 1985)

Table 5.1 Yield of metallothioneins partially purified from gill and digestive tracts of mercury treated mussels using Sephadex G-75 gel filtration chromatography.

Tissue extracts	Total protein (mg)	Acetonic defractionation (mg)	Sephadex G-75 (mg)	% yield
Gill	171	4	3	1.75
Digestive tract	220	9	6	2.72

Chromatographic techniques allow the separation of metallothioneins from other soluble compounds present in tissue homogenates. Given the absence of specific activity of metallothioneins, the criteria used to characterize isolated fractions depend on the physico-chemical properties of these proteins (Amiard and Cosson, 2000) or on the detection of metals associated with them. However, in this study, no significant difference of mercury level between fractions from Sephadex G-75 column was detected, indicating the very low level of mercury in each of metallothionein peak. In addition, mercury can be linked to proteins and causes the analytical and methodological problems (De Wuilloud *et al*., 2002). Therefore, high performance and costly equipments are required for precised measurement of metals at low concentration. Despite constant improvements, these advanced techniques remain

difficult to implement because they necessitate sophisticated analytic instruments and are relatively time-consuming, which is a serious handicap in analysis of a series of samples.

5.3 Determination of metallothionein gene expression in gills and digestive tracts of mercury treated mussels

Synthesis of metallothioneins is regulated at transcriptional level where their coding mRNAs are produced. Therefore, it is possible to measure an increase in the production of these mRNA in response to a metallic contamination of the environment. In this study, the transcriptional or expression level of metallothionein gene in mercury-treated mussel, *P.viridis*, was determined by semi-quantitative RT-PCR. Expression of β-actin, a constitutional gene for cellular structure, was used as reference. Single band of DNA (327bp) was amplified from cDNA while no detectable band was present in the amplification of metallothionein from genomic DNA. Therefore, it limits the interference of the co-amplification of metallothionein from genomic DNA during quantification reaction which causes overestimation of expression level. The study focused on the correlation between the metallothionein mRNA level and mercury concentration exposed to mussels from 2 different locations. For the experiment conducted on mussels from Samutprakarn province, it indicates that there is no significant difference on the expression level of metallothionein gene from mussels treated with mercury. However, there are significant correlation between the expression level of metallothionein gene and the increasing level of mercury concentration used in each treatment within several weeks of the experiment. For the experiment conducted on mussels from Trad province, significant differences on the expression level of metallothionein gene from mussels in respond to mercury treatment were detected from week 2 to week 4. There is also correlation between metallothionein expression level and mercury concentration. This indicates the expression of metallothionein genes from mussels can be induced in respond to mercury level. Similar results were reported in oyster *Crassostrea gigas* and mussel *Mytilus edulis* (Geret *et al.*, 2000).

The difference of metallothionein RNA was significant within short period of time (within 2 week) comparing to the time of exposure of 1 month detected in zebra mussels treated with sub chronic concentration of cadmium (0.2 ppb.) (Tessier and Blais, 1995).

It is interesting to note that at the treatment with high mercury concentration (from 1 to 5 μg/L), the expression of metallothionein tended to decrease, indicating the inhibition of protein expression because of mercury toxic. Similar result was reported in marine flatfish *Scophthalmus maximas* exposed to high dose of cadmium (George, Todd and Wright, 1996). Roseijadi *et al*. (1982) also found that high concentration level of mercuric chloride (5 ppb.) was not effective in inducing enhance mercury tolerance which related to inducing mercury binding protein.

By comparing the normal (control) level of metallothionein expression in mussels from 2 locations, the expression from Samutprakarn province which is in high level of industrial contamination area appeared to be higher than that from Trad which is in low contamination (figure 5.1). The average levels of mercury in both area was shown in table 5.3 This can be preliminarily indicated that the level of

metallothionein in mussels from natural environment is also correlate to the mercury contamination.

Table 5.2 The expression ratio and level of metallothionein gene and β-actin genes in digestive tracts of pre exposure mussels from Samutprakarn and Trad province.

Gene	**The expression level from Samutprakarn province**	**The expression level from Trad province**
MT	239.97	204.39
B-actin	245.09	229.71
MT/B-actin	1.09±0.12	0.89±0.11

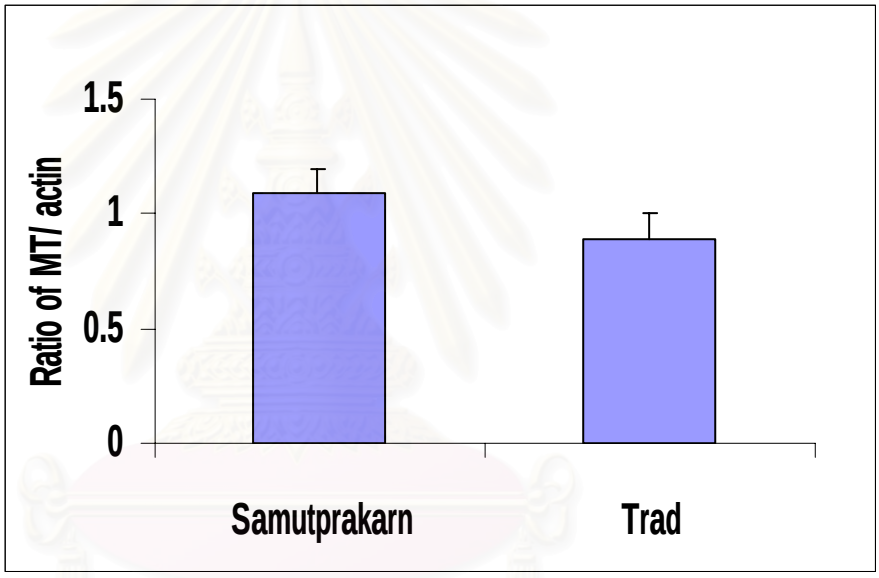

Figure 5.1 Comparison of the expression level of metallothionein gene from digestive tract of mussel from 2 locations, Samutprakarn province and Trad province (control)

Table 5.3 Mercury concentrations in seawater from Samutprakarn and Trad province in Year 1999 (POD, 1999; United Analyst Engineering Consultant LTD., 1999)

Location	**Mercury concentration (ppb.)**
Samutprakarn	0.04-2.2
Trad	0.05-0.09

The minimum amount of mercury treatment (<1μg/L) given significant induction of metallothionein expression is lower than the standard value for the

natural water regulation and even lower than the detection limit of the instruments which can detect mercury at the concentration of higher than 5 μg/L. This indicated that mRNA metallothionein level can be served as biomarker to monitoring mercury contamination at a very low concentration.

5.4 Genetic variation of metallothionein genes

Classification systems of metallothioneins are based on sequence similarities and phylogenetic relationships. This system subdivides the MT superfamily into families, subfamilies, subgroups and isolated isoforms (Binze and Käji, 1999). So far, all metallothioneins are classified into 15 families. Details and characteristics of metallothioneins from each families are shown in table 5.4

Table 5.4 Description of Metallothionein 15 families in organisms.

Family	Pattern	Number of amino acid	Number of cysteine
Family 1:mammal MTs	K-x(1,2)-C-C-x-C-CP-x (2)-C	60-68	20
Family 2: mollusc MTs	C-x-C-x(3)-C-T-G-x(3)-C-x-C-K	64-75	18-23
Family3:crustacean MTs	P-(GD)-P-C-C-x(3,4)-C-x-C	58-60	18
Family4:echinodermata MTs	P-D-x-K-C-(V,F)-C-C-x5-C-x-C-x-C-C-x4-C-C-x(4,6)-C-C	64-67	20
Family5:diptera MTs	C-G-x(2)-C-x-C-x(2)-Q-x (5)-C-x-C- x(2)-D-C-x-C	40-43	10
Family6:nematoda MTs	K-C-C-x (3)-C-C	62 and 74	18
Family7:ciliata MTs	No sequence pattern	105	31
Family8:fungi-I MTs	C-G-C-S-x (4)-C-x-C-x (3,4)-C-x-C-S-x-C	25	7
Family9:fungi-II MTs	-	-	-
Family10:fungi-III MTs	-	-	-
Family11:fungi-IV MTs	C-x-K-C-x-C-x (2)-C-K-C	55-56	9
Family12:fungi–V MTs	-	-	-
Family13:fungi-VI MTs	-	-	-
Family14:procaryota MTs	K-C-A-C-x (2)-C-L-C	53-56	9
Family15:planta MTs	[YFH]-x(5,25)-C-[GA]-[SDPAT]-x (0,1)-C-x-[CYF]	45-84	2 Cys rich region

In this study, PCR products were amplified from first strand cDNA obtained from gills and digestive tracts of mercury treated mussels using MT1 and MT2 primers. These primers were designed from start and stop codons of metallothionein gene in mussels, *P.viridis*. Twelve variant forms of metallothionein genes from PCR-

SSCP were detected. These forms are designated as pvMT1, pvMT2, pvMT3, pvMT4, pvMT5, pvMT6, and pvMT8 from digestive tract and pvMT9, pvMT10, pvMT11, and pvMT12 from gills, respectively. A comparison between amino acid sequences of mussel MTs with those of other species was made on the basis of optimized FASTA scores (Lipman *et al*., 1989). Among MTs from 15 families, very high cysteine conserve was observed from all 12 MT sequences of mercury treated mussels when compared to MT sequences from mollusk MT family, indicating these variant sequences of mussels MT belong to mollusk MT family. The members of mollusk MT family are recognised by the sequence pattern C-x-C-x (3)-C-T-G-x (3)-C-x-C-x (3)-C-x-C-K located at the C-terminal of the sequence. They are single chains of 64-75 amino acid residues, 18-23 cysteine residues, and at least 13 totally conserved. MTs from this family are divided into 4 subforms; mo1, mo2, mog (gastropod MTs), and mo (other mollusk MTs). According to the classification as mentioned above, it is indicated that all variant MT forms from mussels, *P.viridis*, belong to subform of mo. From the result of sequence alignment and phylogenetic analysis, these 12 MT forms can be categorized into 2 main subgroups designated as mop1 and mop2. mop1 consists of 3 isoforms; mop1a, mop1b, and mop1c, respectively. MT sequence of *P. viridis* (Khoo and Patel, 1999) retrieved from GenBank also belongs to the same subgroup of mop1. mop2 consists of 3 isoforms; mop2a, mop2b, and mop2c, respectively. The members of isoform mop1a are pvMT1 and pvMT10. The members of isoform mop1b are pvMT11 and pvMT12. The member of isoform mop1c is pvMT2. The member of isoform mop2a is pvMT7. The member of isoform mop2b is pvMT8. The members of isoform mop2c are pvMT3, pvMT4, pvMT5, pvMT6, and pvMT9. Detail of phylogenetic tree is shown in figure5.2

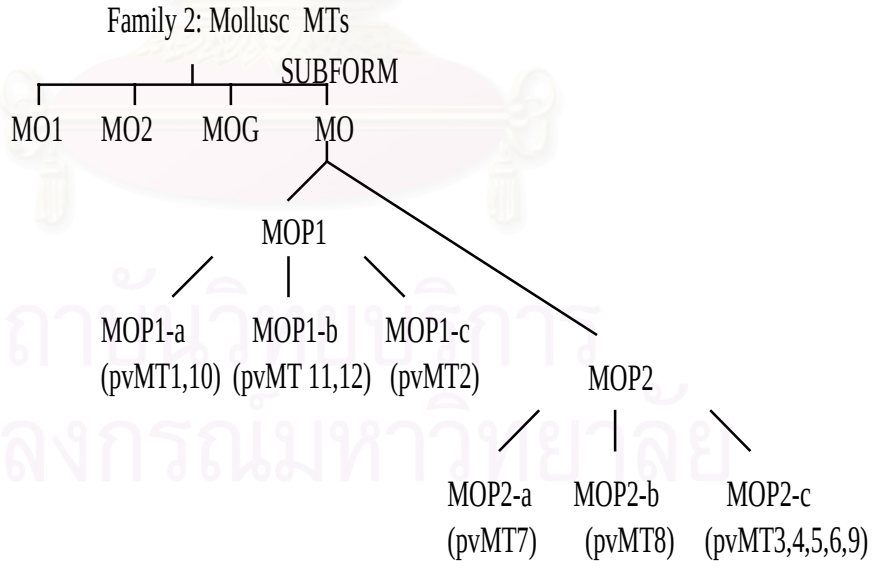

Figure 5.2 Diagram of classification of variant metallothionein gene from gill and digestive tract.

From the results, it can be concluded that 6 novel isoforms of MT from mercury treated mussels, *P.viridis*, have been identified in this study using RT-PCR and PCR-SSCP methods. It should be noted here that all 6 isoforms detected in this experiment was carried out entirely on DNA sequences amplified by primers at start and stop codons and transcribed to protein sequences. Therefore, the difference at or outside start and stop codons can not be detected. It is possible that the exact number of isoforms in mussels might be higher. It is possible that these isoforms arise due to polymorphism within the mussel population as a few numbers of clams were used to prepare the MTs. In most species, MT occurs in several isoforms, which may differ from one another by only a few amino acid positions. All mammals tissues examined thus far usually contain two major MT isoforms, designated as MT-I and MT-II (Kagi and Kojima, 1987a). So far, only a few sequences of molluscan MTs have been characterized. These include MTs in the mussels, *Mytilus edulis* (Mackay *et al*., 1993), *Perna viridis* (Khoo and Patel, 1999) and the oyster *Crassostrea virginica* (Roesijadi *et al*., 1989; Unger *et al*., 1991). To date, the highest number of isoforms found in aquatic species was in mussel, *M. edulis* MT (at least nine Cd-induced isoforms) (Mackay *et al*., 1993) (table 5.5). The reason for this large number of isoforms in marine invertebrates is not clear. Functional differences between MT isoforms have been hypothesized since the discovery of this extensive polymorphism. Suggestions included different specificity of the promoter region of the gene (Schmidt et al., 1985), different metal-binding affinities (Winge and Miklossy, 1982) or simply the need for a large number of copies to facilitate a rapid response when necessary (Olafson *et al*., 1979). Functional specificity between isomers has been proposed, mainly owing to their different modes of expression, or expression in different tissues. Likewise, it has been proposed that different metals are able to induce specific MT isomers. Further investigation of the number and inducibility of the isoforms as well as metal binding characteristics will be necessary for the use of mussel MTs as biomarkers.

Table 5.5 Investigation of metallothionein isoform in mollusc.

Organism	Exposure conditions	Organs/tissue	Isoforms	Methods	Reference
Mytilus edulis	Cd 0.1 mg/l 3 months	Digestive gland	3 (MT-20)	DEAE-cellulose	George *et al*.,(1979)
Mytilus edulis	Cd 0.1 mg/l 3 months	Mantle	3 (MT-20)	DEAE-cellulose	Carpenè *et al*., (1980)
Mytilus edulis	Cd 0.5 mg/l 14 days	Soft part	1 (MT-10)	Acetone precipitation DEAE-cellulose	Frankenne *et al*., (1980)
Mytilus edulis	Cd 0.1 mg/l 3-4 months	Soft part	4 (MT-20) 4 (MT-10)	DEAE-cellulose	Frazier *et al.,* (1985)
Mytilus edulis	Hg 0.005 mg/l 28 days	Gills	3 (MT-20) 6 (MT-20)	DEAE-cellulose HPLC	Roesijadi (1986)
Mytilus edulis	Cd 0.2 mg/l 3-4 months	Soft parts	5 (MT-20) 4 (MT-10)	DEAE-cellulose	Mackay *et al*., (1993)
Mytilus gallopprovincialis	Cd 0.1 mg/l 3 months	Muscle	3 (MT-20)	DEAE-cellulose	Carpenè *et al*., (1983)
Mytilus gallopprovincialis	Cu 0.04 mg/l	Soft part	1 (MT-10)	Acetone precipitation DEAE-cellulose	Viarengo *et al*., (1984)
Mytilus gallopprovincialis	Cd 0.2 mg/l 20 days	Digestive gland, mantle	2 (MT-10-20) 1 (MT-10-20)	DEAE-Sephadex A-25	Pavičić *et al*., (1991)
Crassostrea viriginica	Cd 0.2 mg/l 21 days	Soft part	2 (MT-10)	Acetone precipitation HPLC	Roesijadi *et al*., (1989)
Scapharca inaequivalvis	Cd 0.5 mg/l 28 days	Digestive gland	2 (MT-10)	DEAE-cellulose	Serra *et al.,* (1995)
Dreissena polymorpha	Cd 0.2 mg/l 14 days	Soft part	2 (MT-10)	Acetone precipitation DEAE-cellulose	High *et al*., (1997)

By detecting MT isoforms pattern in SSCP gel from digestive tracts and gills of mercury treated mussels, no significant correlation between isoforms and different tissues and different mercury treatment was observed. All 6 isoforms are detectable from both gills and digestive tracts, indicating no tissue specific isoform in mussels, *P.viridis*. Similar result has been reported in blue mussels, *M. edulis* (Geret and Cosson, 2002). However, when focused on the MT isoforms amplified from gill, 2 main bands of pvMT5 and pvMT6 which are the members of mop2c isoform were detected from mussels treated with mercury while only faint bands of those were detected from control mussels. Similar result was detected in that of digestive tracts. It is therefore possible that some forms of MT in mussels could respond specifically to the level of mercury contamination. However, it should be reminded that SSCP analysis is not designed specifically for quantification analysis. Therefore, further investigation on mercury specific isoforms of MT from mussels is needed.

