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CLONING AND CHARACTERIZATION OF HEAT SHOCK PROTEIN GENES FROM THE HEMOCYTES OF BLACK TIGER PRAWN *Penaeus monodon*

Miss Atchariya Buethong

สถาบันวิทยบริการ
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The experiment was firstly conducted on the cell maintainance of *P. monodon* haemocytes in 3 different media. High viability and relatively high activity of the cells were found in the haemocytes maintained in M199 and the haemocyte were maintained for 4 days with high survival rate whereas the haemocytes maintained in TC100 and Grace's insect media showed high activity but very low viability. Therefore, M199 were further used for maintaining the haemocytes in heat shock experiment. Haemocytes were treated with the temperature of 4, 30, 33, and 35°C for 1 and 2 h. Protein profiles of haemocyte extracts were detected using polyacrylamide gel electrophoresis. The result revealed the difference of peptide bands in samples extracted from different heat treatment. However, the consistency of the results was not satisfied. When Western blot analysis using specific antibodies was carried out, only HSP90 was detected. The sensitivity was also low therefore it was not suitable for quatitative analysis. The investigation on heat induced genes using differential display technique obtained 10 transcript markers. Nine of them were unknown genes and one was identified as vigilin gene. The EST analysis of heat induced haemocyte cDNA library provided DNA sequences of 1090 clones. Of these, 687 clones (63%) were identified genes and 132 clones (12.1%) were reported to be involved in the defense system and homeostasis. Full length sequences of HSP60, HSP70, and HSP90 genes were completed by the combination of techniques. This included RT-PCR, RACE-PCR and cDNA library screening. The results revealed that the ORF of HSP60 was 1731 bp coding for 576 amino acids, HSP70 ORF was 1959 bp coding for 652 amino acids, and HSP90 ORF was bp coding for amino acids. All 3 genes contained a number of specific HSP patterns of their kinds confirming the identity of each gene. The results of this study will be basic knowledge for HSP function investigation and will be useful for biomarker application in stress condition and breeding selection in *P.monodon.*

Field of Study .....Biotechnology.......Student's signature...

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Co-advisor's signature..

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LIST OF ABBREVIATIONS

Abbreviations	Term
aa	Amino acid
AMV	Avian myeloblastic virus
bp	Base pair
BLAST	Basic Local Alignment Search Tool
BSA	Bovine serum albumin
cm	Centimeter
CIAA	Chloroform:isoamyl alcohol
cDNA	Complementary deoxyribonucleic acid
^{O}C	Degree celsius
DEPC-H_2O	Diethylpyrocarbonate treated distilled water
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DW	Distilled water
E. coli	*Escherichia coli*
gm	Gram
GenBank	Genetic databank
hr	Hour
IPTG	Isopropyl-β-D-thiogalactoside
Kb	Kilobase
kDa	Kilodalton
L	Litre
LB	Luria-Bertani medium
2-ME	2-mercaptoethanol
µg	Microgram
µl	Microlitre
mg	Milligram
mM	Millimolar
ml	Millitre
min	Minute
M	Molar
MW	Molecular weight
ng	Nanogram
nm	Nanometer
NCBI	National Center for Biotechnology Information
OD	Optical density
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RT	Room temperature
rpm	Rovolution per minute
sec	Second
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Taq	*Thermus aquaticus*
TBE	Tris borate EDTA buffer

LIST OF ABBREVIATIONS (cont.)

Abbreviations	**Term**
TE	Tris-EDTA buffer
TEMED	N,N,N’,N’-tetraethylenediamine
U	Unit
UV	Ultraviolet
V	Volt
v/v	Volume by volume
w/v	Weight by volume

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

CHAPTER I

INTRODUCTION

Black tiger shrimp, *Penaeus monodon* is the most important species for commercial shrimp culture in Thailand. Currently, infectious diseases are the main problems in shrimp production. Disease outbreak mainly caused by viruses has greatest impact on shrimp culture. As it has been known that shrimp immune system is so different from higher animals. They have no specific antibodies as normally found in vertebrates. To eliminate potential infectious microorganisms, the defense system rely entirely on innate immunity, both cellular and humoral components (Bachere, 2000; Vargas and Yepiz, 1998). In order to find to solution for the disease problems, much attention has been paid to the immune relating proteins found in the haemolymph, haemocyte and in many certain tissues. In response to stressful stimuli, cells increase synthesis of one or more families of stress proteins known as heat shock proteins (HSPs). The expression of HSPs is rapidly up-regulated by various stressors and physiological perturbations (Fink and Goto, 1998). Acute stressors can be both immuno-stimulating and immunosuppressive effects depending on species and physiological status of the animal. Furthermore, induction of HSPs has also been observed after infection of cells with a variety of bacteria or viruses. HSPs perform essential biological functions under both physiological and stressful conditions. General functions attributed to HSPs include preventing protein aggregates under physical stress, serving as molecular chaperones in protein transport between cell organelles and contributing to the folding of nescent and altered proteins. This also includes a role in immunological process. The exposure of organisms to stressors induces the expression of HSPs, as they allow cell survival during and after stress. HSPs are families of proteins, classified according to their apparent molecular weight into four major groups, hsp90, hsp70, hsp60, and low-molecularweight proteins (Santoro, 2000). The elevation in HSP levels was detected in cells under a variety of harmful stimuli. The connection between stress responses and disease resistance has been reported in various numbers of domesticated animals. In shrimps, information concerning the effects of stress on immune functions is scarce. Therefore, the identification and characterization of the genes that involved in defense and

homeostasis are urgently needed in order to prevent and control diseases which are essential for further development of a sustainable shrimp culture.

1.1 Penaeid shrimp biology

Penaeid shrimps are classified into subphylum Crustacea, phylum Arthropoda which is made up of 42,000, predominantly aquatic species, that belong to 10 classes. Within the class Malacostraca, shrimps together with crayfish, lobsters, and crabs, belong to the order Decapoda. The exterior of penaeid shrimp is distinguished by a cephalothorax with a characteristic hard rostrum, and by a segmentd abdomen. In the head region, antennules and antennae perform sensory functions. In the thorax region, the maxillipeds are the first three pairs of appendages, modified for food handling, and the remaining five pairs are the walking legs (pereiopods). Five pairs of swimming legs (pleopods) are found on the abdomen (Baily-Brock and Moss, 1992). A lage part of the cephalothorax in penaeid shrimp is occupied by the hepatopancreas. The main functions of the hapatopancrease are the absorption of nutrients, storage of lipids and production of digestive enzyme (Johnson, 1980). The haemocytes are produced in haematopoietic tissue. This organ is dispersed in the cephalothorax. Organic compounds in the water stimulate the foraging activity of the shrimps. Shrimps slowly chew on the food by means of their mandibles and maxillae (Baily-Brock and Moss, 1992). Shrimp are omnivorous when their food is of poor quality and scare, they will eat any food and have the tendency to become cannibalistic.

1.2 Shrimp culture

Development of shrimp farming

Shrimp farming started more than a century ago in Southeast Asia where farmers raised incidentally wild shrimp crops in tidal fish ponds (Rosenberry, 1997). In 2000, more than 85% of the cultured shrimp production was still raised by farmers in the eastern hemisphere. Thailand was the main shrimp farming country, followed by China, Indonesia and India (Rosenberry, 2001). To a lesser extent, shrimp are produced in Latin America, with Ecuador as the leading country. At present, shrimp

farming is also substantially expanding towards the Middle East and Africa (Rosenberry, 2001).

Important culture species

The most important cultured penaeid shrimp species are the black tiger shrimp (*Penaeus monodon*), Pacific white shrimp (*P. vannamei*), kuruma shrimp (*P. japonicus*), blue shrimp (*P. stylirostris*) and Chinese white shrimp (*P. chinensis*). World shrimp production is dominated by *P. monodon*

P. monodon is the largest, reaching 330 mm or more in body length, and exhibits the highest growth rate of all cultured penaeids (Lee and Wickins, 1992). *P. monodon* can reach a market size up to 25-30 g within 3-4 months after PL stocking in cultured ponds and tolerates a wide range of salinities (Rosenberry, 1997). Those facts together make the black tiger shrimp an interesting species to culture. Although *P. monodon* was normally considered as exceptionally tough, the rapid growth and intensification of its culture industry generated crowding and increased environmental degradation, which made the animals more susceptible to diseases (Lightner, 1983; Johnson, 1989). Nowadays, many disease problems are associated with this important culture and, therefore, *P. monodon* was chosen in the present research as model to study the stress response in relation to the shrimp defense system.

Major constraints in shrimp culture

Rapid growth of commercial shrimp operation may lead to over fishing of wild shrimp broodstocks. In addition, the expansion of shrimp culture is accompanied by local environmental degradation and the occurrence of diseases of both infectious and non-infectious etiologies (Lightner *et al*., 1992). Disease outbreaks, mainly caused by viruses and bacteria and to a lesser extent by rickettsiae, fungi and parasites, may cause losses up to 100% (Johnson, 1989; Lightner *et al*., 1992; Lightner and Redman, 1998).

Up until now, approximately 20 viruses have been described in shrimp culture. The white spot syndrome virus (WSSV) and yellow head virus (YHV) have had the greatest impact on shrimp culture and, at present, still cause the major disease problems (Rosenberry, 2001). Other important viruses are infectious hypodermal and

haemotopoietic necrosis (IHHN) virus, hepatopancreatic parvovirus (HPV), baculoviral midgut gland necrosis (BMN) virus, baculovirus penaei (BP), monodon baculovirus (MBV), lymphoid organ vacuolisation virus (LOVV) and Taura syndrome virus (TSV) (Lightner, 1996). Only a small number of bacterial species have been diagnosed as infectious agents in penaeid shrimp. *Vibrio* spp. are by far the major bacterial pathogens and can cause severe mortalities, particularly in hatcheries. Vibriosis is often considered to be a secondary infection, which usually occurs when shrimp are weakened (Johnson, 1989; Lightner et al., 1992). Primary pathogens can kill even when other environmental factors are adequate, whereas opportunistic pathogens are normally present in the natural environmental of the host and only kill when other physiological or environmental factors are poor.

1.3 Shrimp defense system

Evolution of the immune system

Two systems providing internal defense against infectious agents have been selected during evolution, the innate (natural) and the acquired (adaptive) immune systems. The acquired immune system, which is phylogenetically younger, is found only in vertebrates and operates through lymphocytes. The innate immune system can be found in all multicellular animals and consists of cellular and humoral elements. The most prominent cellular defense reactions against invading microorganisms are phagocytosis, encapsulation, cell-mediated cytotoxicity, and clotting. The humoral defense factors, such as clotting proteins, agglutinins, hydrolytic enzymes and antimicrobial peptides are often produced by and act in conjunction with the defense cells. Even though the immune system of invertebrates has often been described as far less complicated than that of vertebrates, it is still very efficient and complex. Invertebrates have managed to occupy nearly all habitats on earth and consequently, they have to cope with an extremely large variety in pathogens. The efficacy of their defense system is witnessed by their persistent survival through many years of evolution (Millar and Ratcliffe, 1994)

Study of the immune system

The extensive study of vertebrate defense including the origin and development of the different blood cell types results in a fairly uniform scheme of morphological and immuno-functional classification of blood cells. Moreover, purification and characterisation of individual defense proteins explain many of the immune functions. In contrast, the huge diversity of invertebrates and the limited knowledge of their haemocyte lineages make it difficult to categorize haemocytes in morphologically well-defined ontogenic classes. In addition, haemocytes are very reactive cells and undergo considerable transformation when removed from the haemocoel (Bauchau, 1981), thus functional characterisatics of those cells are more difficult to study than vertebrate blood cell functions. Haemocyte activation results in rapid clotting, cellular degranulation, activation of the proPO system and subsequently the production of sticky molecules (Johansson and Soderhall, 1992). The labile nature of several defence proteins and the low quantity of those proteins in the haemolymph also complicate the purification of individual proteins of the invertebrate defence system (Soderhall *et al.*; 1990). During the last few year, considerable progress has been made in utilising different anticoagulants and media to keep the haemocytes closer to their natural state (Bachere, 2000), which has provide opportunities for reliable in vitro functional studies. In addition, the cloning and characterisation of genes during infection or defence stimulation will also lead to a better understanding of the functioning of the defence system (Gross *et al.*, 2001). The combination of different approaches will highly contribute to an improved knowledge.

Haemocyte classification

The haemocytes play an important and central role in the internal defence. The hard cuticle, a physical barrier that also may contain antimicrobial factors can be considered as the external defence in crustaceans. Although until now three different cell types have been commonly described. However, a universally accepted haemocyte classification scheme is not yet available for penaeid shrimp.

Hyaline

In general the hyaline cell is the smallest cell type with a high nucleus to cytoplasm ratio and no or few cytoplamic granules. Primary role in clotting, also involved in phagocytosis.

Granular

The granular cell is the largest cell type with a relatively smaller nucleus and fully packed with granules compared to hyalin cells. They can be distinguished from the semigranulocytes by the presence to numerous, large granules. The granulocytes do not lyse during clotting, but some may dhisce to release their granules when exposed to bacterial invaders.

Semigranular

The semigranular cell is an intermediate between the hyaline and the granular cell (Bauchau, 1981; Soderhall and Cerenius, 1992) with a low nucleus to cytoplasm ratio, and several sub-micron and micron sized granules. Semigranulocytes are distinguished from granulocytes by the central location of the nucleus, a mixture of granule sizes as opposed to a relatively constant size.

Function of the shrimp defense system

The first and essential internal defence process is the recognition of invading micro-organisms which is mediated by the haemocyte and plasma proteins (Vargas-Albores and Yepiz-Plascencia, 2000). The invertebrate immune system presumably recognises large group of pathogens, represented by fixed common molecular patterns, rather than fine structures, specific for particular microbes (Soderhall et al.; 1996). Several types of recognition proteins have been described and are called pattern recognition proteins (PRPs). The PRPs recognise carbohydrate moieties of cell wall components of micro-organisms, like lipopolysaccharides (LPS) or peptidoglycans (PG) from bacteria, or β–1,3-glucans from fungi (Soderhall et al., 1996; Vargas-Albores *et al*., 1996; 1997). Some of the PRPs are lectins and can work directly as agglutinins or opsonins (Soderhall *et al*., 1996). After binding of the PRP ligand with the microbial component, a second site becomes active for cellular binding. Haemocyte activation is generated after this second binding step (Vargas-

Albores and Yepiz-Plascencia, 2000). Recently, the β−1,3-glucan binding protein of *P. monodon* was cloned and sequenced (Sritunyalucksana *et al.*, 2002). The defence proteins that are involed in the defence system have been isolated until now from *P.monodon* are β−1,3-glucan binding protein (Sritunyalucksana *et al.*, 2002), peroxinectin (Sritunyalucksana et al., 2001), Kazal inhibitor (Sritunyalucksana et al., 2001), transglutaminase (H.H. Song (unpublished)), clotting protein (Yeh et al., 1999) and proPO (Sritunyalucksana *et al.*, 1999). After detection of foreign material, haemocytes to the site of invasion by a process of chemotaxis that results in inflammation, which also appears a relevant event in vertebrates. The open circulatory system demands a rapid and efficient defense in which the proteolytic cascades play an important role (Sritunyalucksana and Sorderhall, 2000). The haemocyte are involed in the synthesis, storage and upon activation discharge of proenzymes and substrates of the clotting and proPO cascades (Johansson and Soderhall, 1992; Soderhall *et al.*, 1996; Sritunyalucksana and Sorderhall, 2000). The clotting mechanism entraps foreign material and prevents loss of haemolymph. The transglutaminase (TGase) dependent clotting reaction of crustaceans is best described in the freshwater crayfish *Pacifastacus leniusculus* (Hall et al., 1999). The clotting protein is induced when TGase is released from the haemocytes or tissues.

The proPO activating system in crustaceans is also the most extensively study in the freshwater crayfish *P. leniusculus* (Soderhall *et al.*, 1996; Soderhall and Cerenius, 1998). Proteins of the proPO system occupy a very prominent position in non-selt recognition, haemocyte communication and the production of melanin. Upon activation and degranulation of the haemocytes, the inactive proPO is converted to the active phenoloxidase (PO) by prophenoloxidase activatig enzyme. Malanin is a dark brown pigment that sequesters the pathogens, thus preventing their contact with the host. Melanised matter can often be seen as dark spots in or under the cuticle of arthopods.

Phagocytosis is the internalisation of small foreign particles by individual cells. After ingestion, also shrimp haemocytes like vertebrate blood cells use cytotoxic oxygen radicals to kill the foreign material (Song and Hsieh, 1994; Munoz *et al.*, 2000). If large amounts of particles enter the body or if the they are too large to be internalised several haemocytes will cooperate to seal off the pathogens these

phenomena are called nodule formation and encapsulation, respectively (Soderhall *et al*., 1996).

1.4 Health management

Disease control

Disease can be seen as the resultant of a complex interaction between host, pathogen and environment. The environment of aquatic animals is abounded with infectious microbes. The transmission of disease in this environment is extremely easy, especially under dense culture conditions. Losses, due to diseases whether by slow continuous attrition or by sudden catastrophic epizootics, are serious problems for shrimp culture industry. Correct diagnosis including knowledge of the life cycle and ecology of the pathogens is obviously a critical step in any control programs. Epidemiological surveys of viruses are still marginally performed. However, technologies for quick recognition of pathogens in shrimp culture are developing rapidly and diagnostic probes which can be used in screening of shrimp pathogens (Lightner, 1996)

Also chemotherapy preferably combined with preventive measure, is widely applied in the control of many infectious diseases in aquaculture. However, this type of chemical control should be considered as a last resort because of growing concern for food quality, accumulation of such substances in the environment and increase in the spread of antibiotic or drug resistant pathogenic strains.

In shrimp culture prevention may include environmental manipulation such as the culture of shrimp in salinities below that which certain *Vibrio* pathogens survive. Further more, immuno-stimulants, like –glucan which induce and build up protection against a wide range of diseases, become increasingly important in aquaculture. An immuno-stimulant is a chemical, drug, stressor or action that enhances the defence mechanisms or immune response (Anderson, 1992), thus rendering the animal more resistant to diseases. In cases where disease outbreaks are cyclic and can be predicted, immuno-stimulants may be used in anticipation of events to elevate the non-specific defense mechanism, and thus prevent losses from diseases. However, caution should be taken as a number of the potent immuno-stimulants may suppress or alter certain biological pathways if used inappropriately.

Vaccination and defense stimulation

A vaccine is a compound that induces a specific immune response againt one pathogen. Non-specific immuno-stimulants may be administered together with a vaccine to activate non-specific defence mechanisms as well as to enhance a specific immune response (Aderson, 1992). Different methods have been developed to administer a vaccine to fish and the most efficacious vaccination strategy is by injection. This method is labour intensive and time consuming and it is not feasible in very small animals. Another way of vaccination is by immersion. This method is simple and can be carried out rapidly(Ellis,1988). Nevertheless, it is not efficacious for all diseases, large quantities of vaccine are required and until now the mode of functioning of this method is still no fully understood. The invertebrate defence system is often described as based only on innate immunity, which excludes the possibility of vaccination. Several reports have been published about experiments to enhance the invertebrate defence mechanisms with great potential (Schpiro et al., 1974; Stewart and Zwicker, 1974; Itami and Takahashi, 1991; Alabi, 1999; Vici et al., 2000). As a stimulant, most studies used killed cells, yeast glucans or derived elements or a combination of those two components, which are also widely used for fish (Sakai, 1999). Immuno-stimulation will certainly continue to play an important role in disease control in intensive shrimp culture. Many study (Itami *et al.*, 1994; Sung et al., 1994; 1996; Goarant and Bogio, 2000) deal with the effect of immune stimulation on cellular factors of the defence system of shrimp. A scientific analysis of the underlying mechanisms affecting the efficacy of the stimulant and the constitution of protective defence is required to make effective progress in this field. Obviously, fundamental research on the functioning of the defence system has received less attention than has research from which the results can directly be applied to increase the profit margin either by expansion of the production or by reduction of the costs. However, for efficient and effective research on defence stimulation, practically applicable parameters are needed. These should be based on scientific data and they are of major important to qualify and quantify stimulation of the defense system.

Health parameters

To evaluate the health status of cultured shrimp, farmers nowadays commonly consider a number of variables, including production traits like survival rate, mortality rate, growth rate, feed conversion ratio, size variation and changes in appearance and colour of organs. Also specific stress tests, behavioural, physical and gut content examinations are widely used (Brock and Main, 1994). The occurrence of infectious disease can be detected more specifically by wet-mount microscopy, histopathology, electron microscopy and immuno-cytochemical method (Brock and main, 1994). DNA based technologies, like hybridization with cloned probes and amplyfying sequences by polymerase chain reaction (PCR) are nowadays rapidly expanding (Roch, 1999). However, in comparison with the vertebrates in commercial animal production, there are practically no criteria for specific evaluation of the health status of shrimp and invertebrates in general (Bachere, 2000).

In general, an ideal health parameter reflects a relevant immune function, is related to the health condition is easy to quantify and is found in different species. In order to study the invertebrate internal defence system knowledge and experience of vertebrate immunity is frequently used. However, haemotology one of the principle diagnostic tools of human and veterinary medicine, is so sporadically used as a diagnostic tool in penaeid shrimp pathology. Nevertheless, studies have been carried out in which changes in haemolymph parameters were used to detect physiological variation. Many variables such as total plasma protein content, glucose concentration, alkaline phosphatase activity, clotting time, haemocyte count, prophenoloxidase (proPO) activity, phagocytic index, release of reactive oxygen intermediates and antibacterial activity have been considered as potential health or disease markers in crustaceans. (Stewart et al., 1969; Hose et al., 1984; Persson et al., 1987; Hall and Van Ham, 1998; Rodriguez and le Moullac, 2000). However, only haemolymph clotting time and changes in total haemocyte count are sporadically used by shrimp disease diagnosticians (Lightner, 1996).

In addition, heat shock proteins have recently been demonstrated in shrimp and might also act as a potential health parameter (Gross *et al*., 2001).

The currently available haemolymph markers transiently change shortly after infection or application of the stimulus. Markers that are capable to demonstrate chronic stressors or infections unfortunately still await elucidation. A better understanding of the haemocyte lineages and the haemolymph defence system will facilitate a further development of health parameters.

1.5 Stress responses

Living cells are continually challenged by conditions which cause acute and chronic stress. To adapt to environmental changes and survive different types of injuries, eukaryotic cells have evolved networks of different responses which detect and control diverse forms of stress. One of these response, known as the heat shock response (Santoro, 2000). The observation that an increase in temperature of a few degrees above the physiological level induces the synthesis of a small number proteins in Drosophila salivary glands led to the discovery of a universal protective mechanism which prokaryotic and eukaryotic cells utilize to preserve cellular function and homeostasis (Linquist et al., 1988). The phisiological defence mechanism, known as the heat shock response, involves the rapid induction of a specific set of genes encoding cytoprotective proteins (heat shock protein) (Morimoto et al., 1998). Heat shock proteins synthesis is induced not only by hyperthemia, but can be triggered by a wide variety of toxic conditions which lead to the accumulation of non-native proteins, including alterations in the intracellular redox environment, exposure to heavy metals, amino acid analogs or cytotoxic drugs, glucose deprivation, and virus infection (Feige et al., 1996).

1.6 Heat shock proteins

The heat shock proteins or stress proteins are among the most abundant intracellular proteins. Prokaryotic and eukaryotic cells react to exposition unfavourable conditions of the outer environment by increased synthesis of the stress proteins (Petr Kopecek *et al.*, 2001) . The structure and functions of these proteins are evolutionary highly conserved and they are present in different variations in the cell of all living organisms. Increased synthesis of the stress protein apparently correlates

with an organism's resistance to stress and with a level of own stress (Feder, 1999). An expression of heat shock proteins is induced by many various factors in the cell. These factor include: a) change in temperature, pH, osmolarity and radiation, and b) higher concentration of heavy metal, ethanol, antibiotics, fatty acids and reactive oxygen forms (Petr Kopecek *et al*., 2001). The main functions of stress proteins which are essential for reparation of each living cell which is damaged by stress. They are the participation in protein folding into their correct tertiary structure, incorporation of polypeptides into intracellular membranes or in transport of proteins across those membranes (Van der Vies et a., 1993, Mathew A., and Morimoto, 1998). Although during stress the synthesis of the stress proteins has increased considerably, a lot of the stress proteins are expressed as the constitutive proteins, and they play the significant role even in the cells which are not exposed to the stress factor (Harboe, and Quayle, A. J., 1991).

1.7 Nomenclature and the basic division of heat shock proteins

The study of Tissieres et al. (1974) introduced the term "heat shock proteins", and it belong to the beginning of research on the stress proteins (Tissieres *et al.* 1974). In a context of current knowledge, the term "protein heat shock proteins" especially in eukayotes, is used rather as a historical name. Particularly, it still overlaps with the logically evidently more correct term "stress proteins". This name specifies all the group of proteins generally where expression has increased due to an incidence of the stress factors. An abbreviation for the stress protein(s) "HSP" already remains in use. Sometimes another abbreviation, "HSC' (heat shock cognates), which has been used for the constitutive forms of HSP. Those forms of HSP are also present at non-stressed cells, and in contrast to the majority of other proteins, their intracellular concentrations have been increased during the heat shock.

The term "chaperone" is used very often. This terms point out the functionof the protein directly. It concerns the stress as well as non-stress proteins, which accompany unfolded polypeptides during their cellular transport, and they make passage of protein through the membranes possible or their integration into cellular organelles. A similar, well known term "chaperonin" is the alternative name for the GroEL protein. It is abbreviated as "cpn60" or "HSP60". (Petr Kopecek *et al*., 2001).

The division of HSP into the families is not standardised precisely yet. Earlier dividing of families : HSP90, HSP70, HSP60 and small HSP has been extended step by step to HSP110, HSP100, HSP90, HSP70, HSP60, HSP40 HSP10 and small HSP families (Feder, 1999, Tanguay *et al.*, 1999). Numeric indexing represents the protein molecular masses in kDa. The stress proteins are registered into appropriate families according to their approximate molecular masses, their functions in the cells and their homologies in the primary structures. The major HSPs attention will be dedicated to them are HSP90, HSP70 and HSP60.

HSP90

HSP90 (HSP83) is the most abundant cytosolic protein in the eukaryotic cells. Its homologues were found in the endoplasmic reticula(ER) or higher eukaryotes and in prokaryotic cells. HSP90 have two homologue isoforms those are indicated as alpha and beta and are produced in the same quantity (Parsell, 1993). Under physiological conditions. HPS90 was found in association with several intracellular proteins including calmodulin, actin, tubulin, several kinase, and some receptor proteins (Schwartz *et al.* 1993, Jakob, 1994). In case of the glucocorticotropic receptor, binding of HSP90 leads to an enhancement capability of the receptor to bind to the steriod hormone (Hutchison *et al*., 1994). HSP90 has even chaperone function (Yahara et al., 1998). that is comparable to GroEL function and can suppress assembling into their tertiary structure (Wiech *et al*., 1992). Cytosolic HSP90 aggregates with HSP70 under the stress conditions and it is suggested that interaction occurs of both HSP(s) with unfolded proteins (Jakob, U., Buchner, J., 1994).

HSP70

Proteins in the HSP70 family are known for their ability to bind peptide chains. They act in a protection of the nascent proteins, a protein transport across the membranes, repeated assembling of unfolded proteins and the protein degradation (Craig, 1993; Becker, J., Craig, E. A., 1994; Frydman et al., 1994).

HSP60

The chaperonins (the group of stress proteins belonging to the HSP60 family) have significant roles in polypeptide folding and in protein transport in the cells as well. (Van *et al*., 1993). Generally, chaperonins are able to form stable complexes with proteins, which are imported to chloroplasts and to mitochondria (Gatenby, A, A., Viitanen, P. V., 1994). They perform their chaperone function also in co-operation with the other molecules, e.g. cpn10 and HSP70 (Petr Kopecek *et al*., 2001). Chaperonins are critical for the correct folding of many proteins in the cell, under both normal and stress conditions (Julia *et al*., 2000). HSP60 also has other important functions in an immune response due to its already mentioned immunocominant properties (Dieterle, S., Wolleenhaupt, J. 1996).

1.8 Heat shock proteins and the immune response

HSP can elicit potent specific cellular adaptive immune responses (e.g. CD8+ cytotoxic T-cell effectors or classic CTLs) based on their ability to chaperone atigenic peptide (Srivastava, 2002). By mechanisms that can less well understood, HSPs can also act independent of chaperoned peptides to directly stimulate innate immune response (Multhoff et al., 1997; Basu et al., 2000).

Three major facets of immune activation have been described for various stress proteins. The first involves the appearance of HSP70 and HSP90 on the surface of certain tumor cells or virally infected cells, and second is the is the ability of stress protein-peptide complexes to generate a cytotoxic T-lymphocyte response against cells producing these peptides. The third facet of immune system activation involves HSP-mediated cytokine production (Moseley, 2000; Robert, 2003).

1.9 Dynamics of heat stress protein gene expression and regulation

Regulation of transcription of heat shock protein genes is mediated by the interaction of heat shock factor (HSF) transcription factors with heat shock elements in the heat shock proteins gene promotor regions (Voellmy R, 1994, Morimoto RI *et al*., 1994). In vertebrates, four HSFs have been identified, of which HSF1 and HSF2

are ubiquitously expressed and conserved (Nakai A. and Morimoto RI, 1993., Sarge *et al*., 1991). The main heat shock factor with a role in vertebrates response to phisiological and environmental stress is HSF1 (Sarge KD et al., 1993, Zuo J *et al*., 1994) whereas activity of HSF2 is more selective, and is mostly induced during differentiation and early development. Usually, HSF1 is present in the cytoplasm as a latent monomeric molecule that is unable to bind to DNA. When exposed to stress, an intracellular flux of newly synthesised non-native proteins activates HSF1 (Morimoto RI *et al*., 1994). HSF1 is converted to phosphorylated trimers that have the capacity to bind DNA, and which translocate from the cytoplasm to the nucleus (Figure 2.1) (Pockley G., 2003). HSF2 has the characteristics of a temperature-sensitive protein; it is inactivated when exposed to raised temperature, and sequestered to the cytoplasm, and is thereby prevented from interference with HSF1 activity in stressed cells (Mathew A. *et al*., 2001). The consequences of binding of HSF1 to its target and the events that result in transcription of heat shock protein genes. The induction of heat shock proteins has to be tightly controlled, since their persistent presence would adversely affect protein homoeostasis and intracellular functions, leading to inappropriate growth control and possibly cell death (Pockley G., 2003). One mechanism that regulates heat shock protein expression is the binding of HSP70 to the transactivation domian of HSF1, leading to repression of heat shock gene transcription (Shi et al., 1998). The interaction between HSP70 and HSF1 has no effect on DNA binding or the stress-induced phosphorylation state of HSF1(Shi et al., 1998). A second mechanism regulating heat shock protein binding factor 1(HSBP1) the active trimeric form of HSF1, and HSP70 resulting in inhibition of the capacity of HSF1 to bind to DNA (Satyal et al., 1998). HSBP1 is mainly localised in the nucleus and HSBP1 mRNA is present at high concentrations in various cell lines and animal tissues that are unaffected by heat shock (Satyal et al., 1998).

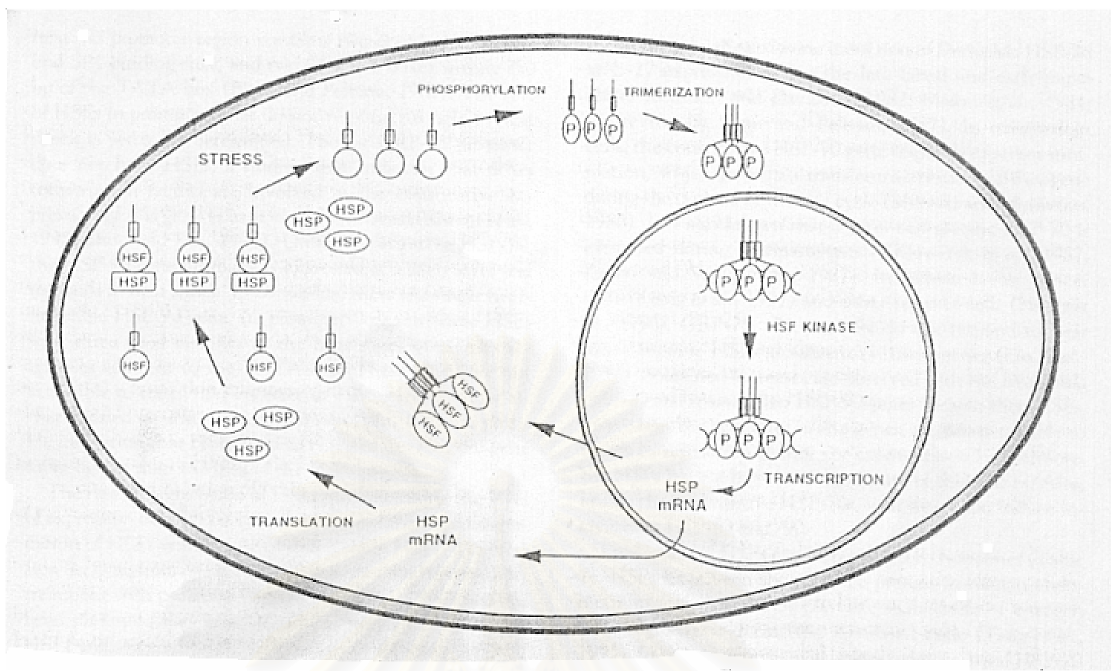

Figure 1.1 Proposed mechanism of stress-induced in HSPs in human and *Drosophila* cells. HSFs residing in the cytosol are normally bound by HSP and are inactive. Under stress, such as heat shock, HSFs separate from HSP, are phosphorylated by protein kinase such as PKC, and form trimers in cytosol that enter the nucleus to bind HSEs in the promoter region of HSP gene. HSF is phosphorylated further, and HSP mRNA is transcribed and leaves the nucleus for cytosol. In cytosol, new HSP is synthesized. HSF returns to the cytosol and is bound once again by HSF.

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1.10 Cell culture

Invertebrate tissue culture began in the early part of this century, Grace (1962) first reported the establishment of four cell strains from insect tissues (Lang *et al*., 2002). However, so far, no continuous cell line from marine crustacean has been established (Shimizu *et al*., 2001). Cell under in vitro conditions are used as exceptionally important tools in a variety of scientific disciplines including biological and medical sciences. In vitro applications may also be used as alternative tools for animal experimentation, for biotechnologycal applications and pathological investigation. With the expansion of intensive aquaculture, viral diseases have

threatened the shrimp aquaculture industry. Shrimp cell culture has therefore gained recent attention for the development of diagnostic reagents and probes for use in the shrimp aquaculture industry. Many reported have been focused on the research of shrimp cell cultures (Luedeman and Lightner, 1992; Lu et al., 1995; Sano, 1998; Walton and Smith, 1999).

In vitro techniques in crustacean biology have become important and sometimes vital tools for the study of crustacean endocrinology and diseases of edible species (Rinkevich, 1999). The study of shrimp haemocytes has involved the clarification of the structure, classification, and separation of haemocytes (Ellender *et al*., 1992) and the examination of the process of phagocytosis and the immune response. Developed primary shrimp cell culture from lymphoid organs of *P. monodon* was successfully in 2x Liebovitz-15 supplemented with 15% fetal bovine serum, 10% shrimp meat extract. These cells can be maintained up to 10 days without changing medium (Kasornchandra and Boonyaratpalin, 1998).

The objectives

To determine protein profiles in heat shock haemocytes

To clone and characterize HSP60, HSP70, and HSP90 genes

To detect heat induced genes in the haemocytes

To determine the expression levels of HSP60, HSP70, and HSP90 genes in heat shock haemocytes

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CHAPTER II

MATERIALS AND METHODS

2.1. Meterials

2.1.1 Chemical

-Absolute ethanol (BOH, England)

-Acetic Acid (Merck, Germany)

-Acrylamide (Sigma Chemical Co., USA)

-Agarose gel (FMC Bioproduct, USA)

-Ammonium persulfate (APS) (Sigma Chemical Co., USA)

-Bacto-agar (Oxoid, England)

-Bacto-yeast extract (Oxoid, England)

-Bio-Rad Protein Assay (Bio-Rad, USA)

-Bis-Acrylamide (Promega, Co., USA)

-Boric acid (Merck, Germany)

-Bovine Serum Albumin (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)

-dATP, dCTP, dGTP, dTTP (100mM)

- Diaminobenzidine (DAB) (Sigma Chemical Co., USA)

- Diethyl pyrocarbonate (DEPC) (Sigma Chemical Co., USA)

Di- sodium hydrogen phosphate (Merck, Germany)

-1,4-Dithio-DL-threitol (DTT) (Fluka, Biochemika, Swizerland)

-Ethidium Bromide (Sigma Chemical Co., USA)

-Ethilene Diamine tretaacetric acid, (EDTA) (Fluka Chemika, Switzerland)

-Formaldehyde (LabScan Asia Co., Thailand)

-Glycine (USB, Amerson Life Science, England)

-Improm –II^{TM} Reverse Transciption System (Promega, USA)

-Isopropanol (LabScam Asia, Co., Thailand)

-Methanol (LabScan Asia Co., Thailand)

-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, D-40724 Hilden)

-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)

2.1.2 Enzyme

-Restriction Enzyme

*ECO*R1 (Promega, Co., USA)

XhoI, (Amersham, UK.)

-DyNAzyme TM II DNA polymerase (Finnzyme, Finland)

-Ribonuclease A (Rnase A) (Promega Co., USA)

-Ribonuclease inhibitor (Rnasin), (Promega, USA).

-Avian myeloblastosis virus reverse transcriptase (AMV-RT), (Promega, USA).

-T4 DNA ligase (Promega Co., USA)

2.1.3 DNA and protein markers

-100 base- pairs DNA ladder (Promega Coperation Medison, USA)

-*Hind III* digested Lamda DNA

-Mid-range protein molecular weight markers, 14.4-97.4 kDa, Promega, USA.

2.1.4 Antibodies

-Mouse Anti-monoclonal antibody anti-HSP60, anti-HSP70 and anti-HSP90 (Stressgen, Canada)

-Anti-HSP60, Anti-HSP70 and Anti-HSP90 (Sigma, Sigma Chemical Co., USA)

-Rabbit anti-mouse immunoglobulin (DAKO, Denmark)

2.1.5 Microorganism

-*Escherichis 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)

- *Escherichis coli* stain XL1-blue MRF'

- *Escherichis coli* strain SOLR

2.1.6 Equipment

-Autoclave : model HICLAVE, HVE-50, HIRAYAMA, Japan

-Automatic micropipate size: P2, P10, P20, P40, P100, P200 and P1000 (Gilsen Medical Electrical S.A., France)

-Camera (Pentax K1000 Asahi Opt. Co, Ltd.)

-Herizontal gel electrophoresis, Sub-cell GT MINI (Bio-rad, USA)

-Laminar flow cabinet (Nuaire Class II, NU-440-300E, USA)

-PCR thermal cycler: PCR sprint (Hybaid)

-Polyacrylamide Electrophoresis, Mini PROTEAN® II Cell (Bio-Rad, USA)

-Polyacrylamide Electrophoresis, PROTEAN® II xi 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)

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2.2 Animals and haemolymph collection

Juvenile *P. monodon* (20-25 g) obtained from local shrimp farm in Pathumtani province were acclimated at least 1 week to the laboratory tanks equiped with air-lift circulating seawater. (salinity at 10 ppt, ambient temperature at 27-28°C). Haemolymph was withdrawn from the ventral part of the haemocoel of the second abdominal segment using a 24 gauche needle and a 1 ml syringe filled with 0.5 ml of 10% sodium citrate pH 7.0 as an anticoagulant. The mixture was kept at 4°C all the time and used freshly.

2.3 Primary haemocyte culture in suspension

The haemolymph collected in 10% sodium citrate pH 7.0, was used for primary cultures. Haemolymph ($1x10^{5}$ cells/flask) was separated and rapidly transferred into 3 culture flasks (25-cm^{2}, Corning). Each flask contained 3 ml of different growth media, M199, TC100 and Grace's insect medium, respectively. Each medium was supplimented with 10% fetal bovine serum, 20 µl of 500 U/µl penicillin, 20µ l of 500 µg/µl streptomycin (Appendix A) and maintained at 28°C. Haemocytes from each flask were observed and examined daily for the percentage of viability of haemocyte using a trypan blue exclusion procedure. The experiment was performed in 2 replications.

2.4 Viability of *P. monodon* haemocytes

2.4.1 Haemocyte count

Cell counting and viability of the haemocyte was determined using a trypan blue exclusion. The method was carried out by mixing 0.1 ml of haemocyte suspension with 0.2 ml of 0.4% trypan blue (prepared in 0.81% sodium chloride and 0.06% potassium phosphate, dibasic). The mixture was transferred to a Neubauer hemocytometer and a count was performed with an inverted microscope.

2.4.2 Haemocyte activity

In addition to the viability of the cultured haemocytes, the enzyme activity was determined by measuring the activity of superoxide dismutase using intracellular superoxide anion (O_2^-) assay. This assay was conducted as described by Song and Hsieh (1994). Reactions were performed in flat-bottomed 96 well microtiter plates containing 200 µl of growth medium. Hamolymph (50 µl) and Hank's balanced salt solution (50 µl) were added to each well ($5x10^5$ haemocytes/well) and incubated at room temperature for 30 min to restore haemocyte adherence and spreading capability. Supernatant was then removed. For haemocyte stimulation, 50 µl of Phorbol Myristate Acetate (PMA) was added. For non-stimulation, 50 µl of Hank's balanced salt solution were added instead. Stimulated and non-stimulated haemocytes were then reacted with 50 µl of nitroblue terrazolium solution (0.3% in Hank's balanced salt solution) for 2 hr at room temperature. The reaction was terminated by removing the solution followed by the addition of absolute methanol. After 2 washes with 70% methanol, the haemocytes were air-dried and soaked with the solutions of 120 µl of KOH (2 M) and 140 µl of dimethyl sulfoxide (DMSO) to dissolve the cytoplasmic formazan. The optical densities of the dissolved cytoplasmic formazan were measured at 630 nm with microplate reader (BioRad). In order to determine the reproducibility of the results, the experiment was performed in 5 replications. The ratios of OD_{630} from stimulated and non-stimulated haemocytes were determined.

2.5 Determination of thermal responses in *P.monodon* haemocytes *in vitro*

The thermal responses in the haemocytes of the shrimps were determined *in vitro* by maintaining the haemocytes in 3 different culture media as described in 3.3 The haemocytes maintained in each medium were thermal-treated at 4, 28, 30, 33 and 35°C for 30, 45, 60, 90 and 120 min. and transferred to ambient temperature (28°C). After remaining at ambient temperature for 2 hr, the total number and viability of haemocytes examined. Protein concentrations of the haemocytes treated with thermal shock were determined using colorimetric method and protein profiles were determined by electrophoretic analysis.

2.5.1 Determination of protein concentration

Upon termination of exposure of haemocyte cells to cold and heat stress. Microcentrifuge tube containing haemolymph and growth medium were incubate at 4°C, 30°C, 33°C and 35°C for 2 hrs and transferred to ambient temperature (28°C) for 2 hrs and the medium aspirated by centrifuge at 3600xg for 2 min. The pellet (haemocyte cells) were washed with 1 ml of 1x PBS pH 7.2 by centrifuge at 3600xg for 2 min. Homogenized haemocyte cells in 50 µl of fresh lysis buffer . Homogenates were centrifuged at 15000 rpm for 30 min to remove unlysed cells and debris. Protein quantitation was determined by colorimetric method, described by Bradford (1976) using Bio-Rad protein assay kit (Bio-Rad, USA). Bovine serum albumin Fraction V (Sigma) with known concentration was used as standard. The protein concentration was calculated by comparing with the protein standard cur ve.

2.5.2 SDS-Polyacrylamide gel electrophoresis

The method used a vertical slab gel apparatus (Bio-Rad, USA, model Mini PROTEIN® II cell) for electrophoresis system. SDS-Polyacrylamide gel was conducted using 12 % (10x7x0.5 cm). Gel preparations was shown in Appendix A

Samples were mixed with 5X 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 200V until the bromophenol blue dye left the gel. Gel was then stained for protein (either with Coomassie or silver stain) or blotted.

2.5.3 Staining

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

2.6 Detection of heat shock proteins by Western blot analysis

Proteins extracted from thermal shock haemocytes were initially separated in 12% SDS-PAGE and heat shock proteins (HSP60, HSP70, and HSP90) were determined by Western blot analysis as previous described by Towbin (1979).

2.6.1 Blotting

Following electrophoresis, the 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 4ºC onto nitrocellulose membrane (Hybond^{TM}-C Pure) using mini-trans-blot electrophoretic transfer cell (Bio-Rad.)

2.6.2 Immunochemical staining

Following gel blotting, transferred 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 with gentle agitation. The membrane was then rinsed 3 times (5 min each) with PBS. Membrane was probed with 1:500 dilution of primary antibody in 1%BSA/PBS for at least 1 h. Mouse monoclonal antibody anti-HSP60 from human (Stressgen, Canada) was used for HSP60 detection, mouse monoclonal antibody anti-HSP70 from human HeLa cells (Stressgen, Canada) was used for HSP70 detection, and mouse monoclonal antibody anti-HSP90 from *Achlya ambisexualis* (water mold) (Stressgen, Canada) was used for HSP90 detection. After primary antibody incubation, the membrane was rinsed again 3 times (5 min each) with PBS before incubating 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 2 times with 0.05 % Tween-20/PBS for 5 min each and 3 times with PBS for 5 min each. Finally, immunoreactive proteins were visualized by soaking the membrane with DAB solution (3 mM DAB, 0.03% w/v Hydrogen peroxide in 50 mM Tris-HCl,

pH 7.6) until peptide bands appeared. The reaction was stopped by washing with distilled water. The membrane was kept in the dark.

2.7 Determination of thermal responses genes in the haemocytes by *in vitro* translation

In vitro translation in the thermal treated haemocytes was carried out. Metabolic labelling was performed as described by Hoffmann and Somero, (1996). The haemocytes were maintained in MEM medium (minimum essential medium without methionine) containing 10 µci/ml of ^{35}S-methionine. The samples were thermal-treated at 4, 28 (ambient temperature), 30, 33 and 35ºC for 2 h. After maintaining at ambient temperature for 2 h, the samples were collected and analysed as described in 2.5.1.

Patterns of protein synthesis during the temperature exposures were examined using 12% SDS-PAGE. Following electrophoresis, the gel was dried and exposed to X-ray film (Kodak Diagnostic film, X-Omat™ K XK-1) in cassettes at -80°C for 1 week. Film was developed as described by manufacture's protocol (Kodak Diagnostic).

2.8 Determinaiton of thermal induced genes in the haemolymph of *P. monodon* using 51$ DUELWUDU\ SULPHG3&5 **(RAP-PCR)**

2.8.1 RNA extraction

Haemocytes obtained from normal or heat-induced shrimps were centrifuged at 3,600xg at 4°C for 2 min.. Supernatant was discarded and haemocyte pellet was resuspended with 50 µl of 10% sodium citrate. One milliliter of TRI REAGENT® (5-10 x 10^6 Cells per 1 ml of Tri reagent) was mixed and maintained at room temperature for 5 min. to permit the complete dissociation of nucleoprotein complex. The mixture was then centrifuged at 12,000x g for 10 min. The aqueous phase (upper phase) was transferred to a new 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 min. then

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centrifuged at 12,000x g for 15 min. at 4°C. The colorless upper aqueous phase containing RNA was transferred to a new 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 min before centrifugation at 12,000x g for 8 min at 4°C. The supernatant was discarded and RNA pellet was washed with 75 % ethanol followed by centrifugation at 7,000x g for 5 min at 4°C. The pellet containing total RNA was air-dried for 3-5 min. and dissolved in DEPC-treated distilled water.

2.8.2 Determination of total RNA concentration

The quantity of total RNA was estimated by measuring 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 A_{260}/A_{280}. The high purity of RNA should be at the ration of A_{260}/A_{280} between 1.6-1.9

2.8.3 First strand cDNA synthesis for RAP-PCR

7KH ILUVW VWUDQG F'1$ ZDV V\QWKHVL]HG IURP ôJ RI WRWDO 51$ H[WUDFWHG IURP WKHUPDO VWUHVV KDHPRF\WHV XVLQJ DQ ,P3URP-,[70] 5HYHUV 7UDQVFULSWLRQ 6\VWHP .LW 3URPHJD 7RWDO 51$ ZDV FRPELQHG ZLWK ôO R DUELWUDU\ SULPHU 8%& DDSSURSULDWH '(3& -WUHDWHG + 2 LQ D ILQDO YROXPH RI ôO 7KH UHDFWLRQ ZDV LQFXEDWHG DW [R]& IRU P LQ DQG LPPHGLDWHO\ SODFHG RQ LFH IRU P LQ 7KHQ [-UHDFWLRQ EXIIHU 0 J&O 173 0L[51DVLQ ZDV DGGHG WR ILQDO FRQFHQWUDWLRQV RI [P0 P0 DQG XQLWV UHVSHFWLYHO\ )LQDOO\ ôO RI ,P3URP -, UHYHUVH WUDQVFULSWDVH ZDV DGGHG DQG JHQWO\ P L[HG E\ SLSHWWLQJ 7KH UHDFWLRQ P L[WXUH ZDV LQFXEDWHG DW [R]& IRU P LQ DQG DW [R]& IRU P LQ 7KH UHDFWLRQ ZDV WHUP LQDWHG E\ LQFXEDWHG DW [R]& IRU P LQ WR WHUP LQDWH UHYHUVH WUDQVFULSWDVH DFWLYLW\ &RQFHQWUDWLRQ DQG URXJK TXDOLW\ RI ILUVW VWUDQGHG F'1$ ZDV VSHFWURSKRWRPHWULFDOO\ H[DPLQHG 2' 2' DQG HOHFWURSKRUHWLFDOO\ DQDO\]HG DJDURVH JHO

One µg of the LUVW VWUDQGHG F'1$ ZDV XVHG DV D WHPSODWH IRU VHFRQG VWUDQGHG V\QWKHVLV F'1$ ZDV DPSOLILHG ZLWK WKH FRPELQDWLRQ RI UBC 268 and either one of DUELWUDU\ SULPHUV 7KH VHTXHQFHV RI WKH

DUELWUDU\ SULPHUV XVHG LQ WKLV VWXG\ ZHUH VKRZQ LQ WDEOH The PCR profile was performed for 40 cycles with predenaturation at 94°C for 3 min, denaturation at 94°C for 30 s, annealing at 36°C for 60 s, and 72°C for 90 s. PCR products were mixed with equal volume of formamide loading buffer and denatured at 95°C for 10 min prior to electrophoresis in a 4.5% polyacrylamide gel. Allele sizes were determined using silver staining according to SILVER SEQUENCETM DNA Staining Reagents (Promega, WI). The gel was dried overnight and photograph under fluoresence light using Camera Pentax K1000 (Asahi Opt. Co., LTD., Japan).

Table 2.1 The sequences of arbitrary primers included in the screening for RAP-PCR analysis

Primer	**Sequence**
UBC 119	ATTGGGCGAT
UBC 122	GTAGACGAGC
UBC 128	GCATATTCCG
UBC 135	AAGCTGCGAG
UBC 158	TAGCCGTGGC
UBC 174	AACGGGCAGC
UBC 228	GCTGGGCCGA
UBC 268	AGGCCGCTTA
UBC 299	TGTCAGCGGT
UBC 459	GCGTCGAGGG

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2.8.4 Denaturating Polyacrylamide gel electrophoresis

Denaturing polyacrylamide gels are used for the separation and purification of single-stranded fragments of DNA. These gels are polymerized in the presence of an agent (urea) that suppresses base pairing in nucleic acids. Denatured DNA migrates through these gels at a rate that is almost completely independent of its base composition and sequence.

The PCR products were electrophoresed on Model SA Adjustable Sequencing Gel Electrophoresis system (GibcoBRL). All gels are assembled using sets of 17 x 32

cm, 0.4-mm spacers and 24 well sharkstooth combs. Polyacrylamide solution, 19:1 acrylamide:bis- acrylamide, containing 1x TBE gel buffer and 7 M Urea, was used to fractionate of single-strand molecules. Amplicons (1 part) were denatured by mixing with 2 parts of denaturing solution (98% (w/v) formamide, 0.025% bromophenol blue, 0.025% xylene cyanol and 10 mM EDTA in water), heating at 95°C for 5 min and placing immediately in iced water. Electrophoresis proceeded at constant watts (40 watts) at room temperature until xylene cyanol run through the end terminal of glass about 15 min, and the gels were silver stained to detect the mobility of the different sizes of fragment DNA.

2.8.5 Silver staining

Gel was removed with on side attached to the glass and placed into the fix-stop solution (10% gracial acetic) for 30 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.

QXWLRQRI ' 1 $ IUDJPHQWV

$IWHUHOHFWURSKRUHVLVWKHGHVLUHG' 1 $ IUDJPHQWZDVH[FLVHGIURP WKHDJDURVHJHOXVLQJ DVWHULOHVFDOSHO DQGSODFHGLQDSUHZHLJKHGPLFURFHQWULIXJHWXEH

Three volumes of the QG buffer was added and mixed by inversion of the tube. The tube was incubated at 50°C for 10 min or until the gel slice was completely dissolved. The mixture was transferred into a QIAquick column inserted in a 2 ml collection tube and centrifuged at 12000 rpm for 1 min. The flow-though solutions was discarded and another 0.5 ml of QG buffer was added to the QIAquick column and centrifuged for 1 min. Then, 0.75 ml of the PE buffer was added to the QIAquick column and centrifuged 12000 rpm for 1 min. The flow-though solutions was discarded. The column was recentrifuged to remove the trace amount of the washing solution. The QIAquick column was placed into a sterile microcentrifuge tube. DNA was eluted out by addition of 10-15 µl of elution buffer (10 mM Tris-HCl, pH 8.5) to

the center of the QIAquick membrane and left for 1-2 min before centrifuged at 12000 rpm for 1 min. Purified DNA from PCR product was cloned and sequenced.

2.9 Determination of partial sequences of HSP genes

2.9.1 Primers

Degenerated primers were designed from the conserved regions of reported HSP genes from the closest species (GenBank accession No AF 254880 for HSP90 and accession No AAB94640.1 for HSP60 and HSP70). Melting temperature (T_m) values of these degenerated primers were calculated as below.

$$Tm = 2 \times (A+T) + 4 \times (C+G)$$

The range of Tm was between 50 to 65°C and the GC content was around 60%. Formation of secondary structure between primers was avoided. Details of the primers were shown in Table 2.2

Table 2.2 Nucleotide sequences and details of oligonucleotide primers used in this study.

Primers	Sequence (5’ 3’)	Polarity
HSP60F1	TCT TTA TTG CGA ACT CCC G	+
HSP60F2	777 * * & $&* * $* * 7& $* *	+
HSP60R1	& 7 7& 7&$* && $ * 7& 7&$* $& $ * 7& & 7 7&	-
HSP60R2	* & & 7 7& $ * 7& 7&$* && $ * $$ 7&$* $&&	-
HSP70F1	&$ $* * & 7&$* $$ $* * $ 7& * &	+
HSP70F2	$7 7&$ $$ 7& * $ $* && 7&$* $& 7&$* * &	+
HSP70R1	$& 7&$* * & 7& 7& $* 7& 7&$* * * $* 77	-
HSP70R2	&& $* 77 7&$* * & $* 7& 7$* $7 $* 7& $* $$	-
HSP90F1	7& $7&* $7*$7 $7& * * $7&* &$ $* 77 &7 * *	+
HSP90F2	CA(CT)AA(CT)GA(CT)GA(CT)GA(AG)CA(AG)TA	+
HSP90R1	C(GT)(AG)TT(CT)TG(ATCG)(CG)(AT)(AG)TC(CT)TC(AG)TG	-
HSP90R2	77 &7 77 &7 7& &7 7& &7 7& $* 7& &7 7&	-

2.9.2 Reverse transcription

Total RNA isolated from the haemocytes was subjected to single stranded cDNA synthesis by the reverse transcription of mRNA to cDNA using oligo dT_{15} primers. The reverse transcription reaction was carried out as follow. The reaction was performed in the final volume of 20 µl, at 42°C, for 90 min using Improm II^{TM} reverse transcription kit condition (1 U of Improm II^{MT} reverse transcription, 2 µl of 1x Improm II^{TM} 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.

2.9.3 PCR amplification of HSP genes

The target cDNA was amplified from single stranded cDNA template by PCR using degenerated primers designed from conserved sequences of reported HSP genes. The reaction mixture of PCR contained 1X PCR buffer (10 mMTris-HCl pH8.8, 50mM KCl, 0.1% TritonX-100), 0.4 mM dNTPs, 1.5 mM $MgCl_2$, 1U of *Taq* DNA polymerase, 500 ng of cDNA template and 0.5 µM of forward and reverse primers.

For HSP60 amplification, the reaction mixture was carried out in the following thermal cycles; 5 cycles of 94ºC for 1 min, 45ºC for 1 min and 72ºC for 1 min, followed by 30 cycles of 94ºC for 1 min, 50ºC for 1 min and 72ºC for 1 min with elongation step at 72ºC for 7 min.

For HSP70 amplification, one cycle of denaturing step at 94ºC for 2 min was initiated, followed by 35 cycles of 94ºC for 30 sec, 50ºC for 30 sec and 72ºC for 1 min. The reaction was ended by the elongation step at 72ºC for 7 min.

For HSP90 amplification, 2 cycles of 94ºC for 1 min, 50ºC for 1 min and 72ºC for 2 min was firstly conducted and followed by 30 cycles of 94ºC for 1 min, 55ºC for 1 min and 72ºC for 2 min. The reaction was ended by the elongation step at 72ºC for 7 min.

PCR products were analyzed by electrophoresis using 1.2% agarose gel in TBE buffer.

2.10 Detection of PCR products by agarose gel electrophoresis

2.10.1 Preparation of agarose gel and electrophoresis

For most applications, agarose gel was prepared at a concentration of 1.2 % (w/v) in 1x tris-borate electrophoresis buffer (TBE) (Appendix A). The mixture was melted in microwave oven until completely dissolved and then poured into the gel tray with an appropriate comb. The gel was left to solidify for at least 30 min at room temperature. The comb was carefully removed and the ge1 was transferred into the electrophoretic chamber. TBE buffer was added to cover the gel to a depth of about 1 mm. Ten microliters of PCR product was thoroughly mixed with 10x loading dye (bromophenol blue) and slowly applied into the gel slots. Five microliters of 100 bp DNA ladder (Promega Coperation Medison, USA), was mixed with 10x loading dye (Appendix A) and applied into the first well as a DNA marker. Electrophoresis was carried out at constant voltage of 100 volts until tracking dye reach about 1 cm from the lower edge of the gel.

2.10.2 Staining of DNA in agarose gel

Following electrophoresis, agarose gel contained DNA was stained by using a fluorescent dye ethidium bromide. The gel was immersed in water containing 0.5 μg/ml of ethidium bromide for about 15 min. at room temperature. The gel was washed shortly with distilled water, then visualized under UV-transiluminator. UV light was absorbed at 260 nm and transmitted to the dye, which was emitted at 590 nm in the red-orange region of the visible spectrum. The visible bands of DNA on the stained gel was photographed using camera Pentax K1000 (Asahi Opt. Co, Ltd.)

2.11 Cloning and sequencing of heat shock protein genes.

2.11.1 DNA preparation

DNA bands of interest separated by agarose gel electrophoresis were removed from the agarose gel using a sterile scalpel and placed in a sterile microcentrifuge

tube. Three volumes of the QG buffer was added and mixed by inversion of the tube. The tube was incubated at 50°C for 10 min or until the gel slice was completely dissolved. The mixture was transferred into a QIAquick column inserted in a 2 ml collection tube and centrifuged at 12000 rpm for 1 min. The flow-though solutions was discarded and another 0.5 ml of QG buffer was added to the QIAquick column and centrifuged for 1 min. Then, 0.75 ml of the PE buffer was added to the QIAquick column and centrifuged 12000 rpm for 1 min. The flow-though solutions was discarded. The column was recentrifuged to remove the trace amount of the washing solution. The QIAquick column was placed into a sterile microcentrifuge tube. DNA was eluted out by addition of 10-15 µl of elution buffer (10 mM Tris-HCl, pH 8.5) to the center of the QIAquick membrane and left for 1-2 min before centrifuged at 12000 rpm for 1 min.

2.11.2 Preparation of competent cell

Competent *E. coli* stain JM109 cells were prepared by calcium chloride method described by Ausubel *et al*. (1989), with some modification. A single colony of *E. coli* was inoculated into 5 ml of LB broth and incubated at 37ºC overnight with shaking. The culture was sub-inoculated by adding 1 ml of the culture into 50 ml of LB broth and incubated at 37ºC until the OD_{600} was approximately 0.4-0.6. The culture was then placed on ice for 30 min and centrifuged at 3,000 rpm for 10 min. The cell pellet was resuspended in 50 ml of chilled 50 mM $CaCl_2$ solution and kept on ice for 45 min. After centrifugation at 3,000 rpm for 10 min, the pellet was resuspended in 2 ml of chilled 0.1 M $CaCl_2$ solution. Glycerol was added to make the final concentration of 15 %. The cell suspension were aliquoted (200 µl each) into a microcentrifuge tube and stored at -80°C for subsequently used.

2.11.3 Cloning

Purified DNA from PCR product of heat shock protein genes was ligated into pGEM®-T Easy Vector (Promega, U.S.A.). The ligation reaction was conducted as follow. 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 (25 ng) of pGEM- T vector was added and following by 1 µl of PCR product. Next, 1 µl T_4 DNA ligase was added and dH_2O was added to 10 µl final volumn.

2.11.4 Transformation of competent cells

Five µl of ligated product were mixed with 200 µl of competent cells which were thawed on ice just before transformation. The mixture was placed on ice for 30 min. The cells were then heat shocked at exactly 42°C for 60 seconds in a water bath and immediately removed and maintained on ice for 5 min. SOC medium (1 ml) was added and incubated at 37°C for 1.5 hr with shaking. Cell Pellet was collected by centrifugation at 5,000 rpm for 1 min and redissolved in 200 µl of LB media. Transformant cells were spreaded on LB agar plate containing 50 µg/ml of ampicillin, 0.5 mM IPTG, and 40 µg/ml of X-gal. The plate was incubated at 37ºC overnight. Individual white colonies of transformed *E.coli* were observed.

2.11.5 Colony PCR

Individual white colony containing recombinant vector was screened for the size of DNA insert by colony PCR using pUC1 (5'–CCGGCTCGTATGTTGTGTGG A–3') and pUC2 (5'–GTGCTGCAAGGCGATTAAGTTGG–3') primers. These primers were at 154 bp upstream and 178 bp downstream of the insertion site. Detail of PCR reaction was shown as follow.

PCR reaction:
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 forward and reverse primer,
1.0 mM dNTP mix, and
0.25 U Taq DNA polymerase.

PCR condition included the initial denaturation step at 94°C for 3 min, followed by 35 cycles of the denaturing step at 94°C for 1 min, the annealing step at 53°C for 90 sec, and the extension step at 72°C for 90 sec. The reaction was ended by the elongation step at 72°C for 7 min.

2.11.6 Plasmid DNA preparation and restriction enzyme digestion

Transformant cells containing DNA target were examined by isolation of plasmid from cell culture and the insert DNA was analyzed by restriction enzyme digestion. Small-scale preparation of plasmid DNA was performed by alkaline lysis mini-preparation method (Brinboim , Doly, 1979) with modification. A single colony of *E. coli* containing recombinant plasmid was inoculated into 3 ml of LB broth containing 50 µg/ml ampicillin and incubated overnight at 37°C with vigorous shaking. The cultured cells were collected by centrifugation at 12,000 rpm for 1 min and the cell pellet was resuspended in 200 µl of GTE buffer (50mM glucose, 25mM Tris-HCl, pH 8.0, and 10mM EDTA). Four hundred µl of freshly prepared lysis buffer (0.2 N NaOH and 1% SDS) were added, gently mixed and placed on ice for 15 min. Two hundred µl of 3 M potassium acetate pH 4.8 was added, gentle mixed and kept on ice for 15 min. After centrifugation at 12,000 rpm, 4°C for 15 min, the supernatant was conducted by phenol chloroform extraction. The plasmid DNA was collected by centrifugation at 12,000 rpm, 4°C for 15 min. After air dried, the DNA was dissolved in 50 µl of TE buffer. One microliter of 10 mg/ml of Rnase A was added and incubated at 37°C for 1 hr. Two µl of plasmid DNA were digested with appropriate restriction enzymes (EcoRI). The digested plasmid DNA was separated by agarose gel electrophoresis in 2.10

Alternatively, plasmid DNA was extracted using QIAprep® Miniprep Kit (QIAGEN GmbH, D-40724 Hilden). Briefly, about 1.5 ml of the inoculated culture was transferred to a microcentrifuge tube and spin for 1 min. at 13,000 rpm. The supernatant was then discarded. It was possible to increase the amount of the cultured cells to the same tube by adding more cell culture and the process was repeated. The pellet of bacterial cells was resuspended in 250 µl of Buffer P1 (Resuspension buffer contain Rnase A) and Vortexed. After adding 250 µl of Buffer P2 (Lysis buffer

contains sodium hydroxide), the tube was inverted gently 4-6 times. Added 350 µl of Buffer N3 (Neutralization buffer contains guanidine hydrochloride) and tube was inverted gently 4-6 times. The tube was centrifuged for 10 min. at maximum speed in tabletop microcentrifuge (13000 rpm). The supernatant (about 850 µl) was carefully collected and applied to the QIAprep column and centrifuged at 13000 rpm for 1 min. The flow-though solution was discarded. The QIAprep column was washed by adding 750 µl of Buffer PE (wash buffer contains ethanol). The QIAquick column was recentrifuged at 13000 rpm for 1 min to remove the trace amount of the washing solution. Place the QIAprep column in a clean 1.5 ml 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 min. and then centrifuge for 1 min.

2.11.7 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 and conducted on ABI 310 and 3100 Department of Medical Science Ministry of Public Health.

2.12 Construction of EST library from heat-induced shrimps

2.12.1 animals and RNA preparation

The experiment was conducted by acclimating 60 giant tiger shrimps in a reared tank provided with supplemental aeration at ambient temperature (27-28°C) for 1 week. Shrimps were treated with heat shock at 35°C for 1 hr and transferred to ambient temperature for 2 hr. Haemolymph from the shrimps was collected in equal volume of 10% sodium citrate and centrifuged at 3600xg for 10 min. at 4°C.

The resulting cell pellet (haemocytes) was homogenized with a glass piston homogenizer in 2 ml of TRI Reagent for total RNA extraction. mRNA was purified using QuickPrep Micro mRNA purification kit (Amersham Biosciences).

2.12.2 library construction

The system of Lambda Uni-ZAP XR Vector (ZAP-cDNA synthesis kit, Stratagene, CA) was used for cDNA library construction. Approximately 15 µg of poly(A)$^+$ mRNA was isolated from total pool of RNA utilized as template for first and second strand cDNA synthesis. The double stranded cDNAs were fractionated by Sepharose CL-2B column and cDNAs greater than 500 bp in size were ligated into the vector and packaged into phages. Mass excision was performed using ExAssist interference-resistant helper phage (Stratagene). Plasmid DNA was extracted using QIAprep kit (Qiagen, CA), digested with restriction enzymes, and separated on a 1.2% agarose gel. The cDNA inserts were single-pass sequenced from the 5' end.

2.13 Screening of ?-ZAP cDNA library

2.13.1 Probe DIG DNA labeling

DNA fragments of HSP60, HSP70 and HSP90 were used as probes for Southern blot hybridization and for library screening. One µl of DNA was added into microcentrifuge tube containing 16 µl of dH_2O. Then, DNA was denatured by heating in boiling water for 10 mins and quickly chilled on ice. DIG-High Prime (4 µl) was added, mixed and centrifuged. The mixture was incubated for 20 hrs at 37 °C. Then, the reaction was stopped by adding 2 µl of 0.2 M EDTA pH 8.0) and/or heating 65°C for 10 mins.

2.13.2 Plaque blotting

Aliquots of the bacteriophage stock was mixed with plating cells (XL1-blue MRF') and plated on soft agarose (NZY agar). The plate was incubated at 37°C until plaques were approximately 0.2-0.5 mm in diameter (approximately 8-10 hr.). Then

the plate was chilled for about 1 hr to set the agarose. Hybridization membrane was cut to size and placed onto the surface of the agarose and left for at least 30 s. The orientation was then marked with a sterile needle. Membrane was removed and placed on a sheet of Whatman 3MM^{TM} filter paper saturated with denaturing solution (Appendix A) for 5 min. The membrane was transferred to a sheet of filter paper saturated with neutralising solution (Appendix A). Membrane was neutralized 2 times for 5 min each. The membrane was dried on filter paper, then fixed the DNA by baking the membrane for 2 hrs at 80°C.

2.13.3 Probe Hybridization

Prior to probe hybridization, appropriate amount of Standard Hybridization solution (20 ml/ 100 cm^2) was pre-warmed to desired temperature (45-65°C) and the membrane was incubated for 30 min with gentle agitation. Then, Dig-labelled probe was denatured by boiling for 5 min and rapidly cooled on ice water. Pre-warmed hybridization solution (2.5 ml/ 100 cm^2) was added and incubated at 65 °C for 3 hrs. Prehybridization solution was poured off and probe hybridization solution was added to the membrane and incubated with gentle agitation for at least 16 hrs.

2.13.4 Stringency washes

Stringency washes were conducted in 2x SSC, 0.1% SDS at room temperature for 2 times, 5 mins each, followed by 0.1x SSC, 0.1% SDS to wash membrane at 65°C for 2 time, 15 mins each.

2.13.5 Immunological Detection

Membrane was rinsed briefly (1-5 min.) in maleic acid buffer then incubated for 30 min. in 100 ml of blocking soluion. Anti- DIG-AP conjugate was diluted to 1:5,000 in blocking solution then the membrane was incubated for 30 min. in 20 ml antibody solution. The membrane was washed twice for 15 mins with 100 ml maleic acid buffer and equilibrated for 2-5 min. in 20 ml of detection buffer then further incubated in 10 ml freshly prepared color solution. The membrane was sealed in a plastic bag or box in the dark (Do not shake). The color was developed within a few

min and the reaction was complete in 16 hrs. Membrane was allowed to expose to light only to monitor color development. The reaction was stopped by washing for 5 min. with TE.

2.13.6 Single clone in vivo excision using ExAssist® Helper phage with SOLR strain

Five hundred µl of SM buffer and 25 µl of chloroform into a sterile were added to microcentrifuge tube. Positive plaque was transferred from the agar plate to microcentrifuge tube and vortexed to release the phage particle into the SM buffer (phage stock). The microcentrifuge tube was incubated at 4°C overnight. The following component was combined in 15 ml polypropylene tube.

- 200 µl of XL1-blue MRF' cells at an A600 of 1.0
- 250 µl of phage stock
- 1 µl of the ExAssist® Helper phage

Following the incubation of the tube at 37°C for 15 min to allow the phage to attach to the cells, 3 ml of LB broth with 30 µl of 1M $MgSO_4$ and 30 µl of 20% (w/v) of moltose were added and further incubated at 37°C with shaking for 2.5-3 hrs. Then the tube was heated at 68°C for 20 min to lyse the lamda phage particle and the cells. The tube was spun at 1000xg for 15 min to pellet cell debris and collect the phagemid supernatant into a sterile tube. To plate the excised phagemid, the following component were combined in 2 of 1.5 ml microcentrifuge tubes

- 200 µl of freshly grown SOLR cells an A600 of 1.0
- 10 µl and 100 µl of phagemid supernatant

The microcentrifuge tube was incubated at 37°C for 15 min and 200 µl of the cell mixture from each microcentrifuge tube was plated on LB ampicillin agar plate (50µg/ml) and the plate was incubated at 37°C overnight.

2.14 Rapid amplication of cDNA ends-polymerase chain reaction (RACE-PCR)

The 5’ ends of HSP genes (HSP60 and HSP90) were amplified using BD SMART (Switching Mechanism At 5’ end of RNA Transcript) technology kit. RACE-ready cDNA was prepared by combining 1 µg of total RNA extraced from haemocytes of *P. monodon* with 1 µl of 10 µM BD SMART II A oligonucleotide for 5’ RACE-PCR and 1 µl of 5’ CDS primer (table 2.3). Sterile H_2O was added to a final volume of 5 µl. The component were mixed and spun briefly. The reaction was incubated at 70°C for 2 min and cooled on ice for 2 min. The reaction tube was spin briefly. Then, 2 µl of 5X first-strand buffer (250 mM Tris-HCl pH 8.3, 375 mM KCl, 30 mM $MgCl_2$), 1 µl of 20 mM DTT, 1 µl of 10 mM dNTP mix and 1 µl of BD PowerScript Reverse Transcriptase were added. The reactions were mixed by gently pipetting and centrifuged briefly to collect the contents at the bottom. The tube was incubated at 42°C for 90 min in an air incubator. The first strand reaction products were diluted with 100 µl of Tricine-EDTA buffer (10 mM Tricine-KOH pH 8.5, 1.0 mM EDTA) and heated at 72°C for 7 min. The first strand cDNA template was stored at -20°C for up to three months.

Gene specific primers (GSP) for 5’ end (anti-sense primer) of HSP60 and HSP90 were designed from HSP genes of *P. monodon* (table 2.3).

The master mix of 5’ RACE was prepared for HSP60 and HSP90. For 50 µl amplification reaction, 34.5 µl of PCR-Grade water, 5 µl of 10X BD Advantage 2 PCR buffer, 1 µl of 10 mM dNTP mix, 1 µl of 50X BD Advantage 2 polymerase mix, 2.5 µl of 5’RACE-Ready cDNA (1:10), 5 µl of 10X Universal primer (UPM) (table 2.3) and 1 µl of 10 µM Gene specific primer were mixed. The reaction was performed as follow

94°C	30 S	
72°C	2 min	5 cycles
94°C	30 S	
70°C	30 S	
72°C	2 min	5 cycles
94°C	30 S	
68°C	30 S	25 cycles
72°C	2 min	

RACE products were electrophoretically analyzed as described in 2.10 then, cloned and sequenced as described in 2.11. Finally, the full length cDNA was constructed.

Table 2.3 Primer sequences for the first strand cDNA synthesis and RACE-PCR

Primers	**Sequence**
SMART II A Oligonucleotide	5'-AAGCAGTGGTATCAACGCAGAGTACGCGGG-3'
5' RACE CDS Primer A	5' – (T)25 V N-3' (N = A, C, G or T; V = A, G or C)
10X Universal Primer A Mix (UPM)	Long : 5'- CTAATACGACTCACTATAGGGCAAGAGTG GTATCAACGCAGAGT-3' Short : 5'-CTAATACGACTCACTATAGGGC-3'
Gene specific primer for HSP60	5'–CAATCTCAGCAGGAGTGGTCACAGGC- 3'
Gene specific primer for HSP90	5' – CGCACCGTGAACGACCCGCCCGCCGA – 3'

2.15 Computational analysis of HSP sequences

DNA sequences were identified by BLAST analysis (Altschul *et al*., 1997) and translated in to amino acid sequences using Genetyx-WIN program (Genetyx-WIN Version 3.2, serial no. 1136275580). The secondary and tertiary structures of putative proteins were analysed by Rasmol (Sayle and Milner-White, 1995) and Swiss-Model (Peitsch and Jongeneel, 1993)

2.16 *In vitro* expression of heat shock protein genes

2.16.1 Semi-quantitative RT-PCR

Reverse transcription was conducted to make first strand cDNA using total RNA extracted from heat-treated haemocytes. The reaction was performed in the final volume of 20 µl, at 42°C, for 90 min using Improm II^{TM} reverse transcription kit condition (1 U of Improm II^{MT} reverse transcription, 2 µl of 1x Improm II^{TM} 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 600, 25 and 50 ng for HSP60, HSP70 and HSP90 kDa, respectively. Primers were designed from heat shock protein genes sequence of black tiger shrimp *P. monodon* (Table 2.4). 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 mM $MgCl_2$, 0.5 µM each of forward and reverse primer, 0.4 mM dNTP mix, and 1 U Taq DNA polymerase. The PCR reaction for HSP 60 kDa, HSP70 kDa and HSP90 kDa gene was performed as follow.

HSP60 kDa gene

Initial denaturation step:	94°C, 3 min	for 1 cycle
Denaturing step:	94°C, 1 min	
Annealing step:	55°C, 1 min	for 28 cycles
Elongation step:	72°C, 1 min	

Extension step: 72°C, 7 min for 1 cycle

HSP70 kDa gene

Initial denaturation step: 94°C, 3 min for 1 cycle

Denaturing step: 94°C, 1 min

Annealing step: 65°C, 1 min for 25 cycles

Elongation step: 72°C, 1 min

Extension step: 72°C, 7 min for 1 cycle

HSP90 kDa gene

Initial denaturation step: 94°C, 3 min for 1 cycle

Denaturing step: 94°C, 1 min

Annealing step: 55°C, 1 min for 26 cycles

Elongation step: 72°C, 1 min

Extension step: 72°C, 7 min for 1 cycle

Table 2.4 Primer sequences for the Semi-quantitative RT-PCR

Primers	Sequence
HSP60F	5’-AGGTTGGTCGTGAGGGTGTC-3’
HSP60R	5’-GAGTCTGGATAGCCTTGCGG-3’
HSP70F	5’-CCTCTATCACTCGTGCTCGC-3’
HSP70R	5’-GTCCCTCTGCTTCTCATCGT-3’
HSP90F	5’-TCCACGAGGATTCCACCAACC-3’
HSP90R	5’-TCGGCATCCGCCTTTGTCTCA-3’
Actin1	5’GGTATCCTCACCCTCAAGTA 3’
Actin2	5’AAGAGCGAAACCTTCATAGA 3’

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

Initial denaturation step:	94°C, 3 min	for 1 cycle
Denaturing step:	94°C, 1 min	
Annealing step:	55°C, 1 min	for 20 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 heat shock protein band was detected and compared with that of beta-actin using Quantity one program (BIO-RAD).

2.17 Statistical Analyses

All the measurements were made in four replicates. The result were analyzed using the ANOVA and Duncan new multiple range test (p <0.05) at 95% confidence level with SPSS program.

CHAPTER III

RESULTS

3.1 Haemocyte cells culture

The viability of haemocytes maintianing in three different culture medium, M199, Grace's insect and TC100 (Fig. 3.1) with 10% fetal bovine serum supplemented, 20 ul of 500 U/µl penicillin, 20 ul of 500 µg/µl streptomycin and maintained at 28°C was detected. The results indicated that haemocyte in M199 media showed the highest viability without changing medium. The percentage of viability of haemocyte in M199 was 96.6%, 94.6%, 91.2% and 82.1% at 24, 48, 72 and 96 h. respectively. The haemocytes were maintained for 1 day in TC100 and Grace's insect media and the viability of haemocytes was 85.5% and 98.8% respectively. (Table 3.1)

Table 3.1 Percentage of viability of haemocyte cell after maintained in growth medium

Time (hr)	**Percentage of viability**		
	M199	**TC100**	**Grace's insect**
24	96.6	85.5	98.8
48	94.6	0	0
72	91.2	0	0
96	82.1	0	0

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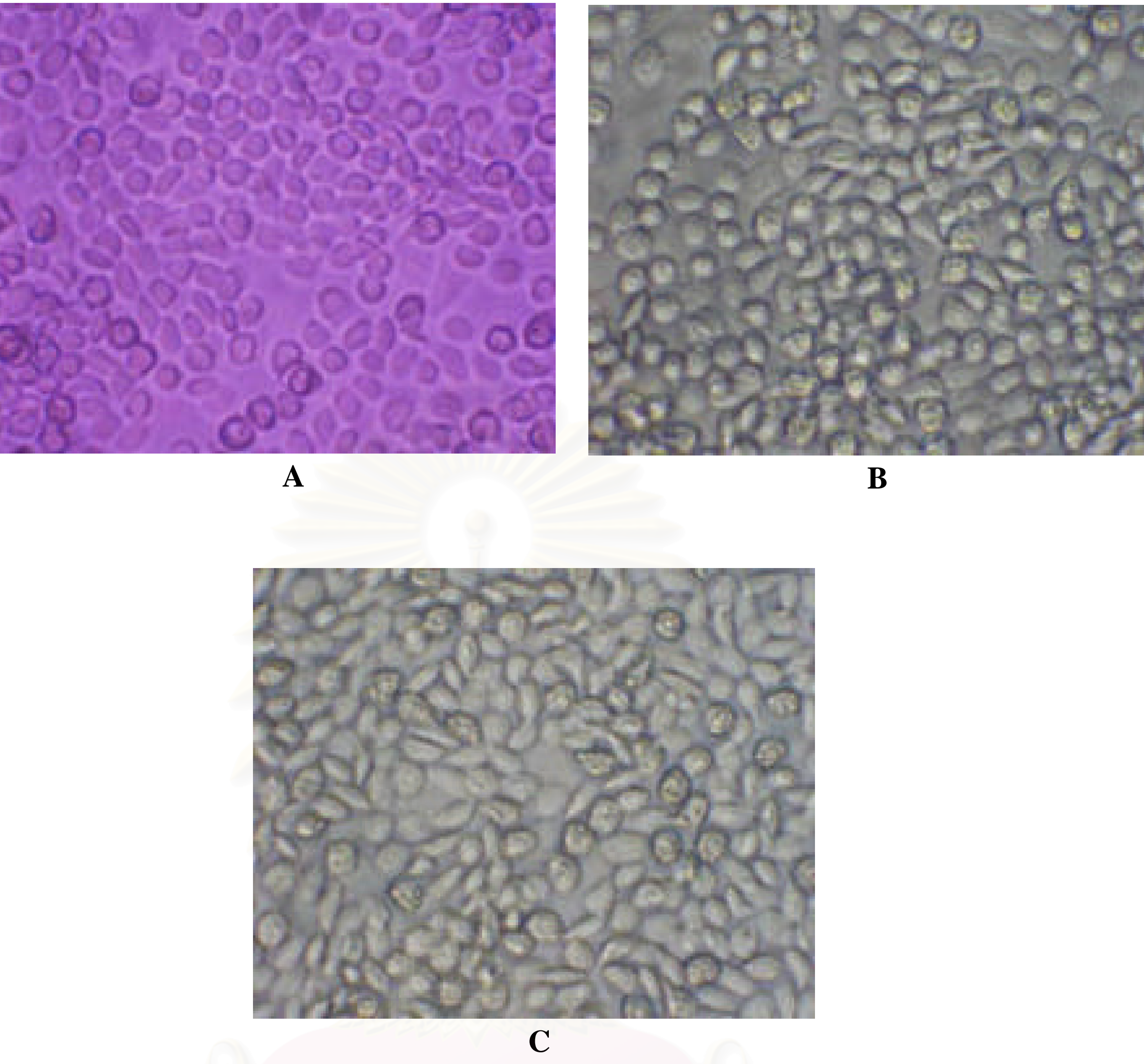

Figure. 3.1 Haemocyte cells of *P. monodon* maintained in M199 (A), Grace's insect (B), and TC100 (C).

3.2 Haemocytes cell activity

In order to monitor the activity of the viable haemocytes in three different growth media, the activity of superoxide dismutase were determined by intracellular superoxide anion (O_2^-) assay. The haemocytes were maintained in M199, TC100 and Grace's insect at 28°C. At 0, 2 and 4 hr of the culture, haemocytes from each treatment was subjected to superoxide anion (O_2^-) assay. The assay was performed by measuring the reduction of NBT in normal haemocytes (basal activity, BA) and haemocytes stimulated with PMA (stimulated activity, SA).

By Compairing the haemocyte activity in all media, there were significant differences between stimulated activity while no significant difference between basal activity was detected from haemocytes in all media at 0, 2, 4 hr ($p<0.05$). The stimulated haemocytes in Grace's insect media obtained highest activity followed by that of M199 and TC100 ($P<0.05$) except at 2 hrs where the stimulated activities of haemocytes in Grace's insect and TC100 were in the same level but were still significant higher than that in M199 ($P<0.05$). (Fig. 3.2).

In addition, the performance of superoxide dismutase in the haemocytes maintained in 3 different growth media were shown by the BA/SA ratio at OD_{630} (Table 3.2). The result indicated that haemocytes maintained in Grace's insect medium also yielded the highest enzyme activity, followed by the activity of the haemocytes in TC100 and M199, respectively. The BA/SA ratios of the haemocytes maintained in all 3 media at 0, 2 and 4 hr were significant lower, respectively. This indicated that the enzyme activity of the haemocyte maintained in all 3 media were decreased corresponding to time of culture.

Table 3.2 NBT reduction of the haemocytes maintained in M199, TC100 and Grace's insect medium

Time (hr)	**M199**			**TC100**			**Grace's insect**		
	BA	**SA**	**Ratio**	**BA**	**SA**	**Ratio**	**BA**	**SA**	**Ratio**
0	0.003 ± 0.001	0.035 ± 0.008	11.66	0.002 ± 0.001	0.039 ± 0.003	19.5	0.0024 ± 0.002	0.054 ± 0.011	22.5
2	0.002 ± 0.0024	0.030 ± 0.003	15.0	0.003 ± 0.0035	0.064 ± 0.007	21.3	0.002 ± 0.001	0.055 ± 0.008	27.5
4	0.001 ± 0.001	0.002 ± 0.001	2.0	0.002 ± 0.0008	0.021 ± 0.002	10.5	0.003 ± 0.001	0.045 ± 0.015	15.0

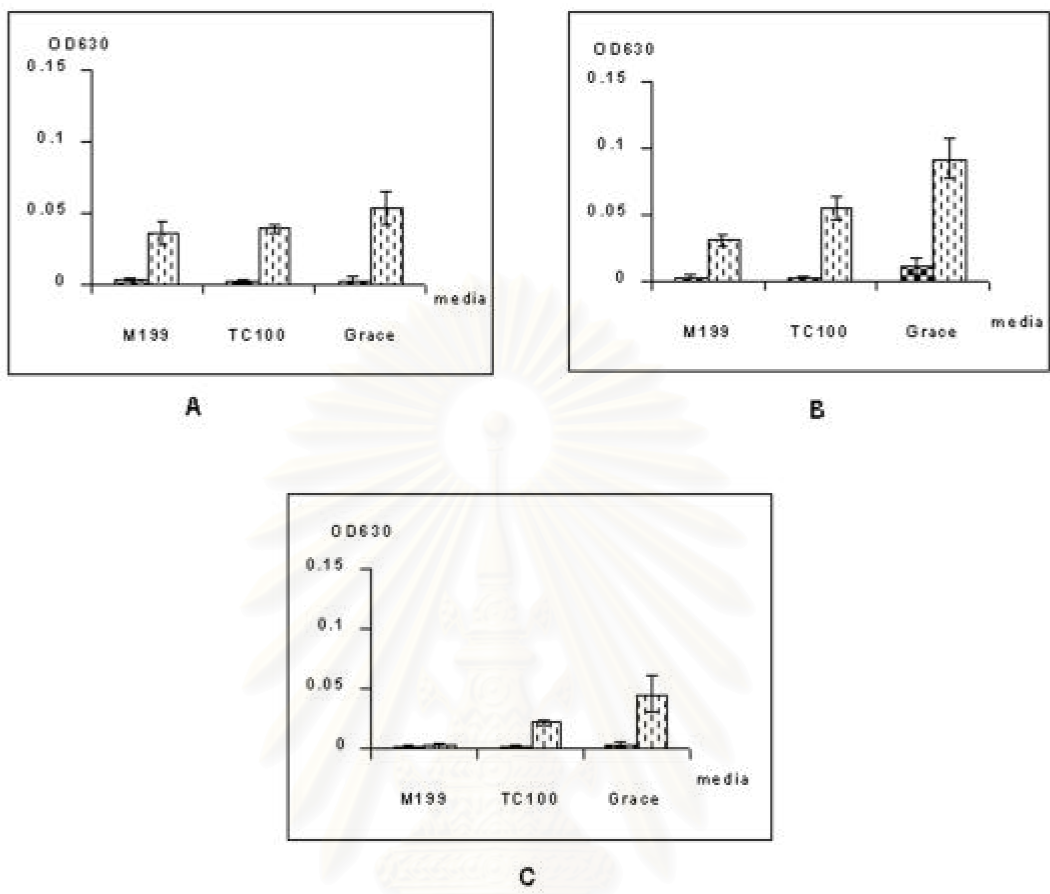

Figure 3.2 NBT reduction of the haemocytes maintained in M199, TC100 and Grace's insect medium. In the absense of PMA In the presence of PMA (30 μg/ml PMA) A. Stimulated haemocyte cell at 0 hr B.Stimulated haemocyte cell which were maintained for 2 h. C. Stimulated haemocyte cell which were maintained for 4 hrs.

It can be concluded from the results that the haemocytes maintained in TC100 and Grace's insect media obtained the high level of enzyme activity but the number of viable haemocytes maintained in both media were decreased dramatically after 24 hrs of incubation. On the other hand, the enzyme activities of haemocytes maintained in M199 was lower but the viability of the haemocytes was considerably high for up to 4 days of incubation without changing the media. For this reason, M199 was further used for *in vitro* experiment.

3.3. The viability of haemocytes treated with thermal shock

There was no significant difference between the viability of the haemocytes from control and thermal shock samples at any temperature and time ($P > 0.05$) (Fig. 3.3 and Table 3.3), indicating that the thermal shock condition used in this experiment was suitable for further *in vitro* experiment.

Table 3.3 The percentage of viability of haemocyte cell after challenge with cold and heat shock at 4, 30, 33 and 35°C for 30, 45, 60, 90 and 120 min

4°C			30°C			33°C			35°C		
Time (Min)	**Control**	**Treatment**	**Time (Min)**	**Control**	**Treatment**	**Time (Min)**	**control**	**treatment**	**Time (Min)**	**Control**	**treatment**
30	96.05	93.38	30	94.02	92.67	30	97.89	98.19	30	96.58	95.37
45	93.73	92.91	45	94.83	95.81	45	97.63	94.50	45	96.64	95.55
60	95.22	93.33	60	94.90	95.51	60	98.17	97.39	60	96.29	93.97
90	93.02	92.40	90	91.67	89.73	90	97.23	94.17	90	96.67	93.14
120	91.97	91.00	120	91.67	86.67	120	96.62	95.94	120	96.24	92.23

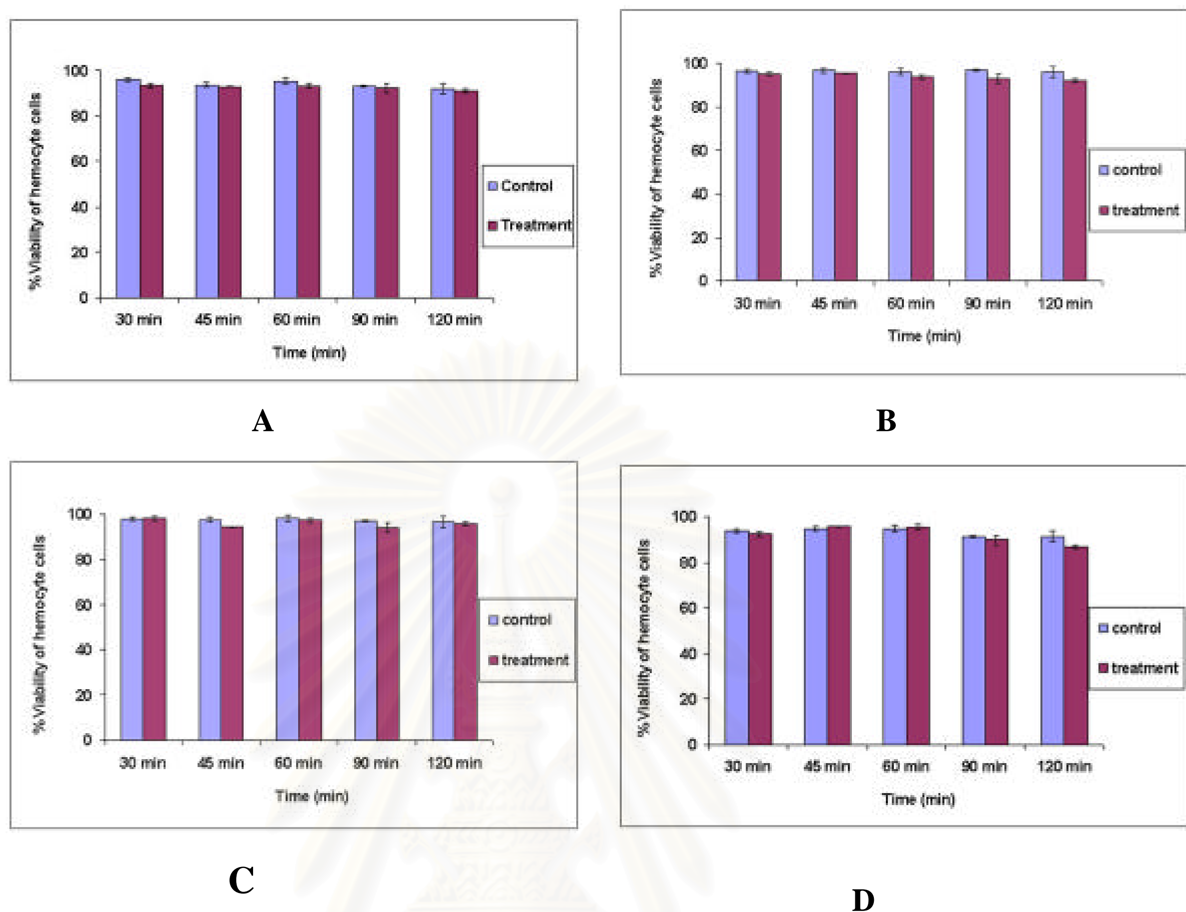

Figure 3.3 The percentage of viability of haemocytes after challenging with cold and heat shock at 4°C (A), 30°C (B), 33°C (C), and 35 °C (D).

3.4. Identification of heat induced proteins by SDS-Polyacrylamide gel electrophoresis

The result of protein profiles extracted from thermal shock haemocytes were shown in figure 3.4. A number of intensified bands were found in samples treated with 4, 30, 33, and 35°C but not present in control sample. Three peptide bands (149, 87 and 42 kDa) were shown in the coomassie stained gel whereas eight peptide bands (149, 121, 106, 87, 65, 62, 55 and 42 kDa) were observed in the gel stained with silver (Fig 3.4b). However, the presence of these different bands was not repeatable in some experiments (picture not shown). Therefore, the response of haemocytes in heat shock condition was no longer detected by this method.

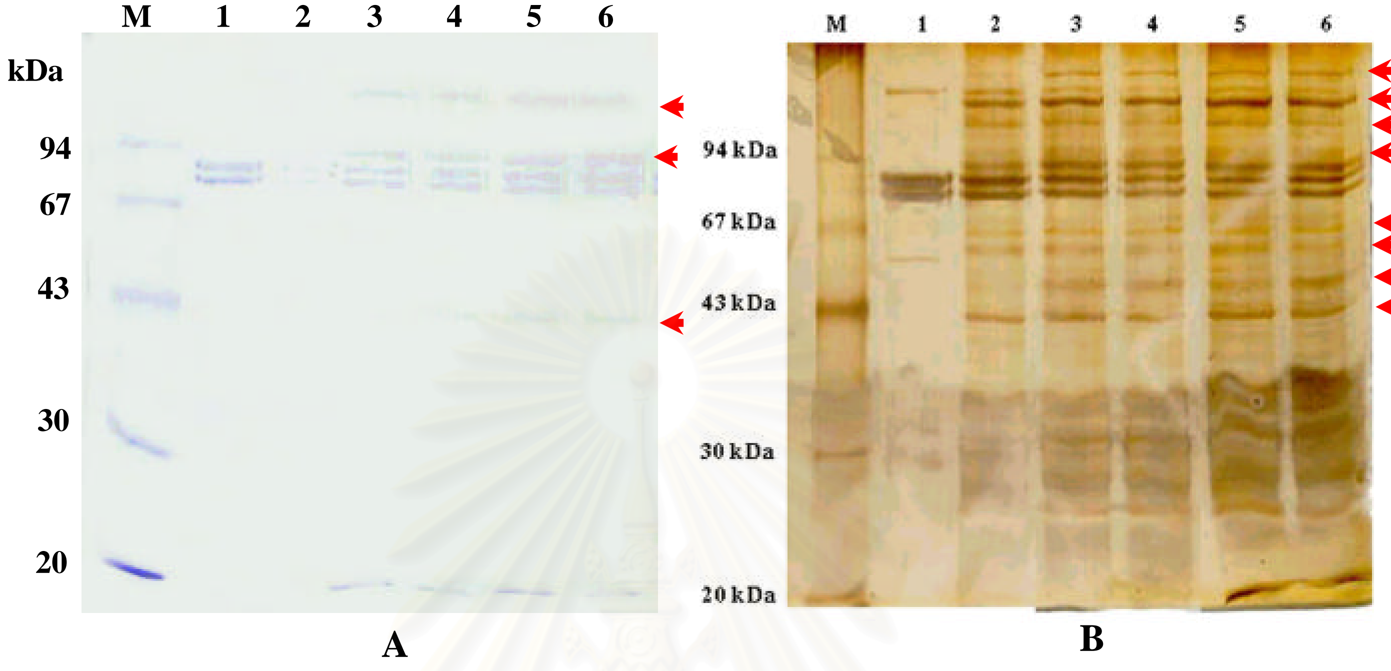

Figure 3.4. Protein profile of samples (5 μg each) on 12% SDS-PAGE stained with Coomassie brilliant blue (A) and silver solution (B)

Lane M = Low molecular weight

Lane 1 = Un-treated haemocyte cell

Lane 2 = Control (haemocyte cell was maintained at 28 °C)

Lane 3 = Haemocyte cell was treated with 30 °C

Lane 4 = Haemocyte cell was treated with 33 °C

Lane 5 = Haemocyte cell was treated with 35 °C

Lane 6 = Haemocyte cell was treated with 4 °C

3.5 Detection of HSPs by Western blot analysis

Only HSP90 was detected by Western blot analysis. The result was shown in figure 3.5. The labelled bands were found at the molecular weight of 82 kDa after detecting with anti-HSP90 monoclonal antibody. The antibody dilution used in this detection was 1:500 which was very low. This revealed the low cross reactivity of the the antibody. There were no corresponding bands of HSP60 and HSP70 found on the gels when mouse anti-human HSP60 and anti-bovine HSP70 monoclonal antibodies were used as primary antibodies detection. This indicated the possibility of low levels of HSP60 and HSP70 and low cross reactivity between the antibodies and target proteins. This caused the low sensitivitie of the reaction beyond possible detectional level of this method.

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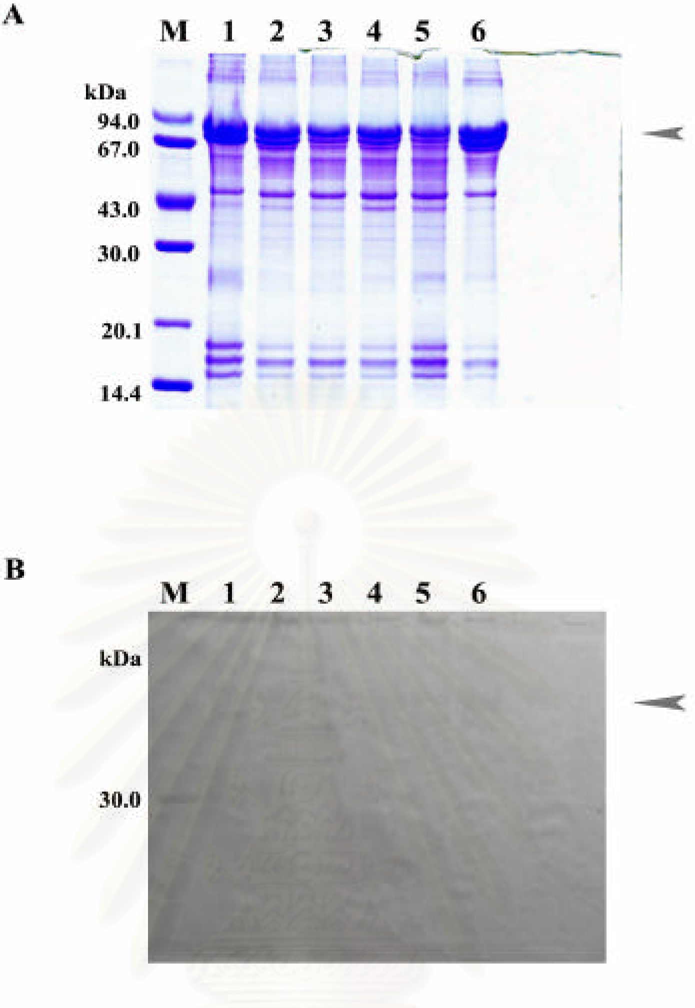

Figure 3.5 Electrophoretic pattern of haemocytes treated with thermal stress. Samples were loaded and run on 2 replications of 12 % SDS-PAGE gels. One gel was stained by Coomassie brilliant blue (A) and the other was subjected to Western blotting, stained by immunochemical method using mouse anti-HSP90 monoclonal antibody, and developed by DAB (B). Black arrows indicated the corresponding bands of HSP90.