CHAPTER VI

CONCLUSIONS

This study focused on the development of the techniques used for the detection of metallothionein. The correlation between metallothionein in animals and mercury level were investigated. The results can be applied for the assessment of mercury contamination in marine environment. Mussel, *P.viridis*, was chosen as bioindicating species because of its ability to accumulate most heavy metals, including mercury. Both proteomic and genomic techniques were applied. Three conventional methods for metallothionein determination were adopted. These included gel electrophoresis, immunochemical analysis using antibody against metallothionein, and chromatographic technique used for identifying mercury specific forms of metallothionein.

The studies indicated that gel electrophoresis and silver staining was not an appropriate method for the determination of metallothionein in mussels treated with low level of mercury. However, this technique could be performed on samples with very high protein concentration. Therefore, it indicates the low sensitivity of this method for the detection of metallothionein in mussels, *P.viridis*.

The quantification of metallothioneins by immunochemical methods (Western blot analysis) using monoclonal antibody specific to rabbit metallothionein was slightly more sensitive than gel electrophoresis and silver staining, but their use was unable to be easily extended because it requires high amount of samples and antibody.

Metallothioneins from gills and digestive tracts of mercury-treated mussels can be successfully purified using Sephadex G-75 gel filtration chromatography. However, only small amount of metallothionein (1.7% yield) is obtained. Mercury specific form of metallothionein was not detectable by this method.

For the genomic techniques, semi-quantitative analysis was conducted for the detection of RNA encoding metallothionein.

The correlation between the metallothionein mRNA level and mercury concentration exposed to mussels from 2 different locations; Samutprakarn province and Trad province. No significant difference on the expression level of metallothionein gene was detected in mercury-treated mussels from Samutprakarn while significant differences on the expression level of metallothionein gene were detected in mercury-treated mussels from Trad. The difference of metallothionein RNA was detected within short period of time (within 2 week) comparing to the time of exposure detected in zebra mussel. In mussels treated with high concentration of mercury (from 1 to 5 μg/L), the expression of metallothionein tended to decrease, indicating the inhibition of protein expression because of mercury toxic.

The expression of metallothionein from mussels induced by low level of mercury (<1μg/L) can be detected using semi-quantitative RT-PCR. This indicated

that mRNA metallothionein level could be served as biomarker for monitoring mercury contamination.

Furthermore, the variant forms of metallothioneins were also detected. PCR products were amplified from first strand cDNA obtained from gills and digestive tracts of mercury treated mussels using MT1 and MT2 primers. These primers were designed from start and stop codons of metallothionein gene in mussels, *P.viridis*.

Variant forms of metallothionein genes from PCR-SSCP were detected at the size of 220 bp. These included 9 forms from gills (pvMT1, pvMT 2, pvMT 3, pvMT 4, pvMT 5, pvMT6, pvMT7, pvMT8 and pvMT 9) and 3 forms from digestive tracts (pvMT10, pvMT11 and pvMT 12).

These 12 variant metallothioneins from mussels, *Perna viridis*, were divided into two main subforms, defined as mop1 and mop2 that contained 6 isoforms, similar to mop1 and mop2 of mollusk metallothioneins. Thus, SSCP technique can be employed for identifying multiple forms of metallothioneins in mussels *P. viridis.*

Finally, further investigation on a particular form of metallothionein that specific to mercury can be carried out and more sensitive technique for assessment of mercury contamination in marine environment can be developed.

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APPENDICES

Appendix A

Reagents

1. Western blot and Native -PAGE

1.1 10% (w/v) Ammonium persulfate

Ammonium persulfate (sigma)	1.0	g

Dissolve in 10 ml of dH_2O.

1.2 Resolving gel buffer: 1.5 M Tris-HCl pH 8.8

Tris (Sigma)	181.7	g

Dissolve in 700 ml of dH_2O, adjust with 5 M HCl to pH 8.8 and adjust to 1000 ml final volume with dH_2O.

1.3 Stacking gel buffer: 0.5 M Tris-HCl pH 6.8

Tris (Sigma)	6.0	g

Dissolve in 40 ml of dH_2O, adjust with 1 M HCl to pH 6.8 and adjust to 100 ml final volume with dH_2O.

1.4 3 30.0% (w/v) Acrylamide-bisacrylamide

Acrylamide (Sigma)	28.5	g
Bis-acrylamide (Sigma)	1.5	g

1.5 10% (w/v) Sodium dodecylsulphate

Sodium dodecylsulphate (Sigma)	10.0	g

Dissolve in 100 ml of dH_2O.

1.6 TEMED (N, N, N', N'-tetramethyl ethlenediamine)

This reagent is commercial available.

1.7 7.5% Resolving gel

Acrylamide-bisacrylamide (28.5: 1.5)	1.25	ml
dH_2O	2.45	ml
Resolving gel buffer	1.25	ml
10% Ammonium persulphate	25	µl
TEMED	2.5	µl

1.8 30.0% Resolving gel

Acrylamide-bisacrylamide (28.5: 1.5)	3.70	ml
Resolving gel buffer	1.25	ml
10% Ammonium persulphate	25	µl
TEMED	2.5	µl

1.9 3.9% Stacking gel

Acrylamide-bisacrylamide (28.5: 1.5)	0.650	ml
dH_2O	3.050	ml
Stacking gel buffer	1.25	ml
10% Ammonium persulphate	50	µl
TEMED	5	µl

1.10 Gradiant gel preparation

Prepare 7.5 % acrylamide and 30 % acrylamide add in each chamber. Then, gradiant gel was performed by mixed these concentrations into mixing chamber and transferred to slab gel gels. Allow to polymerize.

1.11 10X Running buffer: 0.25 M Tris-HCl, 1.92 M glycine, 1% (w/v) SDS, pH 8.3

Tris	30.3	g
Glycine	144.0	g
SDS	10.0	g

Dissolve and adjust to 1000 ml with dH_2O.

1.12 4X Sample buffer: 0.0625 M Tris-HCl pH 6.8, 8% (w/v SDS, 40% (v/v) glycerol and 0.005% Bromophenol blue

SDS	0.8	g
Glycerol	4.0	ml
Stacking gel buffer	5.0	ml
Bromophenol blue	0.5	mg

Dissolve and adjust the volume to 10 ml with dH_2O. One ml of 2-mercaptoethanol (2-ME) was added to 9 ml of 4X sample buffer for reducing condition.

1.13 Staining solution

Coomassie brilliant blue	0.25	g
Methanol	45.0	ml
dH_2O	45.0	ml
Glacial acetic acid	10.0	ml

1.14 Destaining solution

Glacial acetic acid	100	ml
Methanol	300	ml
dH_2O	600	ml

1.15 Transfer buffer: 20 mM Tris-HCl pH 8.3, 150 mM Glycine, 20% (v/v) methanol

Tris (Sigma)	1.211	g
Glycine	5.63	g

Dissolve and adjust to 400 ml with dH_2O. Followed by addition of 100 ml of methanol

1.16 Blocking buffer (1% BSA)

BSA	1	g

Dissolve and adjust to 100 ml with PBS

1.17 Phosphate Buffer Saline (PBS)

NaCl	8	g
KCl	0.2	g
Na_2HPO_4	1.44	g
KH_2PO_4	0.24	g

Dissolve in 800 ml of dH_2O, adjust pH to 6.8 and adjust to 1000 ml final volume with dH_2O.

1.18 Substrate

2. RT-PCR

2.1 0.1% DEPC- dH_2O

Diethyl pyrocarbonate 97%	1.0	ml

Added water to 1000 ml and incubated overnight at 37°C then autoclave

2.2 10X PCR buffer: 500 mM KCl, 100 mM Tris-HCl pH 8.3

2.3 2 M Sodium acetate pH 4.0

Sodium acetate	27.216	g
dH_2O	90	g.

Adjust the pH to 4.0 with glacial acetic acid and adjust the volume to 100 ml with dH_2O.

2.4 25 mM $MgCl_2$

3. Cloning

3.1 Luria-Bertani medium (LB)

Tryptone	10.0	g
NaCl	10.0	g
Yeast extract	5.0	g

Dissolve and adjust the volume to 1,000 ml with dH_2O. And then autoclave.

3.2 TE pH 8.0

1 M Tris-HCl pH 8.0	5.0	ml
0.5 M EDTA pH 8.0	1.0	ml

Adjust the volume to 100 ml with dH_2O.

3.3 Solution I (GTE buffer): 50 mM glucose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0

Glucose	0.9	g
1 M Tris-HCl pH 8.0	2.5	ml
0.5 M EDTA pH 8.0	2.0	ml

Dissolve and adjust the volume to 100 ml.

3.4 Solution II : 0.2 M NaOH, 1% SDS

5 M NaOH	4.0	ml
10% SDS	10.0	ml

Adjust the volume to 100 ml with dH_2O.

3.5 Solution III: 3 M Potassium Acetate pH 4.8

Potassium acetate (CH3COOK)	29.4	g
Glacial acetic acid	40.0	ml

Adjust the pH to 4.8 with glacial acetic acid.

4. SSCP (Single strand conformation polymorphism)

4.1 15% polyacrylamide (acrylamide: bis-acrylamide 37.5:1)

Acrylamide	14.61	g
Bis-acrylamide	0.39	g

Dissolve and adjust the volume to 100 ml with 1X TBE

4.2 Bind silane

95% Ethanol	995	µl
Bind silane	4	µl
Acetic acid	5	µl

4.2 Running buffer (1X TBE)

10X TBE	100	ml

Dissolve and adjust to 1000 ml with dH_2O.

4.3 Fix-stop solution (10% glacial acetic acid)

Glacial acetic acid	100	ml

Dissolve and adjust to 1000 ml with dH_2O.

4.4 Silver nitrate solution (0.1% silver nitrate, 37% formaldehide)

Silver nitrate	1.5	g
Dissolve and adjust to 1.5 L with dH_2O.		
Add 100% formalin before use	2.25	ml

4.5 Developing solution

Na_2CO_3	30	g

Dissolve and adjust to 1000 ml with dH_2O

5. Gel filtration chromatography

5.1 Eluting buffer (20 mM Tris-HCl pH 8.6, 10 mM NaCl)

1 M Tris-HCl pH 8.6	20	ml
1 M NaCl	10	ml

Dissolve and adjust to 1000 ml with dH_2O

Appendix B

pvMT1

Sequences producing significant alignments:	Score (bits)	E Value
gi|18203491|sp|Q9U568|MT_PERVI Metallothionein (MT) >gi|665...	101	5e-21
gi|6652818|gb|AAF22487.1| metallothionein 2 [Perna viridis]	100	7e-21
gi|4104513|gb|AAD02054.1| metallothionein [Perna viridis]	96	2e-19
gi|40994800|emb|CAE11861.1| metallothionein [Mytilus edulis]	83	2e-15
gi|19924225|sp|P80246|MT1A_MYTED Metallothionein 10-Ia (MT-...	82	3e-15
gi|18202100|sp|O62554|MT1B_MYTED Metallothionein 10-Ib (MT-...	82	3e-15
gi|19861705|sp|P80247|MT12_MYTED Metallothionein 10-II (MT-...	81	6e-15
gi|542507|pir||S39416 metallothionein 10-I - blue mussel >g...	80	1e-14
gi|542508|pir||S39417 metallothionein 10-II - blue mussel	80	1e-14
gi|18272157|sp|P80248|MT13_MYTED Metallothionein 10-III (MT...	80	1e-14
gi|18272159|sp|P80249|MT14_MYTED Metallothionein 10-IV (MT-...	79	4e-14
gi|542509|pir||S39418 metallothionein 10-III - blue mussel	78	5e-14
gi|542510|pir||S39419 metallothionein 10-IV - blue mussel >...	77	1e-13
gi|40994788|emb|CAE11855.1| metallothionein [Mytilus edulis]	75	3e-13
gi|18272161|sp|P80252|MT22_MYTED Metallothionein 20-II (MT-...	74	1e-12
gi|40994790|emb|CAE11856.1| metallothionein [Mytilus edulis]	72	3e-12
gi|40994798|emb|CAE11860.1| metallothionein [Bathymodiolus ...	72	3e-12
gi|40994796|emb|CAE11859.1| metallothionein [Bathymodiolus ...	72	3e-12
gi|38348592|emb|CAD56896.1| metallothionein 10 [Bathymodiol...	72	3e-12
gi|19861707|sp|P80253|MT2A_MYTED Metallothionein 20-III iso...	72	3e-12
gi|630806|pir||S39421 metallothionein 20-II - blue mussel	72	5e-12
gi|630807|pir||S39422 metallothionein 20-IIIa - blue mussel	70	1e-11
gi|18202371|sp|P80258|MT2B_MYTED Metallothionein 20-III iso...	70	1e-11
gi|1072470|pir||S47576 metallothionein 20-Ib - blue mussel	70	2e-11
gi|462638|sp|P80251|MT21_MYTED Metallothionein 20-I isoform...	70	2e-11
gi|40994802|emb|CAE11862.1| metallothionein [Mytilus edulis]	67	9e-11
gi|40994792|emb|CAE11857.1| metallothionein [Mytilus edulis]	63	2e-09
gi|40994794|emb|CAE11858.1| metallothionein [Mytilus edulis]	62	5e-09
gi|13786086|gb|AAK39563.1| metallothionein-like protein [An...	55	3e-07
gi|33637528|gb|AAQ23908.1| metallothionein IB [Crassostrea ...	54	8e-07
gi|13194854|gb|AAK15581.1| metallothionein [Crassostrea ang...	54	8e-07
gi|266587|sp|P23038|MT_CRAVI Metallothionein (MT) >gi|10269...	53	2e-06
gi|7406571|emb|CAB85588.1| metallothionein [Crassostrea gig...	53	2e-06
gi|8920353|emb|CAB96402.1| metallothionein [Venerupis (Rudi...	52	4e-06
gi|6687228|emb|CAB64869.1| metallothionein [Crassostrea gigas]	51	6e-06
gi|8920351|emb|CAB96403.1| metallothionein [Venerupis (Rudi...	51	8e-06
gi|22038045|gb|AAM90257.1| metallothionein [Crassostrea vir...	48	5e-05
gi|18073426|emb|CAC82788.1| metallothionein [Crassostrea gi...	47	9e-05
gi|33637544|gb|AAQ23916.1| metallothionein IIH [Crassostrea...	45	6e-04
gi|33637534|gb|AAQ23911.1| metallothionein IIC [Crassostrea...	45	6e-04
gi|33637538|gb|AAQ23913.1| metallothionein IIE [Crassostrea...	45	6e-04
gi|2497868|sp|Q94550|MT_DREPO Metallothionein (MT) >gi|1519...	44	8e-04
gi|18076578|emb|CAC83770.1| metallothionein [Ostrea edulis]	44	8e-04
gi|33637530|gb|AAQ23909.1| metallothionein IIA [Crassostrea...	44	0.001
gi|33637542|gb|AAQ23915.1| metallothionein IIG [Crassostrea...	44	0.001
gi|33637536|gb|AAQ23912.1| metallothionein IID [Crassostrea...	44	0.001
gi|14017405|gb|AAK50565.1| metallothionein [Crassostrea rhi...	44	0.001
gi|33637540|gb|AAQ23914.1| metallothionein IIF [Crassostrea...	44	0.001
gi|8920419|emb|CAB96419.1| metallothionein [Venerupis pulla...	41	0.007
gi|33637532|gb|AAQ23910.1| metallothionein IIB [Crassostrea...	40	0.019
gi|14268534|gb|AAK56498.1| putative metallothionein [Littor...	38	0.073
gi|15148842|gb|AAK84863.1| Cd-metallothionein isoform [Heli...	36	0.28
gi|34536201|dbj|BAC87575.1| unnamed protein product [Homo s...	36	0.28
gi|417326|sp|P33187|MTCD_HELPO Cadmium-metallothionein (CD-...	36	0.28

pvMT2

Sequences producing significant alignments:	Score (bits)	E Value
gi|18203491|sp|Q9U568|MT_PERVI Metallothionein (MT) >gi|665...	100	1e-20
gi|6652818|gb|AAF22487.1| metallothionein 2 [Perna viridis]	100	2e-20
gi|4104513|gb|AAD02054.1| metallothionein [Perna viridis]	95	4e-19
gi|40994800|emb|CAE11861.1| metallothionein [Mytilus edulis]	80	1e-14
gi|19924225|sp|P80246|MT1A_MYTED Metallothionein 10-Ia (MT-...	80	2e-14
gi|18202100|sp|O62554|MT1B_MYTED Metallothionein 10-Ib (MT-...	80	2e-14
gi|19861705|sp|P80247|MT12_MYTED Metallothionein 10-II (MT-...	79	4e-14
gi|542507|pir||S39416 metallothionein 10-I - blue mussel >g...	78	6e-14
gi|542508|pir||S39417 metallothionein 10-II - blue mussel	78	6e-14
gi|18272157|sp|P80248|MT13_MYTED Metallothionein 10-III (MT...	77	8e-14
gi|18272159|sp|P80249|MT14_MYTED Metallothionein 10-IV (MT-...	76	2e-13
gi|542509|pir||S39418 metallothionein 10-III - blue mussel	75	3e-13
gi|542510|pir||S39419 metallothionein 10-IV - blue mussel >...	74	9e-13
gi|18272161|sp|P80252|MT22_MYTED Metallothionein 20-II (MT-...	74	1e-12
gi|40994788|emb|CAE11855.1| metallothionein [Mytilus edulis]	73	2e-12
gi|19861707|sp|P80253|MT2A_MYTED Metallothionein 20-III iso...	72	3e-12
gi|630806|pir||S39421 metallothionein 20-II - blue mussel	72	5e-12
gi|630807|pir||S39422 metallothionein 20-IIIa - blue mussel	70	1e-11
gi|18202371|sp|P80258|MT2B_MYTED Metallothionein 20-III iso...	70	1e-11
gi|40994790|emb|CAE11856.1| metallothionein [Mytilus edulis]	70	2e-11
gi|40994798|emb|CAE11860.1| metallothionein [Bathymodiolus ...	70	2e-11
gi|40994796|emb|CAE11859.1| metallothionein [Bathymodiolus ...	70	2e-11
gi|38348592|emb|CAD56896.1| metallothionein 10 [Bathymodiol...	70	2e-11
gi|1072470|pir||S47576 metallothionein 20-Ib - blue mussel	70	2e-11
gi|462638|sp|P80251|MT21_MYTED Metallothionein 20-I isoform...	70	2e-11
gi|40994802|emb|CAE11862.1| metallothionein [Mytilus edulis]	67	9e-11
gi|40994792|emb|CAE11857.1| metallothionein [Mytilus edulis]	60	1e-08
gi|40994794|emb|CAE11858.1| metallothionein [Mytilus edulis]	59	3e-08
gi|13786086|gb|AAK39563.1| metallothionein-like protein [An...	55	3e-07
gi|33637528|gb|AAQ23908.1| metallothionein IB [Crassostrea ...	52	5e-06
gi|13194854|gb|AAK15581.1| metallothionein [Crassostrea ang...	52	5e-06
gi|266587|sp|P23038|MT_CRAVI Metallothionein (MT) >gi|10269...	50	1e-05
gi|7406571|emb|CAB85588.1| metallothionein [Crassostrea gig...	50	1e-05
gi|8920353|emb|CAB96402.1| metallothionein [Venerupis (Rudi...	49	2e-05
gi|6687228|emb|CAB64869.1| metallothionein [Crassostrea gigas]	49	4e-05
gi|8920351|emb|CAB96403.1| metallothionein [Venerupis (Rudi...	48	5e-05
gi|22038045|gb|AAM90257.1| metallothionein [Crassostrea vir...	45	3e-04
gi|18073426|emb|CAC82788.1| metallothionein [Crassostrea gi...	45	3e-04
gi|2497868|sp|Q94550|MT_DREPO Metallothionein (MT) >gi|1519...	45	6e-04
gi|33637544|gb|AAQ23916.1| metallothionein IIH [Crassostrea...	42	0.004
gi|33637534|gb|AAQ23911.1| metallothionein IIC [Crassostrea...	42	0.004
gi|33637542|gb|AAQ23915.1| metallothionein IIG [Crassostrea...	42	0.004
gi|33637536|gb|AAQ23912.1| metallothionein IID [Crassostrea...	42	0.004
gi|33637538|gb|AAQ23913.1| metallothionein IIE [Crassostrea...	42	0.004
gi|33637540|gb|AAQ23914.1| metallothionein IIF [Crassostrea...	42	0.004
gi|18076578|emb|CAC83770.1| metallothionein [Ostrea edulis]	42	0.005
gi|33637530|gb|AAQ23909.1| metallothionein IIA [Crassostrea...	41	0.007
gi|14017405|gb|AAK50565.1| metallothionein [Crassostrea rhi...	41	0.007

pvMT3

Sequences producing significant alignments:	Score (bits)	E Value
gi|18203491|sp|Q9U568|MT_PERVI Metallothionein (MT) >gi|665...	47	9e-05
gi|6652818|gb|AAF22487.1| metallothionein 2 [Perna viridis]	47	1e-04
gi|18272161|sp|P80252|MT22_MYTED Metallothionein 20-II (MT-...	45	6e-04
gi|40994800|emb|CAE11861.1| metallothionein [Mytilus edulis]	44	0.001
gi|19861707|sp|P80253|MT2A_MYTED Metallothionein 20-III iso...	44	0.001
gi|630806|pir||S39421 metallothionein 20-II - blue mussel	43	0.002
gi|4104513|gb|AAD02054.1| metallothionein [Perna viridis]	42	0.003
gi|19861705|sp|P80247|MT12_MYTED Metallothionein 10-II (MT-...	42	0.004
gi|630807|pir||S39422 metallothionein 20-IIIa - blue mussel	42	0.005
gi|18202371|sp|P80258|MT2B_MYTED Metallothionein 20-III iso...	42	0.005
gi|19924225|sp|P80246|MT1A_MYTED Metallothionein 10-Ia (MT-...	41	0.007
gi|18202100|sp|O62554|MT1B_MYTED Metallothionein 10-Ib (MT-...	41	0.007
gi|1072470|pir||S47576 metallothionein 20-Ib - blue mussel	41	0.009
gi|462638|sp|P80251|MT21_MYTED Metallothionein 20-I isoform...	41	0.009
gi|542508|pir||S39417 metallothionein 10-II - blue mussel	40	0.015
gi|40994802|emb|CAE11862.1| metallothionein [Mytilus edulis]	40	0.019
gi|542507|pir||S39416 metallothionein 10-I - blue mussel >g...	39	0.025
gi|18272159|sp|P80249|MT14_MYTED Metallothionein 10-IV (MT-...	38	0.056
gi|42908149|gb|EAB21172.1| unknown [environmental sequence]	38	0.073
gi|40994788|emb|CAE11855.1| metallothionein [Mytilus edulis]	38	0.073
gi|18272157|sp|P80248|MT13_MYTED Metallothionein 10-III (MT...	37	0.12
gi|18076578|emb|CAC83770.1| metallothionein [Ostrea edulis]	37	0.16
gi|7406571|emb|CAB85588.1| metallothionein [Crassostrea gig...	37	0.16
gi|542510|pir||S39419 metallothionein 10-IV - blue mussel >...	36	0.21
gi|42957281|gb|EAB45609.1| unknown [environmental sequence]	36	0.28
gi|13194854|gb|AAK15581.1| metallothionein [Crassostrea ang...	36	0.28
gi|542509|pir||S39418 metallothionein 10-III - blue mussel	35	0.47
gi|43615149|gb|EAE62292.1| unknown [environmental sequence]	35	0.47
gi|8920351|emb|CAB96403.1| metallothionein [Venerupis (Rudi...	35	0.47
gi|6687228|emb|CAB64869.1| metallothionein [Crassostrea gigas]	35	0.62
gi|34530691|dbj|BAC85954.1| unnamed protein product [Homo s...	35	0.62
gi|44165360|gb|EAH47936.1| unknown [environmental sequence]	35	0.62
gi|40994790|emb|CAE11856.1| metallothionein [Mytilus edulis]	35	0.62
gi|3135045|emb|CAA06552.1| metallothionein 10 IV [Mytilus e...	34	0.81
gi|8920353|emb|CAB96402.1| metallothionein [Venerupis (Rudi...	34	0.81
gi|43902289|gb|EAG05467.1| unknown [environmental sequence]	34	1.1
gi|33637528|gb|AAQ23908.1| metallothionein IB [Crassostrea ...	34	1.1
gi|34534808|dbj|BAC87117.1| unnamed protein product [Homo s...	34	1.1
gi|34534585|dbj|BAC87052.1| unnamed protein product [Homo s...	33	1.4
gi|38348592|emb|CAD56896.1| metallothionein 10 [Bathymodiol...	33	1.4
gi|13786086|gb|AAK39563.1| metallothionein-like protein [An...	33	1.4
gi|40994798|emb|CAE11860.1| metallothionein [Bathymodiolus ...	33	1.4
gi|40994796|emb|CAE11859.1| metallothionein [Bathymodiolus ...	33	1.4
gi|26351279|dbj|BAC39276.1| unnamed protein product [Mus mu...	33	1.4
gi|18073426|emb|CAC82788.1| metallothionein [Crassostrea gi...	33	1.4

pvMT4

Sequences producing significant alignments:	Score (bits)	E Value
gi|18203491|sp|Q9U568|MT_PERVI Metallothionein (MT) >gi|665...	47	9e-05
gi|6652818|gb|AAF22487.1| metallothionein 2 [Perna viridis]	47	1e-04
gi|18272161|sp|P80252|MT22_MYTED Metallothionein 20-II (MT-...	45	6e-04
gi|40994800|emb|CAE11861.1| metallothionein [Mytilus edulis]	44	0.001
gi|19861707|sp|P80253|MT2A_MYTED Metallothionein 20-III iso...	44	0.001
gi|630806|pir||S39421 metallothionein 20-II - blue mussel	43	0.002
gi|4104513|gb|AAD02054.1| metallothionein [Perna viridis]	42	0.003
gi|19861705|sp|P80247|MT12_MYTED Metallothionein 10-II (MT-...	42	0.004
gi|630807|pir||S39422 metallothionein 20-IIIa - blue mussel	42	0.005
gi|18202371|sp|P80258|MT2B_MYTED Metallothionein 20-III iso...	42	0.005
gi|19924225|sp|P80246|MT1A_MYTED Metallothionein 10-Ia (MT-...	41	0.007
gi|18202100|sp|O62554|MT1B_MYTED Metallothionein 10-Ib (MT-...	41	0.007
gi|1072470|pir||S47576 metallothionein 20-Ib - blue mussel	41	0.009
gi|462638|sp|P80251|MT21_MYTED Metallothionein 20-I isoform...	41	0.009
gi|542508|pir||S39417 metallothionein 10-II - blue mussel	40	0.015
gi|40994802|emb|CAE11862.1| metallothionein [Mytilus edulis]	40	0.019
gi|542507|pir||S39416 metallothionein 10-I - blue mussel >g...	39	0.025
gi|18272159|sp|P80249|MT14_MYTED Metallothionein 10-IV (MT-...	38	0.056
gi|40994788|emb|CAE11855.1| metallothionein [Mytilus edulis]	38	0.073
gi|18272157|sp|P80248|MT13_MYTED Metallothionein 10-III (MT...	37	0.12
gi|18076578|emb|CAC83770.1| metallothionein [Ostrea edulis]	37	0.16
gi|7406571|emb|CAB85588.1| metallothionein [Crassostrea gig...	37	0.16
gi|542510|pir||S39419 metallothionein 10-IV - blue mussel >...	36	0.21
gi|13194854|gb|AAK15581.1| metallothionein [Crassostrea ang...	36	0.28
gi|42908149|gb|EAB21172.1| unknown [environmental sequence]	35	0.36
gi|542509|pir||S39418 metallothionein 10-III - blue mussel	35	0.47
gi|8920351|emb|CAB96403.1| metallothionein [Venerupis (Rudi...	35	0.47
gi|6687228|emb|CAB64869.1| metallothionein [Crassostrea gigas]	35	0.62
gi|40994790|emb|CAE11856.1| metallothionein [Mytilus edulis]	35	0.62
gi|3135045|emb|CAA06552.1| metallothionein 10 IV [Mytilus e...	34	0.81
gi|8920353|emb|CAB96402.1| metallothionein [Venerupis (Rudi...	34	0.81
gi|43015761|gb|EAB74632.1| unknown [environmental sequence]	34	0.81
gi|33637528|gb|AAQ23908.1| metallothionein IB [Crassostrea ...	34	1.1
gi|44165360|gb|EAH47936.1| unknown [environmental sequence]	34	1.1
gi|38348592|emb|CAD56896.1| metallothionein 10 [Bathymodiol...	33	1.4
gi|13786086|gb|AAK39563.1| metallothionein-like protein [An...	33	1.4
gi|40994798|emb|CAE11860.1| metallothionein [Bathymodiolus ...	33	1.4
gi|40994796|emb|CAE11859.1| metallothionein [Bathymodiolus ...	33	1.4
gi|18073426|emb|CAC82788.1| metallothionein [Crassostrea gi...	33	1.4
gi|43902289|gb|EAG05467.1| unknown [environmental sequence]	33	1.8
gi|43615149|gb|EAE62292.1| unknown [environmental sequence]	33	1.8
gi|23478566|gb|EAA15617.1| hypothetical protein [Plasmodium...	33	1.8
gi|42957281|gb|EAB45609.1| unknown [environmental sequence]	33	2.3
gi|417326|sp|P33187|MTCD_HELPO Cadmium-metallothionein (CD-...	33	2.3

pvMT5

Sequences producing significant alignments:	Score (bits)	E Value
gi|40994800|emb|CAE11861.1| metallothionein [Mytilus edulis]	40	7e-06
gi|4104513|gb|AAD02054.1| metallothionein [Perna viridis]	36	1e-05
gi|6652818|gb|AAF22487.1| metallothionein 2 [Perna viridis]	36	1e-05
gi|18203491|sp|Q9U568|MT_PERVI Metallothionein (MT) >gi|665...	36	1e-05
gi|19861705|sp|P80247|MT12_MYTED Metallothionein 10-II (MT-...	38	2e-05
gi|19924225|sp|P80246|MT1A_MYTED Metallothionein 10-Ia (MT-...	37	4e-05
gi|18202100|sp|O62554|MT1B_MYTED Metallothionein 10-Ib (MT-...	37	4e-05
gi|18272161|sp|P80252|MT22_MYTED Metallothionein 20-II (MT-...	37	5e-05
gi|542508|pir||S39417 metallothionein 10-II - blue mussel	38	9e-05
gi|19861707|sp|P80253|MT2A_MYTED Metallothionein 20-III iso...	36	1e-04
gi|542507|pir||S39416 metallothionein 10-I - blue mussel >g...	37	2e-04
gi|630806|pir||S39421 metallothionein 20-II - blue mussel	37	2e-04
gi|40994788|emb|CAE11855.1| metallothionein [Mytilus edulis]	34	3e-04
gi|18272159|sp|P80249|MT14_MYTED Metallothionein 10-IV (MT-...	34	3e-04
gi|630807|pir||S39422 metallothionein 20-IIIa - blue mussel	36	4e-04
gi|18202371|sp|P80258|MT2B_MYTED Metallothionein 20-III iso...	36	4e-04
gi|18272157|sp|P80248|MT13_MYTED Metallothionein 10-III (MT...	35	7e-04
gi|1072470|pir||S47576 metallothionein 20-Ib - blue mussel	35	7e-04
gi|462638|sp|P80251|MT21_MYTED Metallothionein 20-I isoform...	35	7e-04
gi|542510|pir||S39419 metallothionein 10-IV - blue mussel >...	34	0.001
gi|40994802|emb|CAE11862.1| metallothionein [Mytilus edulis]	33	0.001
gi|40994790|emb|CAE11856.1| metallothionein [Mytilus edulis]	34	0.003
gi|542509|pir||S39418 metallothionein 10-III - blue mussel	35	0.003
gi|7406571|emb|CAB85588.1| metallothionein [Crassostrea gig...	34	0.009
gi|13194854|gb|AAK15581.1| metallothionein [Crassostrea ang...	34	0.015
gi|38348592|emb|CAD56896.1| metallothionein 10 [Bathymodiol...	33	0.016
gi|40994798|emb|CAE11860.1| metallothionein [Bathymodiolus ...	33	0.016
gi|40994796|emb|CAE11859.1| metallothionein [Bathymodiolus ...	33	0.016
gi|18076578|emb|CAC83770.1| metallothionein [Ostrea edulis]	37	0.026
gi|8920351|emb|CAB96403.1| metallothionein [Venerupis (Rudi...	34	0.027
gi|6687228|emb|CAB64869.1| metallothionein [Crassostrea gigas]	34	0.033
gi|33637528|gb|AAQ23908.1| metallothionein IB [Crassostrea ...	29	0.056
gi|8920353|emb|CAB96402.1| metallothionein [Venerupis (Rudi...	34	0.058
gi|18073426|emb|CAC82788.1| metallothionein [Crassostrea gi...	33	0.093
gi|40994792|emb|CAE11857.1| metallothionein [Mytilus edulis]	28	0.094
gi|40994794|emb|CAE11858.1| metallothionein [Mytilus edulis]	28	0.094
gi|26351279|dbj|BAC39276.1| unnamed protein product [Mus mu...	37	0.16
gi|43902289|gb|EAG05467.1| unknown [environmental sequence]	36	0.21
gi|34534585|dbj|BAC87052.1| unnamed protein product [Homo s...	36	0.28
gi|42957281|gb|EAB45609.1| unknown [environmental sequence]	36	0.28
gi|2497868|sp|Q94550|MT_DREPO Metallothionein (MT) >gi|1519...	32	0.33
gi|43702436|gb|EAF06449.1| unknown [environmental sequence]	35	0.36
gi|42908149|gb|EAB21172.1| unknown [environmental sequence]	34	0.81
gi|43615149|gb|EAE62292.1| unknown [environmental sequence]	34	0.81
gi|3135045|emb|CAA06552.1| metallothionein 10 IV [Mytilus e...	34	0.81