Lane M = Low molecular weight

Lane 1 = Un-treated haemocyte cell

Lane 2 = Control (haemocytes maintained at 28 ^{o}C)

Lane 3 = protein extracted from haemocytes shocked at 30 ^{o}C

Lane 4 = protein extracted from haemocytes shocked at 33 ^{o}C

Lane 5 = protein extracted from haemocytes shocked at 35 ^{o}C

Lane 6 = protein extracted from haemocytes shocked at 4 ^{o}C

3.6 Translation *in vitro* of the genes in thermal shock haemocytes

The haemocytes maintained in MEM medium (minimum essential medium without methionine) containing 10 µci/ml of ^{35}S-methionine. The samples were thermal-treated at 4, 28 (ambient temperature), 30, 33 and 35ºC for 2 h. After remaining at ambient temperature for 2 hr. Then samples were extracted and analyzed by 12% SDS-polyacrylamide gel electrophoresis.

The result of protein profiles in 12% gel stained with silver revealed that 3 major bands (42, 73, and 97 kDa) were detected in the samples from haemocytes treated with both cold (4°C) and heat shocks (30, 33, and 35 ^{o}C) whereas these bands were absent in control samples (Fig. 3.6). After the gel was autoradiographed with X-ray film, no corresponding bands for those target peptides and any other bands were detected (the figure of X-ray film was not shown). The difference of protein bands between thermal shock and normal haemocytes were detected by common silver staining technique but were not detected by radioactive labelling which was a higher sensitive technique. This indicated the existance of the inhibitor within the *in vitro* translation process.

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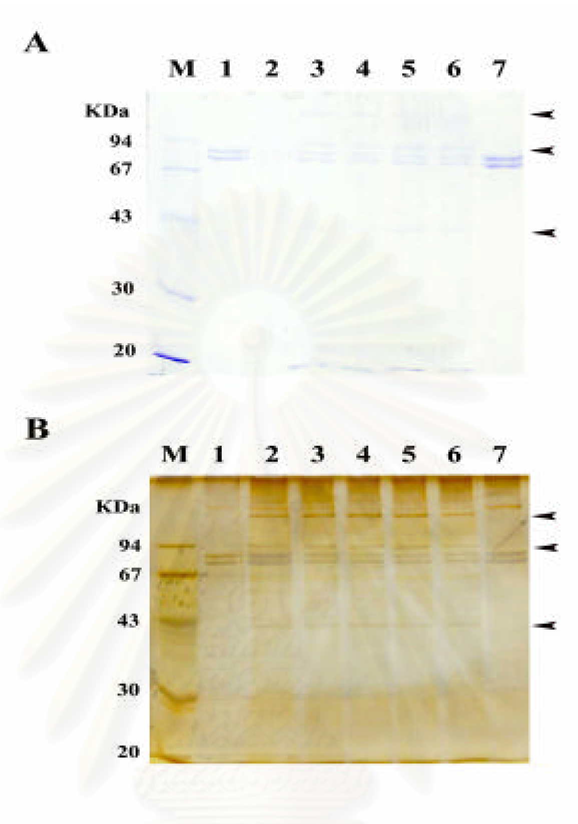

Figure 3.6 Protein profile of samples (2 μg each) on 12% SDS-PAGE. The gels were stained with Coomassie brilliant blue (A) and silver solution (B)

Lane M = Low molecular weight

Lane 1 = Un-treated haemocytes

Lane 2 = protein extracted from haemocytes induced at 4 ^{o}C

Lane 3 = Control (protein extracted from haemocyte maintained at 28 ^{o}C)

Lane 4 = protein extracted from haemocytes induced at 30 ^{o}C

Lane 5 = protein extracted from haemocytes induced at 33 ^{o}C

Lane 6 = protein extracted from haemocytes induced at 35 ^{o}C

Lane 7 = Media MEM at 35 ^{o}C

3.7 Differential expression of thermal induced genes in the haemocytes detected by 51$ DUELWUDU\ SULP HGSROPHUDVH FKDLQUHDFWLRQ (RAP-PCR)

Differential expressed genes were examined using 10 different random primer combinations. The results showed the differential displayed fragments ranging from approximately 280 to 820 bp. The bands smaller than 200 bp were not examined. The result wa shown in table 3.4 and figure 3.7-3.11.

Table 3.4 summary of differential displayed markers obtained from RAP-PCR.

Primer combination	Differential displayed markers (size, bp)
UBC268 and UBC119	430
UBC268 and UBC122	310, 370, 400 and 510
UBC268 and UBC128	300, 305, 400, 405, 480, and 820
UBC268 and UBC268	300, 340, and 820
UBC268 and UBC135	280, 415, 510 and 625
UBC268 and UBC158	450 and 500
UBC268 and UBC174	320, 480, 510 and 600
UBC268 and UBC228	395
UBC268 and UBC299	335 and 475
UBC268 and UBC457	380, 480 and 760

Differential expressed RAP-PCR fragments were purified from each gel and re-amplified using the corresponding primers. Ten fragments were selected, cloned, and sequenced. The result of identified clones was shown in table 3.5. Nine fragments were identified as unknown genes and one fragment contained DNA sequence homologous to the vigilin gene (Table 3.5). Details on DNA sequences of each selected fragments were shown in Figure 3.12.

Table 3.5 Summary of partial gene sequences from RAP-PCR. The percentage identity:similarity, E-value were obtained from BLASTX.

Primer	Closest sp.	E-value	Genes
UBC268_UBC119	-	-	Unknown
UBC268_UBC122	-	-	Unknown
UBC268_UBC128	-	-	Unknown
UBC268_UBC135	-	-	Unknown
UBC268_UBC158	-	-	Unknown
UBC268_UBC174	-	-	Unknown
UBC268_UBC228	-	-	Unknown
UBC268_UBC268	-	-	Unknown
UBC268_UBC299	-	-	Unknown
UBC268_UBC459	*Homo sapiens.*	$4x\ 10^{-43}$	vigilin

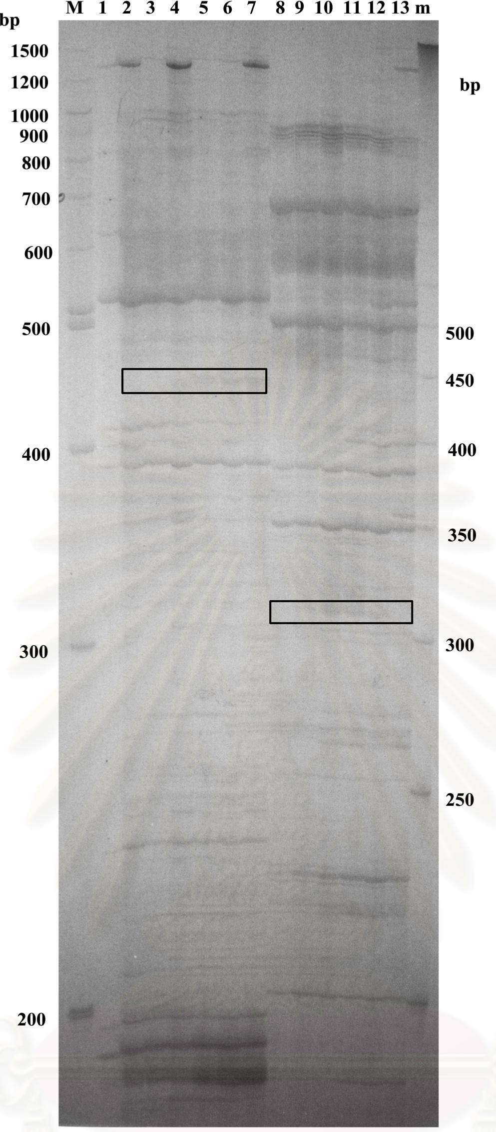

Figure 3.7 Differential display of gene expression in haemocyte of *P. monodon* after thermal stress analyzed on 4.5% denaturing polyacrylamide gel electrophoresis from pirmer UBC119 (Lane 1-6) and UBC122 (Lane 7-12). Boxes indicated that differential expression display were cloned and sequence.

Lane M = 100 bp ladder

Lane 1, 7 = Control

Lane 2, 8 = Cold shock (4°C)

Lane 3, 9 = Shock at room temperature

Lane 4, 10 = Heat shock at 30°C

Lane 5, 11 = Heat shock at 33°C

Lane 6, 12 = Heat shock at 35°C

Lane m = 50 bp ladder

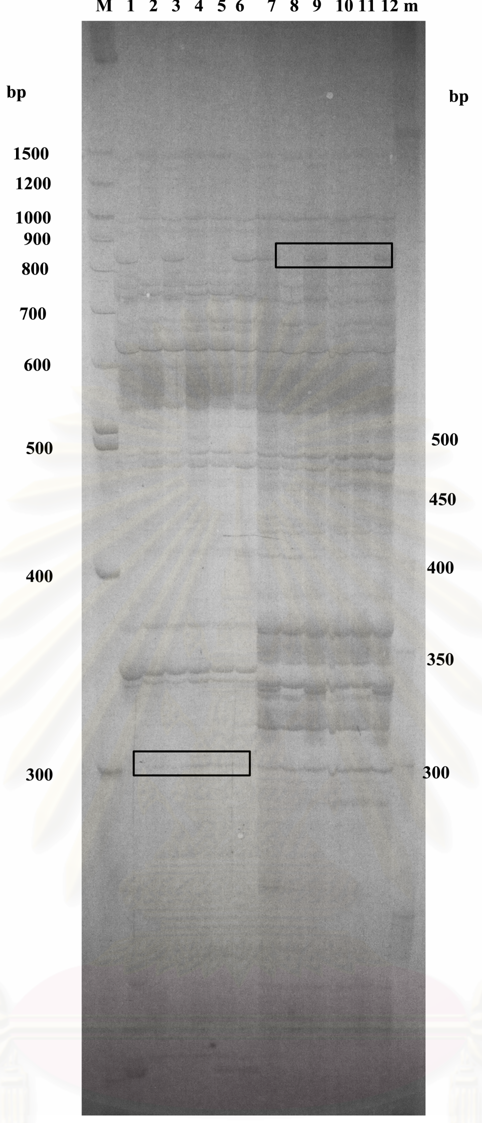

Figure 3.8 Differential display of gene expression in haemocyte of *P. monodon* after thermal stress analyzed on 4.5% denaturing polyacrylamide gel electrophoresis from pirmer UBC128 (Lane 1-6) and UBC268 (Lane 7-12). Boxes indicated that differential expression display were cloned and sequenced.

Lane M = 100 bp ladder

Lane 1, 7 = Control

Lane 2, 8 = Cold shock (4°C)

Lane 3, 9 = Shock at room temperature

Lane 4, 10 = Heat shock at 30°C

Lane 5, 11 = Heat shock at 33°C

Lane 6, 12 = Heat shock at 35°C

Lane m = 50 bp ladder

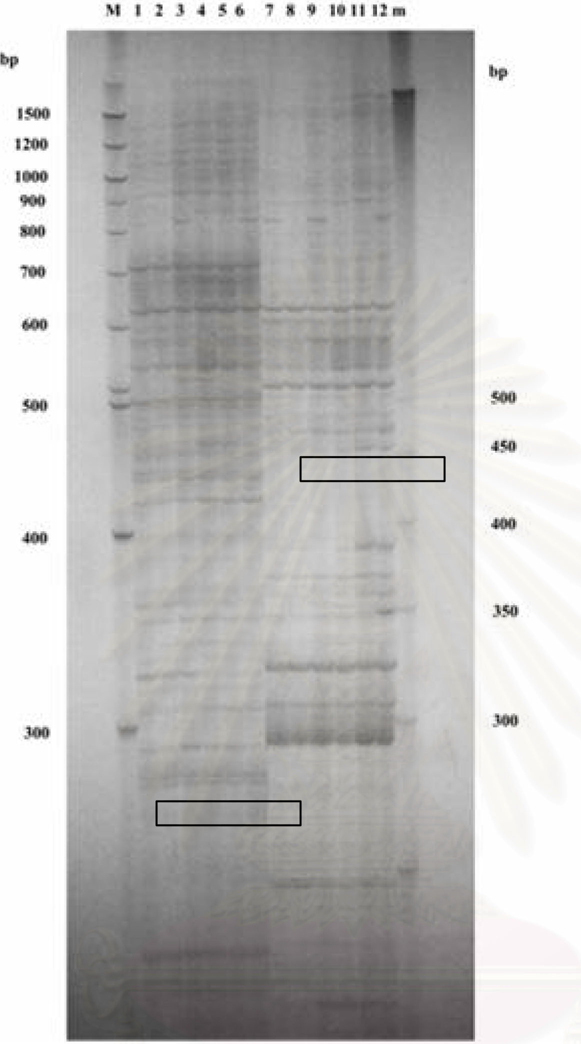

Figure 3.9 Differential display of gene expression in haemocyte of *P. monodon* after thermal stress analyzed on 4.5% denaturing polyacrylamide gel electrophoresis from pirmer UBC135 (Lane 1-6) and UBC158 (Lane 7-12). Boxes indicated that differential expression display were cloned and sequenced.

Lane M = 100 bp ladder

Lane 1, 7 = Control

Lane 2, 8 = Cold shock (4°C)

Lane 3, 9 = Shock at room temperature

Lane 4, 10 = Heat shock at 30°C

Lane 5, 11 = Heat shock at 33°C

Lane 6, 12 = Heat shock at 35°C

Lane m = 50 bp ladder

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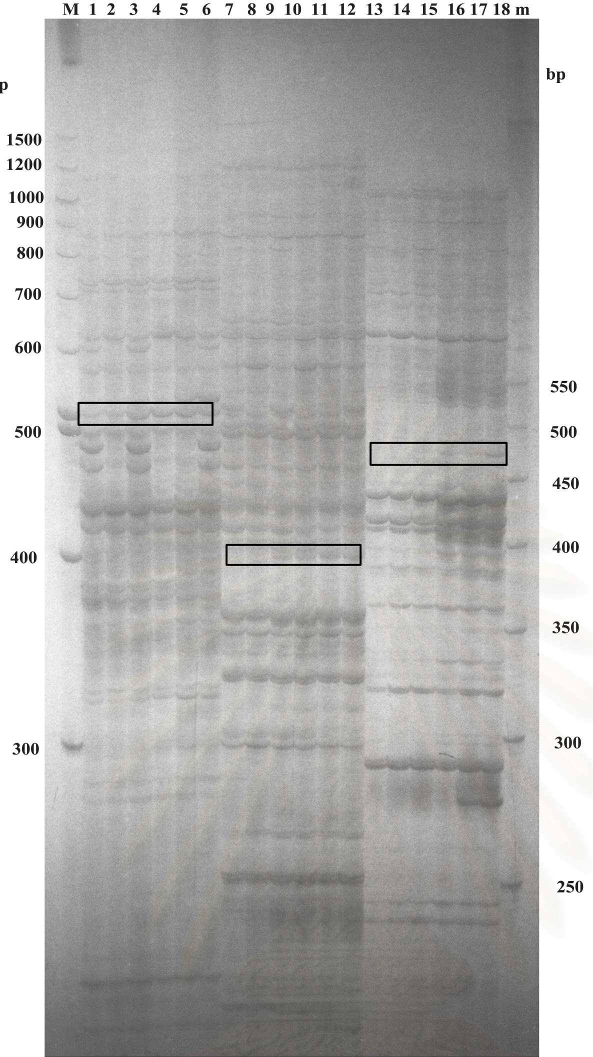

Figure 3.10 Differential display of gene expression in haemocyte of *P. monodon* after thermal stress analyzed on 4.5% denaturing polyacrylamide gel electrophoresis from pirmer UBC174 (Lane 1-6), UBC228 (Lane 7-12) and UBC 299. Boxes indicated that differential display were cloned and sequenced.

Lane M = 100 bp ladder

Lane 1, 7,13 = Control

Lane 2, 8,14 = Cold shock (4°C)

Lane 3, 9,15 = Shock at room temperature

Lane 4, 10,16 = Heat shock at 30°C

Lane 5, 11,17 = Heat shock at 33°C

Lane 6, 12,18 = Heat shock at 35°C

Lane m = 50 bp ladder

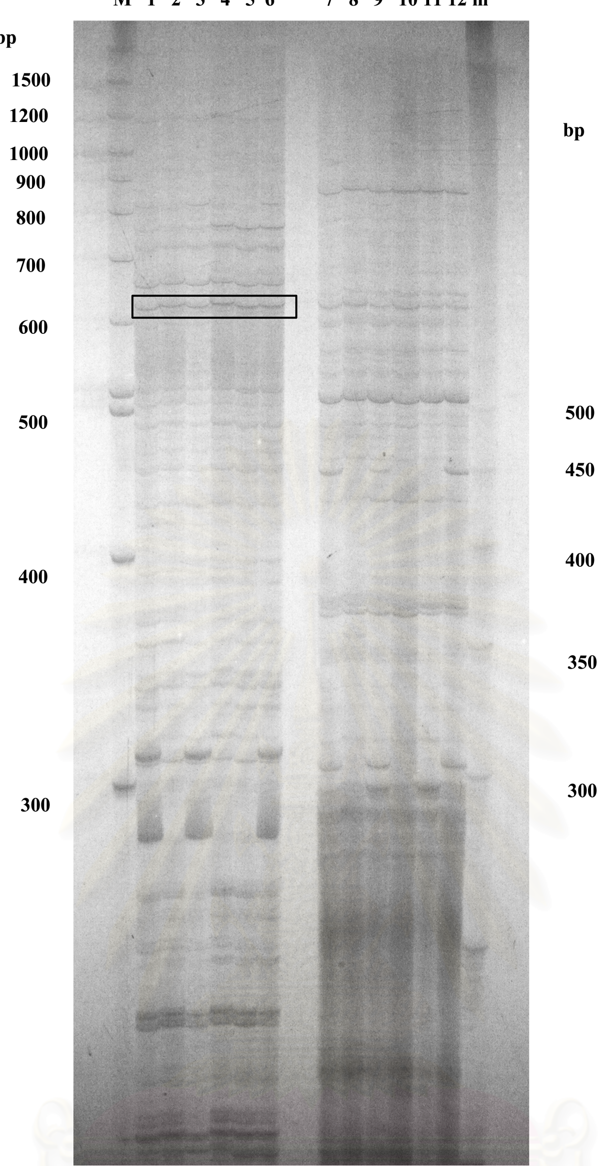

Figure 3.11 Differential display of gene expression in haemocyte of *P. monodon* after thermal stress analyzed on 4.5% denaturing polyacrylamide gel electrophoresis from pirmer UBC457 (Lane 1-6). Box indicated that differential expression display was cloned and sequenced.

Lane M = 100 bp ladder

Lane 1 = Control

Lane 2 = Cold shock (4°C)

Lane 3 = Shock at room temperature

Lane 4 = Heat shock at 30°C

Lane 5 = Heat shock at 33°C

Lane 6 = Heat shock at 35°C

Lane m = 50 bp ladder

UBC268-UBC119 Size 442 bp

AGGCCGCTTACTCAGACACACATTGTGCAATCAGCGATACTGAATCATGTTTAAATTGACATCAGTACT
GACACACTTTTGTCTCGTACAAACATACAGGACTGTGCTGCATACATGATGATGGGATGATTAATGATG
AGTGATGTACTATGAATACCACCTAAATAGGTGGCTTCTTGTCAAGGAACCTCCAATCAGCTTCAGCTA
CTGGAATGCTTGGATTTTAGTTTGCCCACAACAAAAGATTCCATGACTAGAATTAACCGAAGCCCTTGG
NTATTTACAAGAATGTACAACCAAAAGGTTCAGTTAATTGTACTTTGATGTTTAAATAGTACAACAGGC
TATTTAAATAATAACTTCCTTAATAAAATAATATAACTTTACAGCTCGTCATGTTCAAGATCATTATCA
AGATCATCTTCGTCTTCATCGCCCAATA

UBC268_UBC122 Size 321 bp

GTAGACGAGCAGCATGTATAAATATCCAAAATCGCTACCGTGGTTGGAGGCTACGACTGGTATTCCTGA
GAAAGAGGCGAGCGGCAGTAATTATTCAGTCTAATGTCCGAGGAATGTTTGCCAGAGAGGTTGCAAATG
CCCTTCGTGAAATGAAGCGAATCGAGGAGGAACAGCGGAGACGAGAGAAGCTGGAGGAGGAGAGACGAC
AGAGGGAAGAAGAAGCCCTCAAATTGGCAGAGGACGAGGAGAAGAGGAAAGAGCTGGAGGAGGCTCAGA
GGTATGAGCTAGAGAATGCTTGTGGAAATGTAGTTAAGCGGCCTA

UBC268_UBC128 Size 302 bp

GCATATTCCGCTCATAAACAAATACGTCGAGATAAGTATCTAATAACTTGCATTACCTCAAATTGTCGA
GAGCTTTTAATTCCCACGCCATTGCGATGAACGTGCGGTTCACAATGTTCTTTACCAAACGGTTATCAA
AGTGCCTTACCAAGGGCCCTGTCCCCTGCAACCCTGACTCAATTAATGCTTTCCCTGNTTTACATGACA
ATCATTTCGAGTGCTATTTCAGGACTAAAGCACTCGGGGTAGGTGTATGATTCTTTTATGCCTTTTGCT
TTATATATTGTGGAGTAAGCGGCCTA

UBC268_UBC135 Size 279 bp

AGGCCGCTTACATATGAAGATCAGTTCAGGCGAGCGTGTAATCATCTCACTAAAGTGATTCGCTCTACC
CGTAGCAAATACTATACAAAAAAACAAAAACAACAACTAAAGTCTACCTCAAATACCTGCTGGTACACA
TGGAAGGTCATCAATTAAATTCTGAATAGAAATGATACATCTCAGAAACAAACGATATAAGTAGAGGAA
GGTCAGTCTTAAGATCCTATTCAGATTTCTCACTATATCAATGATTATTTCGCTAATGTTGCTCGCAGC
TTA

UBC268_UBC158 Size 459 bp

TAGCCGTGGCAAGAAAGTTACAAGATGTCTTTGAAATGAGATATGCCAAAGTGCCAGAAGATGAACCCT
TCGAAATTCAACCAGCAACAGATTCCGAAGAGTCTGAAAGTGAGAGTGAGAGTGAAACTGACGACTCTG
AGGATGAGAGAGAAAGGAAATTAGTTCAGTTACAGGAGCAGCTACGGCAGGTGCAAGAACAGATGAAGT
TGCTGGTTGAAGAATCTTTAAGAAGAGGGAAAGAAAAGAAGAAAAAGACCAAAAAGAAAAGTAAAGACC
GTGAGAAGATGTTGTCAGAGTTACCCAACGTTCCAAACACAGTTGCCCCCACTGTGAACCAAAATGCCC
CAACGGCAGCAGCTGTCACAACAGCGACAGCTGCTGTTCCTCCTGCAGCGCCCCCACCTGGCCCAGTTC
CTCCTAAACCTAAGAAGAACAAAACTAGTAATAATAAGCGGCCTA

UBC268/UBC459 size 630 bp

CCAATGACCTTGGGATGGTATTCTGGTCAACCTCCACACGCAGGCTGAAGGAGCGGGCCAGGCGGTCCC
TCTCCTCTTCCTCCAGCTGCTGAACCTTTACTCTGAGGGCGGCGCTGGCTCGCTCAGTGTTGGCTGGAG
GGCCAGTGATCCTGATGATGTCACTCTGGTCCTGAGCTGGTGGAACCTGGATGTTGACATCAAACTCAG
TCATCATGTTGCGCACATTCTCTCCCTTCTGACCAATGATGAAGCGATGATGCCTGAAGGGTACGTTCT
CCTCCACAGTGACTGGCACAAGACTCAGCAGAGCATCACGTGCCTTCTCACAGTTATCCTTCTTGCCCA
TGACGCGCACAATGTCTTGAGGCCGGGTGGCAACTTCCCCGTTCACCATTCACCTCTCCATTCACCTGC
CCGTGACTTTCTTCACTGTTGGCATTTACATTTCCACTATCCTTCTCGGGGAACTTGATCTGTACATCG
AACTCGGTGGTTATGGCTTGCACCTTGCTGCCCCTTGGCACCCATGACAGTGCGGTGGAACTTCTGAGG
GATGATCACTTCCATCTGCACCATCTGGTCCAGATCGTTCACGATTTCGGAAATACGGTTCTTGGCTCC
TCGACGCAATCACTAGTGATTCGCGGCCG

UBC268/UBC174 size 510 bp

AGGCGCGCAAATTACCCACTCCCGGCACGGGGAGGTAGTGACGAAAAATACTGTTGCGAGCCCCGAACG
GGGCCTCGCAATTGGAATGAGTACACTTTAAATCCTTGTACGAGGATCGAGTGGAGGGCAAGTCTGGTG
CCAGCCGCCGCGGTAATTCCAGCTCCACTAGCGTATATTAAAGTTGTTGCGGTTGAAACGCTCGTAGTT
TGACTTCTGCTCCGGACCGGCGGTCCGCCTTAGCGGCGGCTACTGCCGGGTTCCGAGCTGTGTCCCCGC
CGGCGCGCACGGGGTTTTTTATGCCCTTAACCGGGTGTCCCCCTGTGGCCGGCACGTTTACTTTGAAAA
AATTAGAGTGCTCAGAGCAGGCTGGTCTTTGCTTACAGCCCGAATGGTCGTGCATGGAATGATGGAACA
GGACCTCGGTTCTATTTTGTCGGTTTTTCGGAACCCGAGGTAATGATTAATAGAAGCAGTCTTCTTCTT
AGAGGGATAAGCGGCCTAATCACTAGT

UBC268/UBC268 size 820 bp

GTACTGAGTAAGAGTAAAGAATCTTTCCTTGAGATTCTTCTTAGAATTCAGAAAGATGCCCGAGGTCAC
ATGTTGACCCATAAATTCTTGAACAAGGGTGAACTTTTCTTCTGCAGTAGACCAGAGATACAATTGTGA
TGGGGCCATGAAAGGATCTACAGTATCATTTGCTGCCATCAAATTTGCTACCAAGAGCAACCAACCTTC
ATCTCCCAGGGTCAAGTCGAGTGTTGAAGCACTATAGGCAGTATCTAGAGACCAAATCACATCTATGGC
ATTGTTATTCAACTTTAACACACTGACTTTTGTTGTTGGAGCAGTATTCACATCAGAAATTGCACCTGC
CGTCACCATGTAGGTGCCACCACTGGTGGTAAAGGATTTTGCATCTGCAATAATTCCTAGTGCTGATCC
CTGTGGCAGGTCCGTTTCCCTATTGTTTAGCAAGATCTTTACAGTACTTGATCCACCATTGGCACAGCT
GCTTATTAGTCCAGTGCCTTCATACCCTTCTGTACTGAAGATGAAAGCAGAGTTTACATCCGTCTCTAT
CTGGCTCATGTTACCGTTATCATCAACTTCATAGAGTGCATATTGCAAAGGGCATCTGCAATCGTAGTC
ACTGCAATCCGCACAGCCAATACTGATGAACCTCCAAATGT

UBC268/UBC299 size 474 bp

AGAGGCAAGGGGAAATTAGCTGGTGGAAATAACCGTTAAAAAAATTTGCATTTTTTTCCCGGCATTCTG
AATGGGGCTTTTAACGGATATAATCCTGGAGGACAGCAGAGTAAAAATGTGTTTGTTTTAGGTCCTATT
CGAAGGAGTAACTAGTTATTTACTTCAAAGCAAAATCAGTTTAGAAAACAAAACAAATCAAAGTAGTTA
CAAGTTATTCACTGACAAAGCAAAACGATAGTAACTAGTTATTCACATAAAAACAATGTAGAAAGCAAA
ACATGGAAGTAGGAACTAGCTATTTAGTTACACGAAACGTATTAACGGGTTAAACGAGGAGTAAACGAC
ATGCAAGGAAGTGAATCGTACCTCAATTACAAGGAGCTACTGAAATCTACATGTCTTGAAGCGCGAACG
GCTGAGAATGTTGACTGTCTGCTTTGTTGTTTCACTTTTCTAAGCGGCCTAATCACTAGT

UBC268/UBC228 size 393 bp

TGTACAATAATGTCGAAAGCCACAAAACACCAGAAAATTTTCTGAAACAACATGTTTACGGTTATTACA
AGGCCCATCCATGCCCCTCTACCGAGGAGATTTTAATTCTCGCCCGAGACAGCAACACTACAAGGCTAA
GACATTTCCTAATTGTTGAAAGCAAAATCTTAAGTCAATCACGAAGGACAGCCTAAATACCCACATCGC
CAACAAGCATCAAAATCATTCGATCAAATCTCTTCATTTAAATTCGAATACCTTAAGCTTTTAGAATAA
ATTTAGCCTTATTTAGCATAGAATACATTTAACATTTCGACACGAAACCGGTTCTTGCCTCGCCCGCAG
CGCCTCTCATAAACGCGACCATAGGTTTTCGGCCCAGCAATCACTAGT

Figure 3.12 Nucleotide sequences of extra intensity bands expressed in 10 primer combination

3.8 EST analysis of cDNA library

Single colony from mass excision was performed by colony PCR (Fig.3.13A). Plasmid DNAs from recombinant clones were extracted, digested with *EcoR*I and *Xho*I, and separated in 1.2% agarose gels (Fig. 3.13 B). The recombinant clones were

unidirectionally sequenced from the 5' end and the sequences were blasted against data in the GenBank using Blast*N* and Blast*X*.

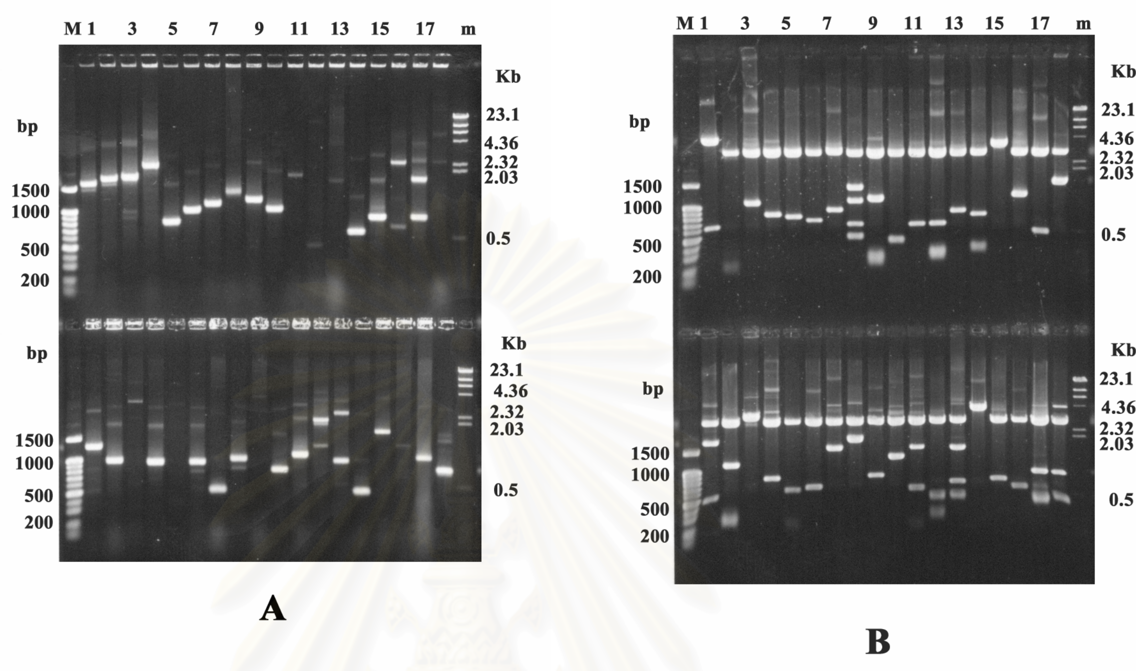

Figure 3.13 A. Colony PCR products from mass excision on 1.2% agarose gel, M = 100 bp ladder m = λ-Hind III marker, Lane 1-18 (Upper and lower) = PCR product **B.** Digested plasmid DNA from recombinant clones M = 100 bp ladder, m = λ-Hind III marker, Lane 1-18 (Upper and lower) = digested plasmid DNA

Haemocyte cDNA library was constructed from heat-stressed *P.monodon*. The titer of the library was 5 x 10^5 pfu/ml. A total of 1090 recombinant clones containing inserts greater than 500 bp in size were unidirectionally sequenced from the 5' terminus. After comparing the sequences against the data in the GenBank, 687 transcripts (63%) significantly matched with the known sequences ($P<10^{-4}$) whereas the remaining sequences were unknown (403 transcripts, 37%) (Table 3.6). The relationship between the number of clone sequenced and the accumulative numbers of new transcripts were shown in Table 3.7 and Figure 3.14. All known transcripts were classified into 12 functional categories. The known transcripts categorized into the

member of defense and homeostasis group were predominated (12.3%) followed by those of gene expression, regulation and protein synthesis group (7.0%) (Table 3.8).

Table 3.6 Summary of ESTs from heat induced haemocyte cDNA library of *P. monodon*.

Experimental animals	Cultured shrimps stressed with 1 h of 35ºC seawater and 2 h of ambient temperature(28ºC).
Tissues	Haemocytes from heat-stressed shrimps
Library titer (pfu/ml)	$5.0x10^5$
Number of cDNA sequences	1090
Number of matched EST(%)	
Number of unmatched EST (%)	

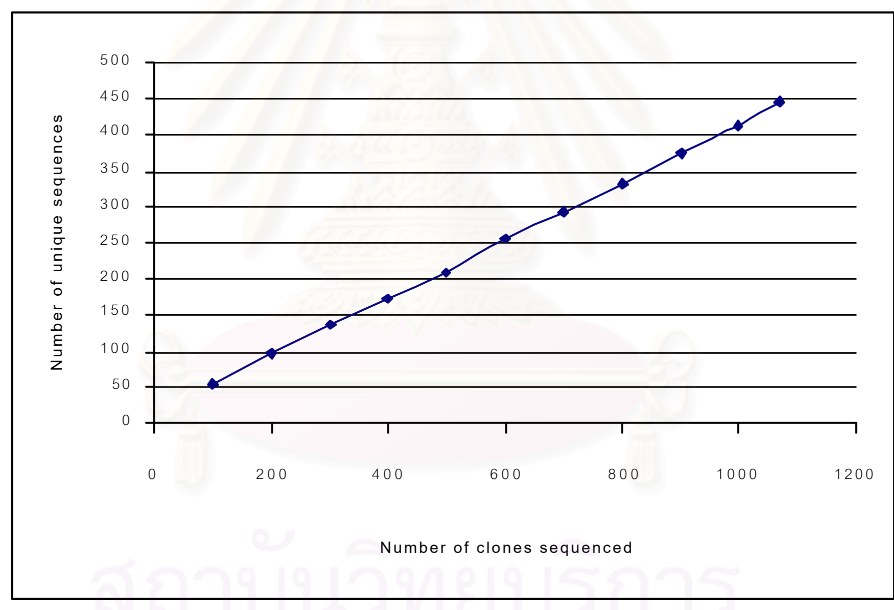

Figure 3.14 Relationship between accumulative number of sequenced ESTs and accumulative numbers of newly identified sequences from the haemocyte cDNA library of heat-stresssed *P. monodon*.

Table 3.7 Number of new genes from Heat shock cDNA library

Clone	Total number of gene	Total number of New gene	% of new gene
50 (clone no.1-55)	31	31	6.93
100 (clone no. 56-109)	54	23	5.14
150 (clone no 110-162)		24	5.36
200 (clone no 163-212)		18	4.02
250 (clone no 213-263)		23	5.14
300 (clone no 264-313)		16	3.57
350 (clone no 314-364)		17	3.80
400 (clone no 365-416)		21	4.69
450 (clone no 417-468)		21	4.69
500 (clone no 469-519)		16	3.57
550 (clone no 520-572)		20	4.47
600 (clone no 573-624)		26	5.81
650 (clone no 625-674)		21	4.69
700 (clone no 675-724)		17	3.80
750 (clone no 725-775)		18	4.02
800 (clone no 776-826)		19	4.25
850 (clone no 827-879)		23	5.14
900 (clone no 880-931)		20	4.47
950 (clone no 932-982)		21	4.69
1000 (clone no 983-1035)		19	4.25
1068 (clone no 1036-1106)		33	7.38
Total		**447**	**100**

Sequence comparison of the EST clones to the DNA databases revealed 60.40% significant match to known genes. Many of these transcripts share similarity with genes involved in basic metabolisms and cellular organization. A total of 1090 expressed sequence tags (ESTs) were found corresponding to defense and homeostatic genes 132 clones (12.1%). Four hundred and thirty two transcripts (39.6%) were unknown. A number of genes were found and classified into 12 functional groups (Table 3.8)

Table 3.8 Classification of genes from haemocytes tress response cDNA library of *P. monodon*

Group	Function	No. Of clones	%
1	Gene expression, regulation and protein synthesis	76	7.0
2	Internal/external structure and motility	50	4.6
3	Metabolism	65	6.0
4	Defense and homeostatis	132	12.1
5	Signaling and communication	20	1.8
6	Cell division/DNA synthesis, repair and replication	34	3.1
7	Ribosomal and rRNA	72	6.6
8	Mitochondria/Protein	60	5.5
9	Transport	16	1.5
10	Miscellaneous function	68	6.2
11	Unidentified (hypothetical) – similar to ther cDNA/DNA	65	6.0
12	Unknown	432	39.6
13	**Total**	**1090**	**100**

3.9 Determination of partial sequences of HSP genes

For HSP60 gene amplification, four primer combinations were used, 5 bands of DNA fragments (850, 749, 300, 280 and 250 bp) were detected (Fig 3.15A). The 749 bp fragments was isolated, cloned and sequenced. The sequence was shown in Figure 3.17.

For HSP70 gene amplification, four primer combinations were also used (Fig.3.15B). Three clear bands of DNA fragments were detected from 3 primer combinations: One band (696 bp) in F1R1 primer combination, 1 band (990 bp) in F1R2 primer combination, and 1 band (612 bp) in F2R1 primer combination. Only smear of DNA was detected in the PCR reaction of F2R2 primer combination. The

696, 990 and 612 bp fragments were isolated, cloned and sequenced. The sequence was shown in Figure 3.18-3.20.

For HSP90 gene amplification, four primer combinations were also used (Fig.3.15C). Two bands of DNA fragments were detected from 2 primer combinations. One band (1261 bp) in F1R2 combination, and 1 band (1186 bp) in F2R2 combination. Only smear of DNA were detected in the PCR reaction of F1R1 and F2R1 combination. The 1261 and 1186 bp fragments were isolated, cloned and sequenced. The sequence was shown in Figure 3.21-3.22.

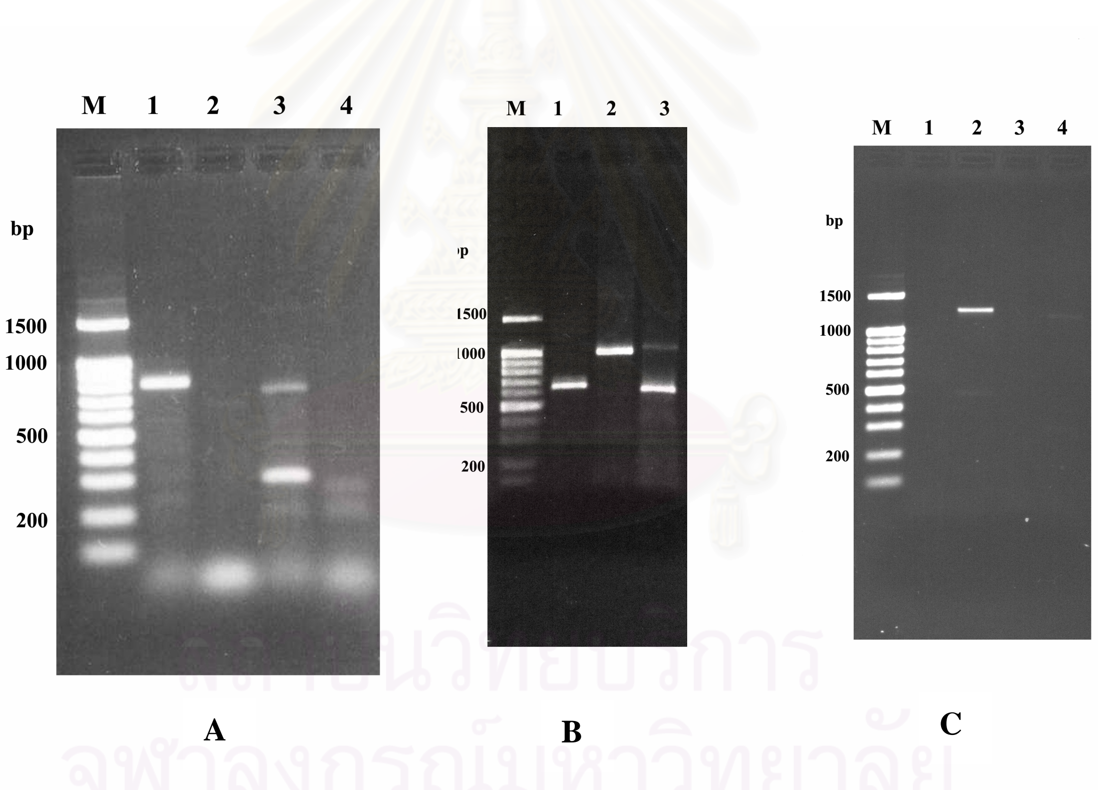

Figure 3.15 PCR product of HSP60 (A), HSP70 (B) and HSP90 (C) analyzed on 1.2% agarose gel.

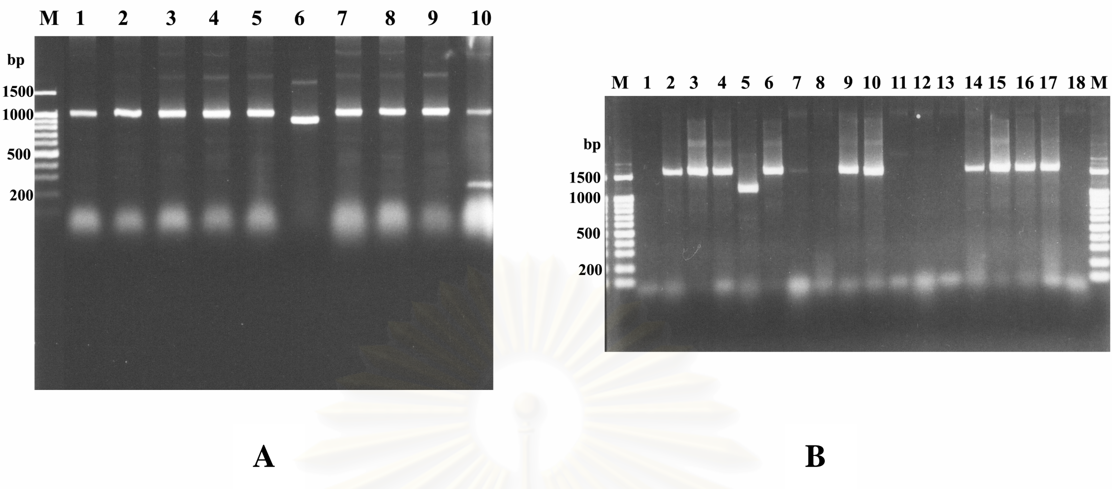

Figure 3.16 Colony PCR of HSP60 (A). Lane 1-10 represented recombinant clones of HSP60, and colony PCR of HSP90 (B). Lane 2-6, 9-10, 14-17 represented recombinant clones of HSP90 analyzed on 1.2 % agarose gel.