pvMT6

Sequences producing significant alignments:	Score (bits)	E Value
gi|18203491|sp|Q9U568|MT_PERVI Metallothionein (MT) >gi|665...	47	9e-05
gi|6652818|gb|AAF22487.1| metallothionein 2 [Perna viridis]	47	1e-04
gi|18272161|sp|P80252|MT22_MYTED Metallothionein 20-II (MT-...	45	6e-04
gi|40994800|emb|CAE11861.1| metallothionein [Mytilus edulis]	44	0.001
gi|19861707|sp|P80253|MT2A_MYTED Metallothionein 20-III iso...	44	0.001
gi|630806|pir||S39421 metallothionein 20-II - blue mussel	43	0.002
gi|4104513|gb|AAD02054.1| metallothionein [Perna viridis]	42	0.003
gi|19861705|sp|P80247|MT12_MYTED Metallothionein 10-II (MT-...	42	0.004
gi|630807|pir||S39422 metallothionein 20-IIIa - blue mussel	42	0.005
gi|18202371|sp|P80258|MT2B_MYTED Metallothionein 20-III iso...	42	0.005
gi|19924225|sp|P80246|MT1A_MYTED Metallothionein 10-Ia (MT-...	41	0.007
gi|18202100|sp|O62554|MT1B_MYTED Metallothionein 10-Ib (MT-...	41	0.007
gi|1072470|pir||S47576 metallothionein 20-Ib - blue mussel	41	0.009
gi|462638|sp|P80251|MT21_MYTED Metallothionein 20-I isoform...	41	0.009
gi|542508|pir||S39417 metallothionein 10-II - blue mussel	40	0.015
gi|40994802|emb|CAE11862.1| metallothionein [Mytilus edulis]	40	0.019
gi|542507|pir||S39416 metallothionein 10-I - blue mussel >g...	39	0.025
gi|18272159|sp|P80249|MT14_MYTED Metallothionein 10-IV (MT-...	38	0.056
gi|40994788|emb|CAE11855.1| metallothionein [Mytilus edulis]	38	0.073
gi|18272157|sp|P80248|MT13_MYTED Metallothionein 10-III (MT...	37	0.12
gi|18076578|emb|CAC83770.1| metallothionein [Ostrea edulis]	37	0.16
gi|7406571|emb|CAB85588.1| metallothionein [Crassostrea gig...	37	0.16
gi|542510|pir||S39419 metallothionein 10-IV - blue mussel >...	36	0.21
gi|13194854|gb|AAK15581.1| metallothionein [Crassostrea ang...	36	0.28
gi|42908149|gb|EAB21172.1| unknown [environmental sequence]	35	0.36
gi|542509|pir||S39418 metallothionein 10-III - blue mussel	35	0.47
gi|8920351|emb|CAB96403.1| metallothionein [Venerupis (Rudi...	35	0.47
gi|6687228|emb|CAB64869.1| metallothionein [Crassostrea gigas]	35	0.62
gi|40994790|emb|CAE11856.1| metallothionein [Mytilus edulis]	35	0.62
gi|3135045|emb|CAA06552.1| metallothionein 10 IV [Mytilus e...	34	0.81
gi|8920353|emb|CAB96402.1| metallothionein [Venerupis (Rudi...	34	0.81
gi|43015761|gb|EAB74632.1| unknown [environmental sequence]	34	0.81
gi|33637528|gb|AAQ23908.1| metallothionein IB [Crassostrea ...	34	1.1
gi|44165360|gb|EAH47936.1| unknown [environmental sequence]	34	1.1
gi|38348592|emb|CAD56896.1| metallothionein 10 [Bathymodiol...	33	1.4
gi|13786086|gb|AAK39563.1| metallothionein-like protein [An...	33	1.4
gi|40994798|emb|CAE11860.1| metallothionein [Bathymodiolus ...	33	1.4
gi|40994796|emb|CAE11859.1| metallothionein [Bathymodiolus ...	33	1.4
gi|18073426|emb|CAC82788.1| metallothionein [Crassostrea gi...	33	1.4
gi|43902289|gb|EAG05467.1| unknown [environmental sequence]	33	1.8
gi|43615149|gb|EAE62292.1| unknown [environmental sequence]	33	1.8
gi|23478566|gb|EAA15617.1| hypothetical protein [Plasmodium...	33	1.8
gi|42957281|gb|EAB45609.1| unknown [environmental sequence]	33	2.3
gi|417326|sp|P33187|MTCD_HELPO Cadmium-metallothionein (CD-...	33	2.3
gi|26351279|dbj|BAC39276.1| unnamed protein product [Mus mu...	33	2.3
gi|15148842|gb|AAK84863.1| Cd-metallothionein isoform [Heli...	33	2.3
gi|2497868|sp|Q94550|MT_DREPO Metallothionein (MT) >gi|1519...	32	3.1

pvMT7

Sequences producing significant alignments:	Score (bits)	E Value
gi|18203491|sp|Q9U568|MT_PERVI Metallothionein (MT) >gi|665...	47	9e-05
gi|6652818|gb|AAF22487.1| metallothionein 2 [Perna viridis]	47	1e-04
gi|18272161|sp|P80252|MT22_MYTED Metallothionein 20-II (MT-...	45	6e-04
gi|40994800|emb|CAE11861.1| metallothionein [Mytilus edulis]	44	0.001
gi|19861707|sp|P80253|MT2A_MYTED Metallothionein 20-III iso...	44	0.001
gi|630806|pir||S39421 metallothionein 20-II - blue mussel	43	0.002
gi|4104513|gb|AAD02054.1| metallothionein [Perna viridis]	42	0.003
gi|19861705|sp|P80247|MT12_MYTED Metallothionein 10-II (MT-...	42	0.004
gi|630807|pir||S39422 metallothionein 20-IIIa - blue mussel	42	0.005
gi|18202371|sp|P80258|MT2B_MYTED Metallothionein 20-III iso...	42	0.005
gi|19924225|sp|P80246|MT1A_MYTED Metallothionein 10-Ia (MT-...	41	0.007
gi|18202100|sp|O62554|MT1B_MYTED Metallothionein 10-Ib (MT-...	41	0.007
gi|1072470|pir||S47576 metallothionein 20-Ib - blue mussel	41	0.009
gi|462638|sp|P80251|MT21_MYTED Metallothionein 20-I isoform...	41	0.009
gi|542508|pir||S39417 metallothionein 10-II - blue mussel	40	0.015
gi|40994802|emb|CAE11862.1| metallothionein [Mytilus edulis]	40	0.019
gi|542507|pir||S39416 metallothionein 10-I - blue mussel >g...	39	0.025
gi|18272159|sp|P80249|MT14_MYTED Metallothionein 10-IV (MT-...	38	0.056
gi|40994788|emb|CAE11855.1| metallothionein [Mytilus edulis]	38	0.073
gi|18272157|sp|P80248|MT13_MYTED Metallothionein 10-III (MT...	37	0.12
gi|42908149|gb|EAB21172.1| unknown [environmental sequence]	37	0.16
gi|18076578|emb|CAC83770.1| metallothionein [Ostrea edulis]	37	0.16
gi|7406571|emb|CAB85588.1| metallothionein [Crassostrea gig...	37	0.16
gi|542510|pir||S39419 metallothionein 10-IV - blue mussel >...	36	0.21
gi|43902289|gb|EAG05467.1| unknown [environmental sequence]	36	0.28
gi|13194854|gb|AAK15581.1| metallothionein [Crassostrea ang...	36	0.28
gi|542509|pir||S39418 metallothionein 10-III - blue mussel	35	0.47
gi|34534585|dbj|BAC87052.1| unnamed protein product [Homo s...	35	0.47
gi|44165360|gb|EAH47936.1| unknown [environmental sequence]	35	0.47
gi|8920351|emb|CAB96403.1| metallothionein [Venerupis (Rudi...	35	0.47
gi|6687228|emb|CAB64869.1| metallothionein [Crassostrea gigas]	35	0.62
gi|40994790|emb|CAE11856.1| metallothionein [Mytilus edulis]	35	0.62
gi|3135045|emb|CAA06552.1| metallothionein 10 IV [Mytilus e...	34	0.81
gi|26351279|dbj|BAC39276.1| unnamed protein product [Mus mu...	34	0.81
gi|42888667|gb|EAB11474.1| unknown [environmental sequence]	34	0.81
gi|8920353|emb|CAB96402.1| metallothionein [Venerupis (Rudi...	34	0.81

PvMT8

Sequences producing significant alignments:	Score (bits)	E Value
gi|18203491|sp|Q9U568|MT_PERVI Metallothionein (MT) >gi|665...	45	5e-04
gi|6652818|gb|AAF22487.1| metallothionein 2 [Perna viridis]	45	6e-04
gi|18272161|sp|P80252|MT22_MYTED Metallothionein 20-II (MT-...	42	0.003
gi|40994800|emb|CAE11861.1| metallothionein [Mytilus edulis]	42	0.005
gi|19861707|sp|P80253|MT2A_MYTED Metallothionein 20-III iso...	41	0.007
gi|630806|pir||S39421 metallothionein 20-II - blue mussel	40	0.011
gi|4104513|gb|AAD02054.1| metallothionein [Perna viridis]	40	0.015
gi|19861705|sp|P80247|MT12_MYTED Metallothionein 10-II (MT-...	40	0.019
gi|630807|pir||S39422 metallothionein 20-IIIa - blue mussel	39	0.025
gi|18202371|sp|P80258|MT2B_MYTED Metallothionein 20-III iso...	39	0.025
gi|19924225|sp|P80246|MT1A_MYTED Metallothionein 10-Ia (MT-...	39	0.033
gi|18202100|sp|O62554|MT1B_MYTED Metallothionein 10-Ib (MT-...	39	0.033
gi|1072470|pir||S47576 metallothionein 20-Ib - blue mussel	39	0.043
gi|462638|sp|P80251|MT21_MYTED Metallothionein 20-I isoform...	39	0.043
gi|542508|pir||S39417 metallothionein 10-II - blue mussel	38	0.073
gi|40994802|emb|CAE11862.1| metallothionein [Mytilus edulis]	37	0.095
gi|542507|pir||S39416 metallothionein 10-I - blue mussel >g...	37	0.12
gi|43902289|gb|EAG05467.1| unknown [environmental sequence]	36	0.21
gi|42908149|gb|EAB21172.1| unknown [environmental sequence]	36	0.28
gi|42957281|gb|EAB45609.1| unknown [environmental sequence]	36	0.28
gi|18272159|sp|P80249|MT14_MYTED Metallothionein 10-IV (MT-...	36	0.28
gi|26351279|dbj|BAC39276.1| unnamed protein product [Mus mu...	36	0.28
gi|34534585|dbj|BAC87052.1| unnamed protein product [Homo s...	35	0.36
gi|40994788|emb|CAE11855.1| metallothionein [Mytilus edulis]	35	0.36
gi|43615149|gb|EAE62292.1| unknown [environmental sequence]	35	0.47
gi|43702436|gb|EAF06449.1| unknown [environmental sequence]	35	0.62
gi|18272157|sp|P80248|MT13_MYTED Metallothionein 10-III (MT...	35	0.62
gi|18076578|emb|CAC83770.1| metallothionein [Ostrea edulis]	34	0.81
gi|7406571|emb|CAB85588.1| metallothionein [Crassostrea gig...	34	0.81
gi|34534808|dbj|BAC87117.1| unnamed protein product [Homo s...	34	1.1
gi|44165360|gb|EAH47936.1| unknown [environmental sequence]	34	1.1
gi|542510|pir||S39419 metallothionein 10-IV - blue mussel >...	34	1.1
gi|13786086|gb|AAK39563.1| metallothionein-like protein [An...	33	1.4
gi|42942451|gb|EAB38234.1| unknown [environmental sequence]	33	1.4
gi|42888667|gb|EAB11474.1| unknown [environmental sequence]	33	1.4
gi|43113148|gb|EAC22595.1| unknown [environmental sequence]	33	1.4
gi|13194854|gb|AAK15581.1| metallothionein [Crassostrea ang...	33	1.4

pvMT9

Sequences producing significant alignments:	Score (bits)	E Value
gi|18203491|sp|Q9U568|MT_PERVI Metallothionein (MT) >gi|665...	47	9e-05
gi|6652818|gb|AAF22487.1| metallothionein 2 [Perna viridis]	47	1e-04
gi|18272161|sp|P80252|MT22_MYTED Metallothionein 20-II (MT-...	45	6e-04
gi|40994800|emb|CAE11861.1| metallothionein [Mytilus edulis]	44	0.001
gi|19861707|sp|P80253|MT2A_MYTED Metallothionein 20-III iso...	44	0.001
gi|630806|pir||S39421 metallothionein 20-II - blue mussel	43	0.002
gi|4104513|gb|AAD02054.1| metallothionein [Perna viridis]	42	0.003
gi|19861705|sp|P80247|MT12_MYTED Metallothionein 10-II (MT-...	42	0.004
gi|630807|pir||S39422 metallothionein 20-IIIa - blue mussel	42	0.005
gi|18202371|sp|P80258|MT2B_MYTED Metallothionein 20-III iso...	42	0.005
gi|19924225|sp|P80246|MT1A_MYTED Metallothionein 10-Ia (MT-...	41	0.007
gi|18202100|sp|O62554|MT1B_MYTED Metallothionein 10-Ib (MT-...	41	0.007
gi|1072470|pir||S47576 metallothionein 20-Ib - blue mussel	41	0.009
gi|462638|sp|P80251|MT21_MYTED Metallothionein 20-I isoform...	41	0.009
gi|542508|pir||S39417 metallothionein 10-II - blue mussel	40	0.015
gi|40994802|emb|CAE11862.1| metallothionein [Mytilus edulis]	40	0.019
gi|542507|pir||S39416 metallothionein 10-I - blue mussel >g...	39	0.025
gi|18272159|sp|P80249|MT14_MYTED Metallothionein 10-IV (MT-...	38	0.056
gi|42908149|gb|EAB21172.1| unknown [environmental sequence]	38	0.073
gi|40994788|emb|CAE11855.1| metallothionein [Mytilus edulis]	38	0.073
gi|18272157|sp|P80248|MT13_MYTED Metallothionein 10-III (MT...	37	0.12
gi|18076578|emb|CAC83770.1| metallothionein [Ostrea edulis]	37	0.16
gi|7406571|emb|CAB85588.1| metallothionein [Crassostrea gig...	37	0.16
gi|542510|pir||S39419 metallothionein 10-IV - blue mussel >...	36	0.21
gi|42957281|gb|EAB45609.1| unknown [environmental sequence]	36	0.28
gi|13194854|gb|AAK15581.1| metallothionein [Crassostrea ang...	36	0.28
gi|542509|pir||S39418 metallothionein 10-III - blue mussel	35	0.47
gi|43615149|gb|EAE62292.1| unknown [environmental sequence]	35	0.47
gi|8920351|emb|CAB96403.1| metallothionein [Venerupis (Rudi...	35	0.47
gi|6687228|emb|CAB64869.1| metallothionein [Crassostrea gigas]	35	0.62
gi|34530691|dbj|BAC85954.1| unnamed protein product [Homo s...	35	0.62
gi|44165360|gb|EAH47936.1| unknown [environmental sequence]	35	0.62
gi|40994790|emb|CAE11856.1| metallothionein [Mytilus edulis]	35	0.62
gi|3135045|emb|CAA06552.1| metallothionein 10 IV [Mytilus e...	34	0.81
gi|8920353|emb|CAB96402.1| metallothionein [Venerupis (Rudi...	34	0.81
gi|43902289|gb|EAG05467.1| unknown [environmental sequence]	34	1.1
gi|33637528|gb|AAQ23908.1| metallothionein IB [Crassostrea ...	34	1.1
gi|34534808|dbj|BAC87117.1| unnamed protein product [Homo s...	34	1.1
gi|34534585|dbj|BAC87052.1| unnamed protein product [Homo s...	33	1.4
gi|38348592|emb|CAD56896.1| metallothionein 10 [Bathymodiol...	33	1.4
gi|13786086|gb|AAK39563.1| metallothionein-like protein [An...	33	1.4
gi|40994798|emb|CAE11860.1| metallothionein [Bathymodiolus ...	33	1.4
gi|40994796|emb|CAE11859.1| metallothionein [Bathymodiolus ...	33	1.4
gi|26351279|dbj|BAC39276.1| unnamed protein product [Mus mu...	33	1.4
gi|18073426|emb|CAC82788.1| metallothionein [Crassostrea gi...	33	1.4
gi|43673025|gb|EAE91572.1| unknown [environmental sequence]	33	1.8
gi|43754695|gb|EAF31775.1| unknown [environmental sequence]	33	1.8
gi|23478566|gb|EAA15617.1| hypothetical protein [Plasmodium...	33	1.8
gi|43169423|gb|EAC50287.1| unknown [environmental sequence]	33	2.3
gi|34536201|dbj|BAC87575.1| unnamed protein product [Homo s...	33	2.3
gi|43702436|gb|EAF06449.1| unknown [environmental sequence]	33	2.3
gi|417326|sp|P33187|MTCD_HELPO Cadmium-metallothionein (CD-...	33	2.3
gi|15148842|gb|AAK84863.1| Cd-metallothionein isoform [Heli...	33	2.3