TTTGGCACGGAGGTCAGGGCACTGATGCTGCAGGGCGTCGACGTCCTCACCGACGCC
GTGGCTGTCACCATGGGCCCCAAGGGTCGAAATGTAATCATTGAGCAGAGCTGGGGC
AGTCCCAAGATCACAAAGGATGGTGTTACAGTTGCAAAGGCTTTGAACTGAAAGACA
AGTTCCAGAACATTGGAGCTAAGTTGGTCCAAGATGTTGCCAACAACACCAATGAAG
AGGCTGGTGATGGAACCACCACGGCCACAGTCCTGGCTCGCACTATTGCAAAGGAAG
GTTTTGACAGGATTAGCAAAGGTGCCAACCCTGTGGAGATCAGGCGTGGAGTTATGT
TGGCCGTGGATGCCATTGTTGCTCACCTGAAGACCCTGTCAAAGCCTGTGACCACTC
CTGCTGAGATTGCTCAGGTTGCAACCATCTCTGCTAATGGAGATATTGAAGTAGGCA
GTCTTATCTCGGCAGCCATGGAGAAGGTTGGTCGTGAGGGTGTCATCACTGTTAAAG
ATGGCAAGACCTTGAAGGATGAGTTGGAGGTCATTGAAGGCATGAAGTTCGATCGCG
GCTACATTTCTCCTTACTTCATAAACTCCAAGCAAGGGAGCTAAGGTTGAATACCCA
GACTGCCTTGTTTTGCTCTCGGAGAAGAAAATTTCTTCTATCCAGTCCCATTATCCC
CAGTGCTAGAACTGGCCAATGCCCCAAAGGAAACCCTCTATTGATCATTGCTGAGGA
CGTCGATGGCGAG

Figure 3.17 Partial sequence of HSP60 genes from HSP60F2 and HSP60R1 primer combination in Black tiger shrimp *P. monodon*

```
CAAGCGACAAAAGATGCAGGGACTATTGCAGGGTTCAAGGTTGAACGAATCATTAAT
GAGCCGACGGCGGCGGCCGTCGCCTATGGTTTAAATGCAAAAAATAACAGCGAAGAA
AAAAATATATTGGTCTTCGACTTCGGCGGTGGCACGTTTGATGTATCCGTCTTGACT
ATGGCCGAAGGGGTGATTGAGGTTAAGGCCACCGCTGGAAATACGCATTTGGGAGGG
GAAGACATCGACGATAAGATGGTGGAACATTTTGTGAGAGAAATCAAGAGGAAATAC
AAAAAAGACATAAGGGACAACAAGCGAGCGCTGAGACGCTTAAAAACCGCATGCGAA
CAAGCCAAGAGAACACTGTCATCGTCTACTCAGGCTGAAATCACACTAGAGTCTCTC
TGCGATGGCATCGATATAAACTTCGTCATGACCCGTGCTAGATTCGACGAGATTTGC
ACGAATCTATTTCAGAGCACTATAGATCTCGTAAAAAANGCTTTAGAAGACGCCAAG
ATGGACAAGAGTTCTATAACGACATCGTGGTTGGGCCGGAGGATCTACCCGCATACC
GAAGGTCCAAAACTGGTCCCGGGCTATTTTTGAAAAAGACCTGACAAATCTATCAAC
CCCCGACGAAACTGTAATCACCTATG
```

Figure 3.18 Partial sequence of HSP70 genes from HSP70F1 and HSP70R1 primer combination in Black tiger shrimp *P. monodon*

```
CAAGCGACAAAAGATGCTGGACAGATTGCAGGATTGAATGTTCTTCGTGTAATTAAT
GAGCCAACTGCTGCTGCTCTTGCCTATGGCATGGACAAAACTGATGATAAAATCATT
GCTGTGTATGACTTGGGTGGTGGAACTTTTGATATCTCTGTCCTGGAAATCCAGAAG
GGTGTCTTCGAAGTGAAGTCTACCAATGGTGATACTTTCCTTGGTGGAGAAGATTTT
GACAATCACCTTGTGAATTTCCTCGCTTCAGAATTTAAGCGAGAGCAAGGTGTTGAT
GTCACAAAGGACAACATGGCAATGCAACGTCTGAAGGAGGCTTCAGAAAAGGCGAAG
ATTGAACTGTCTTCCTCCACACAGACTGACATTAACCTGCCTTACCTTACCATGGAC
GCTTCAGGGCCAAAGCACATGATGTACAAGTTGACCCGATCCAAGTTTGAGAGCATC
GTGGACAAGCTAGTGAAGCGCACTGTGGACCCCTGCCTCAAGGCAATCAAAGATCTG
AATGTGGCAAAGTCTGANATTGAGAAATCATNCNTGTGGGTGGCATGACCAAGATGC
CCAAGGTGGGTACTACTGTGCAAGACATCTTTGGGCCGGGCCCCAAGTAAGTCGGNG
AATCCTGATGAAACTGNGCTGGGGGACCTGCCATNCACGGNGGAGTGCTTGCTNGGG
ATGTAACAAANGTGCCGCTTCTTGATGTACCNCCCTTTTTNTTGGGATTGAGGCCCT
NGGGAGGATCTTGACTTANCTTATCAACNGGAACNCCCCCTTTCCCNCCCCCNAATN
ACAAGGNTTNTTCCCAC
```

Figure 3.19 Partial sequence of HSP70 genes from HSP70F2 and HSP70R1 primer combination in Black tiger shrimp *P. monodon*

ATAAACGAACCGACAGCTGCCGCCATTGCTTATGGCCTAGACAAGAAGAACGTAGGA
ATGGCTGAGCAAAACGTGTTGATCTTCGACCTAGGAGGCGGTACCTTCGACGTGTCC
ATCCTCAGTATCGACGACGGAGTGTTCGAGGTGAAGGCAACAGCCGGCGACACGCAT
TTGGGAGGCGAAGACTTCGATAACAGGATGGTTAGTCACTTCACACAAGAGTTTCAC
AGGAAATACAAGAAGGATCTCACCACCAATAAACGCGCACTTCGACGTCTTCGAACT
GCTTGTGAACGAGCCAAGCGAACTCTCTCTTCCTCCACACAAGCCAGTCTGGAAATT
GATTCTCTCTTCGAAGGCATTGATTTTACACTTCCATCACCCGTGCAAGATTTGAA
GAGCTTTGTTCTGACCTTTTCAGAGGAACTCTACACCCGGTGGAGAAAGCTCTACGA
GATGCTAAGTTAGACAAGACAAGCATCCACGAAATCGTCTTGGTAGGTGGGTCCACA
CGCATCCCCAAAGTGCAAAAACTACTTCAAGATTTCTTCAGTGGGAAAGAACTGAAC
AAGTCCATTAACCCAGATGAAGCTGTTGCTTACGGTGCTGCAGTTCAAGCAGCCATT
TTACGTGGTGATCAGTCCGACACTGTGAAGGGCATGTTACTTCTTGATGTGCTCCCA
CTTTCCATGGGTCTTGAGACAGCTGGAGGAGTCATGACAGTGCTTATTAAGCGCAAT
ACCACAATTCCCACAAAGCAATCTCAGATCTTCACTACATATTCGGACAATCAACCA
GGCGTTCTCATTCAGGTATACGAAGGCGAACGAGCCATGACCAAGGATAATAATTTA
CTGGGCAAGTTTGATCTAAGTGGAATTCCTCCTGCTCCTCGTGGAGTGCCACAGATC
GAAGTCACCTTCGATATTGACGCGAATGG

Figure 3.20 Partial sequence of HSP70 genes from HSP70F2 and HSP70R2 primer combination in Black tiger shrimp *P. monodon*

CGTGGGCTTCTACTCCGCGTACCTGGTGGCCGACAAGGTGACCGTAGTGTCGAGGAA
CAACGACGACGAGCGGTACATCTGGGAGTCGTCGGCGGGCGGGTCGTTCACGGTGCG
CCACGACACCGGTGAACCCATCGGCCGTGGTACAAAGATCACCCTCCACCTGAAGGA
GGACCAGACAGAGTACCTCGAGGAGCGTCGCGTGAAGGAGATCGTGAAGAAGCACTC
GCAATTCATTGGCTATCCCATCAAGCTCCTCGTCGAGAAGGAGAGGGACAAGGAAGT
GTCTGACGATGAGGAAGAGGAGAAAGAGGAGAAGGAAGAGGAAGCAGAGGAGGACAA
GCCCAAAATCGAAGATGTAGGCGAGGACGAAGAAGCCGACAAAGAGAAGGGCGAAGA
CAAGAAGAAAAAGAAGACGGTGAAGGAGAAGTACACGGAGGACGAAGAGCTGAACAA
GACGAAGCCCCTTGGACGCGCACCCCGACGACATCTGAAGGAGGAGTACGGCGAGTT
CTACAAGTCGCTGACCAACGACTGGGAGGACCACCTGGCCGTGAAGCACTTCAGCGT
GGAGGGCCAGCTGAGTTCCGCGCCCTCCTGTTCCTGCCGCGCCGCGCCCCCTTCGAC

CTGTTCGAGAACCGCAAGCAGAAGAACAAGATCAAGCTGTACGTGCGTCGCGTGTTC
ATTATGGAGAACTGCGAGGAACTGATCCCCGAGTACCTGAACTTCATCAACGGTGTC
GTCGACTCCGAGGATCTGCCTCTCAACATCTCTCGTGAGATGCTGCAACAGAACAAG
ATCCTGAAAGTTATCAGGAAGAATCTCGTCAAGAAGACCCTCGAACTTTTTGAAGAA
ATCGTTGACGACAAGGAAAGCTACAAGAAGTTCTACGAAAACTTCTCCAAGAACCTC
AAACTCGGAATCCACGAGGATTCCACCAACCGCAAGAAGCTTGCCGAATTCCTGAGG
TACCACACTTCTGCCTCTGGCGACGAAATGTCCTCCCTCAAGGAGTACGTGTCCCGC
ATGAAGGAGAACCAGAAACACATCTACTTCATCACTGGCGAGACTCGCGAACAGGTG
CAGAACTCTGCCTTCGTGGAGAGGGTGAAGAAGCGCGGCTTCGAGGTCATCTACATG
ACCGAACCCATCGACGAATACTGCGTTCAGCAGCTGAAGGAATACGACGGGAAGCGG
CTTGTCTCGGTGACGAAGGAAGGCCTTGAACTCCCC

Figure 3.21 Partial sequence of HSP90 genes from HSP90F1 and HSP90R2 primer combination in Black tiger shrimp *P. monodon*

CGAGCAGTACATCTGGGAGTCGTCGGCGGGCGGGTCGTTCACGGTGCGCCACGACAC
CGGTGAACCCATCGGCCGTGGTACAAAGATCACCCTCCACCTGAAGGAGGACCAGAC
AGAGTACCTCGAGGAGCGTCGCGTGAAGGAGATCGTGAAGAAGCACTCGCAATTCAT
TGGCTATCCCATCAAGCTCCTCGTCGAGAAGGAGAGGGACAAGGAAGTGTCTGACGA
TGAGGAAGAGGAGAAAGAAGAGAAGGAAGAGGAAGCAGAGGAGGACAAGCCCAAAAT
CGAAGATGTAGGCGAGGACGAAGAAGCCGACAAAGAGAAGGGCGAAGACAAGAAGAA
AAAGAAGACGGTGAAGGAGAAGTACACGGAGGACGAGGAGCTGAACAAGACGAAGCC
CCTGTGGACGCGCAACCCCGACGACATCTCGAAGGAGGAGTACGGCGAGTTCTACAA
GTCGCTGACCAACGACTGGGAGGACCACCTGGCCGTGAAGCACTTCAGCGTGGAGGG
CCAGCTTGAGTTCCGCGCCCTCCTGTTCCTGCCGCGCCGCGCCCCCTTCGACCTGTT
CGAGAACCGCAAGCAGAAGAACAAGATCAAGCTGTACGTGCGTCGCGTGTTCATCAT
GGAG

Figure 3.22 Partial sequence of HSP90 genes from HSP90F2 and HSP90R2 primer combination in Black tiger shrimp *P. monodon*

3.10 Rapid amplication of cDNA ends-polymerase chain reaction (RACE-PCR)

RACE-PCR was carried out using primers designed from Gene Specific Primers (GSPs) were designed anti-sense primer from heat shock protein genes for 5' RACE PCR and used with universal primer (UPM) that recognize the SMART sequence. Fragments of approximately 600 pb for HSP60 and 700 bp RACE products were clone and sequenced (Figure 3.23).

After characterization of RACE product, clone from 5' RACE HSP60 was similar to HSP60 of *Culicoides variipennis* (E-value = $3x10^{-63}$, Score bits = 243). Other transcripts were similar to HSP60 of *Drosophila melanogaster* and chaperonin 60 of *Rattus norvegicus*. For 5'RACE of HSP90, a clone was similar to HSP90 of *Chiromantes haematocheir* (E-value = $6x10^{-77}$ and Score bits = 288). Other transcripts were similar to 90-kDa HSP of *Apis mellifera*. Nucleotide sequences of 5'RACE clones were shown in Figure 3.24.

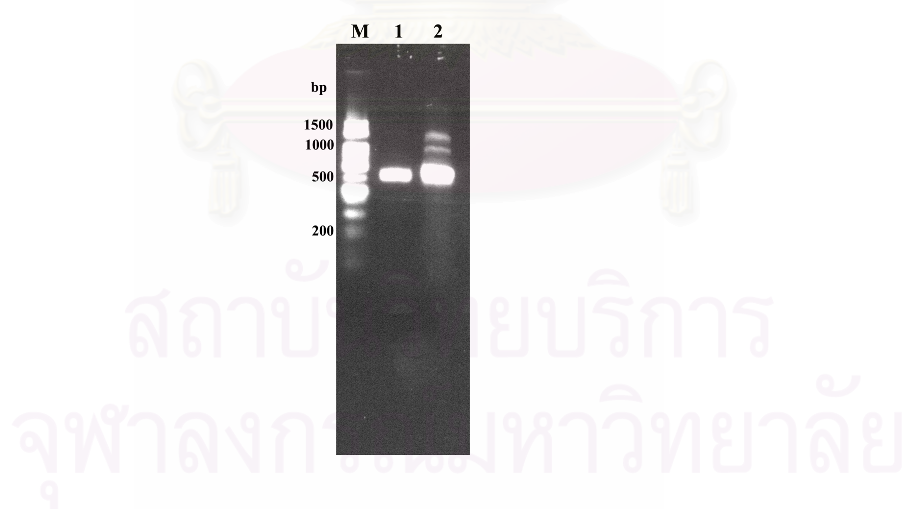

Figure 3.23 5' RACE PCR of HSP60 (lane 1) and 5' RACE PCR of HSP90 (lane 2). Lane M = 100 bp ladder.

A

TTTGACAGGGTCTTCAGGTGAGCAACAATGGCATCCACGGCCAACATAACTCCACGC
CTGATCTCCACAGGGTTGGCACCTTTGCTAATCCTGTCAAACCCTTCCTTTGCAATA
GTACGAGCCAGGACTGTGGCTGTGGTGGTTCCATCACCAGCCTCTTCATTGGTGTTG
TTGGCAACATCTTGGACCAACTTAGCTCCAATGTTCTGGAACTTGTCTTTCAGTTCA
ACAGCCTTTGCAACTGTAACACCATCCTTTGTGATCTTGGGACTGCCCCAGCTCTGC
TCAATGATTACATTTCGACCCTTGGGGCCCATGGTGACAGCCACGGCGTCGGTGAGG
ACGTCGACGCCCTGCAGCATCAGTGCCCTGACCTCCGTGCCAAATTTAACGTCCTTT
GCATAATGTCTTGCCAGGTAGTGCCTTGTGGCCTGGCGAGCGACGGGAGTTCGCAAT
AAAGAGGCTGCGCGATGCATTTTTGTAGGAAGGTTTCAGTGGGAGAGACACACTCTA
CACGATCTCTCAGGCGGCCTCACGGCGTCTAGAATCCCGCGTACTCTGCGTTGATAC
CACTGCTTGCCCTATAGTGAGTCGTATTAGAATCGAATTCCGCGGCCGCCATGGCGG
CCGGGA

B

TATAGATACTCAGCTATGCATCCAACGCGTTGGAGCTTTCCATATGTCGACCTGCAG
GCGGCCGCGAATCACTAGTGATTCTAATACGACTCACTATAGGGCAAGCAGTGGTAT
CAACGCAGAGTACGCGGGGAGCAACAGAAACACGTTCGAGCCGCCGCTGCGTCAGGA
GCTGCGTCAACACATTCCAAAGCCAACAACTTTTGTTCCTTTGTCGGTCAAAGCTTC
ACACATTCCAAAATGGTCGAGGAGACGATGAGCGAGGAGGTGGAGACCTTCGCGTTC
CAGGCGGAGATCGCGCAGCTGATGTCCCTGATCATCAACACCTTCTACAGCAACAAG
GAGATCTTCCTGCGAGAGCTGATCTCGAACTCGTCCGACGCCCTCGACAAGATCCGC
TACGAGTCGCTGACGGACCCGTCCAAGCTGGAGAGCGGCAAGGACCTGTTCATCAAG
CTGGTGCCCAACAAGGACGACCGCACGCTCACCATCATCGACAGTGGCATCGGCATG
ACCAAGGCCGACCTGGTGAACAACCTGGGCACCATCACCAAGTCGGGCACAAAGGCC
TTCATGGAGGCGCTGCAGGCGGGCGCCGACATCTCGATGATCGGCCAGTTCGGCGTG
GGCTTCTACTCCGCGTACCTGGTGGCCGACAAGGTGACCGTAGTGTCGAGGAACAAC
GACGACGAGCAGTACATCTGGGAGTCG

Figure 3.24 Nucleotide sequences of 5’RACE HSP60 (A) and 5’RACE of HSP90 (B).

3.11 Determination of HSP genes from EST library

HSP genes were screened from haemocyte stress response cDNA library using synthesized probe from the DNA fragment of HSP60, HSP70 and HSP90 obtained earlier from partial amplification. Approximately $6.4x10^3$ independent clones from a cDNA library were screened for HSP60 and HSP70 and $8.3x10^3$ screened for HSP90. After immunological detection, 2 positive plaques were found to respond to HSP70 probe (Fig. 3.25). Single colony from single excision was performed by colony PCR. Plasmid DNA from recombinant clones were extracted, digested with *EcoR*I and *Xho*I, and separated in 1.2% agarose gels and unidirect sequenced. Identity of the contiguous HSP70 cDNA with nucleic acid and deduced amino acid sequences was determined using BLASTN and BLASTX . Nucleotide sequences of the clones were shown in Fig. 3.26. No positive plague were obtained when the library was detected with HSP60 and HSP90 probes.

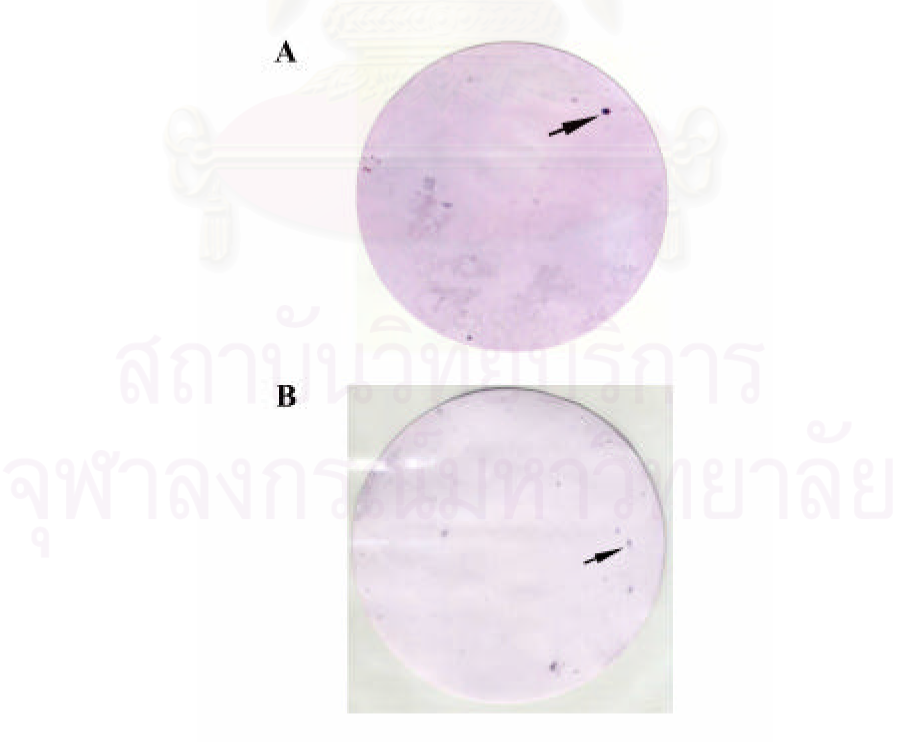

Figure 3.25 Positive plaque screened from haemocyte stress response using HSP70 probe. Black arrow indicated that positive plaque.

Clone 742 bp

TATGACATGATACGCCACNCTCGAAANTTACCTCACTAAAGGGAACAAAAGCTGGAG
CTCCACCGCGGTGGCGGCCGCTCTAGAACTAGTGGATCCCCCGGGCTGCAGGAATTC
GGCACGAGGGAAGAGCGGACGTGTTACAATTAGCTCTTAGGACTATTTAAAAATATC
TAAAATAAGATAAAATGGCAAAGGCACCTGCTGTCGGTATTGATCTGGGAACCACCT
ACTCCTGCGTGGGTGTGTTCCAGCATGGCAAGGTGGAGATCATCGCCAACGACCAGG
GCAACCGCACCACGCCCTCCTACGTCGCCTTCACAGACACAGAGCGTCTGATTGGTG
ACGCCGCCAAGAACCAGGTGGCGATGAACCCCAACAACACTGTATTCGACGCCAAGC
GACTCATCGGCCGCAAATTCGAAGACCACACAGTCCAGAGCGACATGAAGCATTGGC
CCTTCACCATCATCAACGAGAGCACAAAGCCAAAGATCCAGGTAGAGTACAAGGGAG
ACAAGAAGACCTTCTACCCAGAAGAGATCTCCTCGATGGTGCTCATCAAAATGAAGG
AGACCGCCGAGGCTTACCTGGGATCCACAGTGAAGGATGCTGTAGTCACTGTACCTG
CTTACTTCAACGATTCTCAGCGCCAGGCCACCAAGGACGCTGGAACCATCTCGGGTC
TTAATGTGCTGCGTATCATCAACGAACCCACCGCTGCTGCCATCGCCTACGGCCTCG
A

Clone 933 bp

AATNGACATGATACGCACGNCTCGAAATTACCTCACTAAAGGGAACAAAAGCTGGAG
CTCCACCGCGGTGGCGGCCGCTCTAGAACTAGTGGATCCCCCGGGCTGCAGGAATTC
GGCACGAGGCTGAGTGGCATCCCACCTGCTCCCCGTGGCGTGCCTCAGATCGAGGTC
ACCTTCGACATCGACGCCAACGGCATCCTGAACGTATCTGCCGTGGACAAGTCTACT
GGTAAGGAGAACAAGATTACCATCACCAACGACAAGGGTCGCCTCTCCAAGGAGGAG
ATCGAGCGCATGGTGCAGGACGCCGAGAAGTACAAGGCTGACGATGAGAAGCAGAGG
GACCGTATTTCTGCCAAGAACTCCCTCGAGTCTTACTGCTTCAACATGAAGTCGACA
GTTGAGGACGAGAAGTTCAAGGAGAAGATTTCTGAGGAGGACCGCAACAAGATTTTG
GAGACCTGCAACGAGACTATCAAGTGGCTGGACATGAACCAGCTGGGCGAGAAGGAA
GAGTATGAGCACAAGCAGAAGGAGATCGAACAGGTGTGCAACCCCATCATTACCAAG
ATGTACGCTGCTGCTGGTGGTGCTCCTCCAGGCGGCATGCCCGGCGGCTTCCCAGGT
GGTGCCCCAGGTGCTGGCGGTGCTGCTCCCGGTGCTGGTGGTTCCTCCGGACCCACC
ATCGAGGAAGTCGATTAAACGATTCCTCCGCGTCTACTAGTCTCATTGTGAATTGTC
CATGCAAATCGACCCATCGTAGATCATTCCGCATTTTATTTATGATGTTGGTGGCTT
GTGCCATTGGCAGACTTCACATTGCAAGNTTTTCAGTAAACCATTCCAAAATCTGTA
AAACGAATANAAAACCAGCGAAACAANAACAAAACACGGGGGGGCCCGGTAGCCAAT
TCGCCTATATGATCTATTACA

Figure 3.26 Partial sequence of HSP70 genes screening from haemocyte stress response cDNA library using HSP70 probe.

3.12 Determination of complete sequences of HSP genes.

The sequences of HSP genes (HSP60, HSP70 and HSP90) were obtained from 3 methods: RT-PCR, RACE-PCR and EST library screening. For RT-PCR, partial sequences of HSPgenes were obtained from PCR amplifications using degenerated primers. 5' and 3' ends of HSP genes were amplified by RACE-PCR. For EST library screening, cDNA library was constructed using RNA extracted from heat-induced shrimps and a number of EST clones were randomly selected and sequenced. The HSP genes were obtained by the comparison of EST clones with known HSPs. All sequences were aligned with the reported nucleotide sequences in the GenBank database using BLASTN (Appendix D) and BLASTX (Appendix E). Schematic representation of the structure of full length of HSP60, HSP70 and HSP90 genes were shown in Figure 3.27, 3.29 and 3.32. Nucleotide sequences of full length of HSP60, HSP70 and HSP90 were shown in Figure 3.28, 3.31 and 3.33, respectively.

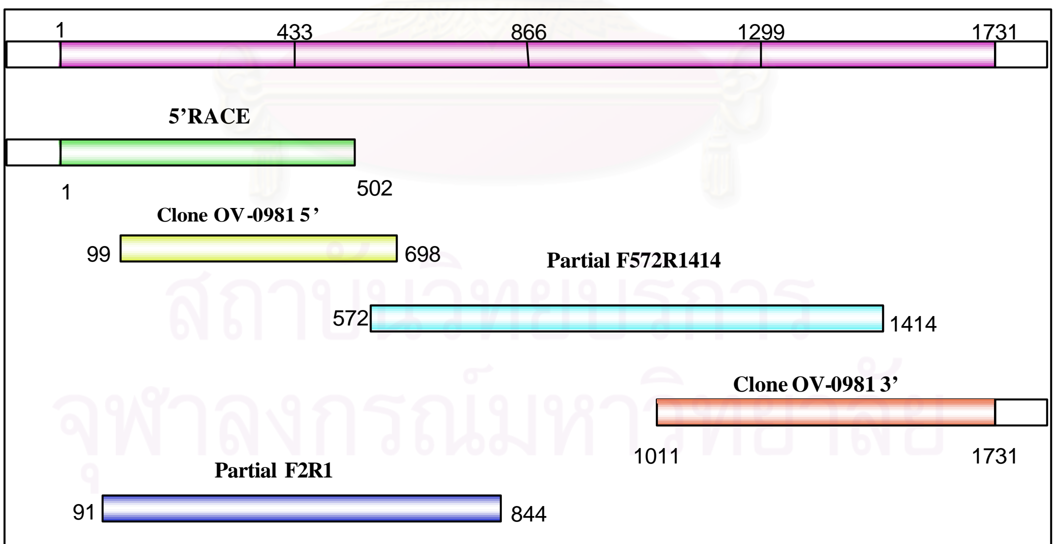

Figure 3.27 Schematic representation of the structure of full length of HSP60 gene. Complete coding sequence (nucleotide 1-1731) constructed from partial sequences of 5' RACE PCR (nucleotide 1-502), ESTs clone OV-0981 5'and 3' end (nucleotide 99-

698 and 1011-1731), partial sequence from F572R1414 primer combination (nucleotice 572-1414). Colorless boxes represent 5’ and 3’ untranslated regions.

TCCCGGCCGCCATGGCGGCCGCGGAATTCGATTCTAATACGACTCACTATAGGGCAAGCAGT
GGTATCAACGCAGAGTACGCGGGATTCTAGACGCCGTGAGGCCGCCTGAGAGATCGTGTAGA
GTGTGTCTCTCCCACTGAAACCTTCCTACAAAA**ATGCATCGCGCAGCCTCTTTATTGCGAAC**
TCCCGTCGCTCGCCAGGCCACAAGGCACTACCTGGCAAGACATTATGCAAAGGACGTTAAAT
TTGGCACGGAGGTCAGGGCACTGATGCTGCAGGGCGTCGACGTCCTCACCGACGCCGTGGCT
GTCACCATGGGCCCCAAGGGTCGAAATGTAATCATTGAGCAGAGCTGGGGCAGTCCCAAGAT
CACAAAGGATGGTGTTACAGTTGCAAAGGCTGTTGAACTGAAAGACAAGTTCCAGAACATTG
GAGCTAAGTTGGTCCAAGATGTTGCCAACAACACCAATGAAGAGGCTGGTGATGGAACCACC
ACAGCCACAGTCCTGGCTCGTACTATTGCAAAGGAAGGGTTTGACAGGATTAGCAAAGGTGC
CAACCCTGTGGAGATCAGGCGTGGAGTTATGTTGGCCGTGGATGCCATTGTTGCTCACCTGA
AGACCCTGTCAAAGCCTGTGACCACTCCTGCTGAGATTGCTCAGGTTGCAACCATCTCTGCT
AATGGAGACATTGAAGTAGGCAGTCTTATCTCGGCAGCCATGGAAAAGGTTGGTCGTGAGGG
TGTCATCACTGTGAAAGATGGCAAGACCTTGAAGGATGAGTTGGAGGTCATTGAAGGCATGA
AGTTCGATCGTGGTTACATTTCTCCTTACTCCATAAACTCCAGCAAGGGAGCTAAGGTTGAA
TACCAAGACTGCCTTGTTTTGCTCTCGGAGAAGAAAATTTCTTCTATCCAGTCCATTATCCC
AGTGCTAGAACTGGCCAATGCCCAAAGGAAACCTCTATTGATCATTGCTGAGGACATTGATG
GAGAAGCCTTGAGCACACTTGTGGTAAACCGCTTGAAGATTGGCCTCCAGGTAGCTGCTGTA
AAAGCTCCAGGCTCTGGTGATAACCGCAAGAATACTCTTCATGACATTGCCATTGCAACAGG
TGCTATTGTCTTCAATGATGAAGCAAGCATGGTCAAGATTGAAGATGTTCAGGTTCATGATC
TTGGCCAGTTGGAGAAGTGCAGATCACAAAGGATGACACACTCCTGTGAAGGGCAAGGGAAA
TACAGTGATATTCAGCGTCGTGTAGAACAAATTAAGGACCAGATTGCTGATAGTTCCTCCGA
GTATAGAAGGAGAAAATGCAGGAGCGTATGGCTCGTCTGGCCTCAGGTGGCAGTTGTGAAGG
TTGGAGGTTCCTCGGAGGTTGAAGTGAACGAGAAGAAGGATCGTGTAAATGATGCTCTGTGT
GCAACAAGGGCTGCAGTTGAAGAGGGCATCGTTCCAGGTGGAGGAGTTGCCTTAATTCGTTG
CCTTCCTGCTTTAGATACTCTCACTCCAAGCAACGAAGACCAGGAGGTTGGCATTGAAATTG
T&&*&$$**&7$7&&$*$&7&&77*&&$&$&7$77*77$*&$$7*&$**7*77*$7*&$7&$*77$77*7&$$&$$**7&$7*
*$$*&77&7**$*$7*77**$7$7*$7*&7*&7$&$**$$&&77&*77$$&&77*7**$$*&$**$$7&$77*$7&&&$&&$$
77*7&&*7$&$*&&&7$$&7*$7*&7*&$ $*7**&77&&&7&&7&$&&$&$*&7*$*$*7*7&77&$*$*$7&&&&
$$**$$*$$&&$*&7**7$7***$**&$7***7**7$7***&**$$7***7**&$7***&**$$7***$**&$7****GCG**
GCATGATGTAAAGCTTCCCATGGATTGGCTAGGAAGGAACTCTTAATTTGTAAACTAACATT
TTTTTGTTATGTACAAAGTTACTTTGGTTCTACAAGAGGTACGGAGAGTACATAGATGCCAC
AGAACTATGTCTAGTTTACAAGAAAATCAATAAGCGGGAGGAAATCTTCAATGTATTTAGAA
GTAACCCCTGGACAGTCTCAGCGAGGAAAAGGGACATGCAAAACATGAAGTTTGCACAAGTC
AAAGGAATTGTTAACCATTCCAAAGAAGTACCTCGGCATACCGAATGTGGTCCATTAATTTC
ATCTTAATCATCTTCATCTTGAATGTTTTGAAGGGTATATGTGCTTCATCTTAGTGTAAATG
TTGAATGTTTGTACAAAGTGTAGATAAGAGAATGTGTAGATAATTTTGTTATAAGGGAAGAT
TTGTTGTTTCCCTAAATGAAATAAGATTTTGAGAAGAAAAAAGAGGGAAAAAA

Figure 3.28 Nucleotide sequence of full sequence HSP60 *P. monodon*, coding sequence illustrated in bold letter.

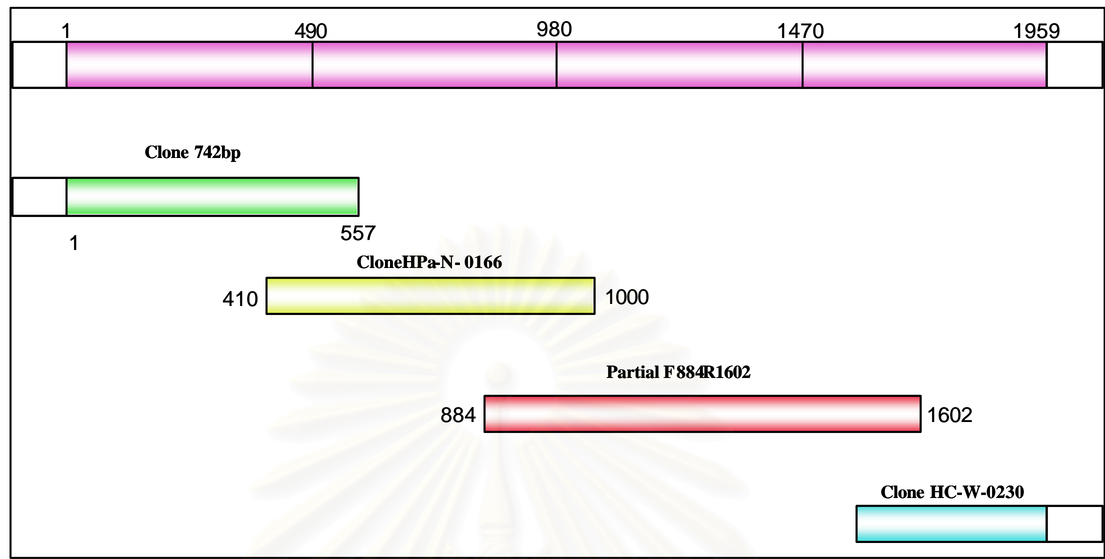

Figure 3.29 Schematic representation of the structure of full length of HSP70 gene. Complete coding sequence (nucleotide 1-1959) constructed from partial sequences of clone 742 bp (nucleotide 1-557), ESTs clone sequence Hpa-N-0166 (nucleotide 410-1000), partial sequence from F884R1602 primer combination (nucleotice 884-1602). Colorless boxes represent 5' and 3' untranslated regions

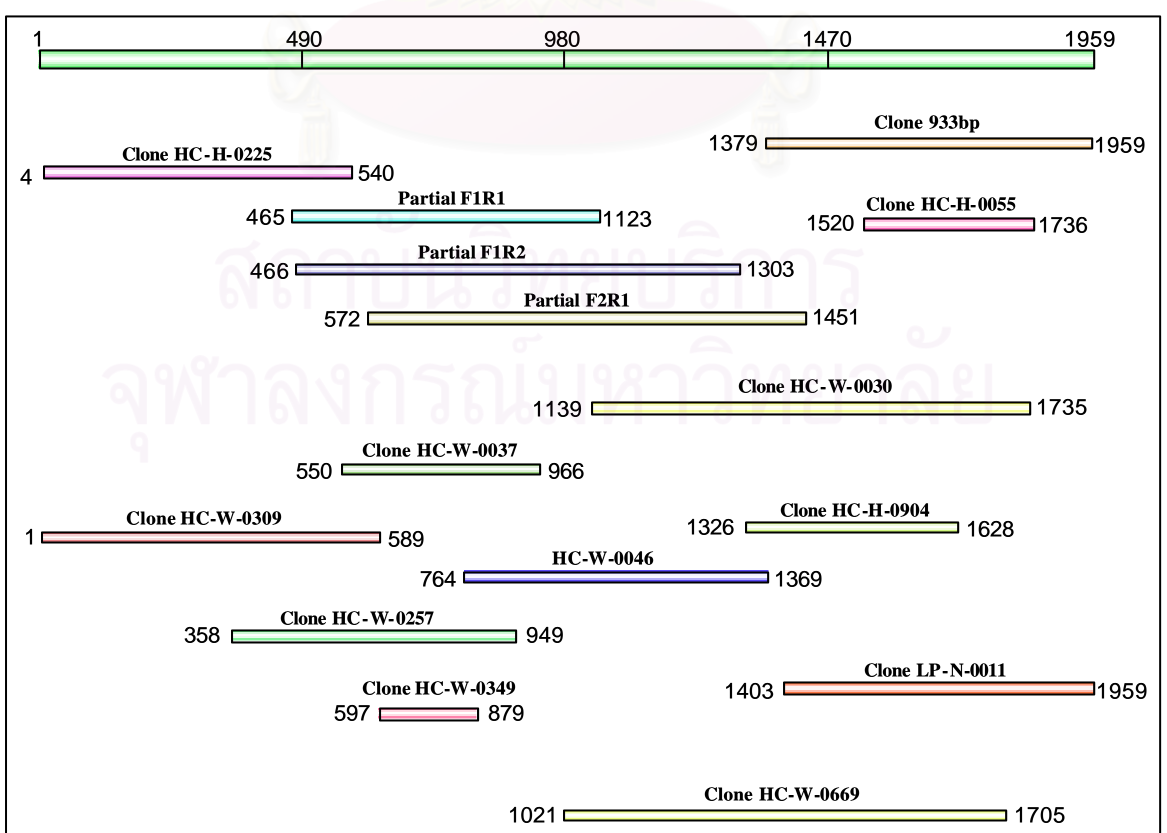

Figure 3.30 Partial sequences of HSP70 gene were blast againt HSP70 of other species.

TATGACATGATACGCCACCTCGAAATTACCTCACTAAAGGGAACAAAAGCTGGAGCTCCACC
GCGGTGGCGGCCGCTCTAGAACTAGTGGATCCCCCGGGCTGCAGGAATTCGGCACGAGGGAA
GAGCGGACGTGTTACAATTAGCTCTTAGGACTATTTAAAAATATCTAAAATAAGATAAA**ATG**
GCAAAGGCACCTGCTGTCGGTATTGATCTGGGAACCACCTACTCCTGCGTGGGTGTGTTCCA
GCATGGCAAGGTGGAGATCATCGCCAACGACCAGGGCAACCGCACCACGCCCTCCTACGTCG
CCTTCACAGACACAGAGCGTCTGATTGGTGACGCCGCCAAGAACCAGGTGGCGATGAACCCC
AACAACACTGTATTCGACGCCAAGCGACTCATCGGCCGCAAATTCGAAGACCACACAGTCCA
GAGCGACATGAAGCATTGGCCCTTCACCATCATCAACGAGAGCACAAAGCCAAAGATCCAGG
TAGAGTACAAGGGAGACAAGAAGACCTTCTACCCAGAAGAGATCTCCTCGATGGTGCTCATC
AAAATGAAGGAGACCGCCGAGGCTTACCTGGGATCCACAGTGAAGGATGCTGTAGTCACTGT
ACCTGCTTACTTCAACGATTCTCAGCGCCAGGCCACCAAGGACGCTGGAACCATCTCGGGTC
TTAATGTGCTGCGTATCATCAACGAACCCACCGCTGCTGCCATCGCCTACGGCCTCGACAAG
AAGGTCGGCGGTGAGCGCAATGTCTTGATCTTCGATCTTGGCGGTGGTACCTTCGATGTGTC
CATCCTTACCATCGAGGATGGTATCTTCGAGGTCAAGTCAACAGCTGGTGACACTCACTTGG
GCGGTGAAGACTTCGACAACCGCATGGTGAACCACTTCATCCAGGAATTCAAGCGCAAGTAC
AAGAAGGACCCAAGTGAGAACAAGCGCTCCCTGCGTCGCCTGCGTACGGCCTGTGAGCGTGC
GAAGCGCACCCTGTCTTCCTCGACACAGGCCAGTGTGGAGATCGACTCCCTCTTCGAAGGTA
TCGACTTCTACACCTCTATCACTCGTGCTCGCTTCGAGGAGCTGTGCGCTGATCTGTTCCGT
GGCACCTTGGAGCCCGTGGAGAAGTCACTCCGTGATGCCAAGATGGACAAGGCCCAGATCCA
CGACATCGTCCTTGTCGGAGGATCCACCCGTATCCCTAAGATCCAGAAGCTCCTGCAGGACT
TCTTCAACGGCAAGGAGTTGAACAAGTCCATCAACCCCGATGAGGCTGTGGCCTACGGCGCC
GCTGTCCAGGCCGCCATTCTGTGCGGTGACAAGTCCGAGGCTGTGCAGGACCTGTTGCTGTT
GGATGTGACCCCCTTGTCCCTGGGTATCGAGACTGCCGGCGGTGTGATGACTGCGCTCATCA
AGCGTAACACCACCATCCCCACCAAGCAGACCCAGACCTTCACCACCTACTCTGACAACCAG
CCAGGTGTGCTCATCCAGGTGTACGAGGGAGAGCGTGCCATGACCAAGGACAACAACCTCCT
GGGTAAGTTCGAGCTGAGTGGCATCCCACCTGCTCCCCGTGGCGTGCCTCAGATCGAGGTCA
CCTTCGACATCGACGCCAACGGCATCCTGAACGTATCCGCCGTGGACAAGTCTACTGGTAAG
GAGAACAAGATTACCATCACCAACGACAAGGGTCGCCTCTCCAAGGAGGAGATCGAGCGCAT
GGTGCAGGACGCCGAGAAGTACAAGGCTGACGATGAGAAGCAGAGGGACCGTATTTCTGCCA
AGAACTCCCTCGAGTCTTACTGCTTCAACATGAAGTCGACAGTTGAGGACGAGAAGTTCAAG
GAGAAGATTTCTGAGGAGGACCGCAACAAGATTTTGGAGACCTGCAACGAGACTATCAAGTG
GCTGGACATGAACCAGCTGGGCGAGAAGGAAGAGTATGAGCACAAGCAGAAGGAGATCGAAC
AGGTGTGCAACCCCATCATTACCAAGATGTACGCTGCTGCTGGTGGTGCTCCTCCAGGCGGC
ATGCCCGGCGGCTTCCCAGGTGGTGCCCCAGGTGCTGGCGGTGCTGCTCCCGGTGCTGGTGG
TTCCTCCGGACCCACCATCGAGGAAGTCGATTAAACGATTCCTCCGCGTCTACTAGTCTCAT
TGTGAATTGTCCATGCAAATCGACCCATCGTAGATCATTCCGCATTTTATTTATGATGTTGG
TGGCTTGTGCCATTGGCAGACTTCACATTGCAAGTTTTCAGTAAACCATTCCAGAAATCTGT
AAAACGAATAAAAAAAAACAGGAAACAAAAAAAAAAAAAAA

Figure 3.31 Nucleotide sequence of full sequence HSP70 *P. monodon*, coding sequence illustrated in bold letter.

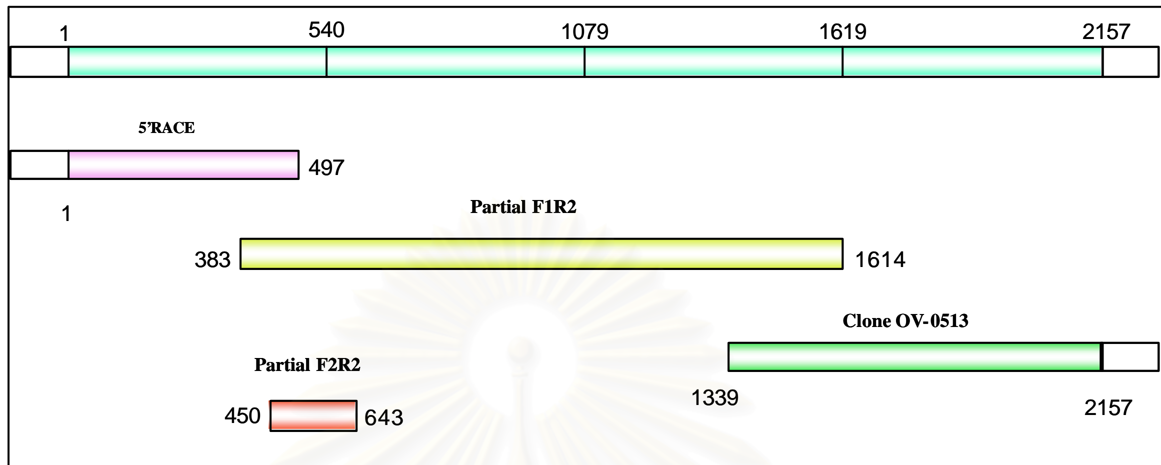

Figure 3.32 Schematic representation of the structure of full length of HSP90 gene. Complete coding sequence (nucleotide 1-2157) constructed from partial sequences of 5' RACE PCR (nucleotide 1-497), partial sequence from F1R2 primer combination (nucleotice 383-1614). ESTs sequence clone OV-0513 (nucleotide 1339-2157), Colorless boxes represent 5' and 3' untranslated regions

TATAGATACTCAGCTATGCATCCAACGCGTTGGAGCTTTCCATATGTCGACCTGCAGGCGGCCGCGAAT
CACTAGTGATTCTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGTACGCGGGGAGCAA
CAGAAACACGTTCGAGCCGCCGCTGCGTCAGGAGCTGCGTCAACACATTCCAAAGCCAACAACTTTTGT
TCCTTTGTCGGTCAAAGCTTCACACATTCCAAA**ATGGTCGAGGAGACGATGAGCGAGGAGGTGGAGACC**
TTCGCGTTCCAGGCGGAGATCGCGCAGCTGATGTCCCTGATCATCAACACCTTCTACAGCAACAAGGAG
ATCTTCCTGCGAGAGCTGATCTCGAACTCGTCCGACGCCCTCGACAAGATCCGCTACGAGTCGCTGACG
GACCCGTCCAAGCTGGAGAGCGGCAAGGACCTGTTCATCAAGCTGGTGCCCAACAAGGACGACCGCACG
CTCACCATCATCGACAGTGGCATCGGCATGACCAAGGCCGACCTGGTGAACAACCTGGGCACCATCACC
AAGTCGGGCACAAAGGCCTTCATGGAGGCGCTGCAGGCGGGCGCCGACATCTCGATGATCGGCCAGTTC
GGCGTGGGCTTCTACTCCGCGTACCTGGTGGCCGACAAGGTGACCGTAGTGTCGAGGAACAACGACGAC
GAGCGGTACATCTGGGAGTCGTCGGCGGGCGGGTCGTTCACGGTGCGCCACGACACCGGTGAACCCATC
GGCCGTGGTACAAAGATCACCCTCCACCTGAAGGAGGACCAGACAGAGTACCTCGAGGAGCGTCGCGTG
AAGGAGATCGTGAAGAAGCACTCGCAATTCATTGGCTATCCCATCAAGCTCCTCGTCGAGAAGGAGAGG
GACAAGGAAGTGTCTGACGATGAGGAAGAGGAGAAAGAGGAGAAGGAAGAGGAAGCAGAGGAGGACAAG
CCCAAAATCGAAGATGTAGGCGAGGACGAAGAAGCCGACAAAGAGAAGGGCGAAGACAAGAAGAAAAAG
AAGACGGTGAAGGAGAAGTACACGGAGGACGAAGAGCTGAACAAGACGAAGCCCCTTGGACGCGCACCC
CGACGACATCTGAAGGAGGAGTACGGCGAGTTCTACAAGTCGCTGACCAACGACTGGGAGGACCACCTG
GCCGTGAAGCACTTCAGCGTGGAGCCAGCTGAGTTCCGCGCCCTCCTGTTCCTGCCGCGCCGCGCCCCC
TTCGACCTGTTCGAGAACCGCAAGCAGAAGAACAAGATCAAGCTGTACGTGCGTCGCGTGTTCATTATG
GAGAACTGCGAGGAACTGATCCCCGAGTACCTGAACTTCATCAACGGTGTCGTCGACTCCGAGGATCTG
CCTCTCAACATCTCTCGTGAGATGCTGCAACAGAACAAGATCCTGAAAGTTATCAGGAAGAATCTCGTC
AAGAAGACCCTCGAACTTTTTGAAGAAATCGTTGACGACAAGGAAAGCTACAAGAAGTTCTACGAAAAC
TTCTCCAAGAACCTCAAACTCGGAATCCACGAGGATTCCACCAACCGCAAGAAGCTTGCCGAATTCCTG
AGGTACCACACTTCTGCCTCTGGCGACGAAATGTCCTCCCTCAAGGAGTACGTGTCCCGCATGAAGGAG
AACCAGAAGCACATCTACTTCATCACTGGCGAGACTCGCGAACAGGTGCAGAACTCTGCCTTCGTGGAG
AGGGTGAAGAAGCGCGGCTTCGAGGTCATCTACATGACCGAACCCATCGACGAATACTGCGTTCAGCAG

CTGAAGGAATACGACGGGAAGCAGCTTGTCTCGGTGACGAAGGAAGGCCTTGAACTCCCCGAGGACGAG
GAGGAGAAAAAGAAGTTCGAGGAACAGAAGACCAAGTTCGAGAACCTGTGCAAGGTAATGAAGGACATT
TTGGACAAGCGCGTTGAGAAGGTGGTGGTGAGCAACCGGCTGGTGACCTCTCCGTGCTGCATCGTGACC
TCCCAGTACGGCTGGACCGCCAACATGGAACGCATCATGAAGGCTCAGGCGCTGAGGGACACCTCGACC
ATGGGCTACATGGCCGCCCAGAAGCACCTTGAGATCAACCCCGACCACAGCATCATCGAAACCCTGAGA
CAAAGGCGGATGCCGAACAAGAACGACAAGTCTGTGAAGGATCTGGTGATGCTGCTGTTCGAGAGCTCC
CTTCTGTCGTCTGGCTTCAGCTTGGAGGACCCAGGTGTCCACGCCAGCCGCATCTACAGAATGATCAAG
CTTGGCCTGGGTATTGACGAGGAGGACGCCCCGATGGAGGAGGCCGAGACCTTGGAGGAGGATATGCCC
CCCCTCGAAGGTGATGACGAGGACGCCTCTCGCATGGAAGAAGTCGATTAAATATTCGTCACAACTTAA
AATATTCACCCATTATATACCAAAGCTAATCATTGTCATTCATTCGGGAACCAAAATACTTCTCTAATG
TTGGTATATTTTGGCTTCTCTGGCTTTCATCATTCCGATCACGCCCAACATTCCATAAGATTTAAACAA
GCATTAGTTTTAGTTATAGACAAAGATATATTCTGTTATAAGGATTTATTCTTTCGTTTATGTAAATAA
TTTGTAACAACTTTGTTACAATAAAACTCGAGCCTCGTGCCGAATTTGGCTCAAGGGTTTATCTGTGGG
AGGTTCCTGTGTTATTGCCCCTAGTCCGTACTCTGAATCTCTGGCTCTGAAATGGATGCTTCACTTGTG
TGTTTATGTCTGTTTTACACAATTATTGTAATGTGACATTTTTTTGAAAATACTGTATTGTCCATTGCC
AATATGTGAGATGGCCATTTTGGGGGCTCTGTAGACTCCATCATAATCCTAAACAGACTTGGTTGCTGG
TTCTCATGAGTTCCTTCTACTTGGTTACCTCATGGCCGGACCTCAACATGGCTGTTGTTCGTCATATAA
CCATCAACATAGTTGTTGGTGATATATCACGGAGTTCCCTCATTGTATCGTATGCTTAAGTATTTTTTT
GTTTTAAAGTGCCATTTGCAGTAAGACTATCTGGTAGGACTGTGTCACGAGCTCAACCAACCAGCATGA
CATGGCATCAAGATCTACTGTTCTAGTGTGTTATACTGCAACGTCTCTCACCTAGTACGCATCTTTGTA
CAAAAATATTAAATATTAATATAGAAAAAAAAAAAAAAAAAA

Figure 3.33 Nucleotide sequence of full sequence HSP90 *P. monodon*, coding sequence illustrated in bold letter.

Full length of HSP60 (2364 bp) was combined with overlapping fragments from RT-PCR, EST transcripts from ovary cDNA libray (S. Klinbunga), and RACE-PCR (Fig.3.27). Sequence analysis revealed an open reading frame of 1731 bp encoding a putative polypeptide of 576 amino acids with a predicted size of 61,129.20 Da and calculated pI of 6.03. Deduced amino acid sequences shared significant identities with mitochondrial Hsp60s from several animals, including the fruit fly (69%), the *Culicoides variipennis* (69%) and *Paracentrotus lividis* (66%). Chaperonins cpn60 signature (AAVEEGIVPGGG) was detected at residue of 427-438. The C-terminus consists of multiple tandem repeats of the Gly-Gly-Met motif (McLennan *et al*., 1993). The deduced amino acid sequence of *P. monodon* HSP60 contained multiple potential sites for phosphorylation by cAMP/cGMP-dependent kinases, protein kinase C and casein kinase II, and also included multiple possible sites of N-glycosylation, myristoylation, and tyrosine sulfation (Table 3.9). Secondary structure of *P.monodon* HSP60 was predicted and shown in Figure 3.35

The full-length cDNA of HSP70 was reconstructed from 3 overlapping ESTs which were obtained from 3 different libraries (haemocytes stress response cDNA libray (N. Puanglarp), haemocytes WSSV infected cDNA libray (A. Pongdara),

hapatopancrease cDNA library (W. Rimphanitchayakit) and Lymphoid organd cDNA library (A. Tassanakajon) and partial sequenced cDNAs (Fig. 3.29). The whole sequence contained 2336 bp with a 1959 bp complete open reading frame (ORF) including the stop codon. Putative protein was composed of 652 amino acid residues. Predicted molecular weight was 71522.85 Da and pI was 5.34. The characterization of the ORF amino acid sequence was accomplished by its comparison to amino acid sequences of other HSP70s, and was found to be most similar to the *Litopenaeus vannamei* HSP70 (94%) (Fig.3.50). The patterns, IDLGTTYS (residue 9-16), IFDLGGGTFDVSIL (residue 197-210), and IVLVGGSTRIPKIQK (residue 334-348) were detected. These patterns matched the 3 signature patterns of HSP70 family. The first consensus pattern ([IV]-D-L-G-T-[ST]-x-[SC]) centered on a conserve pentapeptide found in the N-terminal section; the second pattern ([LIVMF]-[LIVMFY]-[DN]-[LIVMFS]-G-[GSH]-[GS]-[AST]-x(3)-[ST]-[LIVM]-[LIVMFC]) and the third pattern ([LIVMY]-x-[LIVMF]-x-G-G-x-[ST]-x-[LIVM]-P-x-[LIVM]-x-[DEQKRSTA]) were on the conserved regions located in the central part of the sequence. The *P. monodon* HSP70 amino acid sequence GPTIEEVD-stop codon (amino acids 645-652, Fig. 3.36) which matched the carboxy terminal signature of cytosolic/nuclear HSP70 (GP(T/K)(V/I)EEVD-stop codon. Two additional amino acid motifs indicate the cytosolic/nuclear assignment of *P.monodon* HSP70. They are the RARFEEL (amino acids 299-305), typical of non organellar eukaryotic HSP70s, and the bipartite nuclear localization signal (KK and RRLRT; amino acids 250–251 and 261–265, respectively) required for nuclear targeting of cytosolic/nuclear HSP70s. The lack of the motif GPKH, typical of prokaryotic HSP70s, revealed unlikely the possibility of false prokaryotic cloning. CAAT box, a basic element of the transcription machinery, was found at -45 of 5' region. A putative polyadenylation site (AATAAA) was found at 19 bp upstream of the polyA tail. Prosite analysis also revealed the presence of a presumed ATP/GTP binding site (AEAYLGST), at residues 131-138, that has been termed the P-loop (Saraste *et al*., 1990). A complete palindromic HSE (CNNGAANNTTCNNG) was not located in the 5' region of *P.monodon* HSP70 in this study. Secondary structure of P.monodon HSP70 was predicted and shown in Figure 3.37.