pvMT10

Sequences producing significant alignments:	Score (bits)	E Value
gi|18203491|sp|Q9U568|MT_PERVI Metallothionein (MT) >gi|665...	101	5e-21
gi|6652818|gb|AAF22487.1| metallothionein 2 [Perna viridis]	100	7e-21
gi|4104513|gb|AAD02054.1| metallothionein [Perna viridis]	96	2e-19
gi|40994800|emb|CAE11861.1| metallothionein [Mytilus edulis]	83	2e-15
gi|19924225|sp|P80246|MT1A_MYTED Metallothionein 10-Ia (MT-...	82	3e-15
gi|18202100|sp|O62554|MT1B_MYTED Metallothionein 10-Ib (MT-...	82	3e-15
gi|19861705|sp|P80247|MT12_MYTED Metallothionein 10-II (MT-...	81	6e-15
gi|542507|pir||S39416 metallothionein 10-I - blue mussel >g...	80	1e-14
gi|542508|pir||S39417 metallothionein 10-II - blue mussel	80	1e-14
gi|18272157|sp|P80248|MT13_MYTED Metallothionein 10-III (MT...	80	1e-14
gi|18272159|sp|P80249|MT14_MYTED Metallothionein 10-IV (MT-...	79	4e-14
gi|542509|pir||S39418 metallothionein 10-III - blue mussel	78	5e-14
gi|542510|pir||S39419 metallothionein 10-IV - blue mussel >...	77	1e-13
gi|40994788|emb|CAE11855.1| metallothionein [Mytilus edulis]	75	3e-13
gi|18272161|sp|P80252|MT22_MYTED Metallothionein 20-II (MT-...	74	1e-12
gi|40994790|emb|CAE11856.1| metallothionein [Mytilus edulis]	72	3e-12
gi|40994798|emb|CAE11860.1| metallothionein [Bathymodiolus ...	72	3e-12
gi|40994796|emb|CAE11859.1| metallothionein [Bathymodiolus ...	72	3e-12
gi|38348592|emb|CAD56896.1| metallothionein 10 [Bathymodiol...	72	3e-12
gi|19861707|sp|P80253|MT2A_MYTED Metallothionein 20-III iso...	72	3e-12
gi|630806|pir||S39421 metallothionein 20-II - blue mussel	72	5e-12
gi|630807|pir||S39422 metallothionein 20-IIIa - blue mussel	70	1e-11
gi|18202371|sp|P80258|MT2B_MYTED Metallothionein 20-III iso...	70	1e-11
gi|1072470|pir||S47576 metallothionein 20-Ib - blue mussel	70	2e-11
gi|462638|sp|P80251|MT21_MYTED Metallothionein 20-I isoform...	70	2e-11
gi|40994802|emb|CAE11862.1| metallothionein [Mytilus edulis]	67	9e-11
gi|40994792|emb|CAE11857.1| metallothionein [Mytilus edulis]	63	2e-09
gi|40994794|emb|CAE11858.1| metallothionein [Mytilus edulis]	62	5e-09
gi|13786086|gb|AAK39563.1| metallothionein-like protein [An...	55	3e-07
gi|33637528|gb|AAQ23908.1| metallothionein IB [Crassostrea ...	54	8e-07
gi|13194854|gb|AAK15581.1| metallothionein [Crassostrea ang...	54	8e-07
gi|266587|sp|P23038|MT_CRAVI Metallothionein (MT) >gi|10269...	53	2e-06
gi|7406571|emb|CAB85588.1| metallothionein [Crassostrea gig...	53	2e-06
gi|8920353|emb|CAB96402.1| metallothionein [Venerupis (Rudi...	52	4e-06
gi|6687228|emb|CAB64869.1| metallothionein [Crassostrea gigas]	51	6e-06
gi|8920351|emb|CAB96403.1| metallothionein [Venerupis (Rudi...	51	8e-06
gi|22038045|gb|AAM90257.1| metallothionein [Crassostrea vir...	48	5e-05
gi|18073426|emb|CAC82788.1| metallothionein [Crassostrea gi...	47	9e-05
gi|33637544|gb|AAQ23916.1| metallothionein IIH [Crassostrea...	45	6e-04
gi|33637534|gb|AAQ23911.1| metallothionein IIC [Crassostrea...	45	6e-04
gi|33637538|gb|AAQ23913.1| metallothionein IIE [Crassostrea...	45	6e-04
gi|2497868|sp|Q94550|MT_DREPO Metallothionein (MT) >gi|1519...	44	8e-04
gi|18076578|emb|CAC83770.1| metallothionein [Ostrea edulis]	44	8e-04

pvMT11

Sequences producing significant alignments:	Score (bits)	E Value
gi|4104513|gb|AAD02054.1| metallothionein [Perna viridis]	64	9e-16
gi|6652818|gb|AAF22487.1| metallothionein 2 [Perna viridis]	64	9e-16
gi|18203491|sp|Q9U568|MT_PERVI Metallothionein (MT) >gi|665...	64	9e-16
gi|40994800|emb|CAE11861.1| metallothionein [Mytilus edulis]	58	9e-11
gi|19924225|sp|P80246|MT1A_MYTED Metallothionein 10-Ia (MT-...	58	1e-10
gi|18202100|sp|O62554|MT1B_MYTED Metallothionein 10-Ib (MT-...	58	1e-10
gi|19861705|sp|P80247|MT12_MYTED Metallothionein 10-II (MT-...	57	3e-10
gi|542507|pir||S39416 metallothionein 10-I - blue mussel >g...	56	4e-10
gi|542508|pir||S39417 metallothionein 10-II - blue mussel	56	4e-10
gi|18272157|sp|P80248|MT13_MYTED Metallothionein 10-III (MT...	59	6e-10
gi|40994788|emb|CAE11855.1| metallothionein [Mytilus edulis]	58	2e-09
gi|18272159|sp|P80249|MT14_MYTED Metallothionein 10-IV (MT-...	58	2e-09
gi|542509|pir||S39418 metallothionein 10-III - blue mussel	57	2e-09
gi|18272161|sp|P80252|MT22_MYTED Metallothionein 20-II (MT-...	55	5e-09
gi|542510|pir||S39419 metallothionein 10-IV - blue mussel >...	56	6e-09
gi|38348592|emb|CAD56896.1| metallothionein 10 [Bathymodiol...	51	1e-08
gi|40994798|emb|CAE11860.1| metallothionein [Bathymodiolus ...	51	1e-08
gi|40994796|emb|CAE11859.1| metallothionein [Bathymodiolus ...	51	1e-08
gi|40994790|emb|CAE11856.1| metallothionein [Mytilus edulis]	55	1e-08
gi|19861707|sp|P80253|MT2A_MYTED Metallothionein 20-III iso...	54	1e-08
gi|630806|pir||S39421 metallothionein 20-II - blue mussel	53	2e-08
gi|630807|pir||S39422 metallothionein 20-IIIa - blue mussel	52	5e-08
gi|18202371|sp|P80258|MT2B_MYTED Metallothionein 20-III iso...	52	5e-08
gi|1072470|pir||S47576 metallothionein 20-Ib - blue mussel	53	6e-08
gi|462638|sp|P80251|MT21_MYTED Metallothionein 20-I isoform...	53	6e-08
gi|40994802|emb|CAE11862.1| metallothionein [Mytilus edulis]	54	3e-07
gi|40994792|emb|CAE11857.1| metallothionein [Mytilus edulis]	53	2e-06
gi|40994794|emb|CAE11858.1| metallothionein [Mytilus edulis]	53	2e-06
gi|13194854|gb|AAK15581.1| metallothionein [Crassostrea ang...	40	4e-05
gi|33637528|gb|AAQ23908.1| metallothionein IB [Crassostrea ...	38	1e-04
gi|266587|sp|P23038|MT_CRAVI Metallothionein (MT) >gi|10269...	38	2e-04
gi|6687228|emb|CAB64869.1| metallothionein [Crassostrea gigas]	36	5e-04
gi|13786086|gb|AAK39563.1| metallothionein-like protein [An...	36	7e-04
gi|8920351|emb|CAB96403.1| metallothionein [Venerupis (Rudi...	36	7e-04
gi|8920353|emb|CAB96402.1| metallothionein [Venerupis (Rudi...	34	0.003
gi|18073426|emb|CAC82788.1| metallothionein [Crassostrea gi...	33	0.007
gi|18076578|emb|CAC83770.1| metallothionein [Ostrea edulis]	32	0.009
gi|14017405|gb|AAK50565.1| metallothionein [Crassostrea rhi...	38	0.056
gi|33637530|gb|AAQ23909.1| metallothionein IIA [Crassostrea...	38	0.056
gi|22038045|gb|AAM90257.1| metallothionein [Crassostrea vir...	38	0.056

pvMT12

Sequences producing significant alignments:	Score (bits)	E Value
gi|18203491|sp|Q9U568|MT_PERVI Metallothionein (MT) >gi|665...	102	2e-21
gi|6652818|gb|AAF22487.1| metallothionein 2 [Perna viridis]	102	2e-21
gi|4104513|gb|AAD02054.1| metallothionein [Perna viridis]	98	6e-20
gi|40994800|emb|CAE11861.1| metallothionein [Mytilus edulis]	83	2e-15
gi|19924225|sp|P80246|MT1A_MYTED Metallothionein 10-Ia (MT-...	82	3e-15
gi|18202100|sp|O62554|MT1B_MYTED Metallothionein 10-Ib (MT-...	82	3e-15
gi|19861705|sp|P80247|MT12_MYTED Metallothionein 10-II (MT-...	81	6e-15
gi|542507|pir||S39416 metallothionein 10-I - blue mussel >g...	80	1e-14
gi|542508|pir||S39417 metallothionein 10-II - blue mussel	80	1e-14
gi|18272157|sp|P80248|MT13_MYTED Metallothionein 10-III (MT...	80	1e-14
gi|18272159|sp|P80249|MT14_MYTED Metallothionein 10-IV (MT-...	79	4e-14
gi|542509|pir||S39418 metallothionein 10-III - blue mussel	78	5e-14
gi|542510|pir||S39419 metallothionein 10-IV - blue mussel >...	77	1e-13
gi|40994788|emb|CAE11855.1| metallothionein [Mytilus edulis]	75	3e-13
gi|18272161|sp|P80252|MT22_MYTED Metallothionein 20-II (MT-...	74	1e-12
gi|40994790|emb|CAE11856.1| metallothionein [Mytilus edulis]	72	3e-12
gi|40994798|emb|CAE11860.1| metallothionein [Bathymodiolus ...	72	3e-12
gi|40994796|emb|CAE11859.1| metallothionein [Bathymodiolus ...	72	3e-12
gi|38348592|emb|CAD56896.1| metallothionein 10 [Bathymodiol...	72	3e-12
gi|19861707|sp|P80253|MT2A_MYTED Metallothionein 20-III iso...	72	3e-12
gi|630806|pir||S39421 metallothionein 20-II - blue mussel	72	5e-12
gi|630807|pir||S39422 metallothionein 20-IIIa - blue mussel	70	1e-11
gi|18202371|sp|P80258|MT2B_MYTED Metallothionein 20-III iso...	70	1e-11
gi|1072470|pir||S47576 metallothionein 20-Ib - blue mussel	70	2e-11
gi|462638|sp|P80251|MT21_MYTED Metallothionein 20-I isoform...	70	2e-11
gi|40994802|emb|CAE11862.1| metallothionein [Mytilus edulis]	67	9e-11
gi|40994792|emb|CAE11857.1| metallothionein [Mytilus edulis]	63	2e-09
gi|40994794|emb|CAE11858.1| metallothionein [Mytilus edulis]	62	5e-09
gi|13786086|gb|AAK39563.1| metallothionein-like protein [An...	55	3e-07
gi|33637528|gb|AAQ23908.1| metallothionein IB [Crassostrea ...	54	8e-07
gi|13194854|gb|AAK15581.1| metallothionein [Crassostrea ang...	54	8e-07
gi|266587|sp|P23038|MT_CRAVI Metallothionein (MT) >gi|10269...	53	2e-06
gi|7406571|emb|CAB85588.1| metallothionein [Crassostrea gig...	53	2e-06
gi|8920353|emb|CAB96402.1| metallothionein [Venerupis (Rudi...	52	4e-06
gi|6687228|emb|CAB64869.1| metallothionein [Crassostrea gigas]	51	6e-06
gi|8920351|emb|CAB96403.1| metallothionein [Venerupis (Rudi...	51	8e-06
gi|22038045|gb|AAM90257.1| metallothionein [Crassostrea vir...	48	5e-05
gi|18073426|emb|CAC82788.1| metallothionein [Crassostrea gi...	47	9e-05
gi|33637544|gb|AAQ23916.1| metallothionein IIH [Crassostrea...	45	6e-04
gi|33637534|gb|AAQ23911.1| metallothionein IIC [Crassostrea...	45	6e-04
gi|33637538|gb|AAQ23913.1| metallothionein IIE [Crassostrea...	45	6e-04
gi|2497868|sp|Q94550|MT_DREPO Metallothionein (MT) >gi|1519...	44	8e-04
gi|18076578|emb|CAC83770.1| metallothionein [Ostrea edulis]	44	8e-04
gi|33637530|gb|AAQ23909.1| metallothionein IIA [Crassostrea...	44	0.001
gi|33637542|gb|AAQ23915.1| metallothionein IIG [Crassostrea...	44	0.001
gi|33637536|gb|AAQ23912.1| metallothionein IID [Crassostrea...	44	0.001
gi|14017405|gb|AAK50565.1| metallothionein [Crassostrea rhi...	44	0.001
gi|33637540|gb|AAQ23914.1| metallothionein IIF [Crassostrea...	44	0.001

Appendix C

Microassay Procedure for determination of protein

1. Prepare three to five dilutions of a protein standard which is representative of the protein solution to be tested. The linear range of the assay for BSA is 1.2 to 10.0 μg/ml.
2. Pipet 800 μl of each standard and sample solution into a clean, dry test tube. Protein solutions are normally assayed in duplicate or triplicate.
3. Add 200 μl of dye reagent concentrate to each tube and vortex.
4. Incubate at room temperature for at least 5 minutes. Absorbance will increase over time; samples should incubate at room temperature for no more than 1 hour.
5. Measure absorbance at 595 nm.