Complete sequence of *P. monodon* HSP90 was reconstructed from overlapping EST from ovary cDNA library (S. Klinbunga), products of RACE-PCR

and partial sequenced cDNA which displayed sequence similarities with known HSP90 genes (Fig. 3.32). The whole sequence contains 3216 bp. The 3' UTR (819 bp) displayed a polyA tail at their 3' end, confirming that we had obtained the sequences in their integrity at this end. The start and stop codons were located respectively at positions 241 and 2397. The displayed ORFs were 2157 bp long. The polypeptides deduced from the nucleotide sequence comprised 718 amino acids, with a calculated mass of about 83244.30 Da and pI was 5.04. HSP90 proteins family signatures (YSNKEIFLRE) were detected at the amino acid residue 31-40. Glutamic acid-rich region profile was found at residue 216-277. The deduced amino acid sequence contained multiple potential sites for phosphrylation by cAMP/cGMP-dependent kinases, protein kinase C and casein kinase II, and also included multiple possible sites of N-glycosylation, myristoylation, and tyrosine sulfation (Table 3.11). Secondary structure of *P. monodon* HSP90 was predicted and shown in Figure 3.39.

>HSP60

MHRAASLLRTPVARQATRHYLARHYAKDVKFGTEVRALMLQGVDVLTDAVAVTMGPK
GRNVIIEQSWGSPKITKDGVTVAKAVELKDKFQNIGAKLVQDVANNTNEEAGDGTTT
ATVLARTIAKEGFDRISKGANPVEIRRGVMLAVDAIVAHLKTLSKPVTTPAEIAQVA
TISANGDIEVGSLISAAMEKVGREGVITVKDGKTLKDELEVIEGMKFDRGYISPYSI
NSSKGAKVEYQDCLVLLSEKKISSIQSIIPVLELANAQRKPLLIIAEDIDGEALSTL
VVNRLKIGLQVAAVKAPGSGDNRKNTLHDIAIATGAIVFNDEASMVKIEDVQVHDLG
QLEKCRSQRMTHSCEGQGKYSDIQRRVEQIKDQIADSSSEYRRRKCRSVWLVWPQVA
VVKVGGSSEVEVNEKKDRVNDALCATR**AAVEEGIVPGGG**VALIRCLPALDTLTPSNE
DQEVGIEIVRKAIQTPCHTIVSNAGVDASVIVNKVMEASGDVGYDAATGTFVNLVEA
GIIDPTKVVRTALTDAAGVASLLTTAESVITEIPKEEPAGMGGMGGMGGMGGMGGMG
GMGGMM*

Figure 3.34 The deduced amino acid sequence of HSP60 gene. The bold letter indicated that chaperonine cpn60 signature.

```
--HHHHHH------HH-HHHHHHHHHH------HHHHHHHH---HHHHHHH---------
EEEE------------HHHHHHHHHHH-HHHHHHHHHHHH-------------HEHHHHH
HHHH--HHHH-------EHHHHHHHHHHHHHHHHH---------HHHHHHHEE------E
EE-EHHHHHHHH----EEEE------H-HHHHHH------------EE-------EEHHH
HHHHH-H------EEEEEHHHHHHH----HEEEE-----HH--HHHHHHHHHHHHHHHE-
--------------HHHH---EEE-----HEEH--HHH-------H--------------
-----HHHHHHHH--E------H--------EE----HEEEEE----EEEH---------
HHHHHHH------------EEEH----------------HHHHHHHH-------EEE---
----EHEHHHHHHH-----E------EEEHH--------HH-HHHHHHHHHHHHHHHHHH
-HEE------------------------------------
```

Figure 3.35 Secondray structure prediction of HSP60 ; H = helix, E = strand, - = no prediction

>HSP70

```
MAKAPAVGIDLGTTYSCVGVFQHGKVEIIANDQGNRTTPSYVAFTDTERLIGDAAKNQVAMNPNNTVFD
AKRLIGRKFEDHTVQSDMKHWPFTIINESTKPKIQVEYKGDKKTFYPEEISSMVLIKMKETAEAYLGST
VKDAVVTVPAYFNDSQRQATKDAGTISGLNVLRIINEPTAAAIAYGLDKKVGGERNVLIFDLGGGTFDV
SILTIEDGIFEVKSTAGDTHLGGEDFDNRMVNHFIQEFKRKYKKDPSENKRSLRRLRTACERAKRTLSS
STQASVEIDSLFEGIDFYTSITRARFEELCADLFRGTLEPVEKSLRDAKMDKAQIHDIVLVGGSTRIPK
IQKLLQDFFNGKELNKSINPDEAVAYGAAVQAAILCGDKSEAVQDLLLLDVTPLSLGIETAGGVMTALI
KRNTTIPTKQTQTFTTYSDNQPGVLIQVYEGERAMTKDNNLLGKFELSGIPPAPRGVPQIEVTFDIDAN
GILNVSAVDKSTGKENKITITNDKGRLSKEEIERMVQDAEKYKADDEKQRDRISAKNSLESYCFNMKST
VEDEKFKEKISEEDRNKILETCNETIKWLDMNQLGEKEEYEHKQKEIEQVCNPIITKMYAAAGGAPPGG
MPGGFPGGAPGAGGAAPGAGGSSGPTIEEVD
```

Figure 3.36 The deduced amino acid sequence of HSP70 *P. monodon*. The bold letter indicated that the pattern matched the 3 signature of HSP70 family.

```
-------EEE---E-EEEEE-----EEEEE----------EEE----HHHHHHHHH----
-------HHHHHHHH-----------------EEE-------EEEE--------------
-HHHEHHHHHHHHHH-------EEE---------HH-------E---EEEEE-----HHH
HHHH---------EEEEEE-----EEEEEEEE----EEEE--------------HHHHHH
HHHHHHH---------HHHHHHHHHHHHHHHH--------EEEEH--HE--EEEEE----
-HHHHHHHHH-----HHHHHHHHHHH-HH-H-EEEEE------HHHHHHHHHHH------
-------HHHHHHHHHHHHH-----HHHHHHHHE--------EE----HHHHEH------
------EEEE--------EEEEHHH-HHHHHH---H-----------------EEEEE--
-----EEE------------EEE---------HHHHHHHHHHH-----------------
---HHEE--------H-H---------------HHHHH-HH-H-----HHHHHH----HH
H-----HHHHHH
```

Figure 3.37 Secondray structure prediction of HSP70 ; H = helix, E = strand, - = no prediction

```
MVEETMSEEVETFAFQAEIAQLMSLIINTFYSNKEIFLRELISNSSDALDKIRYESLTDPSKLESGKDL
FIKLVPNKDDRTLTIIDSGIGMTKADLVNNLGTITKSGTKAFMEALQAGADISMIGQFGVGFYSAYLVA
DKVTVVSRNNDDERYIWESSAGGSFTVRHDTGEPIGRGTKITLHLKEDQTEYLEERRVKEIVKKHSQFI
GYPIKLLVEKERDKEVSDDEEEEKEEKEEEAEEDKPKIEDVGEDEEADKEKGEDKKKKKTVKEKYTEDE
ELNKTKPLGRAPRRHLKEEYGEFYKSLTNDWEDHLAVKHFSVEPAEFRALLFLPRRAPFDLFENRKQKN
KIKLYVRRVFIMENCEELIPEYLNFINGVVDSEDLPLNISREMLQQNKILKVIRKNLVKKTLELFEEIV
DDKESYKKFYENFSKNLKLGIHEDSTNRKKLAEFLRYHTSASGDEMSSLKEYVSRMKENQKHIYFITGE
TREQVQNSAFVERVKKRGFEVIYMTEPIDEYCVQQLKEYDGKQLVSVTKEGLELPEDEEEKKKFEEQKT
KFENLCKVMKDILDKRVEKVVVSNRLVTSPCCIVTSQYGWTANMERIMKAQALRDTSTMGYMAAQKHLE
INPDHSIIETLRQRRMPNKNDKSVKDLVMLLFESSLLSSGFSLEDPGVHASRIYRMIKLGLGIDEEDAP
MEEAETLEEDMPPLEGDDEDASRMEEVD
```

Figure 3.38 The deduced amino acid sequence of HSP90 *P. monodon*. The bold letter indicated that the signature pattern of HSP90 family.

```
-------HHHHHHHHHHHHHHHHHHEE-------HHHHHEH--------HHHE-------
-------HHEEE---------EEEE--------H-H------EEH---HHHHHHHHH---
-EEE-EEEE-EEEHHEH---HEEE--------EEEE------EEEE------------HE
EE------HHHHHHHHHHHHHH--------H-HHHH------------H--HHHHHHHH-
----------------------H-----------HHHH---------H-H----HHHHHH
H-----HHHHHH--------HHHHHHHH---------HH-------HHEHHHHH-E----
--H-HHHHH-E------------HHHHHHHHHHHHHHHHHH---HHHHHHHHHH------
-HHHHHHHHHHHEEE--------HHHHHHHH--------H--HHHHH-HHHH----EEEE
-----HHE----HHHHHH----EEEE-------HHHH-H------EEEEHHH--------
HHH---HHHHH-HHHHHHHHHH--H-HHHHHEEEE--------EEEE-------HHHHHH
HHH-------HHHHHHH-----------EEHH------------HHHHHHHHHH--E---
------------HHHHHHH-----------HHHHHH-H---------------H----
```

Figure 3.39 Secondary structure prediction of HSP90 (H = helix, E = strand, - = no prediction)

Table 3.9 The deduced amino acid sequence of multiple potential site of HSP60

Potential site	Pattern	Amino acid residue
N-glycosylation site	N[^P][ST][P^]	102-105 : NNTN 229-232 : NSSK
cAMP- and cGMP-dependent protein kinase phosphorylation site	[RK]{2}.[ST]	248-251 : KKIS 308-311 : RKNT
Protein kinase C phosphorylation site	[ST].[RK]	69-71 : SPK 199-201 : TVK 205-207 : TLK 230-232 : SSK 246-248 : SEK 349-251 : SQR
Casein kinase II phosphorylation site	[ST].{2}[DE]	104 TNEE 163 TPAE 199 TVKD 205 TLKD 311 TLHD 379 SSSE 407 SEVE 454 SNED 537 TTAE
N-myristoylation site	G[^EDRKHPFYW].{2}[STAGCN][^P]	76 GVTVAK 111 GTTTAT 142 GVMLAV 182 GSLISA 293 GLQVAA 303 GSGDNR 432 GIVPGG 481 GVDASV 505 GTFVNL 555 GGMGGM 561 GGMGGM 567 GGMGGM
Chaperonins cpn60 signature	A[AS].[DEQ]E.{4}GG[GA]	4 2 7 AAVEEGIVPGGG

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Table 3.10 The deduced amino acid sequence of multiple potential site of HSP70

Potential site	Pattern	Amino acid residue
N-glycosylation site	N[^P][ST][^P]	35 NRTT 64 NNTV 96 NEST 151 NDSQ 360 NKSI 417 NTTI 487 NVSA 575 NETI
cAMP- and cGMP-dependent protein kinase phosphorylation site	[RK]{2}.[ST]	415 KRNT
Protein kinase C phosphorylation site	[ST].[RK]	47 TER 98 STK 138 TVK 153 SQR 259 SLR 320 SLR 340 STR 495 TGK 537 SAK 577 TIK
Casein kinase II phosphorylation site	[ST].{2}[DE]	45 TDTE 66 TVFD 138 TVKD 211 TIED 222 TAGD 265 TACE 286 SLFE 320 SLRD 430 TYSD 489 SAVD 495 TGKE 511 SKEE 551 STVE 563 SEED 573 TCNE 647 TIEE

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Table 3.10 The deduced amino acid sequence of multiple potential site of HSP70 (cont.)

Potential site	Pattern	Amino acid residue
Tyrosine kinase phosphorylation site	[RK].{2,3}[DE].{2,3}Y	517 RMVQDAEKY
N - myristoylation site	G[^EDRKHPFYW].{2}[STAGCN][^P]	8 GIDLGT 162 GTISGL 190 GGERNV 402 GIETAG 408 GVMTAL 616 GAPPGG 624 GGFPGG 632 GAGGAA 639 GAGGSS
Amidation site	G[RK][RK]	74 IGRK
Heat shock hsp70 proteins family signature 1	[IV]DLGT[ST].[SC]	9 IDLGTTYS
Heat shock hsp70 proteins family signature 2	[LIVMF][LIVMFY][DN][LIVMFS]G[GSH][GS][AST].{3}[ST][LIVM][LIVMFC]	197 IFDLGGGTFDVSIL
Heat shock hsp70 proteins family signature 3	[LIVMY].[LIVMF].GG.[ST].[LIVM]P.[LIVM].[DEQKRSTA]	334 IVLVGGSTRIPKIQK

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Table 3.11 The deduced amino acid sequence of multiple potential site of HSP90

Potential site	Pattern	Amino acid residue
Protein kinase C phosphorylation site	SnK SgK TvR TvK SyK TnR SlK SnR TlR SvK	32 - 34 65 - 67 164 - 166 267 - 269 419 - 421 440 - 442 462 - 464 575 - 577 631 - 633 644 – 646
N-glycosylation site	NSSD NKTK NISR NFSK	44 - 47 279 – 282 383 - 386 426 - 42
N-myristoylation site	GIgmTK GMtkAD GAdiSM GGsfTV GTkiTL GVvdSE GVhaSR	88 - 93 90 - 95 118 - 123 160 - 165 176 - 181 373 - 378 668 - 673
Tyrosine kinase phosphorylation site	RnndDer.Y KklaEflrY KrgfEvi.Y	146 - 153 443 - 451 499 - 506
cAMP- and cGMP-dependent protein kinase phosphorylation site	KKhS KKkT	201 - 204 264 - 267
Tyrosine sulfation site	rnndderYiwessag	146 - 160

Table 3.11 The deduced amino acid sequence of multiple potential site of HSP90 (cont.)

Potential site	Pattern	Amino acid residue
Casein kinase II phosphorylation site	TmsE SnkE SltD SklE SgkD TiiD TkaD SddE TvkE TedE SgdE SlkE TkfE SiiE SvkD SleD TleE SrmE	5 - 8 32 - 35 56 - 59 61 - 64 65 - 68 83 - 86 92 - 95 224 - 227 267 - 270 273 - 276 456 - 459 462 - 465 552 - 555 627 - 630 644 - 647 663 - 666 696 - 699 712 - 715
Glutamic acid-rich region profile	Ekerdkevsddeeeekeekeeeaeedkpkie dvgedeeadkekgedkkkktvkekytedeE	216-277
Heat shock hsp90 proteins family signature	YsNKEIFLRE	31 - 40

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The predictions of tertiary structures of *P.monodon* HSPs using Rasmol and Swiss-Model were determined. For HSP60, a single predicted 3D structure of HSP60 was obtained. The protein structure matched with the strong binding peptide domain of HSP60 detected from *Escherichia coli* (PDB ID code = 1mnfL) with 43.2% identity and started from amino acid residue 21 to 553 (Fig. 3.40). The predicted protein structure of HSP70 matched with 3 recognized domains of HSP70 reported in Protein Data Bank. The first domain initiated at amino acid residue 1-389 with 90.1 % identity to 44 KDa ATPase N-terminal fragment domain of HSP70 (PDB ID code = 1ngj_) (Fig. 3.41). The second domain started at amino acid residue 380-560 with 65.1 % identity to the tRNA processing enzyme Rnase PH R86A mutant of HSP70 from *Aquifex aeolicus* (PDB ID code = 1ud0A) (Fig. 3.42). The third domain started at amino acid residue 533-625 with 32.95 % identity to ROD shape-determining protein MREB of HSP70 from *Thermotoga Maritima* (PDB code = 1jceA) (Fig. 3.43). Two predicted 3D structure domains were obtained from HSP90. The first domain was recognized with 88.4 % identity at amino acid residue 4-222 to the N-terminal domain of HSP90 from *Homo sapiens* (PDB code = 1uy8A) (Fig.3.44). The second domain was recognized with 65.25 % identity at amino acid residue 275-538 of HSP90 from *Saccharomyces cerevisiae* (PDB code = 1usvA) (Fig.3.45).

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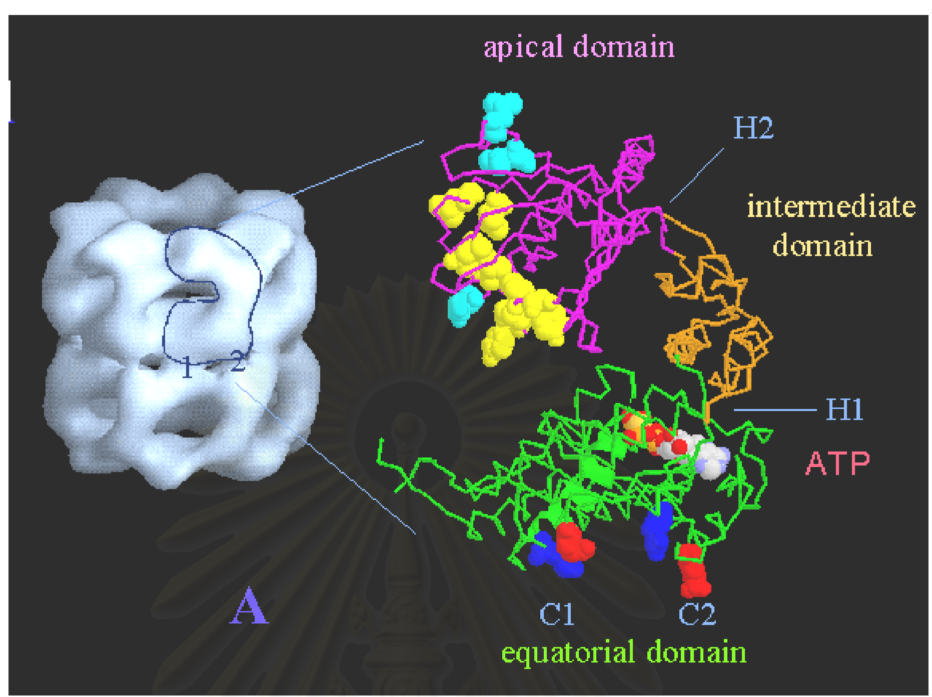


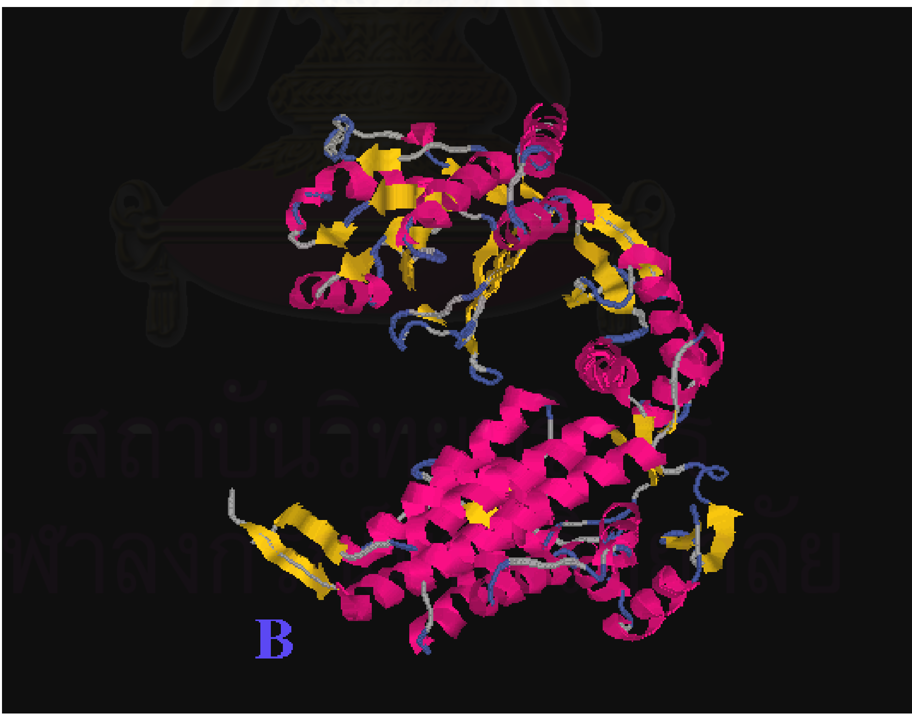


Figure 3.40 The predictions of tertiary structures of HSP60 *P. monodon* (B) compared to the strong binding peptide domain of HSP60 detected from *Escherichia coli* (PDB ID code = 1mnfL) (A).

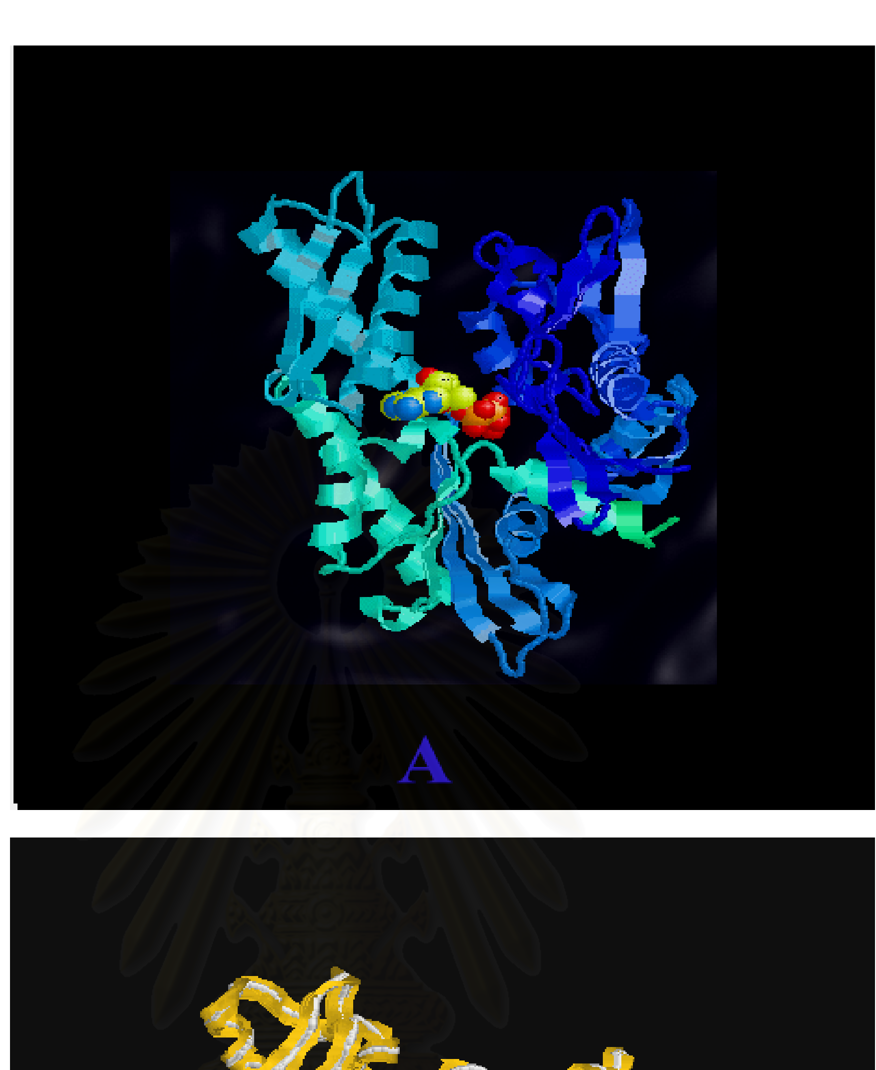


Figure 3.41 The predictions of tertiary structures of HSP70-1 *P. monodon* (B) compared to 44 KDa ATPase N-terminal fragment domain of HSP70 (PDB ID code = 1ngj_) (A)

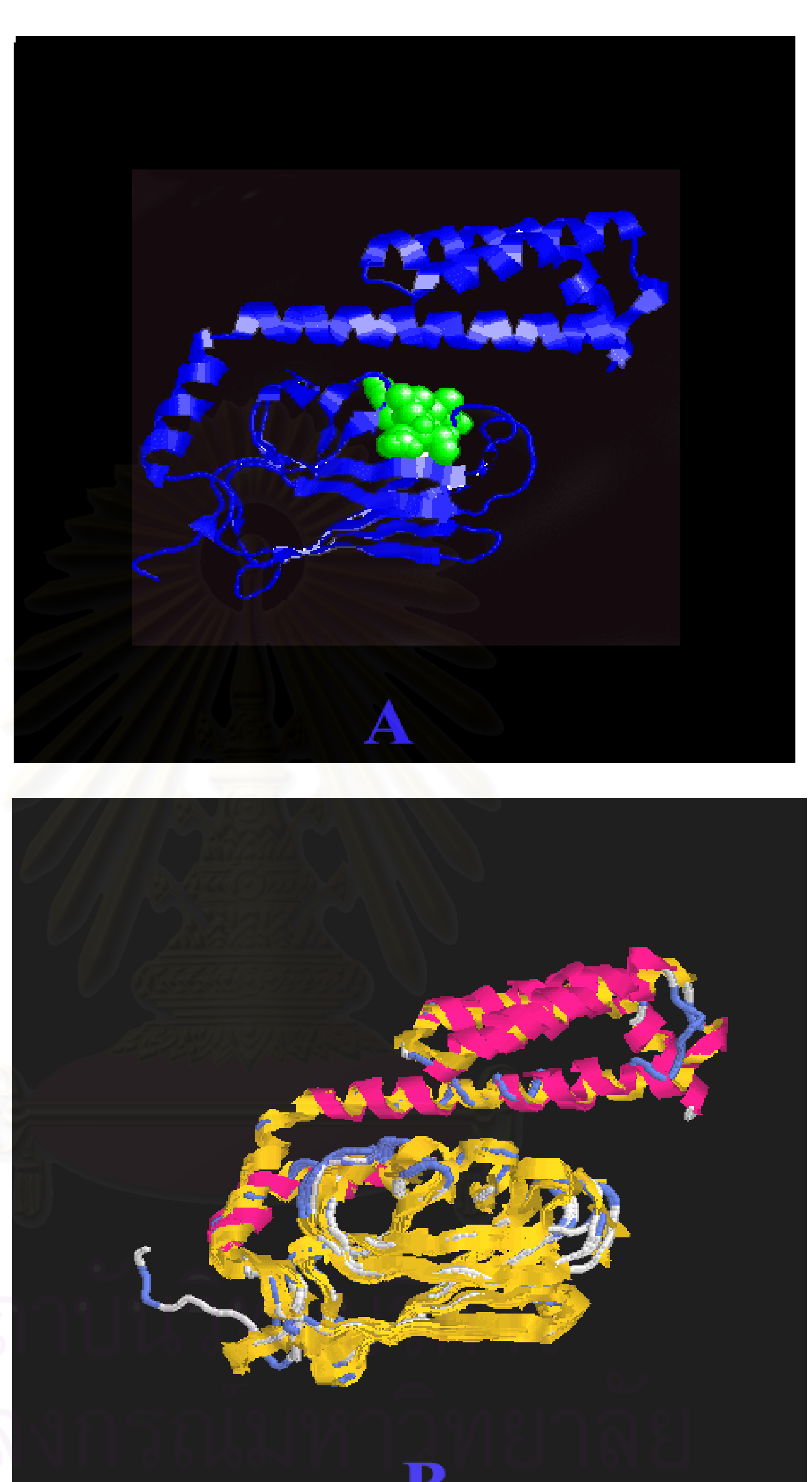
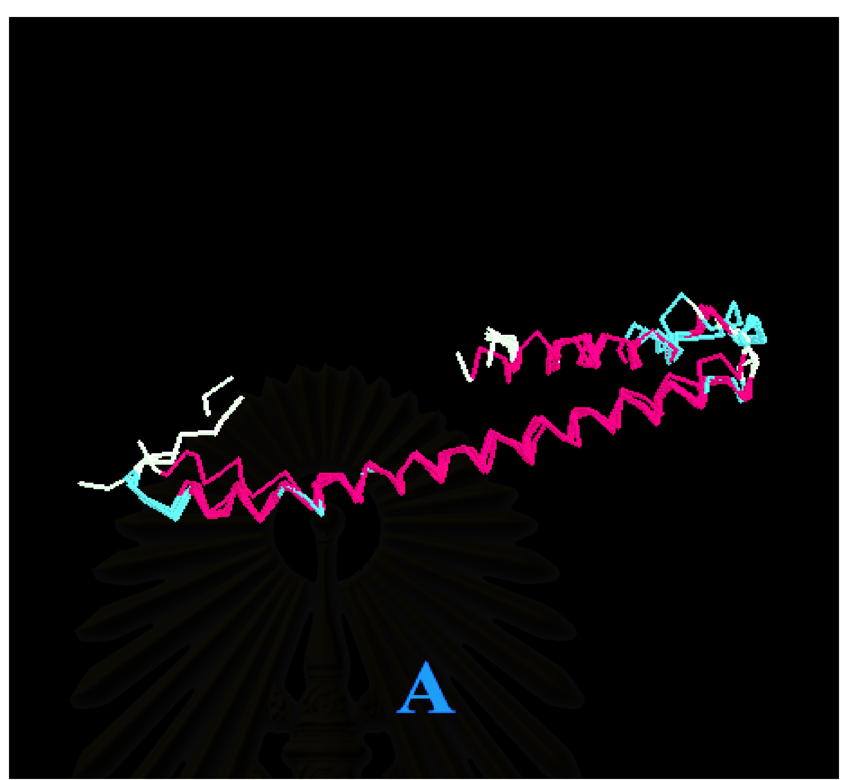


Figure 3.42 The predictions of tertiary structures of HSP70-2 *P. monodon* (B) compared to to the tRNA processing enzyme Rnase PH R86A mutant of HSP70 from *Aquifex aeolicus* (PDB ID code = 1ud0A) (A)


A

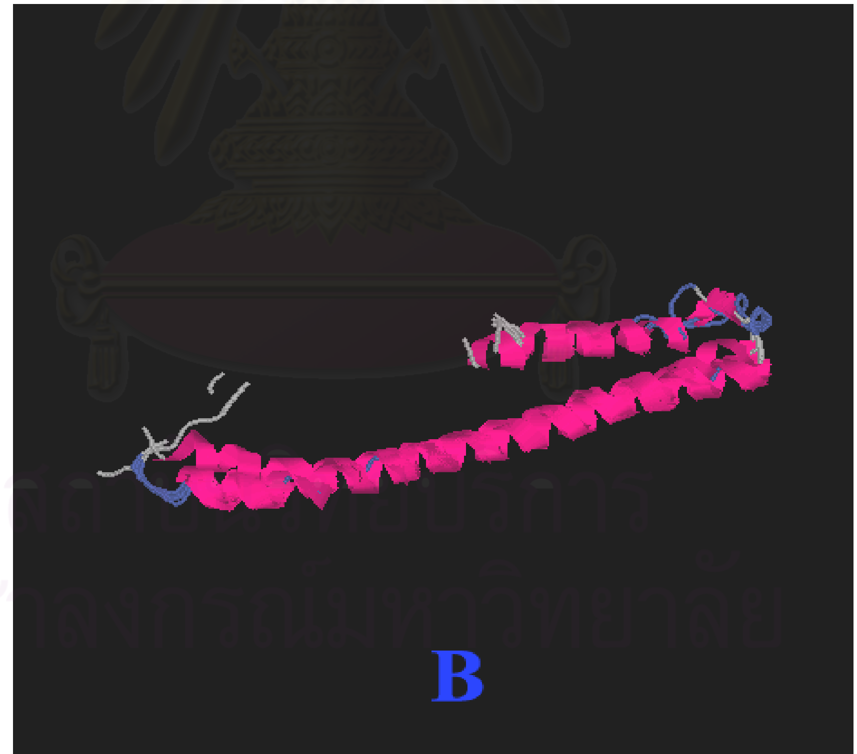
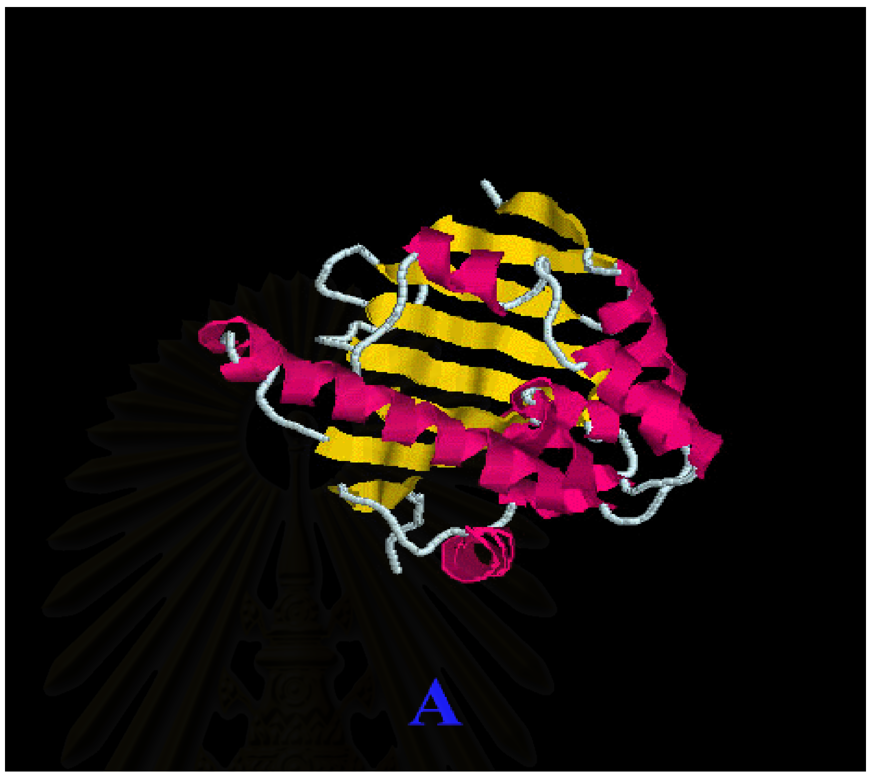

B

Figure 3.43 The predictions of tertiary structures of HSP70-3 *P. monodon* displayed on multiple backbones (A) and cartoon structure (B).



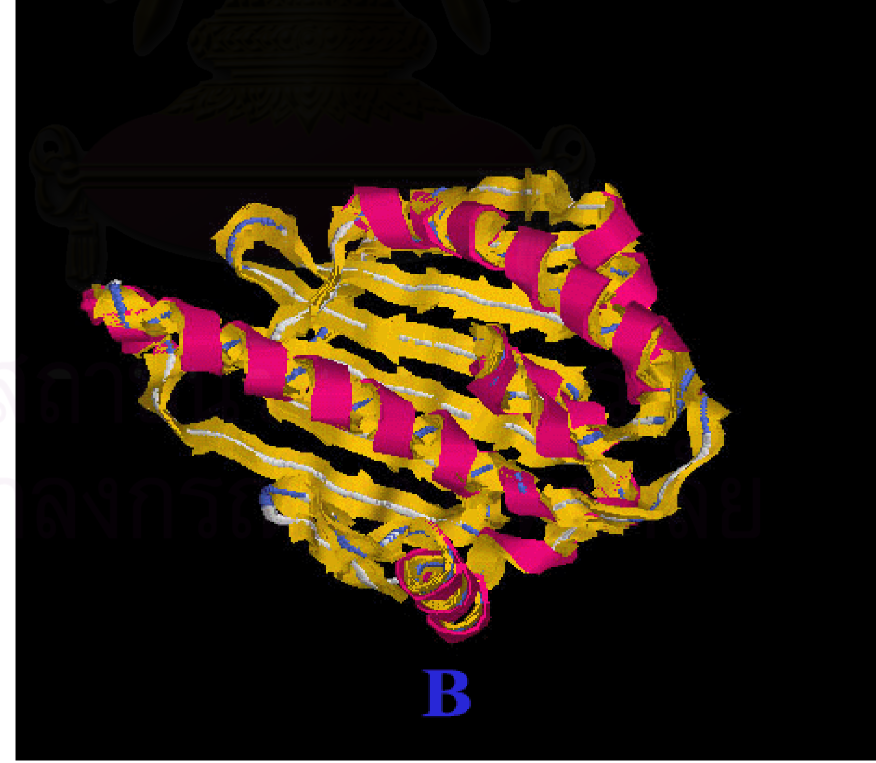


Figure 3.44 The predictions of tertiary structures of HSP90-1 *P. monodon* (B) compared to the N-terminal domain of HSP90 from *Homo sapiens* (PDB code = 1uy8A) (A)

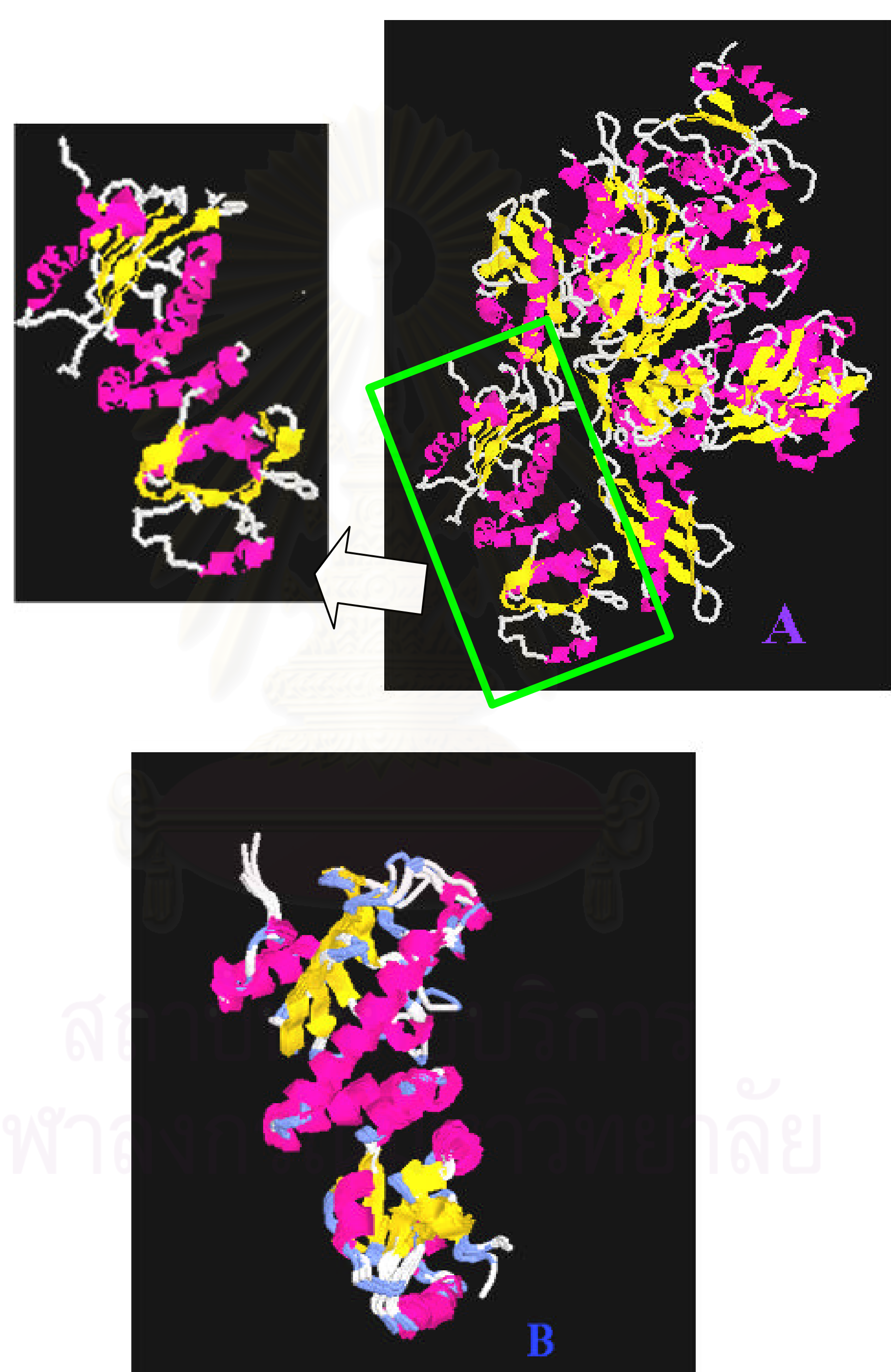


Figure 3.45 The predictions of tertiary structures of HSP90-2 *P. monodon* (A) compared to HSP90 from *Saccharomyces cerevisiae* (PDB code= 1usvA) (B)

The results of phylogenetic analysis of variant HSP70 DNA fragments obtained from *P.monodon* were shown in figure 3.46-3.48. Clones containing 5', 3' and the middle gene regions of HSP70 were separately multiple aligned with the full length HSP cDNA. Sequence divergence between paired of nucleotide sequences were calculated and subjected to phylogenetic analysis using the unweighted pair-group method with arithmetic mean (UPGMA).

A UPGMA dendrogram constructed from sequence divergence between paired 3' gene region indicated allocated investigated clones into 2 groups (HSOC933 and HCW0030 for the first and HSP70 and LPN0011 for the second groups) having sequence divergence of 0.0012 and 0.0000 for between and within groups, respectively (Fig.3.46). This suggested the existence of only one type of investigated clones carrying the 3' region of HSP70. In contrast, two different types (HCW0309 and HSP70 and HCH0225) were observed from the 5' gene region of HSP70 with the sequence divergence of 0.60320 (Fig. 3.47). Additionally, a UPGMA dendrogram constructed from the approximate middle region of *P. monodon* HSP70 allocated investigated clones into several groups having sequencing divergence between 0.1636 (HSP70 and HSPF2R2) – 0.7375 (HSPF1R2 and others; Fig.3.48). High sequence divergence observed between the 5' and approximate middle gene regions suggested that HSP70 should have encoded from more than one locus.

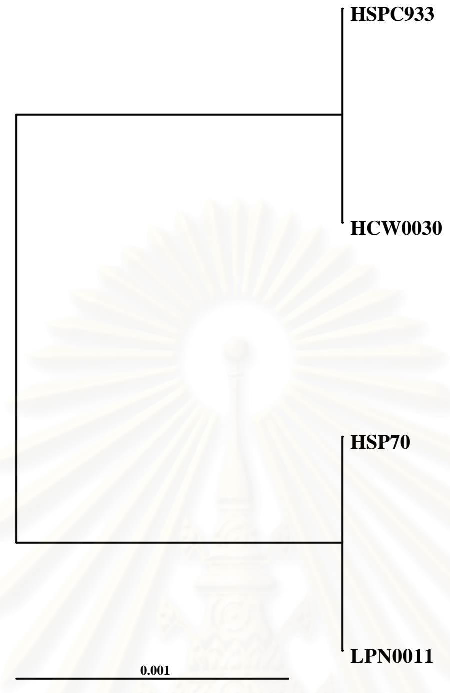


Figure 3.46 A UPGMA dendrogram illustrating relationships between different recombinant clones containing the 3' gene region of HSP70 of *P. monodon*.

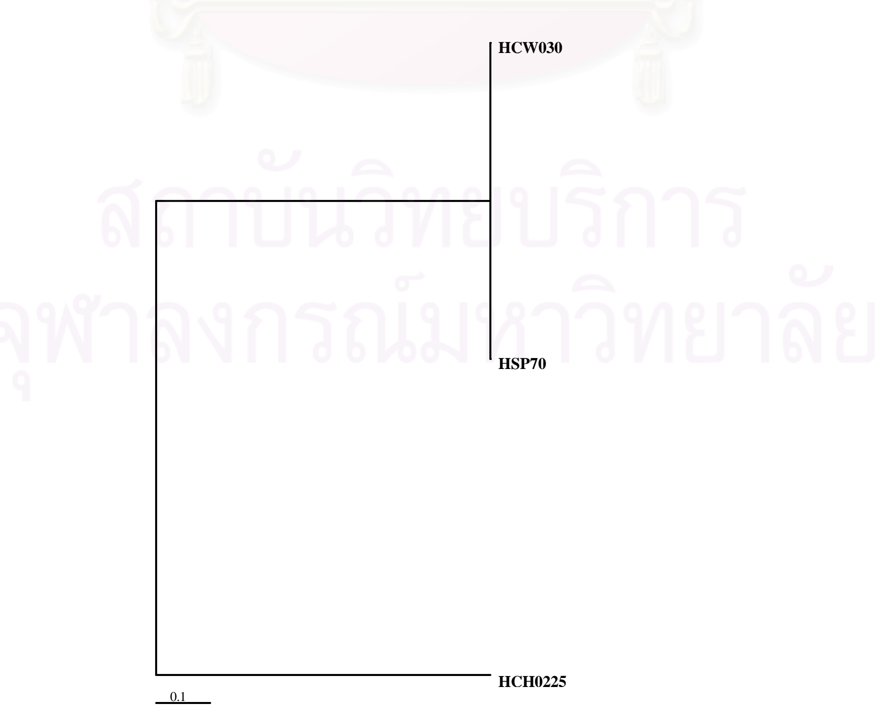


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Figure 3.47 A UPGMA dendrogram illustrating relationships between different recombinant clones containing the 5’ gene region of HSP70 of *P. monodon*.

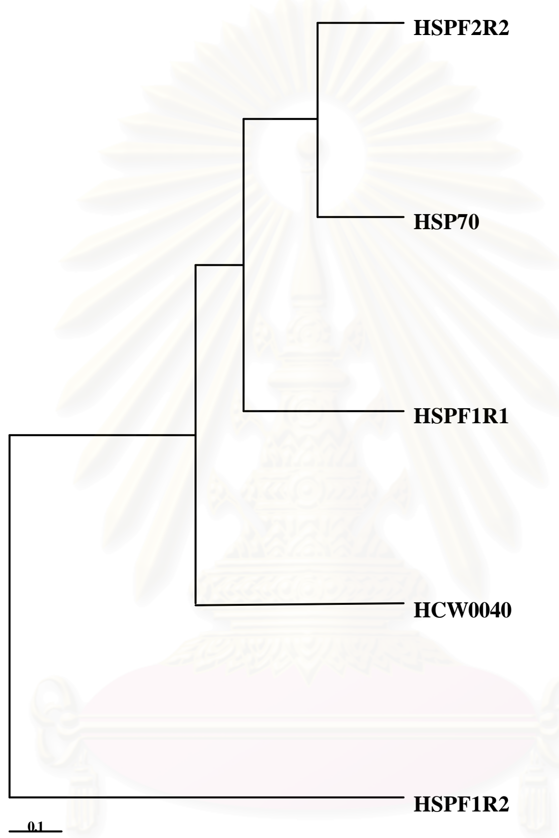

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Figure 3.48 A UPGMA dendrogram illustrating relationships between different recombinant clones containing the middle gene region of HSP70 of *P. monodon*.

```
             ....|....| ....|....| ....|....| ....|....| ....|....|
                 5         15         25         35         45
D.melanoga   MFRLP---VS LAR----SSI SRQLAMRG-- -YAKDVRFGP EVRAMMLQGV
C.variipen   MLRLVGKKVI LRS----PAT KFALAQRAG- -YAKDVRFGP EVRALMLQGV
H.sapiens    MLRLPTVFRQ MRP------V SRVLAPHLTR AYAKDVKFGA DARALMLQGV
P.lividus    MYRISSVLRP LTSRALTPSV NRAVCPHLAR SYAKDIKFGA EARGMMLQGV
P.monodon    MHRAAS---L LRT----PVA RQATRHYLAR HYAKDVKFGT EVRALMLQGV
Clustal Co   * *        :                     ****::**. :.*.:*****

             ....|....| ....|....| ....|....| ....|....| ....|....|
                55         65         75         85         95
D.melanoga   DVLADAVAVT MGPKGRNVII EQSWGSPKIT KDGVTVAKSI ELKDKFQNIG
C.variipen   NILADAVAVT MGPKGRNVIL EQSWGSPKIT KDGVTVAKGI ELKDKFQNIG
H.sapiens    DLLADAVAVT MGPKGRTVII EQSWGSPKVT KDGVTVAKSI DLKDKYKNIG
P.lividus    DLLADAVAVT MGPKGRNVII EQSWGSPKIT KDGVTVAKAV ELKDKWQNIG
P.monodon    DVLTDAVAVT MGPKGRNVII EQSWGSPKIT KDGVTVAKAV ELKDKFQNIG
Clustal Co   ::*:****** ******.**: ********:* ********.: :****::***

             ....|....| ....|....| ....|....| ....|....| ....|....|
                105        115        125        135        145
D.melanoga   AKLVQDVANN TNEEAGDGTT TATVLARAIA KEGFEKISKG ANPVEIRRGV
C.variipen   AKLVQDVANN TNEEAGDGTT TATVLARAIA KEGFEKISKG ANPIEIRRGV
H.sapiens    AKLVQDVANN TNEEAGDGTT TATVLARSIA KEGFEKISKG ANPVEIRRGV
P.lividus    AKLVQDVANN TNEEAGDGTT TATVLARAIA KEGFDNISRG ANPTEIRKGI
P.monodon    AKLVQDVANN TNEEAGDGTT TATVLARTIA KEGFDRISKG ANPVEIRRGV
Clustal Co   ********** ********** *******:** ****:.**:* *** ***:*:

             ....|....| ....|....| ....|....| ....|....| ....|....|
                155        165        175        185        195
D.melanoga   MLAVETVKDN LKTMSRPVST PEEIAQVATI SANGDQAIGN LISEAMKKVG
C.variipen   MLAVDAVKEH LKTLSKNVTT PEEIAQVATI SANGDKAIGQ LISDAMKRVG
H.sapiens    MLAVDAVIAE LKKQSKPVTT PEEIAQVATI SANGDKEIGN IISDAMKKVG
P.lividus    MNAVEVVIKE LQKQSKPVTT PEEIAQVATI SANGDAGIGE LISRAMKKVG
P.monodon    MLAVDAIVAH LKTLSKPVTT PAEIAQVATI SANGDIEVGS LISAAMEKVG
Clustal Co   * **:.:  . *:. *: *:* * ******** *****  :*. :** **::**

             ....|....| ....|....| ....|....| ....|....| ....|....|
                205        215        225        235        245
D.melanoga   RDGVITVKDG KTLTDELEVI EGMKFDRGYI SPYFINSSKG AKVEFQDALL
C.variipen   KEGVITVKDG KTLTDELQVI EGMKFDRGYI SPYFINSSKG AKVEFQDALL
H.sapiens    RKGVITVKDG KTLNDELEII EGMKFDRGYI SPYFINTSKG QKCEFQDAYV
P.lividus    RHGVITVKDG KTLNDELEVI EGLKFDRGYI SPYFINSAKG QKVEFQDALL
P.monodon    REGVITVKDG KTLKDELEVI EGMKFDRGYI SPYSINSSKG AKVEYQDCLV
Clustal Co   :.******** ***.***::* **:******* *** **::**  * *:**. :

             ....|....| ....|....| ....|....| ....|....| ....|....|
                255        265        275        285        295
D.melanoga   LLSEKKISSV QSIIPALELA NAQRKPLVII AEDIDGEALS TLVVNRLKIG
C.variipen   LFSETKISSV QSIIPALELA NTQRKPLVII AEDIDGEALS TLVVNRLKIG
H.sapiens    LLSEKKISSI QSIVPALEIA NAHRKPLVII AEDVDGEALS TLVLNRLKVG
P.lividus    LLSEKKISTI QAIVPALELA NAQRKPLVII AEDVDGEALS TLVLNRLKVG
P.monodon    LLSEKKISSI QSIIPVLELA NAQRKPLLII AEDIDGEALS TLVVNRLKIG
Clustal Co   *:**.***:: *:*:*.**:* *::****:** ***:****** ***:****:*

             ....|....| ....|....| ....|....| ....|....| ....|....|
                305        315        325        335        345
D.melanoga   LQVAAVKAPG FGDNRKSTLT DMAIASGGIV FGDDADLVKL EDVKVSDLGQ
C.variipen   LQVAAVKAPG FGDNRKSTMA DMAIATGGIV FGDEANLVKI EDVQLSDLGK
H.sapiens    LQVVAVKAPG FGDNRKNQLK DMAIATGGAV FGEEGLTLNL EDVQPHDLGK
P.lividus    LQVAAVKAPG FGDNRKNQLH DMAVSTGGMV FGDEAMEVKI EDVQIQDLGQ
P.monodon    LQVAAVKAPG SGDNRKNTLH DIAIATGAIV FNDEASMVKI EDVQVHDLGQ
Clustal Co   ***.******  *****. :  *:*:::*. * *.::.  ::: ***:  ***:

             ....|....| ....|....| ....|....| ....|....| ....|....|
                355        365        375        385        395
D.melanoga   VGEVVITKDD TLLLKGKGKK DDVLRRANQI KDQIEDTTSE YEKEKLQERL
C.variipen   VGEVVITKDD TLLLKGKGTK EHIDRRAEQI RDQIKETTSQ YEKEKLQERL
H.sapiens    VGEVIVTKDD AMLLKGKGDK AQIEKRIQEI IEQLDVTTSE YEKEKLNERL
P.lividus    VGEIAITKDD TLILKGKGKQ EDVDRRVAEI AEQIENTNSE YEREKLNERL
P.monodon    L-EKCRSQRM THSCEGQGKY SDIQRRVEQI KDQIADSSSE YRRRKCRS-V
Clustal Co   : *   ::   :   :*:*    .: :*  :*  :*:  :.*: *.:.* .. :
```

```
            ....|....| ....|....| ....|....| ....|....| ....|....|
                 405        415        425        435        445
D.melanoga  ARLASGVALL RVGGSSEVEV NEKKDRVHDA LNATRAAVEE GIVPGGGTAL
C.variipen  ARLSAGVALL RIGGSSEVEV NEKKDRVTDA LNATRAAVEE GIVPGGGTAL
H.sapiens   AKLSDGVAVL KVGGTSDVEV NEKKDRVTDA LNATRAAVEE GIVLGGGCAL
P.lividus   AKLSDGVAVL KVGGSSDVEV NEKKDRVNDA LNATRAAVEE GIVLGGGTAL
P.monodon   WLVWPQVAVV KVGGSSEVEV NEKKDRVNDA LCATRAAVEE GIVPGGGVAL
Clustal Co    :    **:: ::**:*:*** ******* ** * ******** *** *** **

            ....|....| ....|....| ....|....| ....|....| ....|....|
                 455        465        475        485        495
D.melanoga  LRCIEKLEGV ETTNEDQKLG VEIVRRALRM PCMTIAKNAG VDGAMVVAKV
C.variipen  LRCIPTLKGL KGENEDQKTG IEIVMRALRM PCMTIAKNAG VDGSVVVAKV
H.sapiens   LRCIPALDSL TPANEDQKIG IEIIKRTLKI PAMTIAKNAG VEGSLIVEKI
P.lividus   IRCLPCLQNV PAENADQKIG VEIVRRDLCV PTQTIANNAG VEGALIVEKV
P.monodon   IRCLPALDTL TPSNEDQEVG IEIVRKAIQT PCHTIVSNAG VDASVIVNKV
Clustal Co  :**:  *. :     * **: * :**: : :   *  **..*** *:.:::* *:

            ....|....| ....|....| ....|....| ....|....| ....|....|
                 505        515        525        535        545
D.melanoga  ENQAGDYGYD ALKGEYGNLI EKGIIDPTKV VRTAITDASG VASLLTTAEA
C.variipen  EENQGEYGYD AMNNEYVNMI EKGIIDPTKV VRTALTDASG VASLLTTAEA
H.sapiens   MQSSSEVGYD AMAGDFVNMV EKGIIDPTKV VRTALLDAAG VASLLTTAEV
P.lividus   IDSSEEIGYN AMEGEFVDMV KAGIIDPTKV VRTALMDASG VASLLTTAET
P.monodon   MEASGDVGYD AATGTFVNLV EAGIIDPTKV VRTALTDAAG VASLLTTAES
Clustal Co   :   : **: *   . : ::: : ******** ****: **:* *********

            ....|....| ....|....| ....|....| ....|....
                 555        565        575        585
D.melanoga  VVTEIPKEDG A--PAMPGMG GMGGMGGMGG MGGMM----
C.variipen  VVTEMPKDDK E--VGMPGMG GMGGMGGMGG MGGMNVNCS
H.sapiens   VVTEIPKEEK D--PGMG--- AMGGMG--GG MGGGMF---
P.lividus   VITEIPKEEK E--MPMGGG- GMGGMGGMGG MGGMM----
P.monodon   VITEIPKEEP AGMGGMGGMG GMGGMGGMGG MGGMM----
Clustal Co  *:**:**::        *      .*****  ** ***
```

Figure 3.49 Multiple alignment of deduce amino acid sequences of HSP60 of *P. monodon*

```
            ....|....| ....|....| ....|....| ....|....| ....|....|
                 5          15         25         35         45
P.monodon   MAKAPAVGID LGTTYSCVGV FQHGKVEIIA NDQGNRTTPS YVAFTDTERL
A.mellifer  MAKAPAVGID LGTTYSCVGV FQHGKVEIIA NDQGNRTTPS YVAFTETERL
L.vannamei  MAKAPAVGID LGTTYSCVGV FQHGKVEIIA NDQGNRTTPS YVAFTDTERL
M.rosenber  MAKSAAVGID LGTTYSCVGV FQHGKVEIIA NDQGNRTTPS YVAFTDTERL
A.francisc  MAKAPAIGID LGTTYSCVGV FQHGKVEIIA NDQGNRTTPS YVAFTDTERL
Clustal Co  ***:.*:*** ********** ********** ********** *****:****

            ....|....| ....|....| ....|....| ....|....| ....|....|
                 55         65         75         85         95
P.monodon   IGDAAKNQVA MNPNNTVFDA KRLIGRKFED HTVQSDMKHW PFTIINESTK
A.mellifer  IGDAAKNQVA MNPNNTIFDA KRLIGRRFED PTVQADMKHW PFTVVNDGGK
L.vannamei  IGDAAKNQVA MNPNNTVFDA KRLIGRKFED HTVQSDMKHW PFTIINESTK
M.rosenber  IGDAAKNQVA MNPNNTVFDA KRLIGRKFDD GVVQSDMKHW PFTVINDNTK
A.francisc  IGDAAKNQVA MNPNNTIFDA KRLIGRRFED ATVQSDMKHW PFDVISDGGK
Clustal Co  ********** ******:*** ******:*:*  .**:***** **  ::.:. *

            ....|....| ....|....| ....|....| ....|....| ....|....|
                 105        115        125        135        145
P.monodon   PKIQVEYKGD KKTFYPEEIS SMVLIKMKET AEAYLGSTVK DAVVTVPAYF
A.mellifer  PKIQVVYKGE AKTFFPEEVS SMVLVKMKET AEAYLGKTVS NAVITVPAYF
L.vannamei  PKIQVEYKGD KKTFYPEEIS SMVLIKMKET AEAYLGSTVK DAVVTVPAYF
M.rosenber  PKIQVDYKGE TKTFFPEEIS SMVLIKMKET AEAFLGSTVK DAVITVPAYF
A.francisc  PKVQVEFKGE KKTFAPEEVS SMILVKMKET AEAYLGSPVS NAVITVPAYF
Clustal Co  **:** :**:  *** ***:* **:*:***** ***:**..*. :**:******
```

```
             ....|....| ....|....| ....|....| ....|....| ....|....|
                   155        165        175        185        195
P.monodon    NDSQRQATKD AGTISGLNVL RIINEPTAAA IAYGLDKKVG GERNVLIFDL
A.mellifer   NDSQRQATKD AGTISGLNVL RIINEPTAAA IAYGLDKKTT SERNVLIFDL
L.vannamei   NDSQRQATKD AGTISGLNVL RIINEPTAAA IAYGLDKKVG GERNVLIFDL
M.rosenber   NDSQRQATKD AGTISGLNAL RIINEPTAAA IAYGLDKKVG GERNVLIFDL
A.francisc   NDSQRQATKD AGAIAGLNVL RIINEPTAAA IAYGLDKKTV GEKNVLIFDL
Clustal Co   ********** **:*:***.* ********** ********.  .*:*******

             ....|....| ....|....| ....|....| ....|....| ....|....|
                   205        215        225        235        245
P.monodon    GGGTFDVSIL TIEDGIFEVK STAGDTHLGG EDFDNRMVNH FIQEFKRKYK
A.mellifer   GGGTFDVSIL TIEDGIFEVK STAGDTHLGG EDFDNRMVNH FVQEFKRKYK
L.vannamei   GGGTFDVSIL TIEDGIFEVK STAGDTHLGG EDFDNRMVNH FIQEFKRKYK
M.rosenber   GGGTFDVSIL TIEDGIFEVK STAGDTHLGG EDFDNRMVNH FIQEFKRKYK
A.francisc   GGGTFDVSIL TIEDGIFEVK STAGDTHLGG EDFDNRLVNH FVQEFKRKYK
Clustal Co   ********** ********** ********** ******:*** *:********

             ....|....| ....|....| ....|....| ....|....| ....|....|
                   255        265        275        285        295
P.monodon    KDPSENKRSL RRLRTACERA KRTLSSSTQA SVEIDSLFEG IDFYTSITRA
A.mellifer   KDLTANKRAL RRLRTACERA KRTLSSSTQA SIEIDSLYEG IDFYTSITRA
L.vannamei   KDPSENKRSL RRLRTACERA KRTLSSSTQA SVEIDSLFEG IDFYTSITRA
M.rosenber   KDPSENKRAL RRLRTACERA KRTLSASAQA SIEIDSLYEG TDFYTSVTRA
A.francisc   KDIAVNKRAL RRLRTACERA KRTLSSSTQA SIEIDSLFEG IDFYTSITRA
Clustal Co   ** : ***:* ********** *****:*:** *:*****:**  *****:***

             ....|....| ....|....| ....|....| ....|....| ....|....|
                   305        315        325        335        345
P.monodon    RFEELCADLF RGTLEPVEKS LRDAKMDKAQ IHDIVLVGGS TRIPKIQKLL
A.mellifer   RFEELCADLF RGTLEPVEKS LRDAKMDKAQ IHDIVLVGGS TRIPKIQKLL
L.vannamei   RFEELCADLF RGTLEPVEKS LRDAKMDKAQ IHDIVLVGGS TRIPKIQKLL
M.rosenber   RFEELCGDLF RGTLEPVEKS LRDAKMDKAQ IHDIVLVGGS TRIPKIQKLL
A.francisc   RFEELCADLF RGTLEPVEKS LRDAKMDKGS VHEIVLVGGS TRIPKIQKLL
Clustal Co   ******.*** ********** ********.. :*:******* **********

             ....|....| ....|....| ....|....| ....|....| ....|....|
                   355        365        375        385        395
P.monodon    QDFFNGKELN KSINPDEAVA YGAAVQAAIL CGDKSEAVQD LLLLDVTPLS
A.mellifer   QDFFNGKELN KSINPDEAVA YGAAVQAAIL HGDKSEEVQD LLLLDVTPLS
L.vannamei   QDFFNGKELN KSINPDEAVA YGAAVQAAIL CGDKSEAVQD LLLLDVTPLS
M.rosenber   QDFFNGKELN KSINPDEAVA CGAAVQAAIL CGDKSEAVQD LLLLDVTPLS
A.francisc   QDFFNGKGLN KSITQDEAVA YGAAVQAAIL HGDKSEAVQD LLLLDVAPLS
Clustal Co   ******* ** ***. *****  *********  ***** *** ******:***

             ....|....| ....|....| ....|....| ....|....| ....|....|
                   405        415        425        435        445
P.monodon    LGIETAGGVM TALIKRNTTI PTKQTQTFTT YSDNQPGVLI QVYEGERAMT
A.mellifer   LGIETAGGVM TALIKRNTTI PTKQTQTFTT YADNQPGVLI QVYEGERAMT
L.vannamei   LGIETAGGVM TALIKRNTTI PTKQTQTFTT YSDNQPGVLI QVYEGERAMT
M.rosenber   LGIETAGGVM TALIKRNTTI PTKQTQTFTT YSDNQPGVLI QVYEGERAMT
A.francisc   MGIETAGGVM TVLIKRNTTI PTKQTQTFTT YSDNQPGVLI QVYEGERTMT
Clustal Co   :********* *.******** ********** *:******** *******:**

             ....|....| ....|....| ....|....| ....|....| ....|....|
                   455        465        475        485        495
P.monodon    KDNNLLGKFE LSGIPPAPRG VPQIEVTFDI DANGILNVSA VDKSTGKENK
A.mellifer   KDNNLLGKFE LSGIPPAPRG VPQIEVTFDI DANGILNVSA VDKSTGKENK
L.vannamei   KDNNLLGKFE LSGIPPAPRG VPQIEVTFDI DANGILNVSA VDKSTGKENK
M.rosenber   KDNNLLGKFE LSGIPPAPRG VPQIEVTFDI DANGILNVSA ADKSTGKENK
A.francisc   KDNNLLGKFE LTGIPPAPRG VPQIEVTFDI DANGILNVSA VDKSTGRENK
Clustal Co   ********** *:******** ********** ********** .*****:***
```

```
               ....|....| ....|....| ....|....| ....|....| ....|....|
                   505        515        525        535        545
P.monodon      ITITNDKGRL SKEEIERMVQ DAEKYKADDE KQRDRISAKN SLESYCFNMK
A.mellifer     ITITNDKGRL SKEDIERMVN EAEKYRSEDE KQKETIAAKN GLESYCFNMK
L.vannamei     ITITNDKGRL SKEEIERMVQ DAEKYKADDE KQRDRISAKN SLESYCFNMK
M.rosenber     ITITNDKGRL SKEEIERMVQ EAEKYKADDE KQRDRIAAKN SLESYCFNMK
A.francisc     ITITNDKGRL SKEEIERMVN DAEKYRAEDE KQREVIAAKN SLESYCFNMK
Clustal Co     ********** ***:*****: :****:::** **:: *:*** .*********

               ....|....| ....|....| ....|....| ....|....| ....|....|
                   555        565        575        585        595
P.monodon      STVEDEKFKE KISEEDRNKI LETCNETIKW LDMNQLGEKE EYEHKQKEIE
A.mellifer     STVEDEKLKD KISASDKQVV LDKCNDIIKW LDANQLADKE EYEHKQKELE
L.vannamei     STVEDEKFKE KISEEDRNKI LETCNETIKW LDMNQLGEKE EYEHKQKEIE
M.rosenber     STVEDDKFKD KVPEEDRNKI MEACNDAIKW LDSNQLGEKE EYEHKLKEIE
A.francisc     STMEDEKFKD KLPEADKNTI LDKCNETIKW LDVNQLAEKE EYEEKQKEIE
Clustal Co     **:**:*:*: *:.  *:: : :: **: *** ** ***.:** ***.* **:*

               ....|....| ....|....| ....|....| ....|....| ....|....|
                   605        615        625        635        645
P.monodon      QVCNPIITKM YAAAGGAPPG GMPGGFPGGA PGAGGAAPGA GGSSGPTIEE
A.mellifer     AICNPIVTKL YQGTGGMP-G GMPGGMPGGF PGAGGGAPG- GGASGPTIEE
L.vannamei     QVCNPIITKM YAAAGGAPPG GMPGGFPGGA PGAGGAAPGA GGSSGPTIEE
M.rosenber     QICNPIITKM YQAAGGAPPG GMPGGFPG-A PG-GGAAPG- GGSSGPTIEE
A.francisc     KVCNPIITKL YGQAGGMLAD SLVVWRSS-- --SGCYCSRC WNRQWPNY--
Clustal Co      :****:**: *  :**    . .:   ..      *  ..     . . *.

               ..
               
P.monodon      VD
A.mellifer     VD
L.vannamei     VD
M.rosenber     VD
A.francisc     --
Clustal Co
```