Protein (ug/ml)	A595
0	0
1	0.117
2	0.194
4	0.329
6	0.437

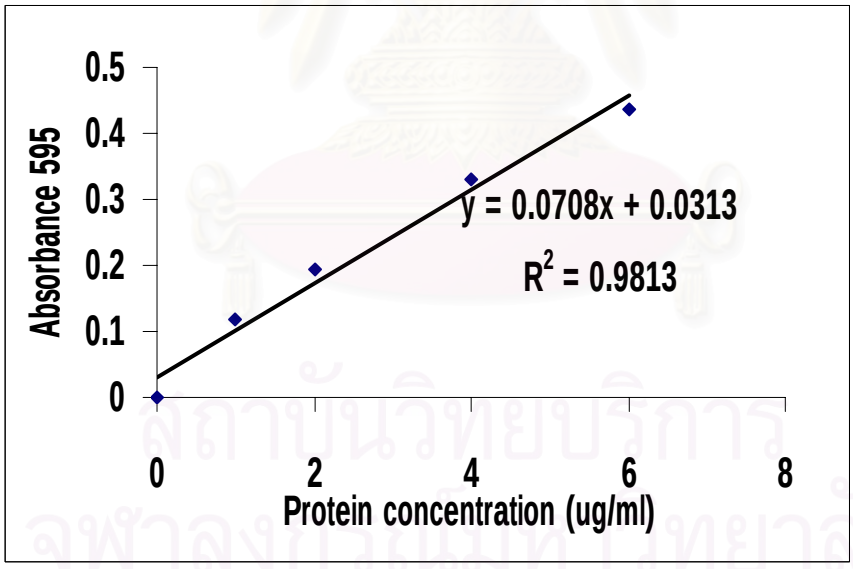


Figure C-1 Standard protein curve for determination protein concentration.

Appendix D

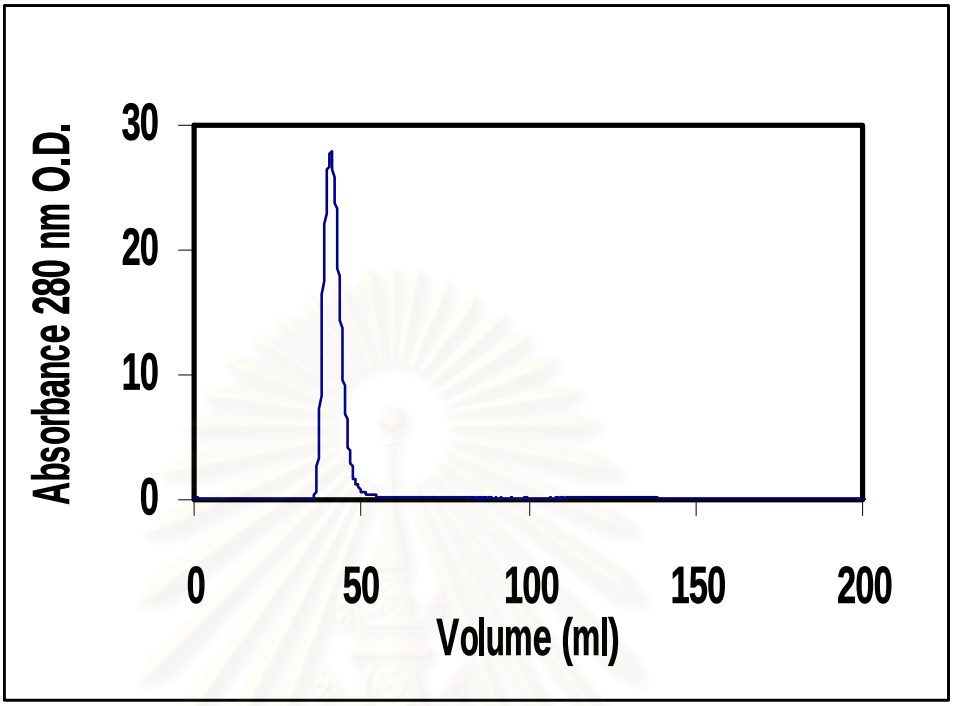


Figure E.1 The elution profile of Sephadex G-75 chromatography of dextran blue

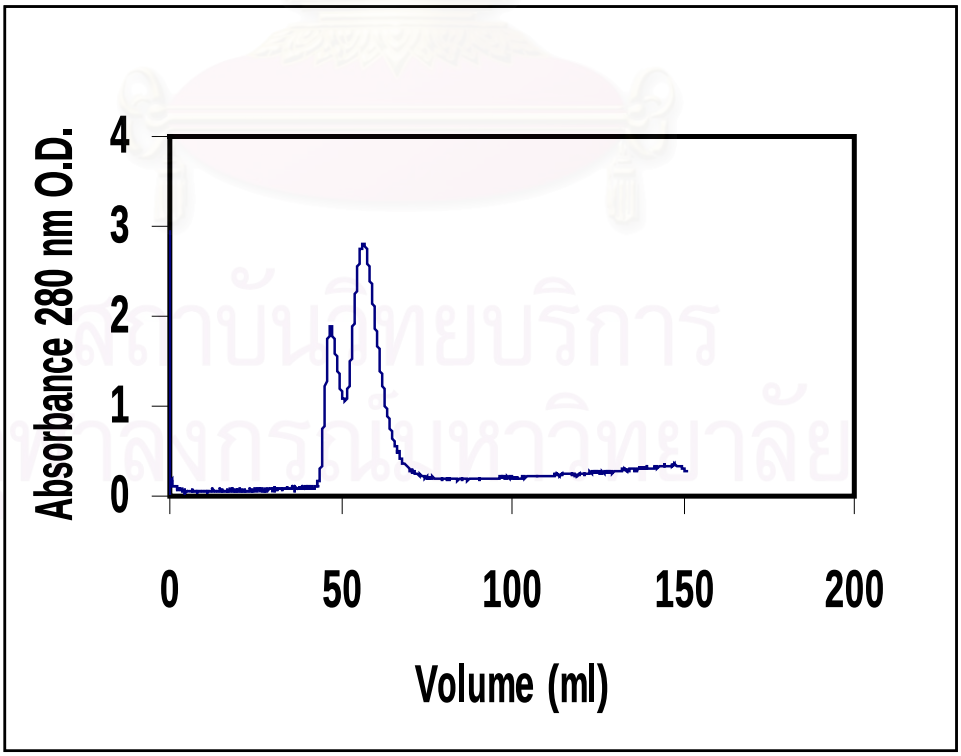


Figure E.2 The elution profile of Sephadex G-75 chromatography of Bovine Serum Albumin (BSA)

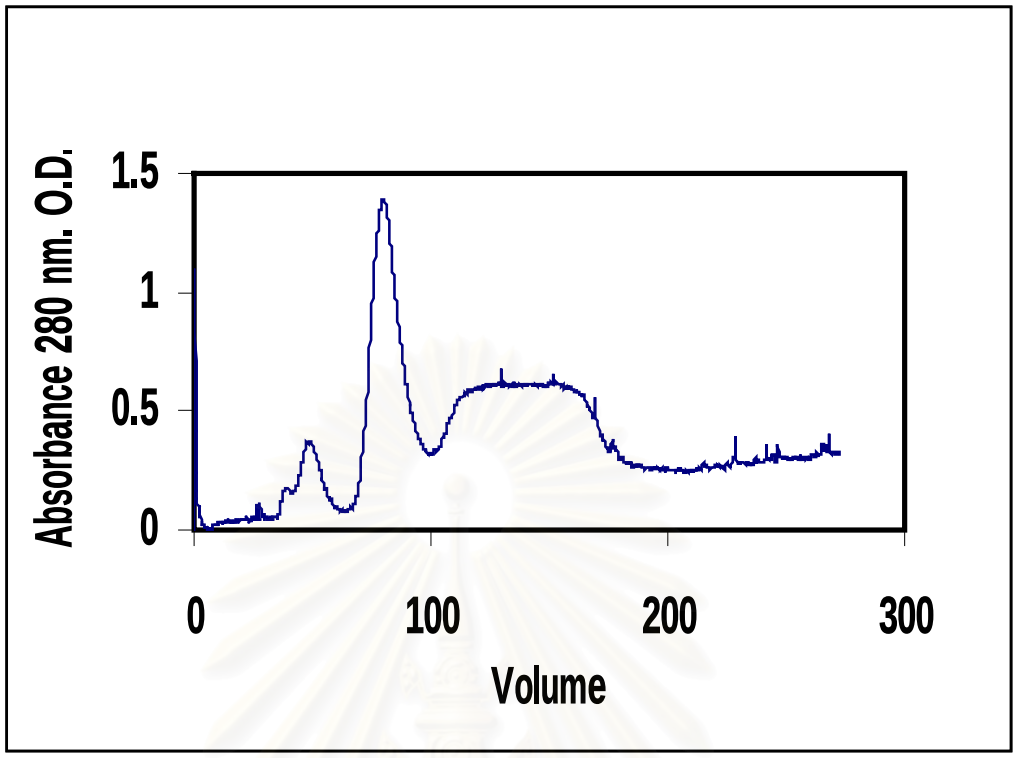


Figure E.3 The elution profile of Sephadex G-75 chromatography of cytochrome C

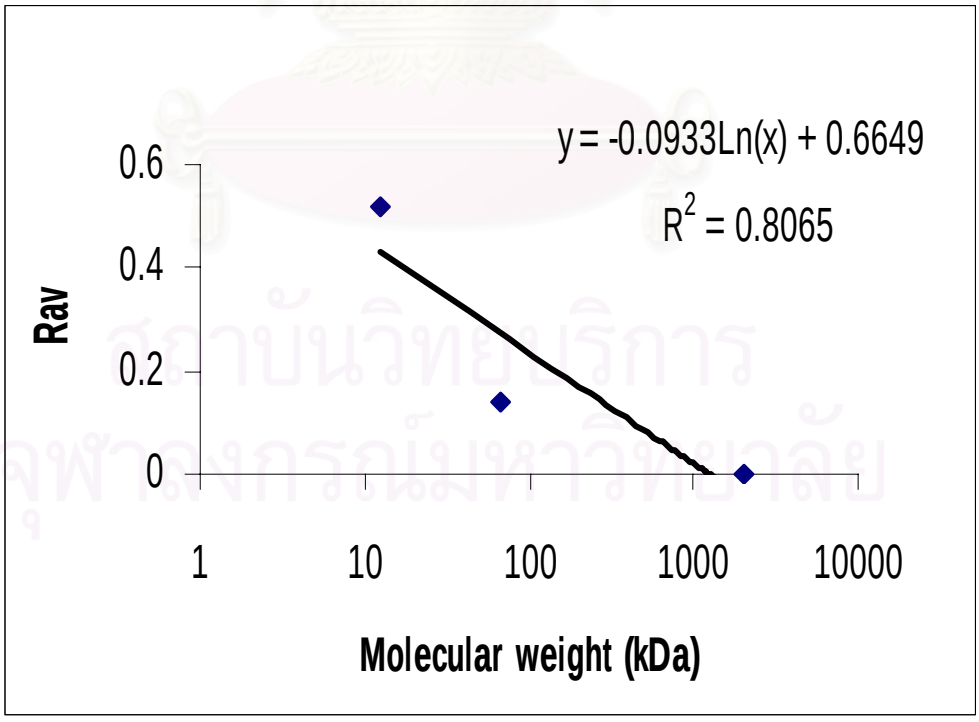


Figure E.4 The calibration curve of Sephadex G-75 column.

Appendix E

Table F.1 Total RNA concentration from digestive tract *Perna viridis* were exposed mercuric chloride for 7 weeks.

Week	Sample	O.D.260	O.D.280	Ratio	Concentration (ng/μl)
0	mussel 1	1.379	0.673	2.049	11032
	mussel 2	1.448	0.707	2.048	11584
	mussel 3	1.905	0.970	1.964	15240
	mussel 4	0.878	0.432	2.032	7024
	mussel 5	1.823	0.892	2.044	14584
	mussel 6	2.708	1.385	1.955	21664
	mussel 7	2.428	1.275	1.904	29136
	mussel 8	2.504	1.247	2.008	30048
	mussel 9	2.410	1.241	1.942	19280
1	Control 1	2.162	1.242	1.741	17296
	Control 2	2.845	1.61	1.767	11380
	0.1 μg/l 1	2.518	1.406	1.791	30216
	0.1 μg /l 2	2.586	1.492	1.733	20688
	0.5 μg /l 1	2.052	1.141	1.752	24624
	0.5 μg /l 2	2.381	1.371	1.737	19048
	1.0 μg /l 1	2.257	1.292	1.747	18056
	1.0 μg /l 2	2.62	1.749	1.749	20960
	5.0 μg /l 1	2.277	1.754	1.754	18216
	5.0 μg /l 2	2.343	1.372	1.708	18744
2	Control 1	1.719	0.915	1.879	13752
	Control 2	1.941	1.094	1.774	15528
	0.1 μg /l 1	1.702	.979	1.739	6808
	0.1 μg /l 2	2.404	1.329	1.809	19232
	0.5 μg /l 1	1.494	.808	1.845	11928
	0.5 μg /l 2	2.061	1.159	1.778	16488
	1.0 μg /l 1	1.906	1.045	1.824	15248
	1.0 μg /l 2	2.131	1.198	1.779	17048
	5.0 μg /l 1	1.388	0.760	1.826	11104
	5.0 μg /l 2	1.066	0.637	1.673	8528
3	Control 1	1.989	1.157	1.719	7956
	Control 2	2.845	1.61	1.767	11380
	0.1 μg /l 1	1.764	1.016	1.736	14112
	0.1 μg /l 2	1.989	1.148	1.733	15912
	0.5 μg /l 1	2.195	1.228	1.787	17560
	0.5 μg /l 2	1.633	.950	1.719	13064
	1.0 μg /l 1	1.641	0.95	1.717	13128
	1.0 μg /l 2	2.409	1.361	1.77	19272
	5.0 μg /l 1	2.681	1.519	1.765	10724
	5.0 μg /l 2	2.231	1.283	1.739	8924

Table F.1 (Continue) Total RNA concentration from digestive tract *Perna viridis* were exposed mercuric chloride for 7 weeks.

Week	Sample	O.D.260	O.D.280	Ratio	Concentration (ng/μl)
4	Control 1	2.678	1.568	1.708	10712
	Control 2	1.938	1.086	1.785	15504
	0.1 μg /l 1	2.599	1.514	1.717	10396
	0.1 μg /l 2	1.726	.993	1.738	13808
	0.5 μg /l 1	1.631	0.873	1.868	13048
	0.5 μg /l 2	1.730	.994	1.740	13840
	1.0 μg /l 1	1.749	0.95	1.824	15248
	1.0 μg /l 2	1.948	1.093	1.782	15584
	5.0 μg /l 1	2.554	1.456	1.754	10216
	5.0 μg /l 2	1.095	.640	1.711	8760
5	Control 1	1.579	0.935	1.689	12632
	Control 2	1.897	1.114	1.703	15176
	0.1 μg /l 1	2.586	1.492	1.733	20688
	0.1 μg /l 2	1.812	1.079	1.679	14496
	0.5 μg /l 1	2.195	1.253	1.752	17560
	0.5 μg /l 2	1.375	0.837	1.643	11000
	1.0 μg /l 1	2.287	1.317	1.737	27444
	1.0 μg /l 2	1.733	1.028	1.686	13864
	5.0 μg /l 1	2.193	1.273	1.723	17544
	5.0 μg /l 2	2.132	1.252	1.703	17056
7	Control 1	1.911	1.069	1.788	7644
	Control 2	1.944	1.116	1.742	7776
	0.1 μg /l 1	2.496	1.43	1.745	9984
	0.1 μg /l 2	2.125	1.21	1.756	8500
	0.5 μg /l 1	1.955	1.077	1.815	15640
	0.5 μg /l 2	2.500	1.397	1.790	10000
	1.0 μg /l 1	1.749	.950	1.84	13992
	1.0 μg /l 2	1.948	1.093	1.782	15584
	5.0 μg /l 1	1.519	0.826	1.839	12152
	5.0 μg /l 2	1.095	0.640	1.711	8760

Table F.2 Single strand cDNA concentrations from digestive tracts *Perna viridis* were exposed mercuric chloride for 7 weeks.