Figure 3.50 Multiple alignment of deduce amino acid sequences of HSP70 of *P. monodon*

```
               ....|....| ....|....| ....|....| ....|....| ....|....|
                   5          15         25         35         45
S.solar        MPEEMRQ--- --EEEAETFA FQAEIAQLMS LIINTFYSNK EIFLRELISN
C.haematoc     MPEEATM--- ---EDVETFA FQAEIAQLMS LIINTFYSNK EIFLRELISN
S.frugifer     MPEEMQT--D --VAEVETFA FQAEIAQLMS LIINTFYSNK EIFLRELISN
P.monodon      MVEETMS--- ---EEVETFA FQAEIAQLMS LIINTFYSNK EIFLRELISN
G.gallus       MPEAVQTQDQ PMEEEVETFA FQAEIAQLMS LIINTFYSNK EIFLRELISN
Clustal Co     * *          :.**** ********** ********** **********

               ....|....| ....|....| ....|....| ....|....| ....|....|
                   55         65         75         85         95
S.solar        ASDALDKIRY ESLTDPTKLD NGKELKIDVI PNVEERTLTL IDTGIGMTKA
C.haematoc     SSDALDKIRY ESLTDPSKLE SGKELFIKLV PNKNDRTLTI IDSGVGMTKA
S.frugifer     SSDALDKIRY ESLTDPSKLD SGKELYIKII PNKSEGTLTI IDTGIGMTKA
P.monodon      SSDALDKIRY ESLTDPSKLE SGKDLFIKLV PNKDDRTLTI IDSGIGMTKA
G.gallus       SSDALDKIRY ESLTDPSKLD SGKDLKINLI PNKHDRTLTI VDTGIGMTKA
Clustal Co     :********* ******:**: .**:* *.:: **  : ***: :*:*:*****
```

```
            ....|....| ....|....| ....|....| ....|....| ....|....|
               105        115        125        135        145
S.solar     DLINNLGTIA KSGTKAFMEA LQAGADISMI GQFGVGFYSA YLVAERVTVI
C.haematoc  DLVNNLGTIA KSGTKAFMEA LQAGADISMI GQFGVGFYSA YLVADKVTVV
S.frugifer  DLVNNLGTIA KSGTKAFMEA LQAGADISMI GQFGVGFYSC YLVADRVTVH
P.monodon   DLVNNLGTIT KSGTKAFMEA LQAGADISMI GQFGVGFYSA YLVADKVTVV
G.gallus    DLVNNLGTIA KSGTKAFMEA LQAGADISMI GQFGVGFYSA YLVAEKVTVI
Clustal Co  **:******: ********** ********** *********. ****::***

            ....|....| ....|....| ....|....| ....|....| ....|....|
               155        165        175        185        195
S.solar     TKHNDDEQYI WESSAGGSFT VKVDTGEPML RGTKVILHMK EDQTEYVEEK
C.haematoc  SRNNDDEQYV WESSAGGSFT VRTDHGEPVG RGTRITLHLK EDQTEYLEER
S.frugifer  SKHNDDEQYM WESSAGGSFT VRPDPGEPLG RGTKIVLHIK EDLTEYLEEH
P.monodon   SRNNDDERYI WESSAGGSFT VRHDTGEPIG RGTKITLHLK EDQTEYLEER
G.gallus    TKHNDDEQYA WESSAGGSFT VRLDNGEPLG RGTKVILHLK EDQTEYLEER
Clustal Co  :::****:*  ********** *: * ***:  ***:: **:* ** ***:**:

            ....|....| ....|....| ....|....| ....|....| ....|....|
               205        215        225        235        245
S.solar     RVKEVVKKHS QFIGYPITLF VEKEREKEIS DDEEEKAEEE KEEKE--AED
C.haematoc  RIKEIVKKHS QFIGYPIKLL VEKERDKEVS DDEEEEKEEE EKDEE---ED
S.frugifer  KIKEIVKKHS QFIGYPIKLM VEKEREKELS DDEAEE-EKK EDEKE---DD
P.monodon   RVKEIVKKHS QFIGYPIKLL VEKERDKEVS DDEEEEKEEK EEEAE---ED
G.gallus    RIKEIVKKHS QFIGYPIRLF VEKERDKEVS DDEAEEKEEE KEEKEEKTED
Clustal Co  ::**:***** ******* *: *****:**:* *** *: *:: :.: *   :*

            ....|....| ....|....| ....|....| ....|....| ....|....|
               255        265        275        285        295
S.solar     -KPKIEDVGS DDEEDSKDKD -KKKTKKIKE KYIDQEELNK TKPIWTRNPD
C.haematoc  EKPKIEDVGE DEDADKKEGG --KKKKTVKE KYTEDEELNK TKPLWTRNPD
S.frugifer  -KPKIEDVGE DDEEDKKD-- -KKKKKTIKE KYTEDEELNK TKPIWTRNAD
P.monodon   -KPKIEDVGE DEEADKEKGE DKKKKKTVKE KYTEDEELNK TKPLG-RAPR
G.gallus    -KPEIEDVGS DEEEEKKDGD -KKKKKKIKE KYIDEEELNK TKPIWTRNPD
Clustal Co   **:*****. *:: :.:.    **.*.:** ** :***** ***:  * .

            ....|....| ....|....| ....|....| ....|....| ....|....|
               305        315        325        335        345
S.solar     DITMEEYGEF YKSLTNDWEE HLAVKHFSVE GQLEFRALLF IPRRAPFDLF
C.haematoc  DISQEEYGEF YKSLTNDWED HLAVKHFSVE GQLEFRALLF LPRRAPFDLF
S.frugifer  DITQEEYGDF YKSLTNDWED HLAVKHFSVE GQLEFRALLF VPRRAPFDLF
P.monodon   RHLKEEYGEF YKSLTNDWED HLAVKHFSVE -PAEFRALLF LPRRAPFDLF
G.gallus    DITNEEYGEF YKSLTNDWED HLAVKHFSVE GQLEFRALLF VPRRAPFDLF
Clustal Co      ****:* *********: **********    ******* :*********

       ....|....| ....|....| ....|....| ....|....| ....|....|
               355        365        375        385        395
S.solar     ENKKKKNNIK LYVRRVFIMD SCEELIPEYL NFVRGVVDSE DLPLNISREM
C.haematoc  ENRKQKNKIK LYVRRVFIME NCEELIPEYL NFLNGVVDSE DLPLNISREM
S.frugifer  ENKKRKNNIK LYVRRVFIMD NCEDLIPEYL NFIKGVVDSE DLPLNISREM
P.monodon   ENRKQKNKIK LYVRRVFIME NCEELIPEYL NFINGVVDSE DLPLNISREM
G.gallus    ENRKKKNNIK LYVRRVFIMD NCEELIPEYL NFMRGVVDSE DLPLNISREM
Clustal Co  **:*:**:** *********: .**:****** **:.****** **********

            ....|....| ....|....| ....|....| ....|....| ....|....|
               405        415        425        435        445
S.solar     LQQSKILKVI RKNIVKKCME LFGELAEDRE NYNKFYDGFS KNLKLGIHED
C.haematoc  LQQNKILKVI RKNLVKKALE LFEELIEDKD NYKKFYENFS KNIKLGIHED
S.frugifer  LQQNKILKVI RKNLVKKCLE LFEELAEDKE NYKKYYEQFS KNLKLGIHED
P.monodon   LQQNKILKVI RKNLVKKTLE LFEEIVDDKE SYKKFYENFS KNLKLGIHED
G.gallus    LQQSKILKVI RKNLVKKCLE LFTELAEDKE NYKKFYEQFS KNIKLGIHED
Clustal Co  ***.****** ***:*** :* ** *: :*:: .*:*:*: ** **:*******
```

```
             ....|....| ....|....| ....|....| ....|....| ....|....|
                  455        465        475        485        495
S.solar      SQNRKKLSEL LRYHSSQSGD ELTSLTEYLT RMKDNQKSIY YITGESKDQV
C.haematoc   STNRKKLAEF LRYHTSASGD EMSSLKDYVS RMKENQKQIY YITGESGSRC
S.frugifer   SQNRSKLADL LRYHTSASGD EACSLKEYVS RMKENQKHIY YITGENRDQV
P.monodon    STNRKKLAEF LRYHTSASGD EMSSLKEYVS RMKENQKHIY FITGETREQV
G.gallus     SQNRKKLSEL LRYYTSASGD EMVSLKDYCT RMKENQKHVY YITGETKDQV
Clustal Co   * **.**::: ***::* *** *   **.:* : ***:***  :*  :****. .:

             ....|....| ....|....| ....|....| ....|....| ....|....|
                  505        515        525        535        545
S.solar      ANSAFVERVR KRGFEVLYMT EPIDEYCVQQ LKEFDGKTLV SVTKEGLELP
C.haematoc   TTAAFVERVK KRGFEVVYMV EPIDEYCVQQ LKEYGGKQLV SVTKEGLELP
S.frugifer   ANSSFVERVK KRGYEVVYMT EPIDEYVVQQ MREYDGKTLV SVTKEGLELP
P.monodon    QNSAFVERVK KRGFEVIYMT EPIDEYCVQQ LKEYDGKQLV SVTKEGLELP
G.gallus     ANSAFVERLR KHGLEVIYMI EPIDEYCVQQ LKEFEGKTLV SVTKEGLELP
Clustal Co     .::****:: *:* **:**  ****** ***  ::*: ** ** **********

             ....|....| ....|....| ....|....| ....|....| ....|....|
                  555        565        575        585        595
S.solar      EDEEEKKKMD EDKTKFENLC KLMKEILDKK VEKVTVSNRL VSSPCCIVTS
C.haematoc   EDDDEKKKLE EQKAKFENLC KVVKDILDKR VEKVVVSNRL VTSPCCIVTS
S.frugifer   EDEEEKKKRE EDKVKFEGLC KVMKNILDNK VEKVVVSNRL VESPCCIVTA
P.monodon    EDEEEKKKFE EQKTKFENLC KVMKDILDKR VEKVVVSNRL VTSPCCIVTS
G.gallus     EDEEEKKKQE EKKAKFENLC KIMKDILEKK VEKVVVSNRL VTSPCCIVTS
Clustal Co   **::****  : *.*.***.** *::*:**::: ****.***** * *******:

             ....|....| ....|....| ....|....| ....|....| ....|....|
                  605        615        625        635        645
S.solar      TYGWTANMER IMKAQALRDN STMGYMMAKK HLEINPDHPI VETLRQKADL
C.haematoc   QYGWTANMER IMKAQ-LRDT STMGYMAAKK HLEINPDHSI IETLRQKADA
S.frugifer   QYGWSANMER IMKAQALRDT STMGYMAAKK HLEINPDHSI VETLRQKAEA
P.monodon    QYGWTANMER IMKAQALRDT STMGYMAAQK HLEINPDHSI IETLRQRRMP
G.gallus     TYGWTANMER IMKAQALRDN STMGYMAAKK HLEINPDHSI IETLRQKAEA
Clustal Co    ***:***** ***** ***. ****** *:* ********.* :*****:

             ....|....| ....|....| ....|....| ....|....| ....|....|
                  655        665        675        685        695
S.solar      DKNDKAVKDL VILLFETALL SSGFSLDDPQ THSNRIYRMI KLGLGIDDDE
C.haematoc   DKNDKSVKDL VMLLFESALL SSGFTLEDPG VHAGRIYRMI KLGLGIDEDD
S.frugifer   DKNDKAVKDL VILLYETALL SSGFTLDEPQ VHASRIYRMI KLGLGIDEDE
P.monodon    NKNDKSVKDL VMLLFESSLL SSGFSLEDPG VHASRIYRMI KLGLGIDEED
G.gallus     DKNDKSVKDL VILLYETALL SSGFSLEDPQ THANRIYRMI KLGLGIDEDD
Clustal Co   :****:**** *:**:*::** ****:*::*  .*:.****** *******:::

             ....|....| ....|....| ....|....| ..
                  705        715        725
S.solar      VIPEEPTSAP APDEIPPLEG D-DDASRMEE VD
C.haematoc   APAEDNAETA E--EMPPLE- DEEDTSRMEE VD
S.frugifer   PIQVEESSAG D---VPPLEG DADDASRMEE VD
P.monodon    APMEEAETLE E--DMPPLEG DDEDASRMEE VD
G.gallus     TAAEEASPAV T-EEMPPLEG D-DDTSRMEE VD
Clustal Co        :          :**** * :*:***** **
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Figure 3.51 Multiple alignment of deduce amino acid sequences of HSP90 of *P. monodon*

3.13 *In vitro* Expression of HSP genes

3.13.1 Semi-quantitative PCR conditions

The expression of HSP genes in thermal treated haemocytes were determined by semi-quantitative RT-PCR using β-actin as reference. The sequence of heat shock protein 60, 70 and 90 genes were retrieved from *P. monodon* full length sequence in above experiment. Primers for amplifying HSP60, HSP70 and HSP90 were then designed as in Fig. 3.52, 3.53 and 3.54. Primers for β-actin designed from DNA sequence of *P. monodon* from GenBank (AF1000987) (Fig. 3.55). Appropriate condition for semi-quantitative RT-PCR for HSP60, HSP70 and HSP90 genes and β-actin genes were conducted by adjusting magnesium cholide concentration, template concentration, and the number of PCR cycle. For HSP and β-actin amplifications, total RNA extracted from thermal treated haemocyte cells were subjected to first strand cDNAs production by reverse transcription using oligod(T) primer. Double strand cDNA of heat shock protein gene and β-actin genes were then amplified using first strand cDNA as template at optimum annealing temperature 55°C, 65°C and 55°C for heat shock 60 primers (HSP60F and HSP60R), heat shock 70 primers (HSP70F and HSP70R) and heat shock 90 primers (HSP90F and HSP90R), respectively. For actin1 and actin2 primers, the annealing temperature were used at 55°C. Optimum magnesium concentration was used at 1.5mM. Various concentration of template and the number of PCR cycle used in PCR reaction were verified. The PCR products were determined by 1.2% 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 result indicated that the condition of using cDNA template at 600 ng with 28 PCR cycles were suitable for the amplification of heat shock 60 gene (Fig. 3.56, 3.57) while the appropriate condition for heat shock 70 and 90 gene amplification were at the template concentration of 25 ng with 25 cycles and 50 ng with 26 cycles, repectively (Fig.. 3.58 -3.61). For β-actin gene, the appropriate condition was as same as the template concentration of heat shock proteins (25 ng) with 20 cycles (Fig. 3.62-3.63).

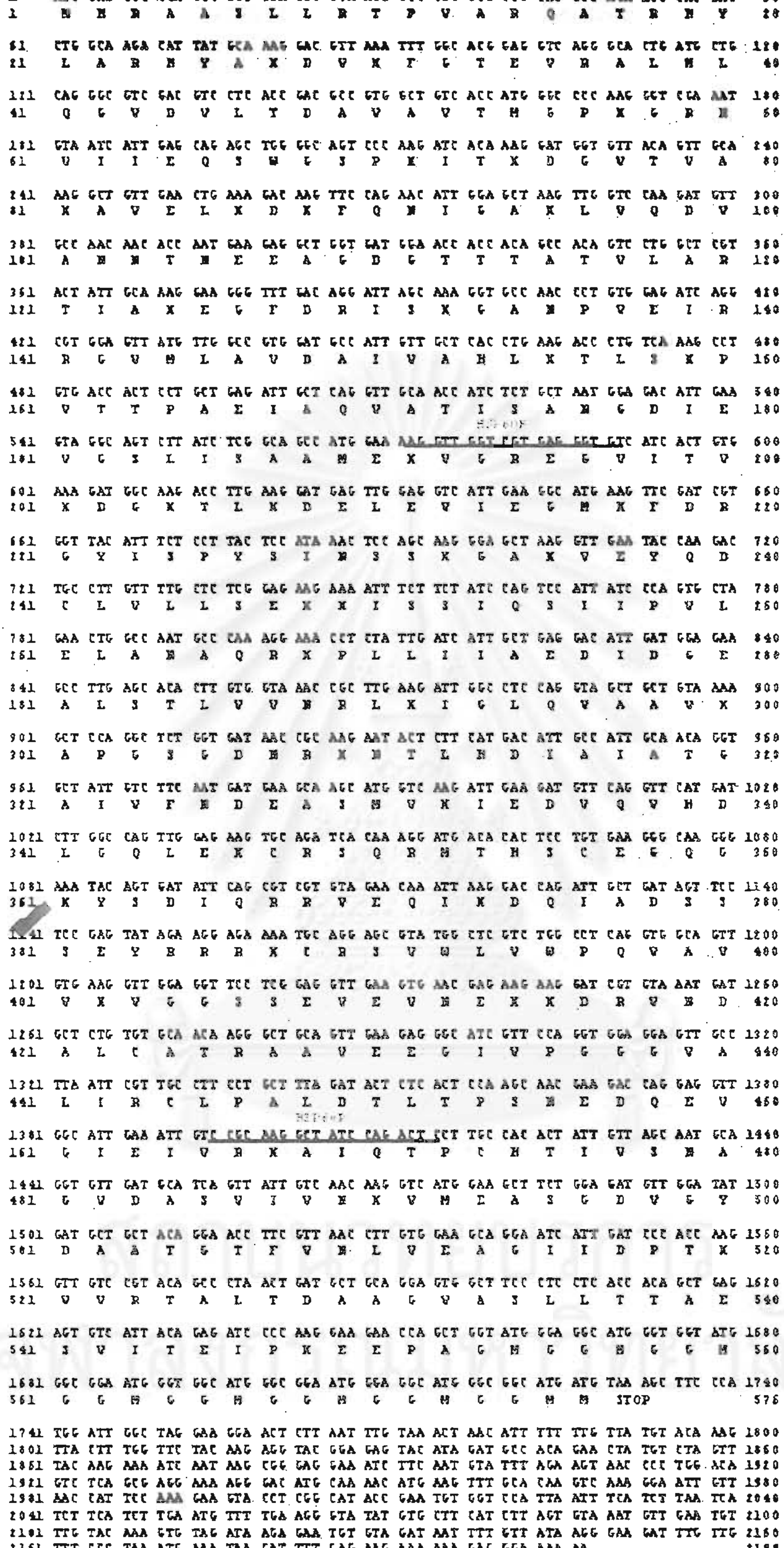

Figure 3.52 Nucleotide sequence of HSP60 gene .The highlight show the position of HSP60F and HSP60R primer.

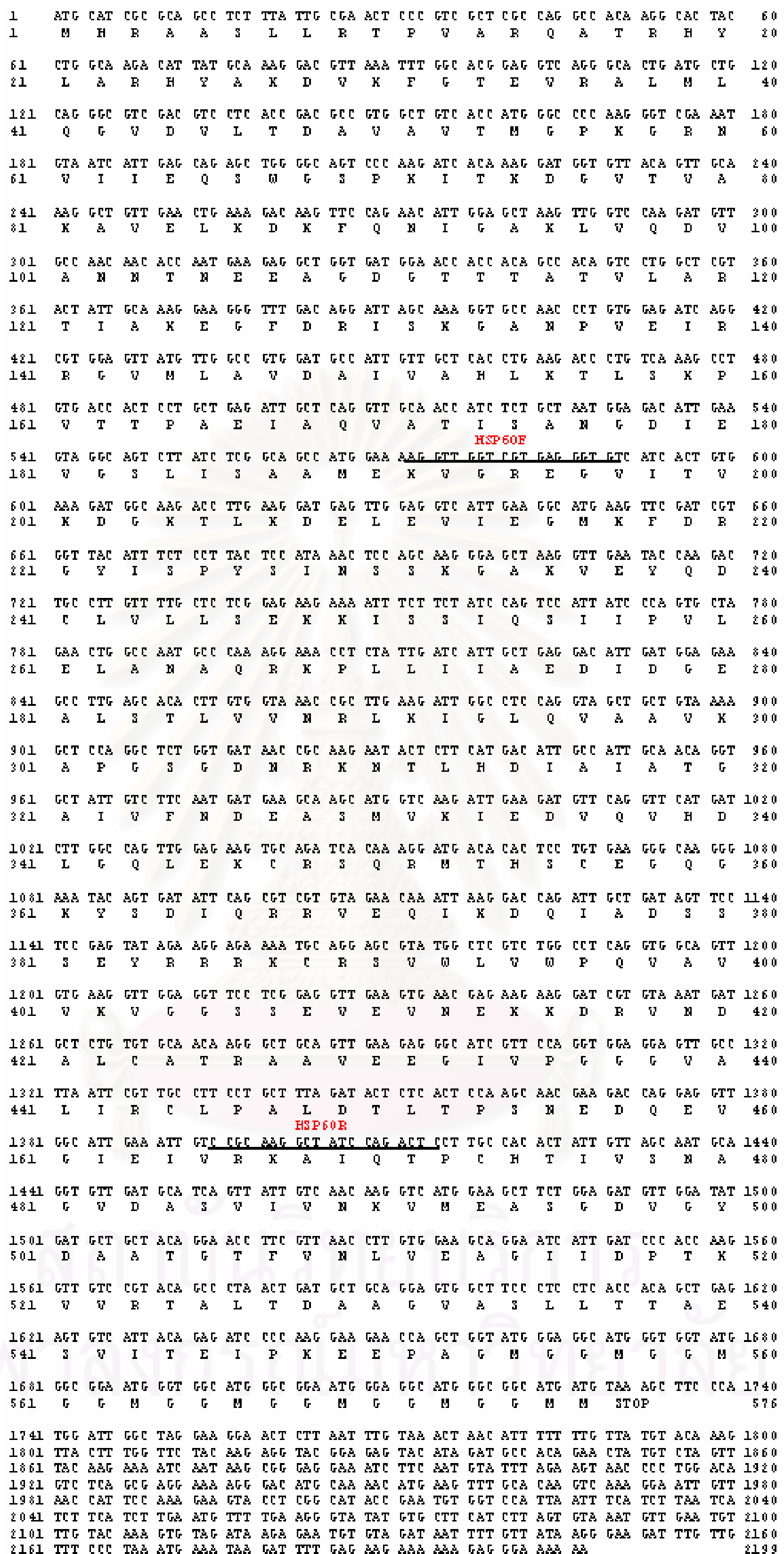

Figure 3.52 Nucleotide sequence of HSP60 gene .The highlight show the position of HSP60F and HSP60R primer.

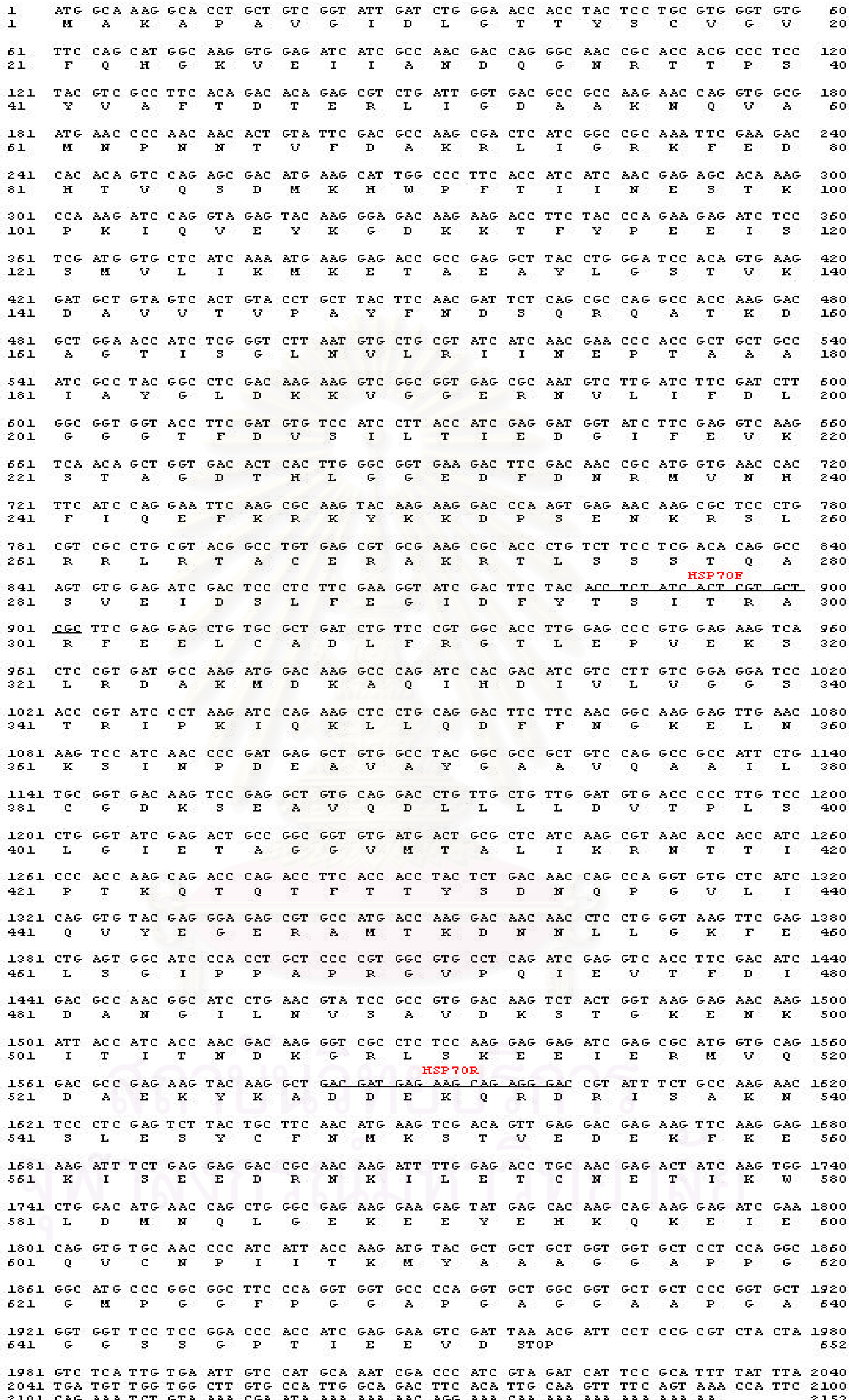

Figure 3.53 Nucleotide sequence of HSP 70 gene .The highlight show the position of HSP70F and HSP70R primer

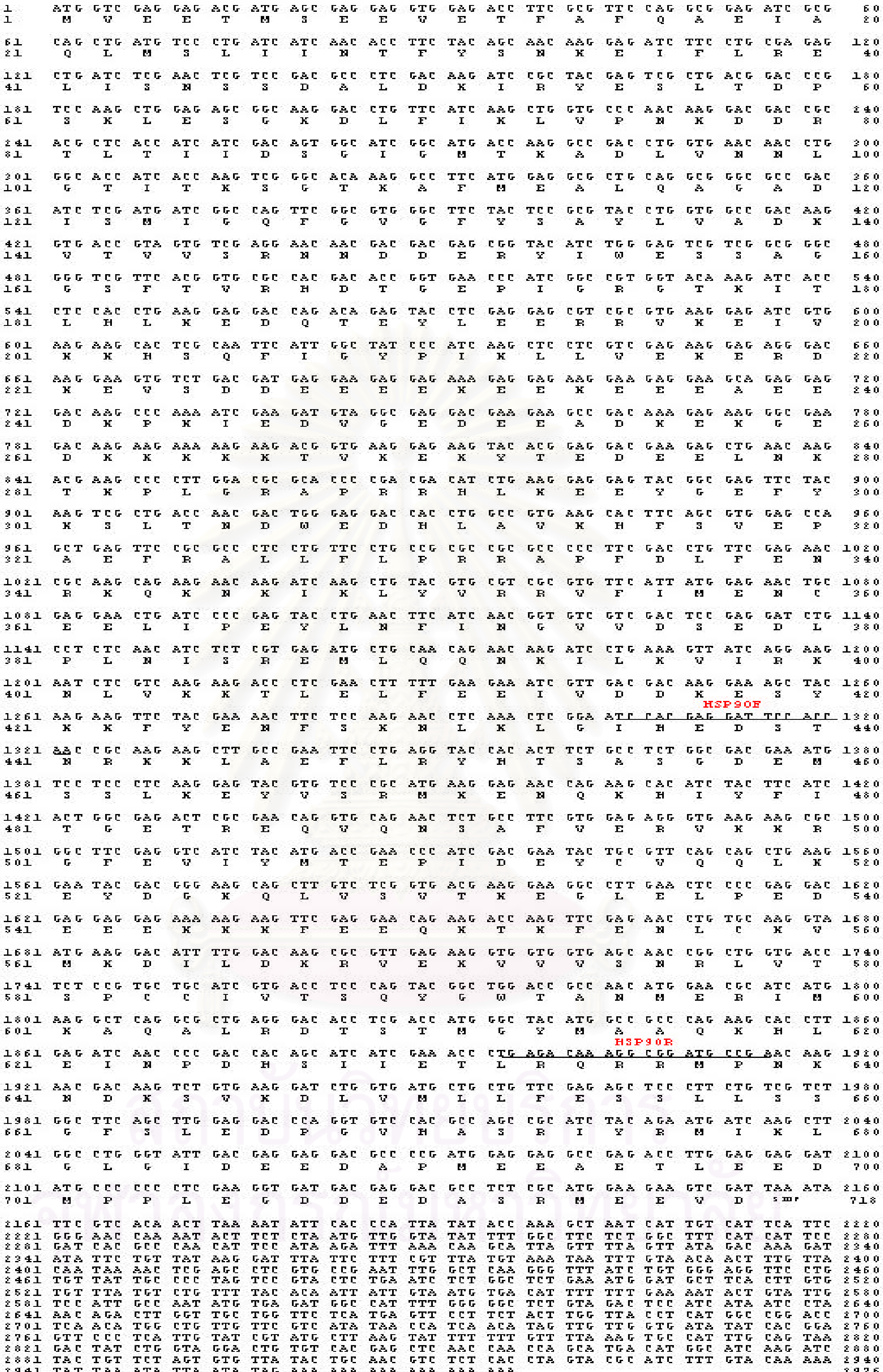

Figure 3.54 Nucleotide sequence of HSP90 gene .The highlight show the position of HSP90F and HSP90R primer.

1 GGGACTGGGGTACTCCTACACTCATAAACCAACGACATCATGTGTGACGACGAGGATCTTACTGC 65

66 CCTTGTGGTTGACAATGGCTCCGGCCTTTGCAAGGCCGGCTTCGCCGGAGACGACGCCCCTCGTG 130

131 CCGTCTTCCCCTCCATCGTCGGCCGTGCCCGTCATCAGGGTGTGATGGTCGGTATGGGTCAGAAG 195

Actin 1

197 GACGCCTACGTCGGTGATGAGGCCCAGAGCAAACGT**GGTATCCTCACCCTCAAGTA**CCCCATTGA 260

261 ACACGGTATCATCACCAACTGGGATGACATGGAGAAGATCTGGTACCATACTTTCTACAATGAGC 325

326 TCCGTGTTGCCCCCGAGGAGTCCCCCACACTTCTCACTGAGGCTCCCCTCAACCCCAAGGCCAAC 390

391 CGTGAGAAGATGACTCAGATCATGTTCGAGTCCTTCAATGTACCTGCCACTTACATTACCATCCA 455

456 GGCTGTGCTCTCCCTCTACGCCTCTGGTCGTACTACCGGTGAGGTTTGCGACTCTGGTGATGGTG 520

Actin 2

521 TGACTCACTTTGTCCCCG**TCTATGAAGGTTTCGCTCTT**CCTCATGCTATCCTTCGTCTCGATCTT 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 3.55 Nucleotide sequence of Beta-actin gene .The highlight show the position of Actin 1 and Actin 2 primer.

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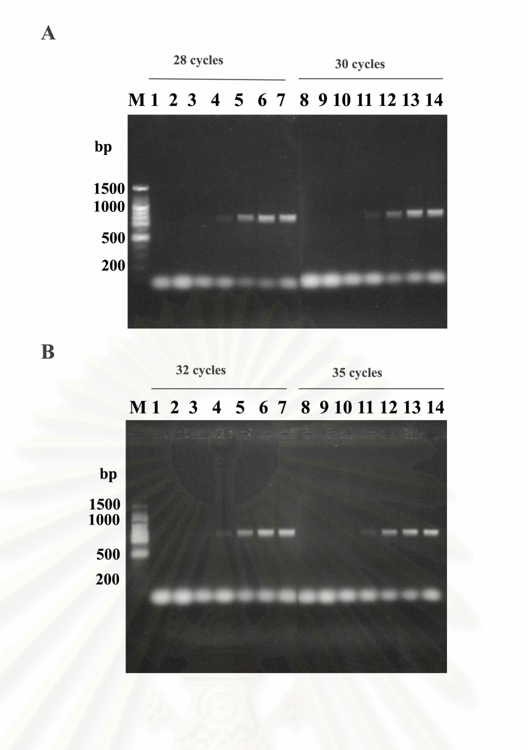

Figure 3.56 PCR products of heat shock protein 60 gene amplification determined on 1.2 % agarose gel and stained with Ethidium bromide. PCR reaction was conducted on 28 (lane 1-7), 30 (lane 8-14) (A), 32 (lane 1-7) and 35 cycles (lanes 8-14) (B). The template concentration in each reaction was 10, 100, 200, 300, 400, 600 and 800 ng, respectively (lane 1-7). A 100 bp DNA standard was shown in lane M.

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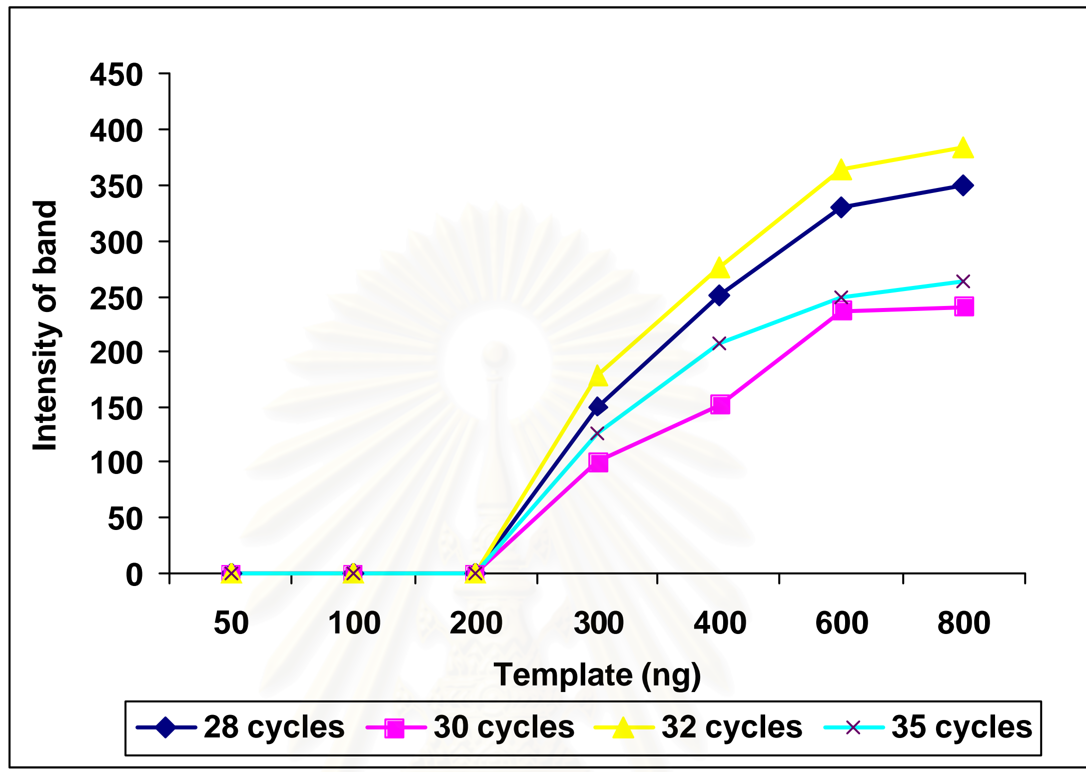

Figure 3.57 Relationship between PCR products of heat shock protein 60 gene amplified from haemocyte cell of *P. monodon* and various amount of DNA template used in PCR reaction.

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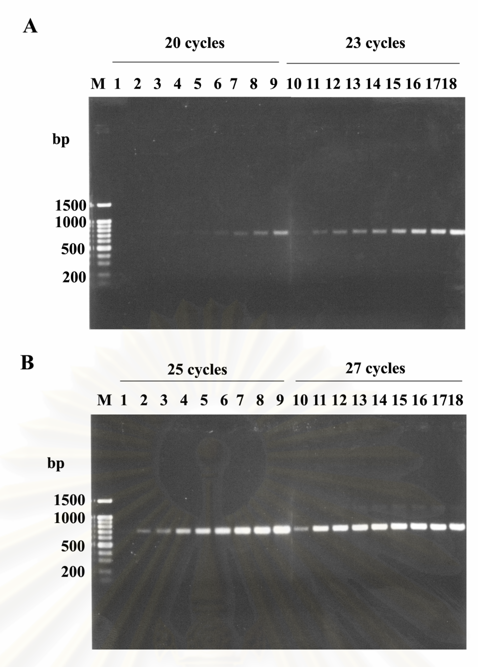

Figure 3.58 PCR products of heat shock protein 70 gene amplification determined on 1 % agarose gel and stained with Ethidium bromide. PCR reaction was conducted on 20 (lane 1-9), 23 (lane 10-18) (A), 25 (lane 1-9) and 27 cycles (lanes 10-18) (B). The template concentration in each reaction was 10, 50, 75, 100, 125, 150, 200, 250, andb 300 ng, respectively (lane 1-9). A 100 bp DNA standard was shown in lane M.

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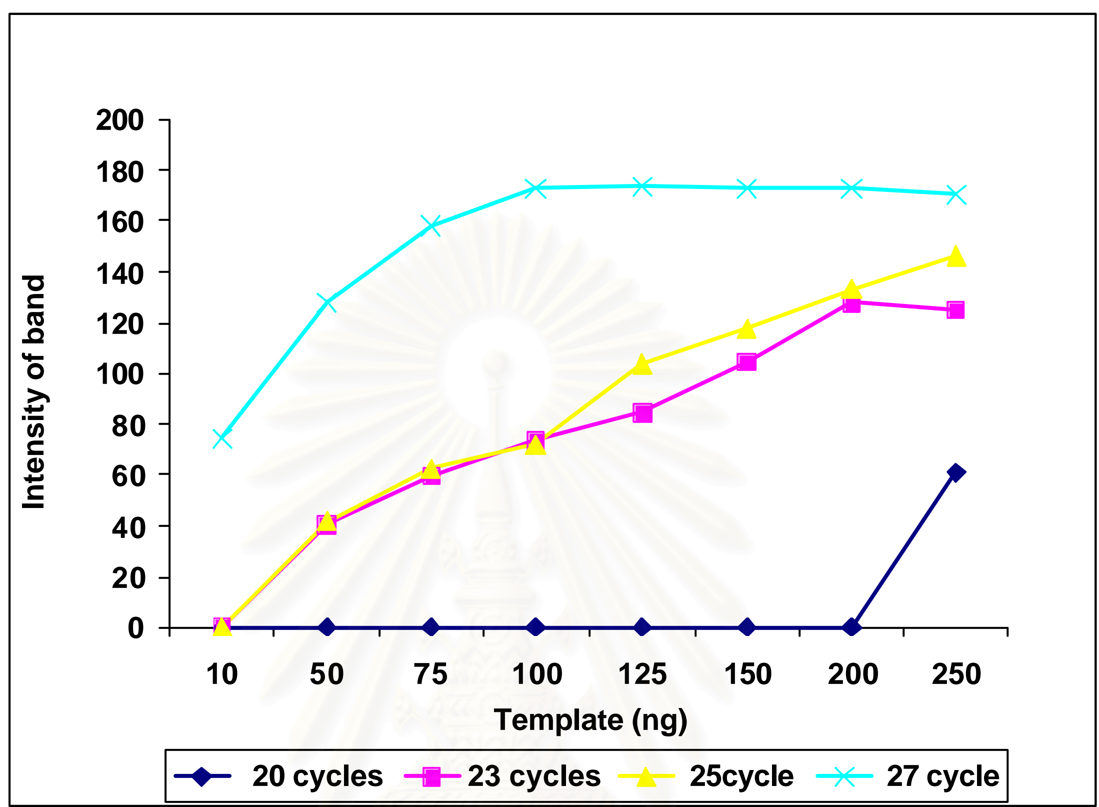

Figure 3.59 Relationship between PCR products of heat shock protein 70 gene amplified from haemocyte cell of *P. monodon* and various amount of DNA template used in PCR reaction.

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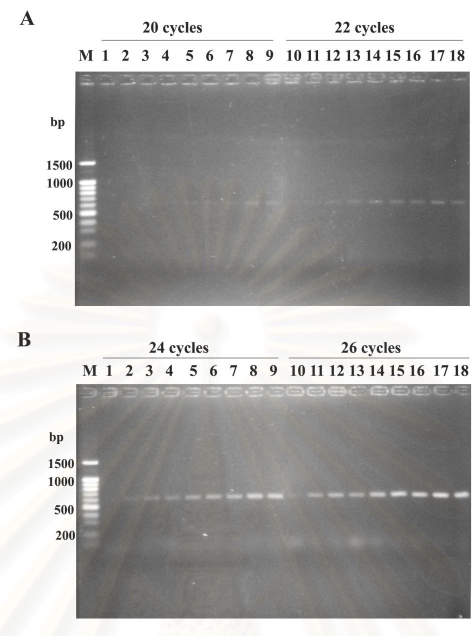

Figure 3.60 PCR products of heat shock protein 90 gene amplification determined on 1.2 % agarose gel and stained with Ethidium bromide. PCR reaction was conducted on 20 (lane 1-9), 22 (lane 10-18) (A), 24 (lane 1-9) and 26 cycles (lanes 10-18) (B). The template concentration in each reaction was 10, 25, 50, 75, 100, 125, 150, 200, and 250 ng, respectively (lane 1-9). A 100 bp DNA standard was shown in lane M.

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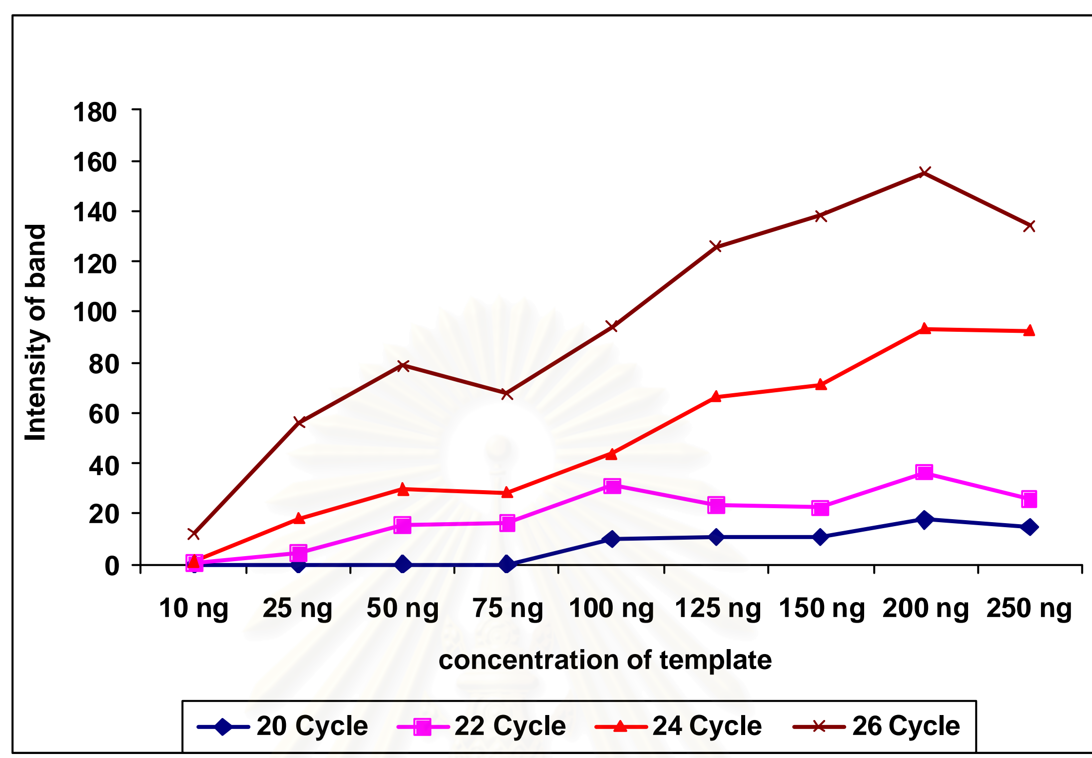

Figure 3.61 Relationship between PCR products of heat shock protein 90 gene amplified from haemocyte cell of *P. monodon* and various amount of DNA template used in PCR reaction

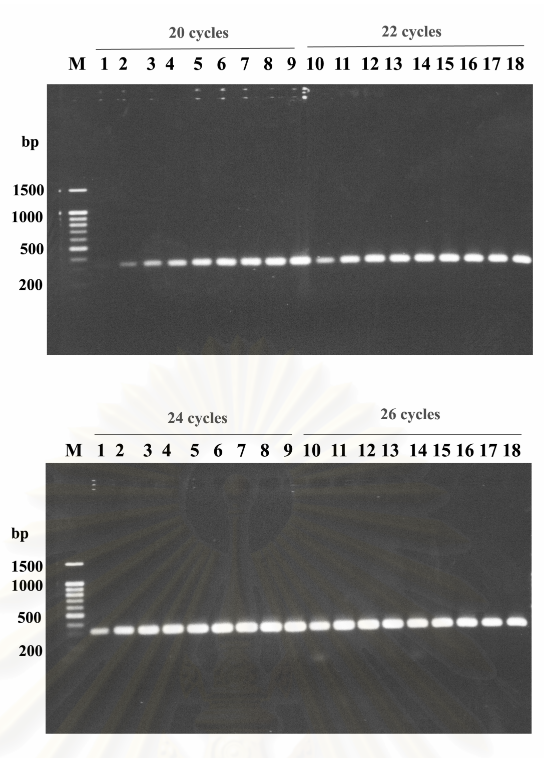

Figure 3.62 PCR products of β-actin gene amplification determined on 1.2 % agarose gel and stained with Ethidium bromide. PCR reaction was conducted on 20 (upper lane 1-9), 22 (upper lane 10-18), 24 (lower lane 1-9) and 26 cycles (lower lanes 10-18). The template concentration in each reaction was 5, 10, 25, 50, 75, 100, 125, 150, and 200 ng, respectively (lane 1-9). A 100 bp DNA standard was shown in lane M.

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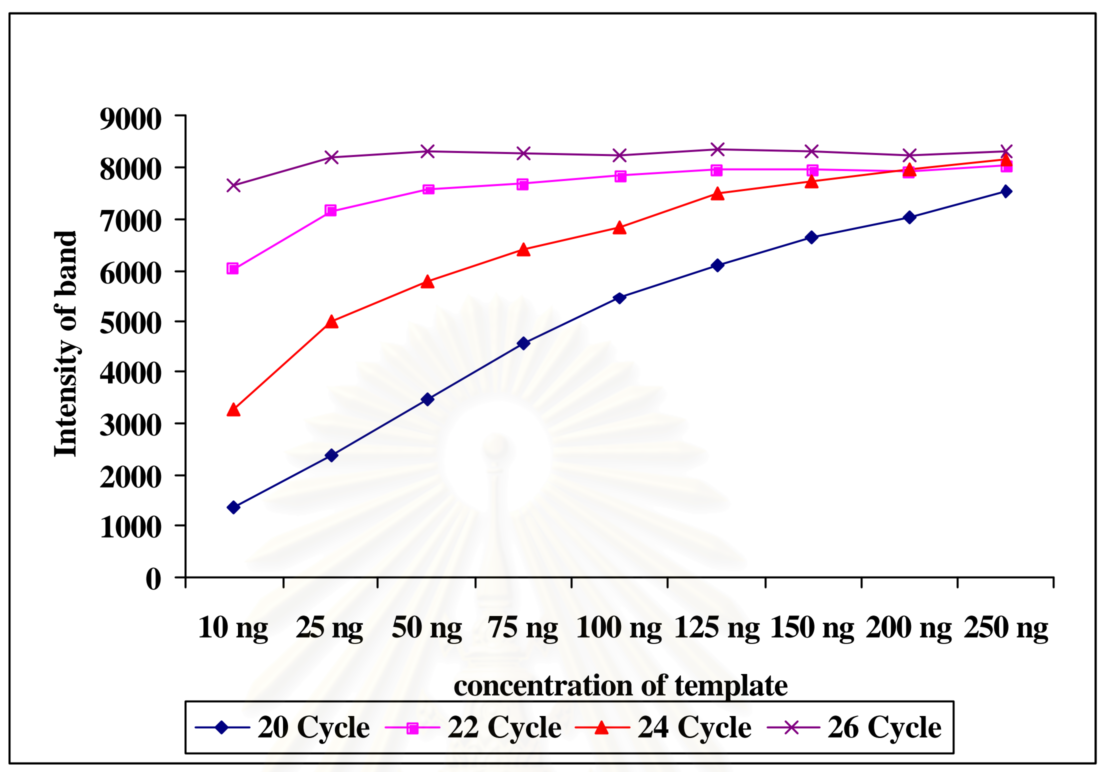

Figure 3.63 Relationship between PCR products of β-actin gene amplified from haemocyte cell and various amount of DNA template used in PCR reaction.

3.13.2 Expression level of HSP60 gene

After haemocytes were treated with thermal stress for 1 and 2 h, the significant difference of the expression level of HSP60 gene was not detected in response to any thermal treated haemocytes (*p*>0.05) The result of expression levels was provided in Table 3.12-3.13 and Figure 3.64-3.67.

Table 3.12 The expression level of heat shock protein 60 gene and β-actin gene and the expression ratio of heat shock protein 60 gene and β-actin genes in haemocyte cell after treated with various temperature for 1 hrs.

Genes	Temperature (°C)				
	28	**30**	**33**	**35**	**4**
HSP60	466.20	397.23	540.50	489.36	381.40
Actin	544.55	491.67	503.95	522.33	396.33
HSP60/Actin	1.32 ±	1.41 ±	2.14 ±	1.80 ±	1.30 ±

Table 3.13 The expression level of heat shock protein 60 gene and β-actin gene and the expression ratio of heat shock protein 60 gene and β-actin genes in haemocyte cell after treated with various temperature for 2 hrs.

Genes	Temperature (°C)				
	28	**30**	**33**	**35**	**4**
HSP60	297.5532	341.028	376.0925	461.2124	243.7809
Actin	366.5472	348.6033	330.5639	351.9881	343.3613
HSP60/Actin	0.84± 0.35	1.00± 0.54	1.12 ±	1.34 ±	0.76 ±

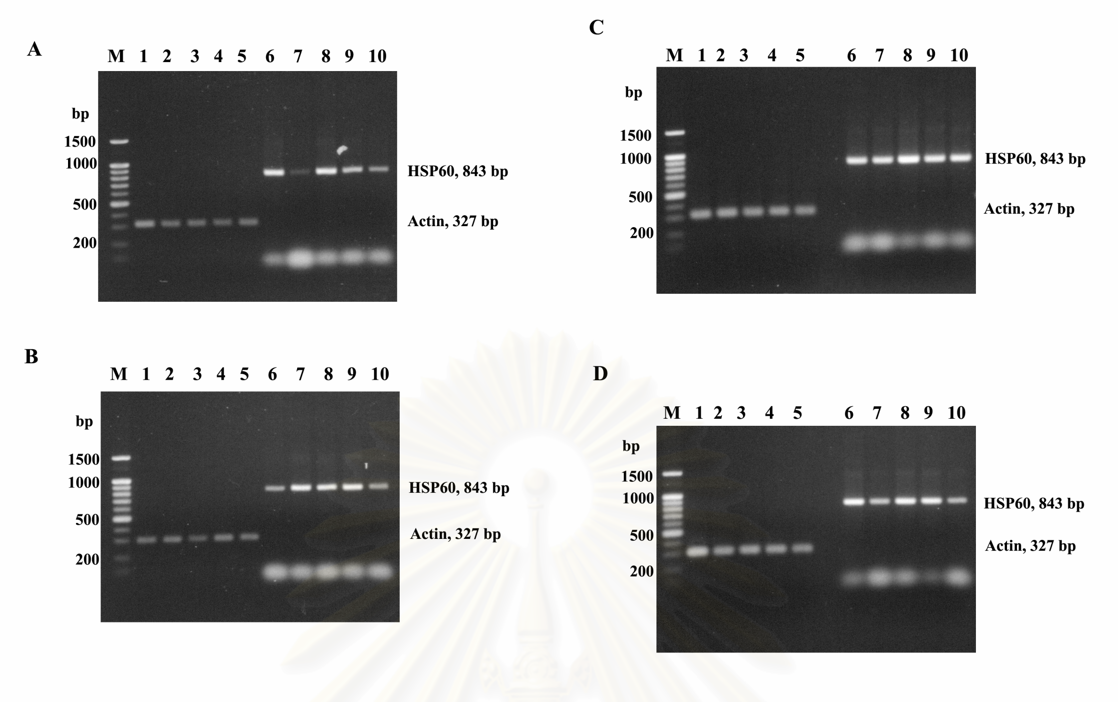

Figure 3.64 The expression levels of heat shock protein 60 gene from haemocyte cell after treated with various thermal stress for 1 hr in comparison with ß-actin. Samples were obtained from 4 replications (A, B, C, and D) and analysed by 1.2% agarose gel electrophoresis.

Lane M = 100 base pair ladder

Beta-actin gene

Lane 1 = untreated haemocyte cell (28 ^{o}C)

Lane 2 = expression level of haemocyte cell treated with 30 ^{o}C

Lane 3 = expression level of haemocyte cell treated with 33 ^{o}C

Lane 4 = expression level of haemocyte cell treated with 35 ^{o}C

Lane 5 = expression level of haemocyte cell treated with 4 ^{o}C

Heat shock protein 60 gene

Lane 6 = untreated haemocyte cell (28 ^{o}C)

Lane 7 = expression level of haemocyte cell treated with 30 ^{o}C

Lane 8 = expression level of haemocyte cell treated with 33 ^{o}C

Lane 9 = expression level of haemocyte cell treated with 35 ^{o}C

Lane 10= expression level of haemocyte cell treated with 4 ^{o}C

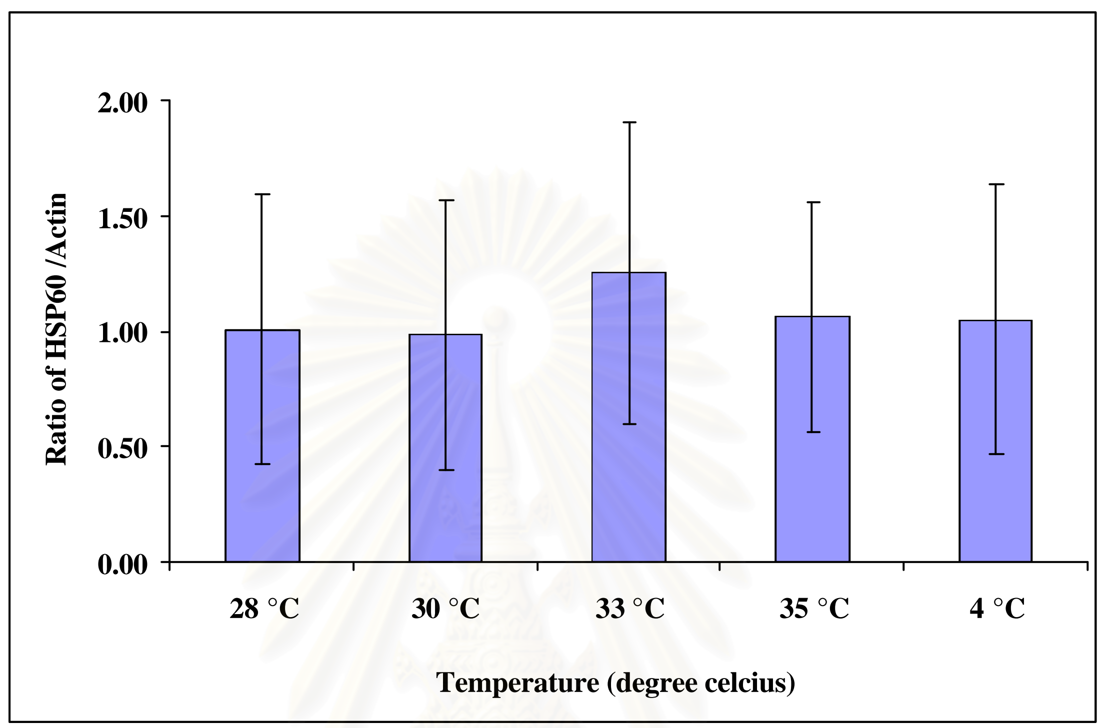

Figure 3.65 The expression ratio of heat shock protein 60 gene and β-actin gene in haemocyte cell after treated with various temperature for 1 hr.

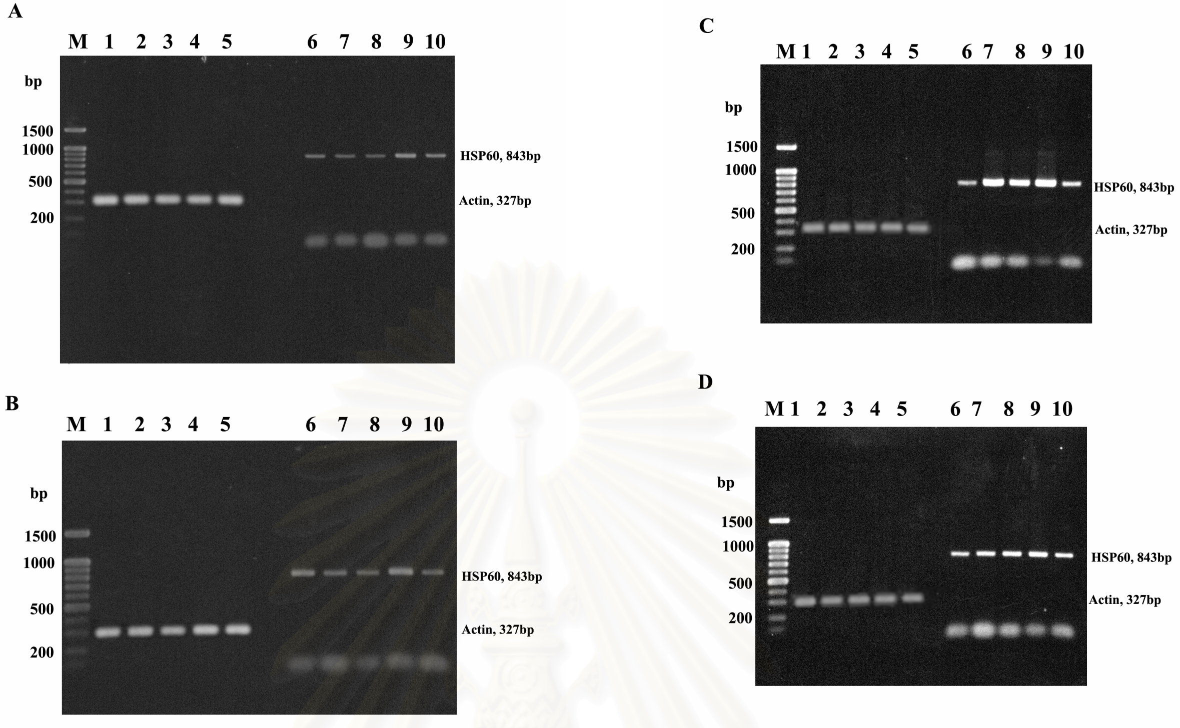

Figure 3.66 The expression levels of heat shock protein 60 gene from haemocyte cell after treated with various thermal stress for 2 hrs in comparison with ß-actin. Samples were obtained from 4 replications (A, B, C, and D) and analysed by 1.2% agarose gel electrophoresis.

Lane M = 100 base pair ladder

Beta-actin gene

Lane 1 = untreated haemocyte cell (28 ^{o}C)

Lane 2 = expression level of haemocyte cell treated with 30 ^{o}C

Lane 3 = expression level of haemocyte cell treated with 33 ^{o}C

Lane 4 = expression level of haemocyte cell treated with 35 ^{o}C

Lane 5 = expression level of haemocyte cell treated with 4 ^{o}C

Heat shock protein 60 gene

Lane 6 = untreated haemocyte cell (28 ^{o}C)

Lane 7 = expression level of haemocyte cell treated with 30 ^{o}C

Lane 8 = expression level of haemocyte cell treated with 33 ^{o}C

Lane 9 = expression level of haemocyte cell treated with 35 ^{o}C

Lane 10= expression level of haemocyte cell treated with 4 ^{o}C

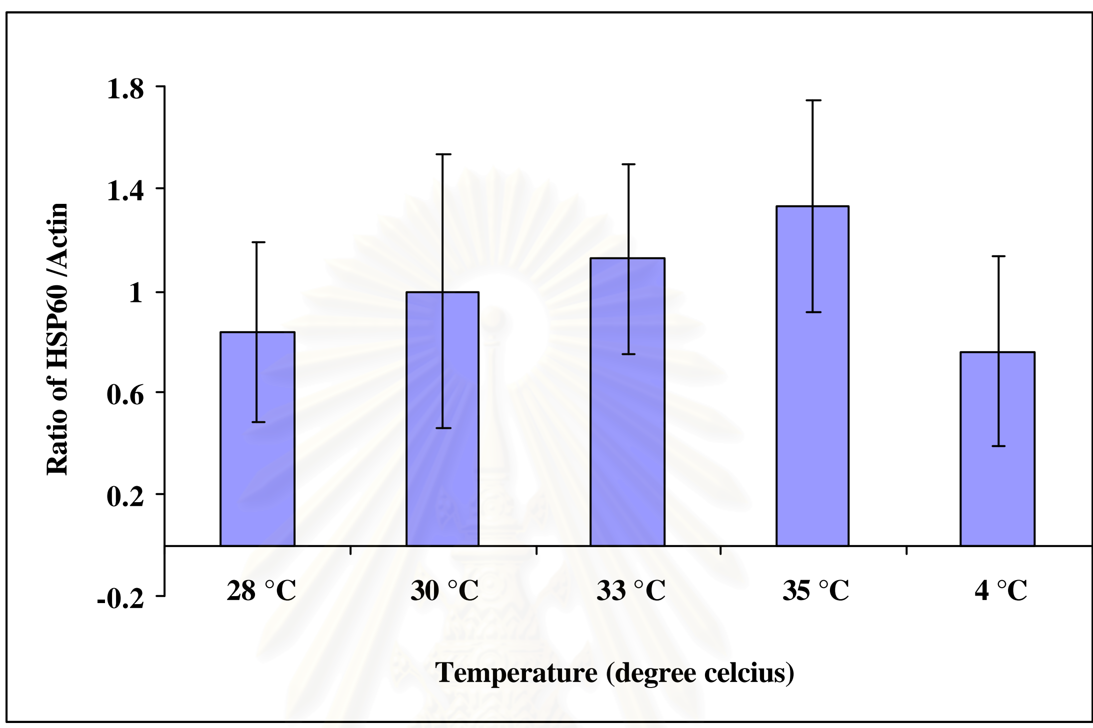

Figure 3.67 The expression ratio of heat shock protein 60 gene and β-actin gene in haemocyte cell after treated with various temperature for 2 hrs.

3.13.3 Expression level of HSP70 gene

The results of HSP70 gene expression in thermal treated haemocytes were shown as Figure 3.68-3.71. After treated with thermal stress for 1 h, no significant difference on the expression level of HSP70 gene was detected ($p>0.05$). (Table 3.14 and figure 3.68-3.69). After 2 h of post exposure, significant difference on the expression level of HSP70 gene between control and thermal treated haemocytes were detected ($p<0.05$) (Table 3.15 and Fig. 3.70-3.71). The haemocytes thermally shocked at 33 and 35°C revealed higher expression level of HSP70 gene than that of the haemocytes thermally shocked at 28°C and 30°C. At 4°C shock, the result also revealed the higher expression level of HSP70 gene when compared to control. There was no difference of HSP70 expression from the haemocyted thermally shocked at 28 and 30 °C.

Table 3.14 The expression level of heat shock protein 70 gene and β-actin gene and the expression ratio of heat shock protein70 gene and β-actin gene in haemocyte cell after treated with various temperature for 1 hr

Genes	Temperature (°C)				
	28	**30**	**33**	**35**	**4**
HSP70	414.12	380.02	419.07	438.22	368.45
Actin	544.55	491.67	503.95	522.33	396.33
HSP70/Actin	0.81 ±	0.83 ±	0.89 ±	0.88 ±	0.95 ±

Table 3.15 The expression level of heat shock protein 70 gene and β-actin gene and the expression ratio of heat shock protein70 gene and β-actin genes in haemocyte cell after treated with various temperature for 2 hrs

Genes	Temperature (°C)				
	28	**30**	**33**	**35**	**4**
HSP70	300.9371	327.8573	370.7802	388.4079	345.3506
Actin	366.547229	348.6033	330.5639	351.9881	343.3613
HSP70/Actin	0.83 ±	0.94 ±	1.12 ± 0.04	1.10 ±	1.02 ±

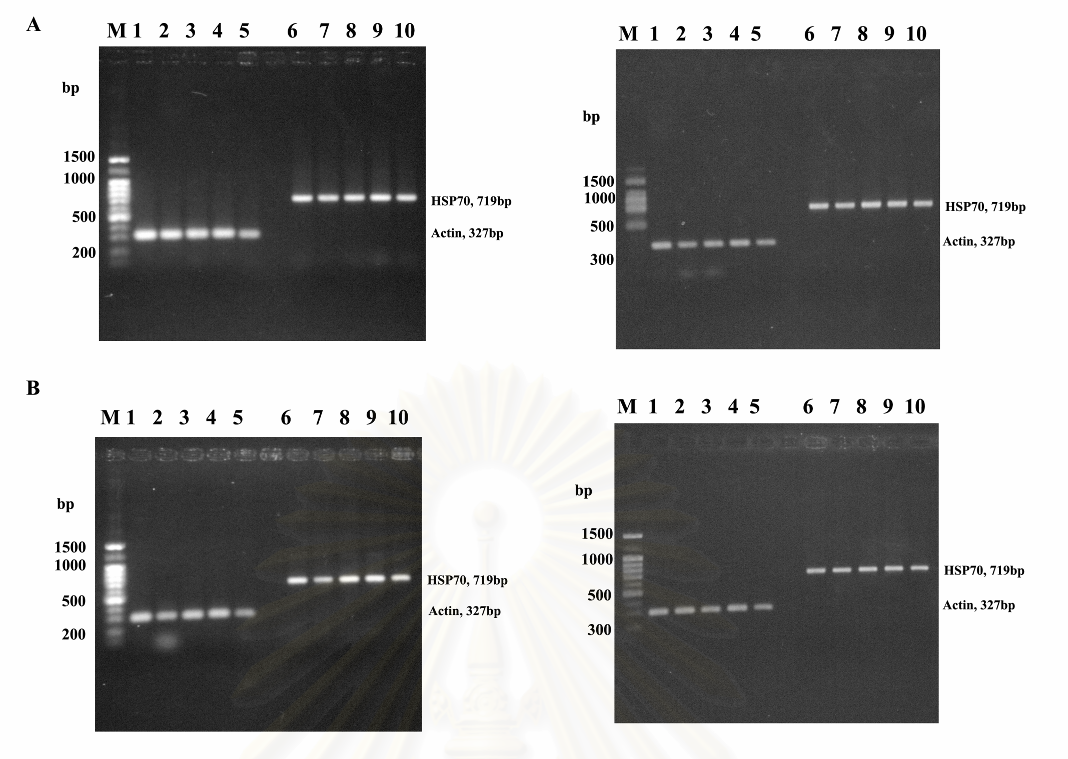

Figure 3.68 The expression levels of heat shock protein 70 gene from haemocyte cell after treated with various thermal stress for 1 hr in comparison with ß-actin. Samples were obtained from 4 replications (A, B, C, and D) and analysed by 1.2% agarose gel electrophoresis.

Lane M = 100 base pair ladder

Beta-actin gene

Lane 1 = untreated haemocyte cell (28 ^{o}C)

Lane 2 = expression level of haemocyte cell treated with 30 ^{o}C

Lane 3 = expression level of haemocyte cell treated with 33 ^{o}C

Lane 4 = expression level of haemocyte cell treated with 35 ^{o}C

Lane 5 = expression level of haemocyte cell treated with 4 ^{o}C

Heat shock protein 70 gene

Lane 6 = untreated haemocyte cell (28 ^{o}C)

Lane 7 = expression level of haemocyte cell treated with 30 ^{o}C

Lane 8 = expression level of haemocyte cell treated with 33 ^{o}C

Lane 9 = expression level of haemocyte cell treated with 35 ^{o}C

Lane 10= expression level of haemocyte cell treated with 4 ^{o}C

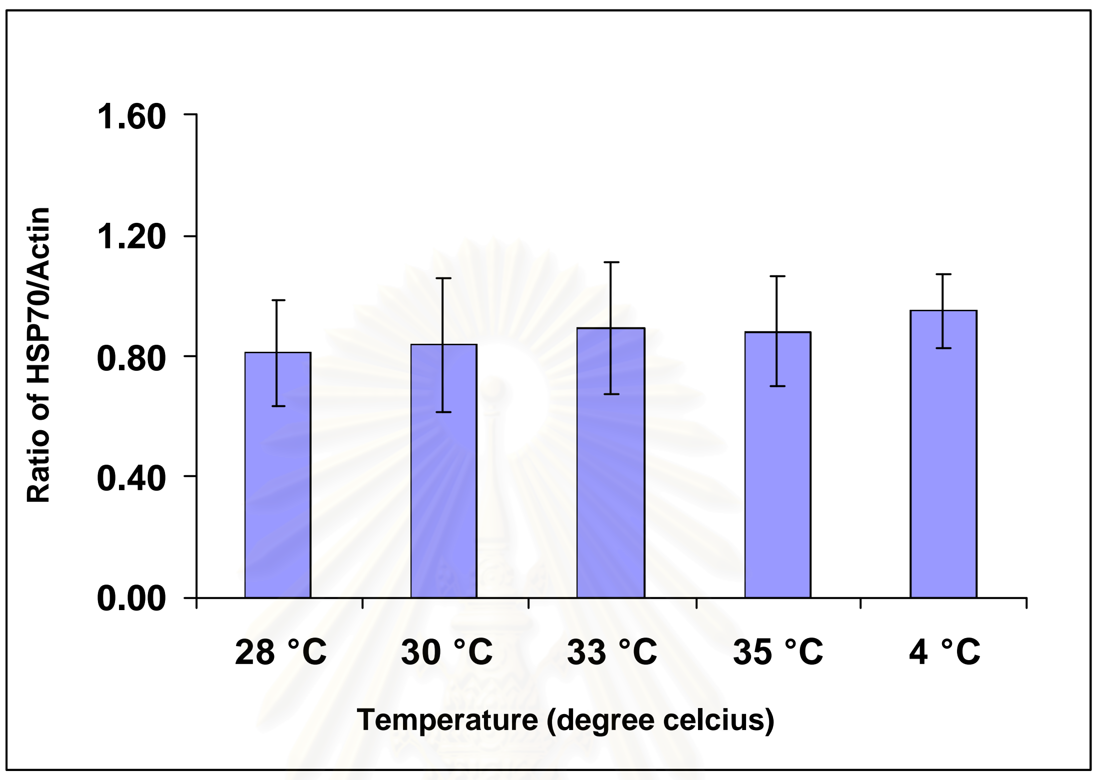

Figure 3.69 The expression ratio of heat shock protein 70 gene and β-actin gene in haemocyte cell after treated with various temperature for 1 hr.

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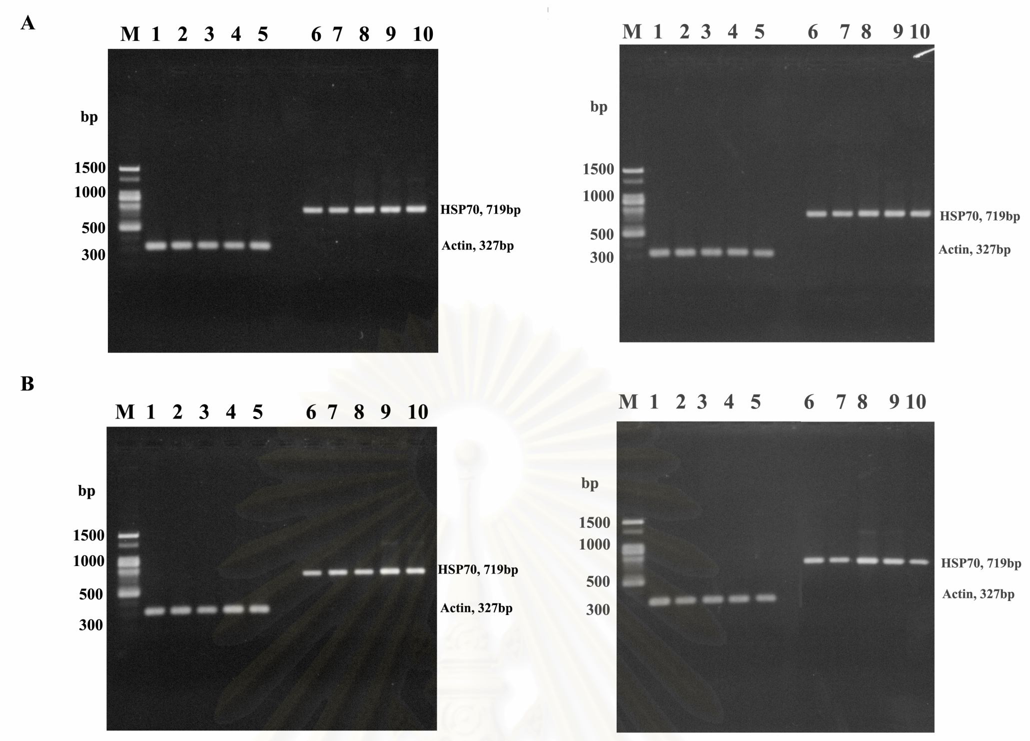

Figure 3.70 The expression levels of heat shock protein 70 gene from haemocyte cell after treated with various thermal stress for 2 hrs in comparison with ß-actin. Samples were obtained from 4 replications (A, B, C, and D) and analysed by 1.2% agarose gel electrophoresis.

Lane M = 100 base pair ladder

Beta-actin gene

Lane 1 = untreated haemocyte cell (28 ^{o}C)

Lane 2 = expression level of haemocyte cell treated with 30 ^{o}C

Lane 3 = expression level of haemocyte cell treated with 33 ^{o}C

Lane 4 = expression level of haemocyte cell treated with 35 ^{o}C

Lane 5 = expression level of haemocyte cell treated with 4 ^{o}C

Heat shock protein 70 gene

Lane 6 = untreated haemocyte cell (28 ^{o}C)

Lane 7 = expression level of haemocyte cell treated with 30 ^{o}C

Lane 8 = expression level of haemocyte cell treated with 33 ^{o}C

Lane 9 = expression level of haemocyte cell treated with 35 ^{o}C

Lane 10= expression level of haemocyte cell treated with 4 ^{o}C

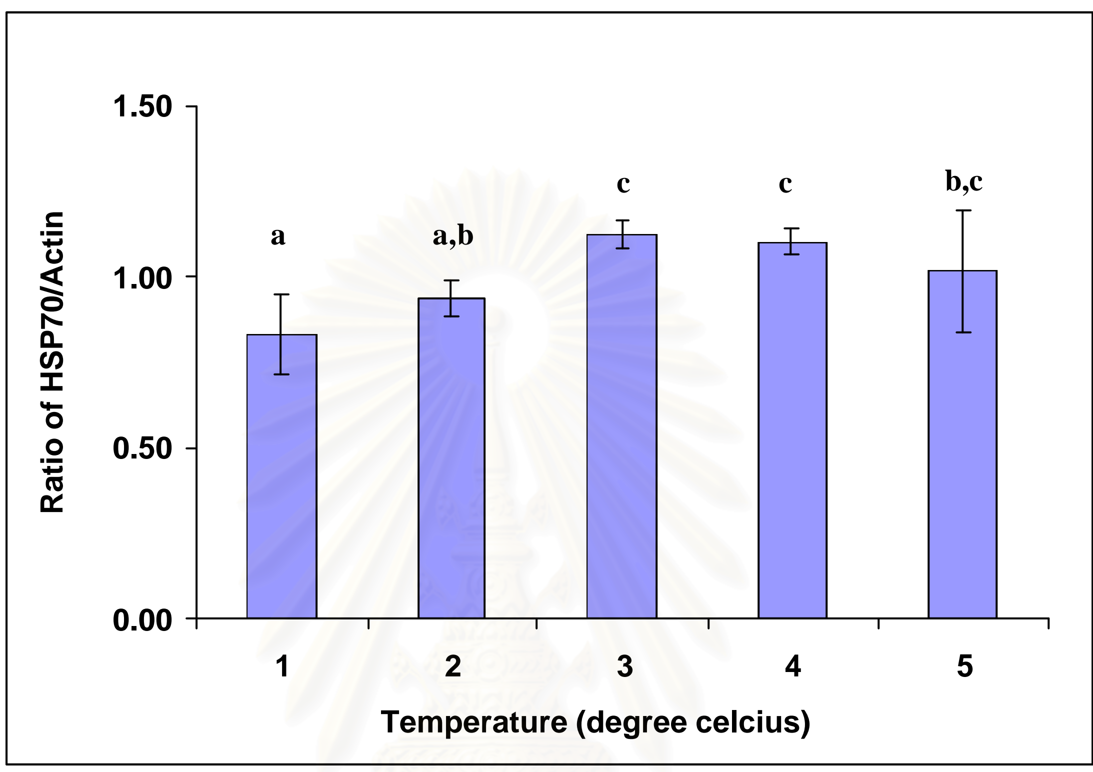

Figure 3.71 The expression ratio of heat shock protein 70 gene and β-actin gene in haemocyte cell after treated with various temperature for 2 hrs.

3.13.4 Expression level of HSP90 gene

The results of thermal treated haemocytes were shown in Figure 3.72-3.75 and Table 3.16-3.17. Significant difference ($p<0.05$) on the expression level of HSP90 gene between haemocytes of control (28 °C) and thermal treated haemocytes after treated with thermal stress for 1 h post exposure were detected (Table 3.16 and Fig. 3.74-3.75). The expression level at 33°C shock was higher than 28 and 30°C shocks, respectively. For 35°C shock, the expression level was higher than 28°C but in the same level as 30°C and 4°C shocks. After treated with thermal stress for 2 hrs, the expression level of HSP90 gene was clearly higher than that at 28, 30, and 33°C shocks. For 4°C shock, the expression was detected at the same level as 35 °C shock but no significant different level to that of 30 and 33 °C shocks.

Table 3.16 The expression level of heat shock protein 90 gene and β-actin gene and the expression ratio of heat shock protein90 gene and β-actin genes in haemocyte cell after treated with various temperature for 1 hr

Genes	Temperature (°C)				
	28	**30**	**33**	**35**	**4**
HSP90	69.86	84.22	163.97	157.94	95.38
Actin	544.55	491.67	503.95	522.33	396.33
HSP90/Actin	0.13±	0.19 ±	0.36 ±	0.32 ±	0.24 ±

Table 3.17 The expression level of heat shock protein 90 gene and β-actin gene and the expression ratio of heat shock protein 90 gene and β-actin genes in haemocyte cell after treated with various temperature for 2 hrs

Genes	Temperature (°C)				
	28	**30**	**33**	**35**	**4**
HSP90	106.41	115.63	117.66	166.36	137.50
Actin	366.55	348.60	330.56	351.99	343.36
HSP90/Actin	0.29 ±	0.34 ±	0.36 ±	0.48 ±	0.41± 0.07

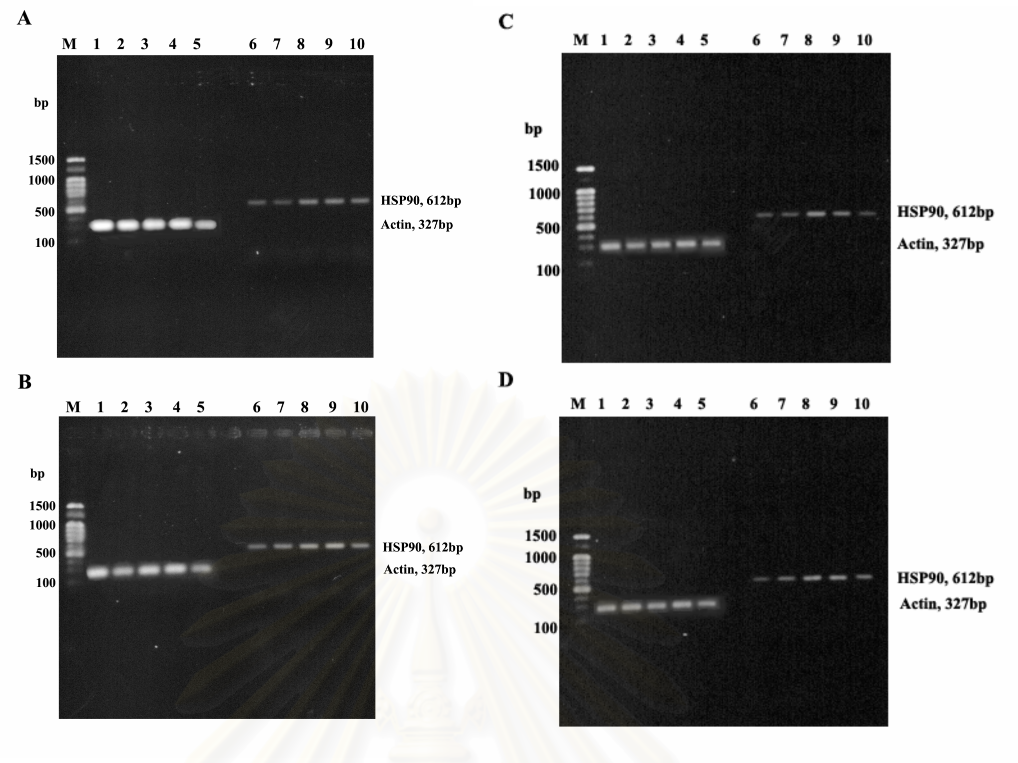

Figure 3.72 The expression levels of heat shock protein 90 gene from haemocyte cell after treated with various thermal stress for 1 hr in comparison with ß-actin. Samples were obtained from 4 replications (A, B, C, and D) and analysed by 1.2% agarose gel electrophoresis.

Lane M = 100 base pair ladder

Beta-actin gene

Lane 1 = untreated haemocyte cell (28 ^{o}C)

Lane 2 = expression level of haemocyte cell treated with 30 ^{o}C

Lane 3 = expression level of haemocyte cell treated with 33 ^{o}C

Lane 4 = expression level of haemocyte cell treated with 35 ^{o}C

Lane 5 = expression level of haemocyte cell treated with 4 ^{o}C

Heat shock protein 90 gene

Lane 6 = untreated haemocyte cell (28 ^{o}C)

Lane 7 = expression level of haemocyte cell treated with 30 ^{o}C

Lane 8 = expression level of haemocyte cell treated with 33 ^{o}C

Lane 9 = expression level of haemocyte cell treated with 35 ^{o}C

Lane 10= expression level of haemocyte cell treated with 4 ^{o}C

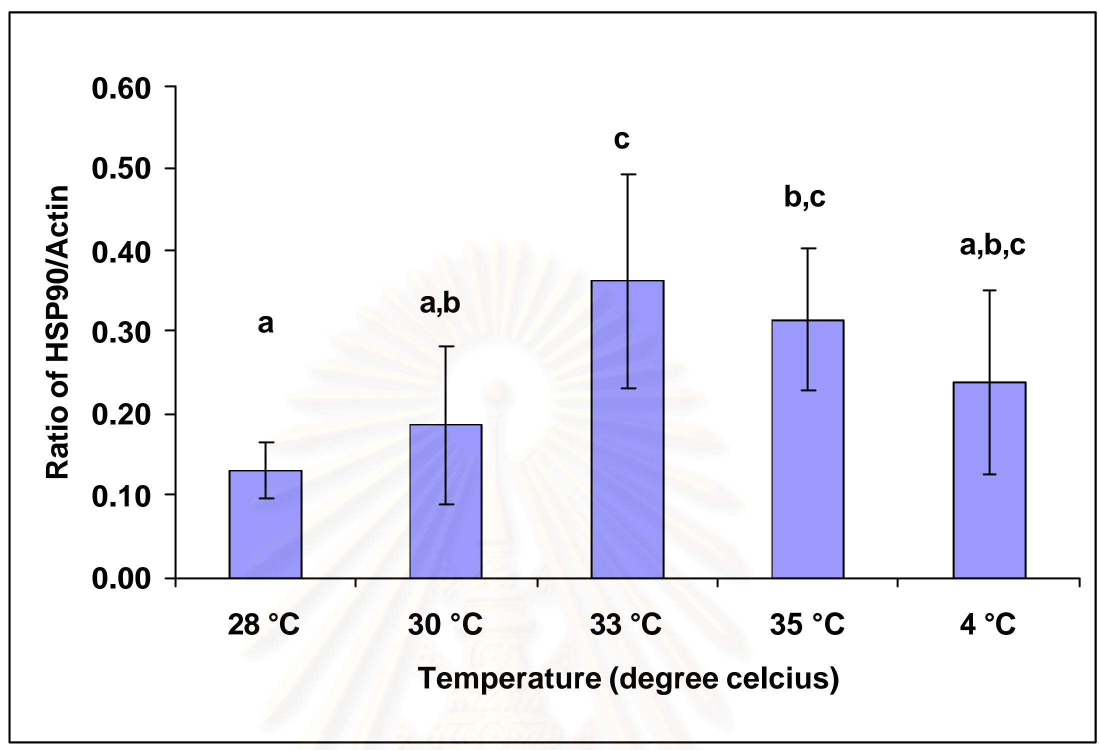

Figure 3.73 The expression ratio of heat shock protein 90 gene and β-actin gene in haemocyte cell after treated with various temperatures for 1 hr.

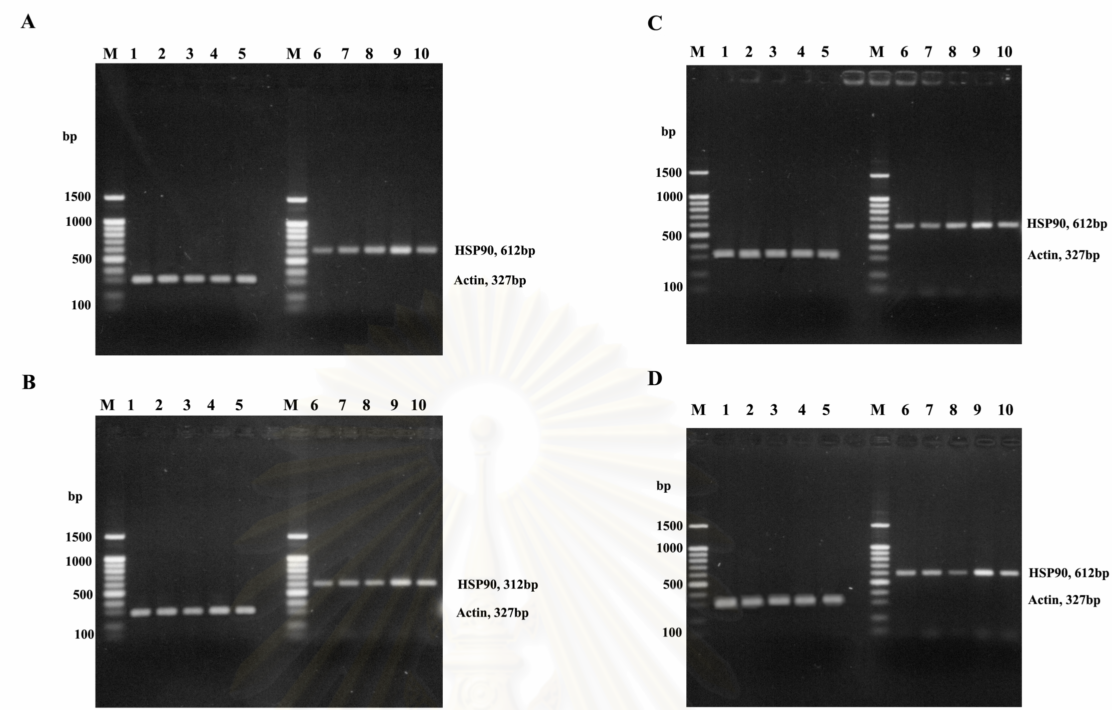

Figure 3.74 The expression levels of heat shock protein 90 gene from haemocyte cell after treated with various thermal stress for 2 hrs in comparison with ß-actin. Samples were obtained from 4 replications (A, B, C, and D) and analysed by 1.2% agarose gel electrophoresis.

Lane M = 100 base pair ladder

Beta-actin gene

Lane 1 = untreated haemocyte cell (28 °C)

Lane 2 = expression level of haemocyte cell treated with 30 °C

Lane 3 = expression level of haemocyte cell treated with 33 °C

Lane 4 = expression level of haemocyte cell treated with 35 °C

Lane 5 = expression level of haemocyte cell treated with 4 °C

Heat shock protein 90 gene

Lane 6 = untreated haemocyte cell (28 °C)

Lane 7 = expression level of haemocyte cell treated with 30 °C

Lane 8 = expression level of haemocyte cell treated with 33 °C

Lane 9 = expression level of haemocyte cell treated with 35 °C

Lane 10= expression level of haemocyte cell treated with 4 °C

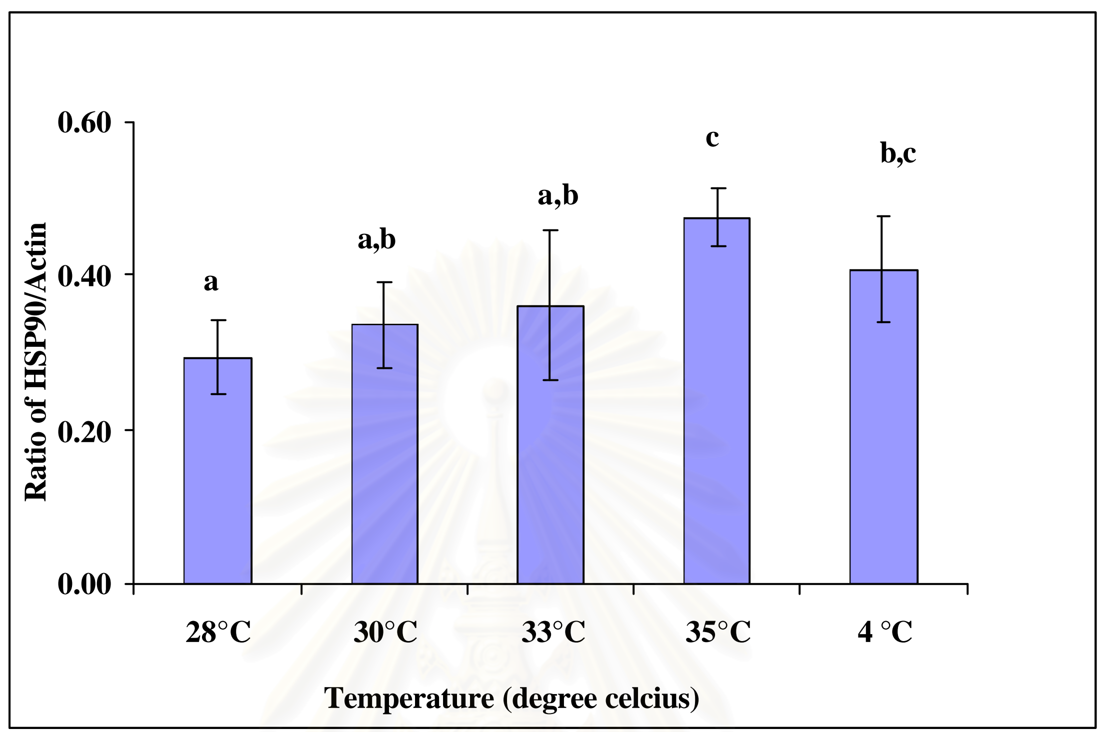

Figure 3.75 The expression ratio of heat shock protein 90 gene and β-actin gene in haemocyte cell after treated with various temperature for 2 hrs

CHAPTER IV

DISCUSSION

4.1 Haemocyte maintenance

The viability of *P.monodon* haemocytes maintained at 28^{o}C in M199, Grace's insect and TC100 with fetal bovine serum and antibiotics supplements revealed significant difference of maintenance period between haemocytes in each media. Haemocytes survived more than 4 days in M199 medium with high survival rate (>80%) while complete mortality of the haemocytes maintained in Grace's insect and TC100 media was detected after 1 day. In term of cell activity, however, haemocytes maintained in Grace's insect and TC100 media yielded significant higher enzyme activity. The activity of the viable haemocytes was analysized based on the activity of superoxide dismutase which was performed by measuring the ratio of the reduction of NBT in normal haemocytes (basal activity, BA) and haemocytes stimulated with PMA (stimulated activity, SA) at OD_{630}.

A number of studies on shrimp cell culture have been reported (Chen *et al*. 1986, 1988, and 1989; Hu *et al*., 1990; Diamant, 1990; Luedeman and Lightner, 1992; Fraser and Hall, 1999; and Fan and Wang, 2002). Primary monolayer cultures were established from haematopoietic tissue (Chen *et al*., 1988) and lymphoid organ (Chen and Kou, 1989) using double strength L-15 medium and various serum, haemolymph and muscle tissue extract supplements. Itami *et al*. (1989) found medium 199 to be superior to L-15 for the maintenance of *P. japonicus* lymphoid organ primary cultures. Hu *et al*. (1990) also used serum -supplemented 199 for the in vitro culture of hepatopancreas tissue from *P. orientalis*. Rosenthal and Diamant (1990) successfully initiated primary cultures of haematopoietic tissue, hepatopancreas and ovary from *P. semisulcatus* with the same basal medium supplemented with serum and shrimp haemolymph. Luedeman and Lightner (1992) and Fraser and Hall (1999) were able to obtain cultures of ovarian cells from *P. stylirostris, P. vannamei* and *P. monodon* using modified Grace's Insect Medium or L-15 media supplemented with hybridoma quality fetal bovine serum.

In this study, M199 media was further used for *in vitro* experiments of the haemocytes because it provided a considerable viability of the haemocytes for long period of time (up to 4 days) without changing the media.

An *in vitro* experiment on thermal shock revealed that haemocytes maintained in M199 media could tolerate temperatures at 4, 30 33, and 35 °C for at least 2 hrs without significant change in the number of viable cells.

This indicated the appropriate non-lethal conditions of thermal shock were used in this study.

4.2 Detection of HSPs

Enormous amount of literature available on the HSP response in a variety of organisms, however, very little is known about the HSP response in aquatic invertebrates, especially in penaeid shrimps.

In this study, silver stained SDS-PAGE gels revealed different protein profiles in thermal shock and un-shock samples. Less number of proteins in normal haemocytes was clearly observed when compared to the thermal shock haemocytes. The detection of protein accumulation in the haemocytes using SDS-PAGE revealed a number of peptide bands (149, 121, 106, 87, 65, 62, 55 and 42 kDa) appeared in thermal shock haemocytes but absent in un-shocked haemocytes. Among those differential apparent bands, the major bands at 83 and 75 kDa, present in thermal treated haemocytes were in agreement with the result reported in the investigation of HSP25 and HSP86 in the pleopods of *P.monodon* (Elisabeth *et al.*, 2002) and others (Rochelle *et al.*, 1991; Sheller *et al.*, 1998)

An increase in HSP70 and HSP90 was observed following thermal stress in crayfish (*Procambarus clarkii*) (Rochelle et al., 1991; Sheller *et al.*, 1998), encysted brine shrimp (*Artemia*) (Clegg *et al.*, 2000b; Frankenberg *et al.*, 2000), and *Homarus americanus* (Chang *et a* ., 1999). In oyster haemocytes (*Crassostrea virginica*), three different isoforms of HSP30 (HSP32, HSP34 and HSP37), HSP45 and HSP85 were detected in vitro by autoradiograph of radioactive proteins after hyperthermal shock from 20 to 41°C (Tirard *et al.*, 1995).

In this study, the protein profile were also examined using autoradiography. No radioactively labelled band was detected on X-ray film. In comparison to the

results of many authors, one possibility was the amounts of ^{35}S-methionine used in the protocol. It was ranged from 10 to 400 µci/ml (Buckley *et al.*, 2001; Tedeschi and Ciavarra, 1997; Wood *et al.*, 1999). Only minimal amount of ^{35}S-methionine (10 µ ci/ml) was used in this study. Another possibility would probably involve the existence of some inhibitors in the reaction.

The results from Western blotting analysis of the haemocyte lysates from the shrimps from control, cold and heat shock experiments showed a considerable signal of cross reaction of monoclonal anti-HSP90 antibody and the proteins at 82 kDa. The increase of HSP90 accumulation after thermal shock due to the temperature level, indicating that HSP90 was induced by both cold and heat shock. The detection of HSP60 and HSP70 in the haemocytes delivered no positive results which were presumably caused by low cross reactivity and low sensitivity of the antibodies.