Week	Sample	O.D.260	O.D.280	Ratio	Concentration (ng/μl)
0	mussel 1	0.276	0.175	1.577	1380
	mussel 2	0.272	0.171	1.591	1360
	mussel 3	0.268	0.169	1.586	1340
	mussel 4	0.266	0.170	1.565	1330
	mussel 5	0.279	0.179	1.559	1395
	mussel 6	0.257	0.162	1.586	1285
	mussel 7	0.275	0.177	1.554	1375
	mussel 8	0.266	0.171	1.556	1330
	mussel 9	0.263	0.166	1.584	1315
1	Control 1	0.276	0.171	1.614	1380
	Control 2	0.314	0.207	1.517	1570
	0.1 μg /l 1	0.293	0.183	1.601	1465
	0.1 μg /l 2	0.280	0.170	1.647	1400
	0.5 μg /l 1	0.283	0.178	1.590	1415
	0.5 μg /l 2	0.281	0.179	1.57	1405
	1.0 μg /l 1	0.304	0.189	1.608	1520
	1.0 μg /l 2	0.266	0.163	1.632	1330
	5.0 μg /l 1	0.284	0.180	1.578	1420
	5.0 μg /l 2	0.293	0.186	1.575	1465
2	Control 1	0.247	0.149	1.658	1235
	Control 2	0.247	0.149	1.658	1235
	0.1 μg /l 1	0.258	0.155	1.665	1290
	0.1 μg /l 2	0.267	0.162	1.648	1335
	0.5 μg /l 1	0.269	0.162	1.66	1345
	0.5 μg /l 2	0.319	0.191	1.670	1595
	1.0 μg /l 1	0.263	0.158	1.665	1315
	1.0 μg /l 2	0.267	0.160	1.669	1335
	5.0 μg /l 1	0.267	0.162	1.648	1335
	5.0 μg /l 2	0.279	0.164	1.701	1395
3	Control 1	0.238	0.148	1.608	1190
	Control 2	0.250	0.156	1.603	1250
	0.1 μg /l 1	0.256	0.159	1.61	1280
	0.1 μg /l 2	0.243	0.146	1.664	1215
	0.5 μg /l 1	0.237	0.146	1.623	1185
	0.5 μg /l 2	0.265	0.167	1.587	1325
	1.0 μg /l 1	0.251	0.157	1.599	1255
	1.0 μg /l 2	0.253	0.159	1.591	1265
	5.0 μg /l 1	0.265	0.164	1.616	1325
	5.0 μg /l 2	0.271	0.165	1.642	1355

Table F.2 (Continue) Single strand cDNA concentrations from digestive tracts *Perna viridis* were exposed mercuric chloride for 7 weeks.

Week	Sample	O.D.260	O.D.280	Ratio	Concentration (ng/µl)
4	Control 1	0.261	0.156	1.673	1305
	Control 2	0.279	0.175	1.594	1395
	0.1 µg /l 1	0.266	0.157	1.669	1330
	0.1 µg /l 2	0.272	0.164	1.659	1360
	0.5 µg /l 1	0.271	0.159	1.704	1355
	0.5 µg /l 2	0.286	0.168	1.702	1430
	1.0 µg /l 1	0.271	0.163	1.663	1355
	1.0 µg /l 2	0.263	0.158	1.665	1315
	5.0 µg /l 1	0.259	0.156	1.660	1295
	5.0 µg /l 2	0.266	0.158	1.684	1330
5	Control 1	0.314	0.207	1.517	1570
	Control 2	0.272	0.174	1.563	1360
	0.1 µg /l 1	0.280	0.170	1.647	1400
	0.1 µg /l 2	0.289	0.180	1.606	1445
	0.5 µg /l 1	0.289	0.184	1.571	1445
	0.5 µg /l 2	0.358	0.233	1.536	1790
	1.0 µg /l 1	0.341	0.218	1.564	1705
	1.0 µg /l 2	0.323	0.212	1.524	1615
	5.0 µg /l 1	0.306	0.191	1.602	1530
	5.0 µg /l 2	0.288	0.176	1.636	1440
7	Control 1	0.236	0.147	1.605	1180
	Control 2	0.268	0.161	1.665	1340
	0.1 µg /l 1	0.265	0.171	1.550	1325
	0.1 µg /l 2	0.268	0.164	1.634	1340
	0.5 µg /l 1	0.274	0.170	1.612	1370
	0.5 µg /l 2	0.264	0.164	1.610	1320
	1.0 µg /l 1	0.271	0.163	1.663	1355
	1.0 µg /l 2	0.263	0.158	1.684	1315
	5.0 µg /l 1	0.267	0.172	1.552	1335
	5.0 µg /l 2	0.266	0.158	1.684	1330

Table F.3 The total RNA concentration from digestive tracts *Perna viridis* were treat with various mercuric chloride concentration for 1 month.

Week	Sample	O.D.260	O.D.280	Ratio	Concentration (ng/μl)
0	mussel1	2.765	1.375	2.011	22120
	mussel2	2.102	1.073	1.959	25224
	mussel3	2.923	1.478	1.978	23384
	mussel4	2.156	1.064	2.026	17248
	mussel5	2.427	1.206	2.012	19416
1	Control 1	0.526	0.269	1.955	2104
	Control 2	0.684	0.414	1.652	2736
	Control 3	1.004	0.605	1.66	4016
	Control 4	0.949	0.583	1.628	3796
	Control 5	2.252	1.296	1.738	9008
	0.1 μg /l 1	1.578	0.901	1.751	12624
	0.1 μg /l 2	2.047	1.191	1.719	8188
	0.1 μg /l 3	1.023	0.618	1.655	4092
	0.1 μg /l 4	1.159	0.699	1.658	4636
	0.1 μg /l 5	2.580	1.482	1.741	10320
	0.5 μg /l 1	2.821	1.606	1.757	11284
	0.5 μg /l 2	1.659	0.962	1.725	13272
	0.5 μg /l 3	1.885	1.081	1.744	15080
	0.5 μg /l 4	1.405	0.839	1.675	5620
	0.5 μg /l 5	2.000	1.156	1.730	8000
	1.0 μg /l 1	1.187	0.719	1.651	4748
	1.0 μg /l 2	2.221	1.281	1.734	8884
	1.0 μg /l 3	1.955	1.132	1.727	7820
	1.0 μg /l 4	2.101	1.200	1.751	16808
	1.0 μg /l 5	1.843	1.063	1.734	14744
	5.0 μg /l 1	2.939	1.663	1.767	11756
	5.0 μg /l 2	1.574	0.910	1.730	12592
	5.0 μg /l 3	2.078	1.199	1.733	8312
	5.0 μg /l 4	1.550	0.911	1.701	12400
	5.0 μg /l 5	2.963	1.699	1.744	11852
2	Control 1	1.180	0.698	1.691	4720
	Control 2	2.226	1.272	1.750	8904
	Control 3	1.567	0.914	1.714	12536
	Control 4	1.677	0.993	1.689	13416
	Control 5	1.945	1.138	1.709	
	0.1 μg /l 1	1.677	1.003	1.672	13416
	0.1 μg /l 2	2.403	1.383	1.738	19224
	0.1 μg /l 3	2.362	1.347	1.754	18896
	0.1 μg /l 4	2.674	1.532	1.745	21392
	0.1 μg /l 5	1.665	0.988	1.685	13320

Table F.3 (continue) The total RNA concentration from digestive tracts *Perna viridis* were treat with various mercuric chloride concentration for 1 month

Week	Sample	O.D.260	O.D.280	Ratio	Concentration (ng/μl)
2	0.5 μg /l 1	1.960	1.130	1.735	15680
	0.5 μg /l 2	2.687	1.538	1.747	21496
	0.5 μg /l 3	2.272	1.309	1.736	18176
	0.5 μg /l 4	2.389	1.349	1.771	28668
	0.5 μg /l 5	1.873	1.088	1.722	14984
	1.0 μg /l 1	1.040	0.647	1.607	4160
	1.0 μg /l 2	1.908	1.139	1.675	7632
	1.0 μg /l 3	2.084	1.215	1.715	16672
	1.0 μg /l 4	2.998	1.720	1.743	11992
	1.0 μg /l 5	2.360	1.338	1.764	18880
	5.0 μg /l 1	2.751	1.536	1.791	22008
	5.0 μg /l 2	2.512	1.444	1.740	20096
	5.0 μg /l 3	2.131	1.170	1.821	17048
	5.0 μg /l 4	2.923	1.657	1.764	11692
	5.0 μg /l 5	1.852	1.020	1.816	14816
3	Control 1	1.608	0.939	1.712	6432
	Control 2	1.858	1.058	1.756	7432
	Control 3	2.467	1.388	1.777	19736
	Control 4	1.657	0.953	1.739	13256
	Control 5	1.776	1.038	1.711	7104
	0.1 μg /l 1	1.661	0.959	1.732	13288
	0.1 μg /l 2	2.591	1.439	1.801	20728
	0.1 μg /l 3	2.614	1.481	1.765	10456
	0.1 μg /l 4	1.816	1.053	1.725	14528
	0.1 μg /l 5	1.803	1.002	1.799	7212
	0.5 μg /l 1	1.747	0.890	1.963	13976
	0.5 μg /l 2	1.464	0.759	1.929	11712
	0.5 μg /l 3	2.344	1.226	1.912	18752
	0.5 μg /l 4	2.774	1.462	1.897	22192
	0.5 μg /l 5	2.899	1.630	1.779	11596
	1.0 μg /l 1	1.899	0.996	1.907	15192
	1.0 μg /l 2	2.160	1.140	1.895	17280
	1.0 μg /l 3	1.987	1.020	1.948	7948
	1.0 μg /l 4	1.483	0.790	1.877	11864
	1.0 μg /l 5	2.606	1.465	1.779	10424
	5.0 μg /l 1	1.880	1.004	1.873	15040
	5.0 μg /l 2	2.383	1.238	1.925	19064
	5.0 μg /l 3	0.977	0.525	1.861	11724
	5.0 μg /l 4	1.521	0.837	1.817	6084
	5.0 μg /l 5	1.630	0.856	1.904	13040

Table F.3 (continue) The total RNA concentration from digestive tracts *Perna viridis* were treat with various mercuric chloride concentration for 1 month

Week	Sample	O.D.260	O.D.280	Ratio	Concentration (ng/μl)
4	Control 1	1.649	0.961	1.716	13192
	Control 2	1.735	1.004	1.728	6940
	Control 3	1.865	1.087	1.716	7460
	Control 4	1.306	0.782	1.670	5224
	Control 5	1.858	1.081	1.719	7432
	0.1 μg /l 1	1.362	0.814	1.673	5448
	0.1 μg /l 2	2.532	1.448	1.749	10128
	0.1 μg /l 3	2.298	1.323	1.737	9192
	0.1 μg /l 4	2.230	1.278	1.745	17840
	0.1 μg /l 5	1.586	0.926	1.713	12688
	0.5 μg /l 1	2.352	1.352	1.740	9408
	0.5 μg /l 2	2.089	1.201	1.739	16712
	0.5 μg /l 3	2.398	1.371	1.749	9592
	0.5 μg /l 4	1.650	0.966	1.708	13200
	0.5 μg /l 5	1.534	0.912	1.682	6136
	1.0 μg /l 1	2.585	1.474	1.754	10340
	1.0 μg /l 2	1.846	1.069	1.727	14768
	1.0 μg /l 3	2.837	1.600	1.773	11348
	1.0 μg /l 4	1.317	0.784	1.68	5268
	1.0 μg /l 5	2.269	1.292	1.756	9076
	5.0 μg /l 1	2.553	1.465	1.743	20424
	5.0 μg /l 2	1.890	1.097	1.723	15120
	5.0 μg /l 3	1.570	0.923	1.701	6280
	5.0 μg /l 4	1.903	1.100	1.730	15224
	5.0 μg /l 5	2.913	1.657	1.758	11652

Tables F.4 Single strand cDNA concentration from digestive tracts *Perna viridis* were treated with were treat with various mercuric chloride concentrations for 1 month

Week	Sample	O.D.260	O.D.280	Ratio	Concentration (ng/μl)
0	mussel1	0.258	0.164	1.573	1290
	mussel2	0.271	0.174	1.557	1355
	mussel3	0.271	0.172	1.576	1355
	mussel4	0.267	0.166	1.608	1335
	mussel5	0.264	0.166	1.590	1320
1	Control 1	0.281	0.172	1.634	1405
	Control 2	0.263	0.164	1.604	1315
	Control 3	0.263	0.165	1.594	1315
	Control 4	0.278	0.183	1.519	1390
	Control 5	0.274	0.171	1.602	1370
	0.1 μg/l 1	0.270	0.168	1.607	1350
	0.1 μg/l 2	0281	0.170	1.653	1405
	0.1 μg/l 3	0.261	0.165	1.582	1305
	0.1 μg/l 4	0.282	0.186	1.516	1410
	0.1 μg/l 5	0.253	0.166	1.524	1265
	0.5 μg/l 1	0254	0.155	1.639	1270
	0.5 μg/l 2	0.266	0.160	1.663	1330
	0.5 μg/l 3	0.257	0.161	1.596	1285
	0.5 μg/l 4	0.286	0.186	1.538	1430
	0.5 μg/l 5	0.270	0.170	1.588	1350
	1.0 μg/l 1	0.273	0.173	1.578	1365
	1.0 μg/l 2	0.258	0.161	1.602	1290
	1.0 μg/l 3	0.265	0.166	1.596	1325
	1.0 μg/l 4	0.288	0.179	1.609	1440
	1.0 μg/l 5	0.266	0.165	1.612	1330
	5.0 μg/l 1	0.266	0.167	1.593	1330
	5.0 μg/l 2	0.253	0.157	1.611	1265
	5.0 μg/l 3	0.276	0.176	1.568	1380
	5.0 μg/l 4	0.272	0.177	1.537	1360
	5.0 μg/l 5	0.273	0.175	1.560	1365
2	Control 1	0.261	0.162	1.611	1305
	Control 2	0.275	0.173	1.590	1375
	Control 3	0.290	0.181	1.602	1450
	Control 4	0.293	0.183	1.601	1465
	Control 5	0.273	0.168	1.625	1365
	0.1 μg/l 1	0.288	0.180	1.600	1440
	0.1 μg/l 2	0.278	0.172	1.616	1390
	0.1 μg/l 3	0.309	0.193	1.601	1545
	0.1 μg/l 4	0.301	0.189	1.593	1505
	0.1 μg/l 5	0.297	0.189	1.571	1485

Tables F.4 (continue) Single strand cDNA concentration from digestive tracts *Perna viridis* were treat with were treat with various mercuric chloride concentrations for 1 month

Week	Sample	O.D.260	O.D.280	Ratio	Concentration (ng/μl)
2	0.5 μg/l 1	0.279	0.171	1.632	1395
	0.5 μg/l 2	0.297	0.188	1.580	1485
	0.5 μg/l 3	0.287	0.177	1.621	1435
	0.5 μg/l 4	0.310	0.205	1.512	1550
	0.5 μg/l 5	0.298	0.194	1.536	1490
	1.0 μg/l 1	0.277	0.171	1.620	1385
	1.0 μg/l 2	0.286	0.178	1.607	1430
	1.0 μg/l 3	0.293	0.186	1.575	1465
	1.0 μg/l 4	0.286	0.180	1.589	1430
	1.0 μg/l 5	0.303	0.191	1.586	1515
	5.0 μg/l 1	0.285	0.174	1.638	1425
	5.0 μg/l 2	0.278	0.173	1.607	1390
	5.0 μg/l 3	0.291	0.187	1.556	1455
	5.0 μg/l 4	0.296	0.185	1.600	1480
	5.0 μg/l 5	0.280	0.174	1.609	1400
3	Control 1	0.236	0.146	1.616	1180
	Control 2	0.252	0.159	1.585	1260
	Control 3	0.295	0.200	1.475	1475
	Control 4	0.290	0.194	1.495	1450
	Control 5	0.281	0.186	1.511	1405
	0.1 μg/l 1	0.256	0.165	1.552	1280
	0.1 μg/l 2	0.292	0.192	1.521	1460
	0.1 μg/l 3	0.278	0.182	1.527	1390
	0.1 μg/l 4	0.305	0.204	1.495	1525
	0.1 μg/l 5	0.290	0.190	1.526	1450
	0.5 μg/l 1	0.254	0.160	1.588	1305
	0.5 μg/l 2	0.285	0.191	1.492	1425
	0.5 μg/l 3	0.267	0.176	1.517	1335
	0.5 μg/l 4	0.288	0.192	1.500	1440
	0.5 μg/l 5	0.288	0.192	1.500	1440
	1.0 μg/l 1	0.261	0.167	1.563	1305
	1.0 μg/l 2				
	1.0 μg/l 3	0.282	0.186	1.516	1410
	1.0 μg/l 4	0.276	0.183	1.508	1380
	1.0 μg/l 5	0.316	0.220	1.436	1580
	5.0 μg/l 1	0.268	0.176	1.523	1340
	5.0 μg/l 2	0.298	0.197	1.513	1490
	5.0 μg/l 3	0.271	0.175	1.549	1355
	5.0 μg/l 4	0.262	0.177	1.480	1310
	5.0 μg/l 5	0.287	0.191	1.503	1435

Tables F.4 (continue) Single strand cDNA concentration from digestive tracts *Perna viridis* were treat with were treat with various mercuric chloride concentrations for 1 month

Week	Sample	O.D.260	O.D.280	Ratio	Concentration (ng/μl)
4	Control 1	0.232	0.136	1.706	1160
	Control 2	0.232	0.139	1.669	1160
	Control 3	0.226	0.140	1.614	1130
	Control 4	0.229	0.136	1.684	1145
	Control 5	0.204	0.116	1.759	1020
	0.1 μg/l 1	0.213	0.122	1.746	1065
	0.1 μg/l 2	0.221	0.132	1.674	1105
	0.1 μg/l 3	0.227	0.137	1.657	1135
	0.1 μg/l 4	0.260	0.159	1.635	1300
	0.1 μg/l 5	0.245	0.146	1.678	1225
	0.5 μg/l 1	0.261	0.162	1.611	1305
	0.5 μg/l 2	0.232	0.140	1.657	1160
	0.5 μg/l 3	0.234	0.143	1.636	1170
	0.5 μg/l 4	0.228	0.138	1.652	1140
	0.5 μg/l 5	0.235	0.139	1.691	1175
	1.0 μg/l 1	0.229	0.141	1.624	1145
	1.0 μg/l 2	0.236	0.139	1.698	1180
	1.0 μg/l 3	0.250	0.157	1.592	1250
	1.0 μg/l 4	0.242	0.153	1.582	1210
	1.0 μg/l 5	0.258	0.157	1.643	1290
	5.0 μg/l 1	0.253	0.158	1.601	1265
	5.0 μg/l 2	0.258	0.168	1.536	1290
	5.0 μg/l 3	0.256	0.163	1.571	1280
	5.0 μg/l 4	0.232	0.142	1.634	1160
	5.0 μg/l 5	0.248	0.149	1.664	1240

Appendix F

F.1 Statistic of metallothionein gene expression of mussel from Samutprakarn

Oneway 1 week

ANOVA

MT

	Sum of squares	df	Mean square	F	Sig.
Between groups	1.101	4	.275	.779	.584
Within groups	1.765	5	.253		
Total	**2.866**	**9**			

Post Hoc Tests

Homogeneous Subsets

MT

Duncan[a]

CONC	N	Subset for alpha = .05
		1
1.00	2	.8800
2.00	2	.9775
3.00	2	1.5300
4.00	2	1.6150
5.00	2	1.6500
Sig.		.263

Means for groups in homogeneous subsets are displayed.
[a] Uses Harmonic Mean Sample Size = 2.000.

Correlations

Correlations

		CONC	MT
CONC	Pearson Correlation	1.000	.575*
	Sig. (1-tailed)	.	.041
	N	10	10
MT	Pearson Correlation	.575*	1.000
	Sig. (1-tailed)	.041	.
	N	10	10

* Correlation is significant at the 0.05 level (1-tailed).

F.1 Statistic of metallothionein gene expression of mussel from Samutprakarn (continue)

Oneway 2 weeks

ANOVA

MT

	Sum of squares	df	Mean square	F	Sig.
Between groups	3.917	4	.979	3.629	.095
Within groups	1.349	5	.270		
Total	**5.267**	**9**			

Post Hoc Tests

Homogeneous Subsets

MT

Duncan[a]

CONC	N	Subset for alpha = .05	
		1	2
1.00	2	1.2602	
2.00	2	1.9142	1.9142
5.00	2	2.1042	2.1042
3.00	2	2.1907	2.1907
4.00	2		3.2030
Sig.		.145	.063

Means for groups in homogeneous subsets are displayed.
[a] Uses Harmonic Mean Sample Size = 2.000.

Correlations

Correlations

		CONC	MT
CONC	Pearson Correlation	1.000	.580*
	Sig. (1-tailed)	.	.039
	N	10	
MT	Pearson Correlation	.580*	1.000
	Sig. (1-tailed)	.039	3
	N	10	10

* Correlation is significant at the 0.05 level (1-tailed).

F.1 Statistic of metallothionein gene expression of mussel from Samutprakarn (continue)

Oneway 3 weeks

ANOVA

MT

	Sum of squares	df	Mean square	F	Sig.
Between groups	.732	4	.183	.601	.679
Within groups	1.524	5	.305		
Total	**2.256**	9			

Post Hoc Tests

Homogeneous Subsets

MT

Duncan[a]

CONC	N	Subset for alpha = .05
		1
1.00	2	1.7563
5.00	2	2.1747
2.00	2	2.3552
3.00	2	2.3797
4.00	2	2.5486
Sig.		.223

Means for groups in homogeneous subsets are displayed.
[a] Uses Harmonic Mean Sample Size = 2.000.

Correlations

Correlations

		CONC	MT
CONC	Pearson Correlation	1.000	.662*
	Sig. (1-tailed)	.	.037
	N	8	8
MT	Pearson Correlation	.662*	1.000
	Sig. (1-tailed)	.037	.
	N	8	8

* Correlation is significant at the 0.05 level (1-tailed).

F.1 Statistic of metallothionein gene expression of mussel from Samutprakarn (continue)

Oneway 4 weeks

ANOVA

MT

	Sum of squares	df	Mean square	F	Sig.
Between groups	29.556	4	7.389	2.773	.147
Within groups	13.321	5	2.664		
Total	**42.877**	**9**			

Post Hoc Tests

Homogeneous Subsets

MT

Duncan[a]

CONC	N	Subset for alpha = .05	
		1	2
1.00	2	1.6621	
2.00	2	4.4699	4.4699
5.00	2	5.0870	5.0870
3.00	2	5.5838	5.5838
4.00	2		6.8376
Sig.		.069	.220

Means for groups in homogeneous subsets are displayed.
[a] Uses Harmonic Mean Sample Size = 2.000.

Correlations

Correlations

		CONC	MT
CONC	Pearson Correlation	1.000	.630*
	Sig. (1-tailed)	.	.026
	N	10	10
MT	Pearson Correlation	.630*	1.000
	Sig. (1-tailed)	.026	.
	N	10	10

* Correlation is significant at the 0.05 level (1-tailed).

F.1 Statistic of metallothionein gene expression of mussel from Samutprakarn (continue)

Oneway 5 weeks

ANOVA

MT

	Sum of squares	df	Mean square	F	Sig.
Between groups	3.132	4	.783	2.439	.177
Within groups	1.605	5	.321		
Total	**4.737**	**9**			

Post Hoc Tests

Homogeneous Subsets

MT

Duncan[a]

CONC	N	Subset for alpha = .05	
		1	2
1.00	2	.6202	
2.00	2	1.5093	1.5093
5.00	2	1.7319	1.7319
4.00	2	2.0004	2.0004
3.00	2		2.2501
Sig.		.067	.261

Means for groups in homogeneous subsets are displayed.
[a] Uses Harmonic Mean Sample Size = 2.000.

Correlations

Correlations

		CONC	MT
CONC	Pearson Correlation	1.000	.558*
	Sig. (1-tailed)	.	.047
	N	10	10
MT	Pearson Correlation	.558*	1.000
	Sig. (1-tailed)	.047	3
	N	10	10

* Correlation is significant at the 0.05 level (1-tailed).

F.1 Statistic of metallothionein gene expression of mussel from Samutprakarn (continue)

Oneway 7 weeks

ANOVA

MT

	Sum of squares	df	Mean square	F	Sig.
Between groups	.177	4	4.425E-02	0.135	.932
Within groups	1.636	5	.327		
Total	**1.813**	**9**			

Post Hoc Tests

Homogeneous Subsets

MT

Duncan[a]

CONC	N	Subset for alpha = .05
		1
5.00	2	1.8007
1..00	2	1.8044
4.00	2	1.8530
3.00	2	2.0110
2.00	2	2.1395
Sig.		.585

Means for groups in homogeneous subsets are displayed.
[a] Uses Harmonic Mean Sample Size = 2.000.

Correlations

Correlations

		CONC	MT
CONC	Pearson Correlation	1.000	-.098
	Sig. (1-tailed)	.	.394
	N	10	10
MT	Pearson Correlation	-.098	1.000
	Sig. (1-tailed)	.394	.
	N	10	10

F.2 Statistic of metallothionein gene expression of mussel from Trad

Oneway 1 week

ANOVA

MT

	Sum of squares	df	Mean square	F	Sig.
Between groups	3.409E-02	4	8.522e-03	.977	.442
Within groups	0.174	20	8.721E-03		
Total	**0.209**	**24**			

Post Hoc Tests

Homogeneous Subsets

MT

Duncan[a]

CONC	N	Subset for alpha = .05
		1
1.0	5	.9061
4.00	5	.9101
2.00	5	.9767
3.00	5	.9814
5.00	5	.9907
Sig.		.212

Means for groups in homogeneous subsets are displayed.
[a] Uses Harmonic Mean Sample Size = 5.000.

Correlations

Correlations

		CONC	MT
CONC	Pearson Correlation	1.000	.159
	Sig. (1-tailed)	.	.447
	N	25	25
MT	Pearson Correlation	.159	1.000
	Sig. (1-tailed)	.447	.
	N	25	25

F.2 Statistic of metallothionein gene expression of mussel from Trad (continue)

Oneway 2 weeks

ANOVA

MT

	Sum of squares	df	Mean square	F	Sig.
Between groups	.373	4	9.334E-02	3.255	.033
Within groups	.574	20	2.868E-02		
Total	**.947**	**24**			

Post Hoc Tests

Homogeneous Subsets

MT

Duncan[a]

CONC	N	Subset for alpha = .05	
		1	2
1.00	5	1.1303	
5.00	5	1.2516	1.2516
2.00	5	1.3429	1.3429
3.00	5		1.3808
4.00	5		1.4926
Sig.		.074	.050

Means for groups in homogeneous subsets are displayed.
[a] Uses Harmonic Mean Sample Size = 5.000.

Correlations

Correlations

		CONC	MT
CONC	Pearson Correlation	1.000	.623**
	Sig. (1-tailed)	.	.003
	N	20	20
MT	Pearson Correlation	.623**	1.000
	Sig. (1-tailed)	.003	.
	N	20	20

** Correlation is significant at the 0.01 level (2-tailed).

F.2 Statistic of metallothionein gene expression of mussel from Trad (continue)

Oneway 3 weeks

ANOVA

MT

	Sum of squares	df	Mean square	F	Sig.
Between groups	.141	4	3.526E-02	3.656	.022
Within groups	.193	20	9.645E-03		
Total	**.334**	**24**			

Post Hoc Tests

Homogeneous Subsets

MT

Duncan[a]

CONC	N	Subset for alpha = .05		
		1	2	3
1.00	5	.9055		
2.00	5	.9729	.9729	
3.00	5	1.0183	1.0183	1.0183
5.00	5		1.0791	1.0791
4.00	5			1.1169
Sig.		.100	.120	.148

Means for groups in homogeneous subsets are displayed.
[a] Uses Harmonic Mean Sample Size = 5.000.

Correlations

Correlations

		CONC	MT
CONC	Pearson Correlation	1.000	.601**
	Sig. (1-tailed)	.	.001
	N	25	25
MT	Pearson Correlation	.601**	1.000
	Sig. (1-tailed)	.001	.
	N	25	25

** Correlation is significant at the 0.01 level (2-tailed).

F.2 Statistic of metallothionein gene expression of mussel from Trad (continue)

Oneway 4 weeks

ANOVA

MT

	Sum of squares	df	Mean square	F	Sig.
Between groups	.250	4	6.253E-02	4.351	.011
Within groups	.287	20	1.437E-02		
Total	**.538**	**24**			

Post Hoc Tests

Homogeneous Subsets

MT

Duncan[a]

CONC	N	Subset for alpha = .05	
		1	2
1.00	5	.8646	
2.00	5	.8996	
5.00	5	.9708	
3.00	5	.9938	
4.00	5		1.1533
Sig.		.133	1.00

Means for groups in homogeneous subsets are displayed.
[a] Uses Harmonic Mean Sample Size = 5.000.

Correlations

Correlations

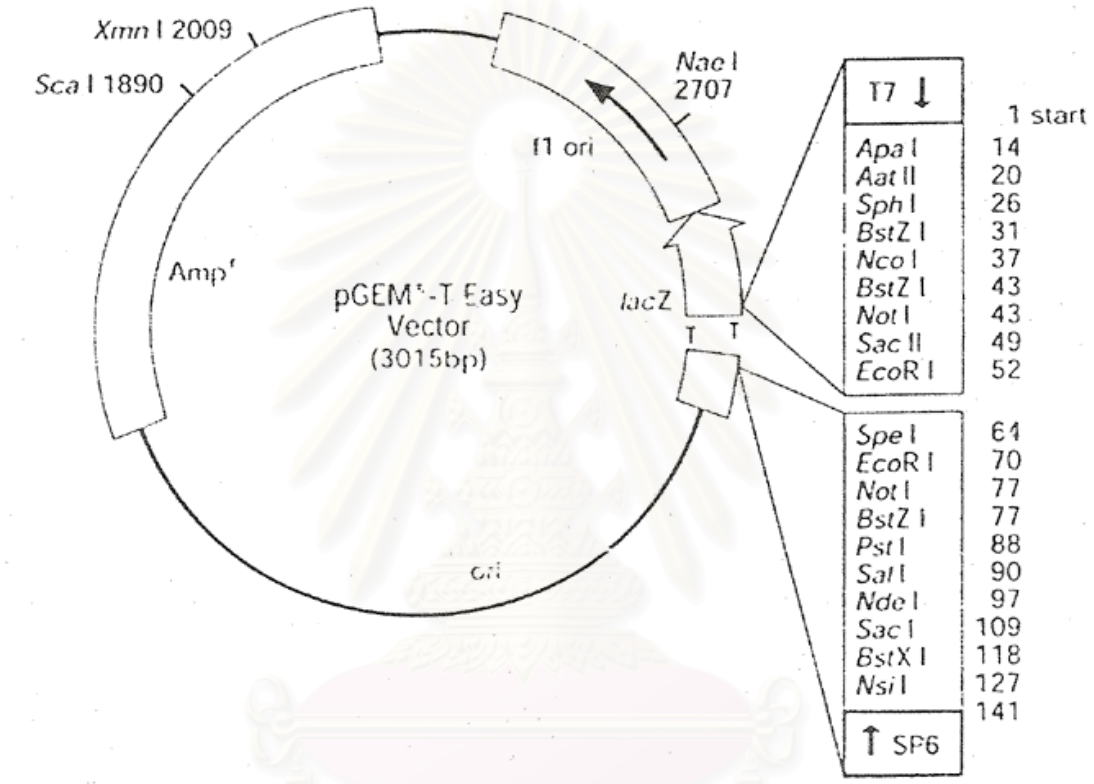
		CONC	MT
CONC	Pearson Correlation	1.000	.450*
	Sig. (1-tailed)	.	.024
	N	25	25
MT	Pearson Correlation	.450*	1.000
	Sig. (1-tailed)	.024	.
	N	25	25

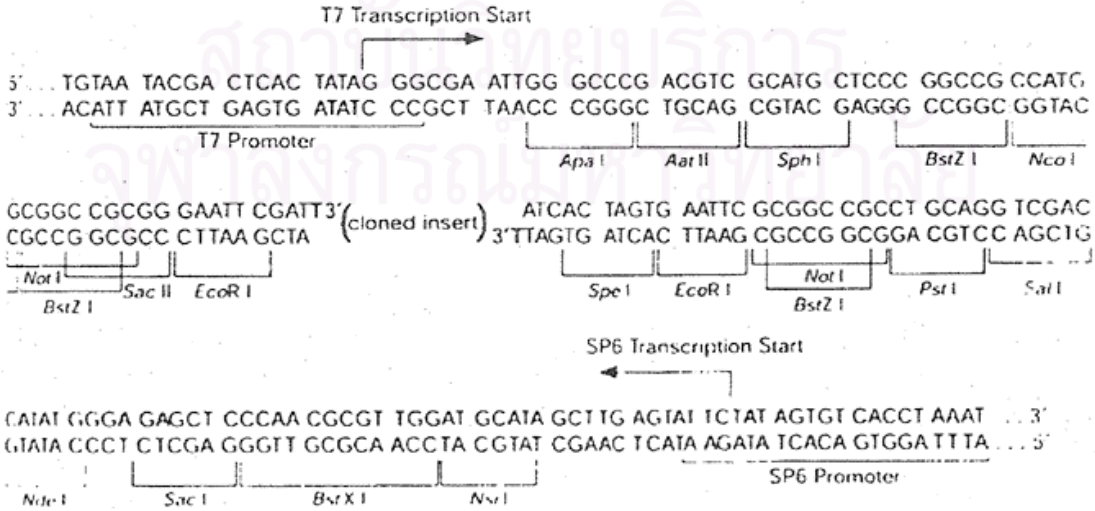
* Correlation is significant at the 0.05 level (1-tailed).

Appendix G

Retriction mapping of pGEM® T-easy Vector

pGEM® T-easy Vector





BIOGRAPHY

Miss Chinda Parsont was born on November 28, 1978 in Pathumthani, Thailand. She graduated with the degree of Bachelor of Science in Marine Science from Chulalongkorn University in 1999. She has studied for a degree of Master of Science at the Inter-Department of Environmental Science, Chulalongkorn University since 2000.