Although, HSP90 in *P.monodon* can be determined by the cross reactivity of monoclonal antibody against water mold HSP90, the successful dilution used in this study was considerably low (1:500). Therefore, it is not so practical to perform quantitative analysis (ELISA) using this antibody with large number of samples.

In addition, to precisely determine the levels of HSP90 or other HSPs in the samples, a homologous antibodies and a calibration curve from a pure HSP are required. To date, no homologous antibodies and pure HSP standards have been produced for *P.monodon*. Although, the induction of HSP60 and HSP70 by thermal shock were not detected in this experiment, in most organisms studied so far, HSP70 proteins are among the most prominent proteins induced by heat, and these proteins do play a central role in tolerance to high temperatures, as they allow cell survival during and after thermal stress (reviewed by Parsell and Lindquist, 1993). A number of investigations have focused on the HSP70 family as the majority of HSPs in Crustacean. Many studies have reported that members of the HSP70 family commonly show up-regulation during times of stress (Rochelle *et al.*, 1991; Dunlap and Matsumura, 1997; Frankenberg *et al.*, 2000). The result in Western blotting described by Elisabeth et al., (2002) showing the presence of an immuno-reactive protein to mouse anti-human HSP70 IgG1 monoclonal antibody at a mass of 86 kDa in pleopod samples of *P.monodon* but it was not sensitive enough to detect differences in response to stress. However, an ELISA was reported to detect the significantly

RAP-PCR applied in this study was based upon the use of reverse transcribed RNA as a template to identify differentially expressed genes in a manner analogous to that of arbitrarily primed PCR (RAP-PCR, Welsh and McClelland, 1992), which used genomic DNA as a template. RAP-PCR has proven to be a powerful method for the detection and isolation of differentially expressed genes in several systems including tumor cells (Wong *et al*., 1993, Nelson *et al*., 1996), human brain cells (Dalal *et al*., 1996), and rat glial cells (Sakai *et al*., 1997).

In this study, ten differential expressed DNA fragments from the PCR products amplified by 10 different random primer combinations were obtained. Sequence analyses revealed that 9 sequences were identified as protein of unknown genes and 1 sequence was identified to encode a putative protein known as vigilin, a high density lipoprotein-binding protein.

Vigilin is a 150 kDa protein containing 14 copies of the hnRNP K homology (KH) domain, a highly conserved RNA binding motif (Siomi, Choi, Siomi, Nussbaum, & Dreyfuss, 1994). Vigilin specifically binds HDL molecules and may function in the removal of excess cellular cholesterol (Kugler *et al*., 1996). The protein is expressed in a wide variety of cell types and tissues, and its relative abundance is sensitive to the growth and differentiation states of cultured cells. It is expressed primarily as a 150-kD membrane-bound protein localized in the cytoplasm of cells and appears to undergo processing to form a 110-kD protein that binds HDL on ligand blots and that is localized, at least partially, to the plasma membrane (Chiu *et al*., 1997). It is most likely that vigilin plays a role in RNA transport or metabolism (Vollbrandt *et al*., 2004). Vigilin has been found in human (Plenz, Gan, Raabe, & Müller, 1993), chicken (Schmidt *et al*., 1992), *Xenopus laevis* (Dodson & Shapiro, 1997), *Drosophila melanogaster* (Cortes & Azorin, 2000), *Caenorhabditis elegans* (Weber, Wernitzing, Hager, Harata, & Park, 1997) and *Saccharomyces cerevisiae* (Lang & Fridovich-Keil, 2000). Although the exact function of vigilin is unknown, its expression in plaque macrophages suggests a role for this molecule in atherogenesis (Chiu *et al*., 1997 and Kozarsky *et al*., 1997).

Recent reports provided strong evidence that vigilin might be involved in the inhibition of the proliferation of human breast cancer cells (Cao *et al*., 2004). More evidences are needed to confirm the involvement of this vigilin-like protein to the thermal response. Complete sequence, quantitative analysis of gene expression in

different tissues, and specific function activities of this gene in *P.monodon* will be very helpful to understand more about shrimp molecular activity.

4.4 EST library analysis

A number of cDNA libraries have been constructed from shrimps during the past few years. In ESTs of cDNA libraries constructed from haemocyte of *Litopenaeus vannamei* and *Litopenaeus setiferus* (Gross *et al.,* 2001), it was found that immune genes and genes of potentail immune function were prominent in both haemocyte libraries (27.6% in *L. setiferus* and 21.2% in *L. vannamei*) whereas the defense and homeostasis genes in this study was 12.1%. Among those genes categorized as immune or potentially immune in function, the antibacterial peptides dominate in the haemocyte libraries (82.2% of immune function genes in *L. setiferus* and 73.1% in *L. vannamei*) which were similar to result in this study.

EST library created from cephalothorax, eyestalk, and pleopod tissue of the black tiger shrimp (*P. monodon*) revealed that significant database matches were found for 48 of 83 nuclear genes sequenced from the cephalothorax library, 22 of 55 nuclear genes from the eyestalk library, and 6 of 13 nuclear genes from the pleopod library. The putative identities of these genes reflected the expected tissue specificity. A few sequences matched anonymous EST or genomic sequences, and others contained mini-satellite or microsatellite repeat sequences. The remainder, 31 from the cephalothorax library, 25 from the eyestalk library, and 5 from the pleopod library, were unknown genes (Lehnert *et al*., 1999).

EST approach in haemocytes of the normal and white spot syndrome virus (WSSV) infected kuruma prawn (Penaeus japonicus) was investigated. Of 635 clones obtained from the normal library, 284 (44.7%) significantly matched sequences in GenBank, and of 370 clones obtained from WSSV-infected library, 174 (47.0%) significantly matched sequences in the database. One hundred fifty-two deduced proteins were newly identified. Of these, 28 types were involved in biodefence. The putative defense proteins accounted for 2.7% of total ESTs in a normal shrimp library and 15.7% of the total ESTs in an infected library. (Rojtinnakorn *et al*., 2002)

Another EST library was constructed from haemocytes of *P. monodon* to identify genes associated with immunity in this economically important species. The

number of clones was approximately 4 x 10^5. Of these, 615 clones were sequenced and analyzed. Significant homology to known genes was found in 51%, the remaining sequences (49%) did not match any sequence in GenBank. Approximately 8.9% were identified as putative immune-related genes. A heat shock protein (cpn10 homologue) are reported. (Supungul *et al*., 2002)

In this study, EST library was constructed in order to identify stress-related genes expressed in the haemocytes of *P.monodon*. The EST library revealed a total of 1090 expressed sequence tags (ESTs) from haemocytes stress response cDNA library were found corresponding to defense and homeostatic genes 132 clones (12.1%), 2 clones (0.18%) of ESTs homologues of HSP 70 and 130 clones of other stress related genes chaperonins, ubiquitin, anti-lipopolysaccharide factor, antimicrobial peptide, transglutaminase, cyclophilin, ferritin, chelonianin, glutathione, lysozyme, penaeidin, perlucin, profilin, protease inhibitor, proteinase inhibitor, serine proteas, superoxide dismutase, transglutaminase, prophenoloxidase, serine proteas inhibitor and thymosin were found.

4.5 Sequences and characterization of HSP genes

HSP60 or chaperonin family was considered to be a ring complex family. The term GroEL is used for HSP60 found in prokaryotes, chloroplasts, and mitochondria whereas HSP60 and its homologs are found in the eukaryotic cytosol. Many of the HSP60s are also known as chaperonins (cpn60). They are ring-shaped oligomeric protein complexes with a large central cavity in which nonnative proteins can bind. In bacteria, at least, HSP60 require a cochaperonin, GroES (cpn10), for full function. The availability of a high-resolution crystallographic structure, in conjunction with mutagenesis studies, has helped in the elucidation of the details of the reaction cycle. However, there are still many points of controversy, reflecting the complexity of the mechanism of this large chaperone.

The HSP70 are a family of molecular chaperones that are involved in protein folding and several other cellular functions and that exhibit weak ATPase activity. Genomic sequences for HSP70 gene were elucidated in fish, including rainbow trout, *Oncorhynchus mykiss* (Kothary *et al*., 1984), medaka, *Oryzias latipes* (Arai *et al*., 1995), zebrafish (Lele *et al*., 1997), pufferfish, *Fugu rubripes* (Lim and Brenner,

1999) and tilapia, *Oreochromis mossambicus* (Molina *et al*., 2000). The HSP70 chaperones are composed of two major functional domains. The NH_2-terminal, highly conserved ATPase domain binds ADP and ATP very tightly (in the presence of Mg^{2+} and K^{1+}) and hydrolyzes ATP, whereas the COOH terminal domain is required for polypeptide binding. The HSP70 family is very large, with most organisms having multiple members. The crystallographic structures of human HSP70 ATPase domain have been determined (Flaherty *et al*., 1990; Sriram *et al*., 1997; and Ogata *et al*., 1996)

Members of the HSP90 family are highly conserved, essential proteins found in all organisms from bacteria to humans. Mammalian HSP90 was reported to be dimmers in active forms. Although there are a number of similarities between the activities of HSP90 and HSP70, the former has several identified specific interactions, for example, with cytoskeleton elements, signal transduction proteins including steroid hormone receptors, and protein kinases such as the mitogen-activated protein kinase system (Fink 1999).

The encoded sequences of HSP60, HSP70 and HSP90 identified from *P.monodon* were highly conserved when compared to reported HSPs from various species whereas the untranslated regions were relatively different from others. A major mechanism of HSPs stress-related transcription induction, operates through binding of regulatory proteins, the trimeric heat shock factors (HSFs) to HSP70 5' flanking heat shock element (HSEs), located upstream of the TATA box (Bienz nad Pelham, 1987; Morimoto, 1993). In this study, the TATA box and the complete HSE regions were not found. The polyadenylation pattern was only found in HSP70 but not in HSP60 and HSP90. The difference between the 5' and 3' regions of *P. monodon* HSPs may provide the new information for the different gene function and regulation of these HSPs in invertebrates.

No difference between the PCR products of HSPs amplified from cDNA and genomic DNA indicating that *P. monodon* HSPs contained no intron. This result was in agreement with HSPs reported in most non-mammalian speices. However, these HSP products were not amplified from the whole genes. Therefore, complete genomic sequence analyses on these HSPs will be required for precise conclusion.

Various numbers of HSP forms have been reported in many organisms. HSP90s have been reported to contain 2 major cytoplasmic isoforms (Csermely *et al*., 1998); a major inducible form (HSP90a) and a minor constitutive form (HSP90ß). Recent reported has added another isoform (HSP90N) to HSP90 family. This new isoform is associated with cellular transformation (Grammatikakis *et al*., 2002). Functional differences between HSP90 isoforms in cell differentiation have also been reported in various organisms. This includes the regulatory role in muscular cell differentiation of zebrafish (Lele *et al*., 1999), the function of HSP90a in the stability of the cyclin-dependent kinases against thermal stress (Nakai, and Ishikawa, 2001) and the major role in trophoblast differentiation of HSP90ß (Voss *et al*., 2000). HSP90 has been reported to involve cell survival and the various pathways leading to cell death, such as apoptosis or necrosis (Sreedher and Csermely, 2004).

In this study, a single form of the complete cDNA sequences have been verified from each of HSPs. However, the varieties of the DNA sequences from different DNA fragments were observed, indicating the existence of isoforms of these HSPs in *P. monodon*.

4.6 The expression of HSP genes

Reported studies on the induction values from each of these protein families (HSP60, HSP70, HSP90) in aquatic invertebrates were ranged from zero to several hundred fold higher than found in controls (Hofmann 1999; Feder and Hofmann 1999). It has been shown in zebrafish (*Danio rerio*) that the transcriptional regulation of HSP genes, in response to heat shock, was also mediated by Heat shock factors (HSFs) (Rabergh *et al*., 2000). Most of the HSP genes do not contain introns. Therefore the mRNA is rapidly translated into nascent proteins within minutes of stress exposure. In the unstressed cell, there is a constitutive production of HSPs, which are required in various aspects of protein metabolism to maintain cellular homeostasis (Fink and Goto, 1998).

The levels of HSP in control animals were relatively stable in this study. It demonstrated that common stress such as handling during experiment did not elicit the HSP responses. This result was in agreement with a number of reports. It has been demonstrated in rainbow trout that handling stress does not alter levels of hepatic HSP70 (Vijayan *et al*., 1997), and levels of muscle, gill, heart and hepatic HSP60

(Washburn *et al*., 2002). Recently, Zarate and Bradley (2003) showed that common forms of hatchery-related stressors (exposure to anesthesia, formalin, hypoxia, hyperoxia, capture stress, crowding, feed deprivation and cold stress) did not alter levels of gill HSP30, HSP70 and HSP90 in Atlantic salmon (*Salmo salar*).

Heat induction in *P.monodon* was not reflected in the content of HSP60. This result was surprising since this family of stress proteins have been known to play a major role in the heat response of a wide variety of organisms (Bukau and Horwich, 1998; Karlin and Brocchieri, 1998; Kiang and Tsokos, 1998; Feder, 1999; Feder and Hofmann, 1999; Krebs, 1999; Nollen *et al*., 1999). Although heat response and the increase of HSP60 levels were not correlated in induced and uninduced shrimps (controls), a higher level of HSP60 was observed when compared to HSP70 and HSP90 (Fig. 4.1). From the result, it can be assumed that the HSP60 detected in this study was the non-induced form or factors other than HSP60 must be involved in the process.

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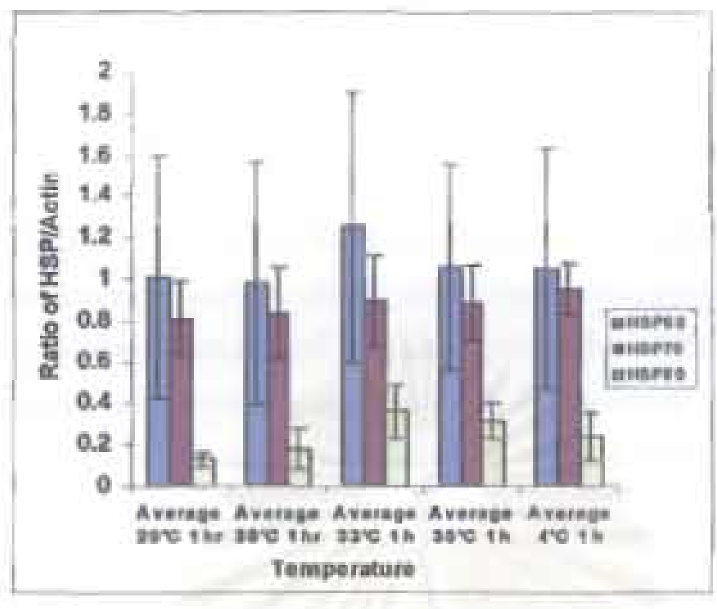

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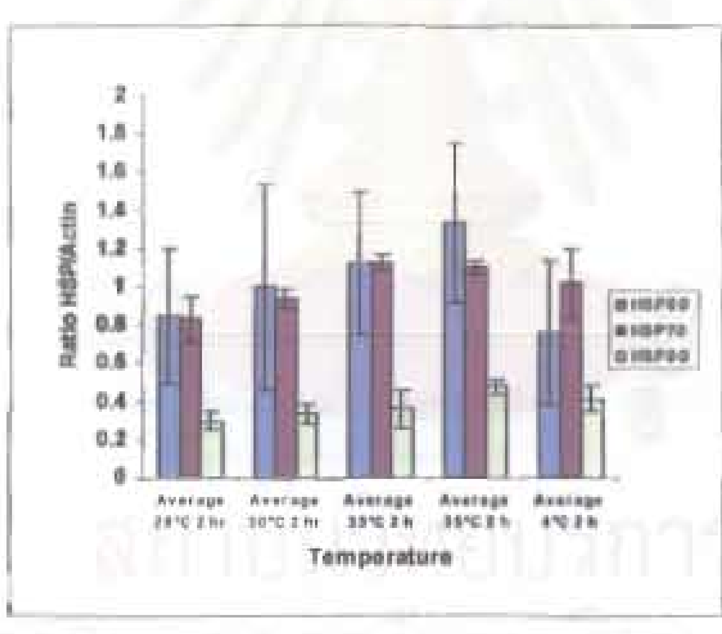

B

Figure 4.1 The comparison of the expression ratio of HSP60, 70 and 90 gene in haemocyte cells of *P. monodon* at various temperatures after treated with thermal stress for 1 hr (A) and 2 hrs (B).

Among the major HSP families, HSP60 is less understood in terms of stress responses, especially in aquatic organisms. Members of this family in eukaryotes are required for normal mitochondrial functions in terms of importing proteins and folding them into their proper functional conformations (Ryan *et al.* 1997). Elevated HSP60 was correlated with decreased survival of amphipods (*Ampelisca abdita*) in sediments contaminated by high levels of polyaromatic hydrocarbons (Werner *et al.* 1998). The alga *Isochrysis galbana* also displayed significantly elevated HSP60 levels on exposure to crude oil fractions and individual hydrocarbons (Wolfe *et al.* 1999). In addition, the results with *Mitilus galloprovincialis* demonstrated that HSP60 might prove to be useful as an additional marker of stress induced by exposure to hydrocarbons (Sanders *et al.* 1992; Sanders and Martin 1993), copper exposures in gill and mantle (Sanders *et al.* 1991; Sanders and Martin 1993), and long-term exposure in contaminated field sites (Sanders and Martin 1993; Lundebye *et al.* 1997).

The regulation of HSP70 gene expression has been reported to occur mainly at the transcriptional level (Fink and Goto, 1998). Studies have demonstrated increased levels of hsp70 in various tissues in fish exposed to pathogens (Forsyth *et al.*, 1997). The later study revealed that rainbow trout infected with a bacterial pathogen (*Vibrio anguilarum*) increased levels of hsp70 in hepatic and head kidney tissues prior to clinical signs of the disease.

The HSP90 gene transcription is known to be less dependent upon the heat stress than other genes of the HSP family (Buchner, 1999) and the uninduced level of expression is quite important in a number of tissues, e.g. in porcine tissues (Huang *et al.*, 1999). However, the elevation of HSP90 level in this study was quite high when compared to other HSPs (Fig. 4.1). Elevated HSP90 expression is found in rat brain, liver, and lung after oral dosing with polycyclic halogenated hydrocarbons and chlorinated or organophosphate pesticides (Bagchi *et al.* 1996). In a previous work of Helgen and Fallon (1990), it revealed that the expression of HSPs occurred only above 41°C in *S. frugiperda* cells, i.e. 13°C over normal growing conditions. The result of Northern blot analysis also confirmed that the heat-inducibility of the *S. frugiperda* HSP90 gene required a temperature at least 14°C above normal growing conditions. A similar observation was made for the Lepidoptera *Manduca sexta*

(Fittinghoff and Riddiford, 1990) or the Orthoptera *Locusta migratoria* (Whyard *et al.*, 1986). The response of HSP90 in this study was induced by lower temperature above normal condition. This maybe because of the ecology of these insects which were in the warm climatic conditions where they develop or aquatic or marine invertebrates are more sensitive to the temperature than terrestrial invertebrates.

There were a few studies related to the physiological and cellular stress responses *in vivo*. In mammals, it was known that HSPs were involved in the immune response. HSPs have been known to involve in the immuno-suppression in a number of fish. For adult fish, it was found in Pacific salmon, *Oncorhynchus spp.* that all larvae died after spawning because the lack of ability to clear cortisol from the circulation after stress (Stein-Behrens and Saplosky, 1992). The investigation on the consequences of a 15 min disturbance on immune parameters (the number of circulating haemoccytes, reactive oxygen species production, migratory and phagocytic activities) in Oysters, *Crassostrea gigas* showed that all immune functions were significantly downregulated during stress (Lacoste *et al.*, 2002). A transient period of immunostimulation was observed 30-240 min after the end of disturbance. These results suggest that stress can exert a profound influence on invertebrate immune functions.

HSPs have proven useful as part of a suite of biochemical markers of xenobiotic exposure in molluscs. HSP inductions are markers of multiple stress exposures, whereas specific proteins are generally responsive to a limited group of xenobiotic exposures. HSPs therefore cannot indicate exposure to any specific stressor without direct observation under carefully controlled conditions. When combined with additional physiological observations, HSPs can, however, be indicative of the severity of the stress exposure. It is also important to note that some animals may not show a heat shock response. Hofmann *et al.* (2000) showed that hsp70 was not induced by temperature stress in the Antarctic fish *Trematomus bernacchi*. Thus, generalizations about the HSP response cannot be made unequivocally, and more knowledge is needed in order to use a specific HSP family as an indicator of stress.

Cells treated with a non-lethal heat stress develop transient resistance to a subsequent lethal heat stress, an adaptive phenomenon termed thermotolerance. Studies on hsp70 in larvae and adults of three species of *Drosophila* from different thermal environments (Krebs 1999), and on marine snails from different locations in the intertidal zone (Tomanek and Somero 1999) confirmed the possibility of themotolerance induction, while also uncovering the difficulty of establishing causal connections between levels of stress proteins, thermotolerance and ecological setting. Induction of cytoprotective HSP70 is associated with increased survival following heat stress. However, the role of HSP70 in the development of thermotolerance is unclear. It is commonly assumed that the synthesis of stress proteins is intended for the cell's survival and adaptation to adverse conditions. The existence of induced tolerance would seem to be supported by the short-term treatment with mild stress which results in an increase in tolerance against a subsequent, normally lethal, dose of the same stress. The most direct interpretation of these results is that the presence of stress proteins, previously induced by mild stress, increases cell tolerance. However, the relationship between the induction of individual stress proteins and the acquisition of stress tolerance is not always evident.

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CHAPTER V

CONCLUSION

1. Haemocytes maintained in M199 medium revealed high survival rate (>80%) for more than 4 days while complete mortality of the haemocytes maintained in Grace's insect and TC100 media was detected after 1 day. Despite the higher activity of haemocytes maintained in TC100 and Grace's insect media, these 2 media showed the disadvantage in short time maintenance of the *P. monodon* haemocytes. Therefore, it was more appropriate to use M199 for maintaining *P. monodon* haemocytes in *in vitro* experiment.

2. The thermal shock at 4, 30, 33, or 35°C for 2 h provided no lethal effect to the *P. monodon* haemocytes maintained in M199 culture media. There has significant difference between the protein concentrations of haemocyte extracts ($P<0.05$)..

3. The determination of thermal response in the haemocytes by distinguishing the protein accumulation pattern in the protein extracts of haemocytes was not achieved because the protein profiles detected by SDS-PAGE did not provide the reliable results. The result of *in vitro* translation using ^{35}S methionine also revealed no detectable protein pattern.

4. Western blot analysis using cross reactivity of the monoclonal antibody raised by HSPs from different species revealed positive result on the detection of HSP90. However, the sensitivity of the antibody was very low. Therefore, it was not possible to quantify the level of HSP90 in different treatments. On the other hand, the presences of HSP60 and HSP70 were not detectable by this method.

5. Ten differential expressed DNA fragments were obtained from RAP-PCR conducted on heat induced Haemocytes. Nine sequences were identified as protein of unknown genes and 1 DNA fragment was identified as vigilin, a high density lipoprotein-binding protein.

6. EST library of the haemocytes from heat induced *P. monodon* was constructed. Of 1090 clones obtained from the randomly selected sequence analysis, 63% were identified as known genes and 12.1% was the genes related to defense and homeostasis. A number of HSPs were also identified.

7. Complete sequences of HSP60, HSP70 and HSP90 genes were obtained. The structure analyses of these HSP genes confirmed their identities. Some variant forms of these HSP genes were also observed.

8. HSP60 was composed of 1731 bp ORF encoding a putative polypeptide of 576 amino acids with a predicted size of 61,129.20 Da and calculated pI of 6.03. Deduced amino acid sequences shared significant identities (69%) with mitochondrial Hsp60s from many animals.

9. HSP70 was composed of 1959 bp ORF encoding a putative polypeptide of 652 amino acids with a predicted size of 71,522.85 Da and calculated pI of 5.34. Deduced amino acid sequences shared significant identities (94%) with Hsp70s from *P. vanamei*.

10. HSP90 was composed of 2157 bp ORF encoding a putative polypeptide of 718 amino acids with a predicted size of 83,244.30 Da and calculated pI of 5.04. Deduced amino acid sequences shared significant identities (67%) with Hsp90 from *Salmo salar*

11. *In vitro* detection of HSP gene expression revealed that the expression of HSp70 and HSP90 genes were induced by heat shock after 2 hrs of post exposure while the expression of HSP60 genes showed no correlation with the heat shock.

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APPENDICES

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Appendix A

Reagents

1. 10% (w/v) Ammonium persulfate

Ammonium persulfate (sigma)	1.0	g

Dissolve in 10 ml of dH_2O.

2. Resolving gel buffer : 3 M Tris-HCl pH 8.8

Tris (Sigma)	36.3	g

Dissolve in 40 ml of dH_2O, adjust with 1 M HCl to pH 8.8 and adjust to 100 ml final volume with dH_2O.

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.

4. 30.8% (w/v) Acrylamide-bisacrylamide

Acrylamide (Sigma)	30.0	g
Bis-acrylamide (Sigma)	0.8	g

5. 10% (w/v) Sodium dodecylsulphate

Sodium dodecylsulphate (Sigma)	10.0	g

Dissolve in 100 ml of dH_2O.

6. TEMED (N, N, N', N'-tetramethyl ethlenediamine)

This reagent is commercial available.

7. 10% Resolving gel

Acrylamide-bisacrylamide (30:0.8)	3.33	ml
dH_2O	5.245	ml
Resolving gel buffer	2.5	ml
10% SDS	100	µl
10% Ammonium persulphate	75	µl
TEMED	5	µl

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8. 3.85% Stacking gel

Acrylamide-bisacrylamide (30:0.8) 0.50 ml
dH_2O 2.43 ml
Stacking gel buffer 1.0 ml
10% SDS 40 µl
10% Ammonium persulphate 30 µl
TEMED 3 µl

9. 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.

10. 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. Add 1 ml of 2-mercaptoethanol (2-ME) to 9 ml of 4X sample buffer for reducing condition.

11. Staining solution

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

12. Destaining solution

Glacial acetic acid 100 ml
Methanol 300 ml
dH_2O 600 ml

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13. 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

Dissolved and adjusted to 400 ml with dH_2O, followed by the addition of 100 ml of methanol

14. Blocking buffer (1% BSA)

BSA	1	g

Dissolve and adjust to 100 ml with PBS

15. 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.

16 Substrate

3,3' Diaminobenzamidine (DAB)	0.054	g
1 M Tris-HCl pH 7.6	2.5	ml
3% H_2O_2	0.5	ml
Added dH_2O to	50	ml

17. 0.1% DEPC- dH_2O

Diethyl pyrocarbonate 97%	1.0	ml

Add dH_2O to 1000 ml and incubated overnight at 37^oC then autoclav

18. M Sodium acetate pH 4.0

Sodium acetate	27.216	g
dH_2O	90	ml

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

19. Luria-Bertani medium (LB broth)

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 adjust pH to

pH 7.0 with 5 N NaOH, and then autoclave.

20. LB agar (per liter)

NaCl	10.0	g
Trytone	10	g
Yeast extract	5	g
Agar	20	g

Add dH_2O to a final volume of 1 liter. Adjust to pH 7.0 with

5 N NaOH and autoclave. Pour into petri dishes (~25ml/100-mm plate)

21. LB Ampicillin agar (per liter)

prepare 1 liter of LB agar. Autoclave and cool to 55°C

Add 50 ml of filter-sterilized ampicillin

Pour into petri dishes (~25ml/100-mm plate)

22. 1X TAE buffer

40 mM Tris-acetate

1 mM EDTA

23. 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.

24. SOB medium (per liter)

Bacto-tryptone	20	g
Yeast extract	5	g
NaCl	0.5	g

25. SM buffer (per liter)

NaCl	5.8	g
$MgSO_4.7H_2O$	2	g
1 M Tris-Cl pH 7.5	50	ml
2% gelatin	5	ml

26. Ampicillin

Stock solution 25 mg/ml of the sodium salt of ampicillin in dH_2O.

Sterilize by filtration and store in aliquots at –20°C

27. 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.

28. 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.

29. 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.

30. 3 M Sodium acetate (pH 5.2)

Sodium acetate.$3H_2O$	408.1	g
Dissolve with dH_2O	800	ml

Adjust pH to 5.2 with glacial acetic acid

Adjust volume to 1000 ml with dH_2O

31. Ethidium bromide 10 mg/ml

Ethidium bromide	1	g
Add dH_2O	100	ml

Stir on a magnetic for several hours to ensure that the dye has dissolved. Wrap the container in aluminium foil or transfer to a dark bottle and store at 4°C

32. 5x Tris-Borate (5XTBE)

Tris base	54	g
Boric acid	27.5	g
EDTA 0.5 M pH 8.0	20	ml
Dissolve with dH_2O	1000	ml

33. Gel loading buffer Type II

10X buffer

bromophenol blue	0.25%
Xylene cyanol	0.25%
Ficoll (type 400) in dH_2O	25%

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34. IPTG (20% w/v, 0.8 M)

IPTG	2	g
Dissolving in dH_2O	8	ml
Adjust volume with dH_2O to	10	ml

Sterile by passing it through a 0.22 μM disposable filter.

Aliquots solution and store at –20°C

35. X-gal solution (2% w/v)

X-gal is 5-bromo-4-chloro-3-indolyl-B-d-galactoside.

Make a stock solution by dissolved X-gal in dimethylformamide at a concentration of 20 mg/ml solution. Wrap the tube containing the solution in aluminum foil to prevent damage by light and store at –20°C.

36. NZY agar

NZY agar	22	g
Dissolve in dH_2O	800	ml
Adjust pH to 7.5 with 5 N NaOH		
Adjust volume with dH_2O to	1000	ml

37. 20x SSC

NaCl	175	g
Sodium citrate	88	g
Dissolving in dH_2O	800	ml
Adjust pH to 7.0 with NaOH		
Adjust volume with dH_2O to	1000	ml

38. Denaturing solution

1.5 M NaCl

0.5 M NaOH

39. Nuetralsing solution

1.5 M NaCl

0.5 M Tris-Cl pH 7.2

0.001 M EDTA

40. Maleic acid buffer (0.1 M maleic acid, 0.15 M NaCl)

Maleic acid	11.6	g
5 M NaCl	30	ml
Dissolving in dH_2O	800	ml

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Adjust pH to 7.5 with NaOH (solid)

41. 10x Blocking solution (10% w/v in maleic buffer)

Blocking reagent	10	g
Dissolve in maleic buffer	50	ml
Heat in a microwave and adjust volume to	100	ml

Autoclave and store at 4°C . The solution remains opaque.

42. Washing buffer

Maleic buffer	1000	ml
Tween-20	3	ml

43. Detection buffer (0.1M Tris-HCl pH 9.5, 0.1M NaCl, 50 mM $MgCl_2$)

1 M Tris-HCl pH 9.5	50	ml
5 M NaCl	10	ml
0.5 M $MgCl_2$	50	ml
Adjust volume with dH_2O to	500	ml

44. Color substrate solution for anti DIG

NBT solution	45	µl
X-phosphate solution	35	µl
Detection buffer	10	ml

45. Hank solution

50 mM KCl

3 mM KH_2PO_4

1.39 M NaCl

80 mM $Na_2H_2PO_4$

56 mM Glucose

46. Hank salt solution (H0) pH 7.2

Hank solution	100	ml
Hepes	2.6	g
NaCl	190 mM	
Adjust volume with dH_2O to	1000	ml

Autoclave at 121°C for 15 min.

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47. Hank salt solution (H1) pH 7.2

Hank solution 100 ml

Hepes 2.6 g

NaCl 190 mM

$CaCl_2$ 12 mM

$MgCl_2$ 26 mM

Adjust volume with dH_2O to 1000 ml

48. Culture medium M199

culture medium M199 1.1 g

Fetal bovine serum 10 ml

Penicillin (500u/µl) 20 µl

Streptomycin (500µg/µl) 20 µl

Dissolve with dH_2O 90 ml

Adjust pH to 7.6 with $NaHCO_3$ and adjust volume to 100 ml

Sterile by passing it through a 0.22 µM store at 4°C

49. Culture medium TC100

Culture medium TC100 2.04 g

Fetal bovine serum 10 ml

Penicillin (500u/µl) 20 µl

Streptomycin (500µg/µl) 20 µl

Dissolve with dH_2O 90 ml

Adjust pH to 7.6 with $NaHCO_3$ and adjust volume to 100 ml

Sterile by passing it through a 0.22 µM store at 4°C

50. Culture medium Grace's insect medium

Grace's insect medium 4.63 g

Fetal bovine serum 10 ml

Penicillin (500u/µl) 20 µl

Streptomycin (500µg/µl) 20 µl

Dissolve with dH_2O 90 ml

Adjust pH to 6.5 with $NaHCO_3$ and adjust volume to 100 ml

Sterile by passing it through a 0.22 µM store at 4°C

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Appendix B

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.

3URWHLQ XJ PO	$

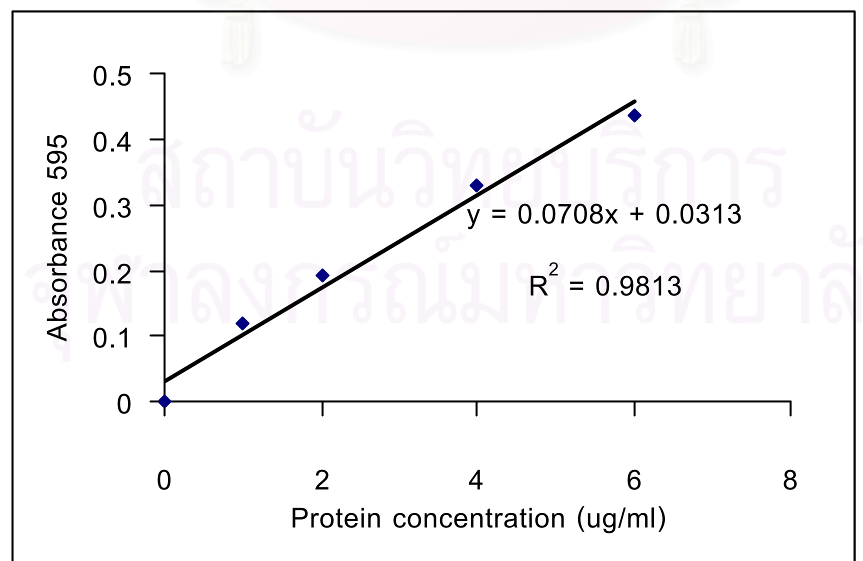
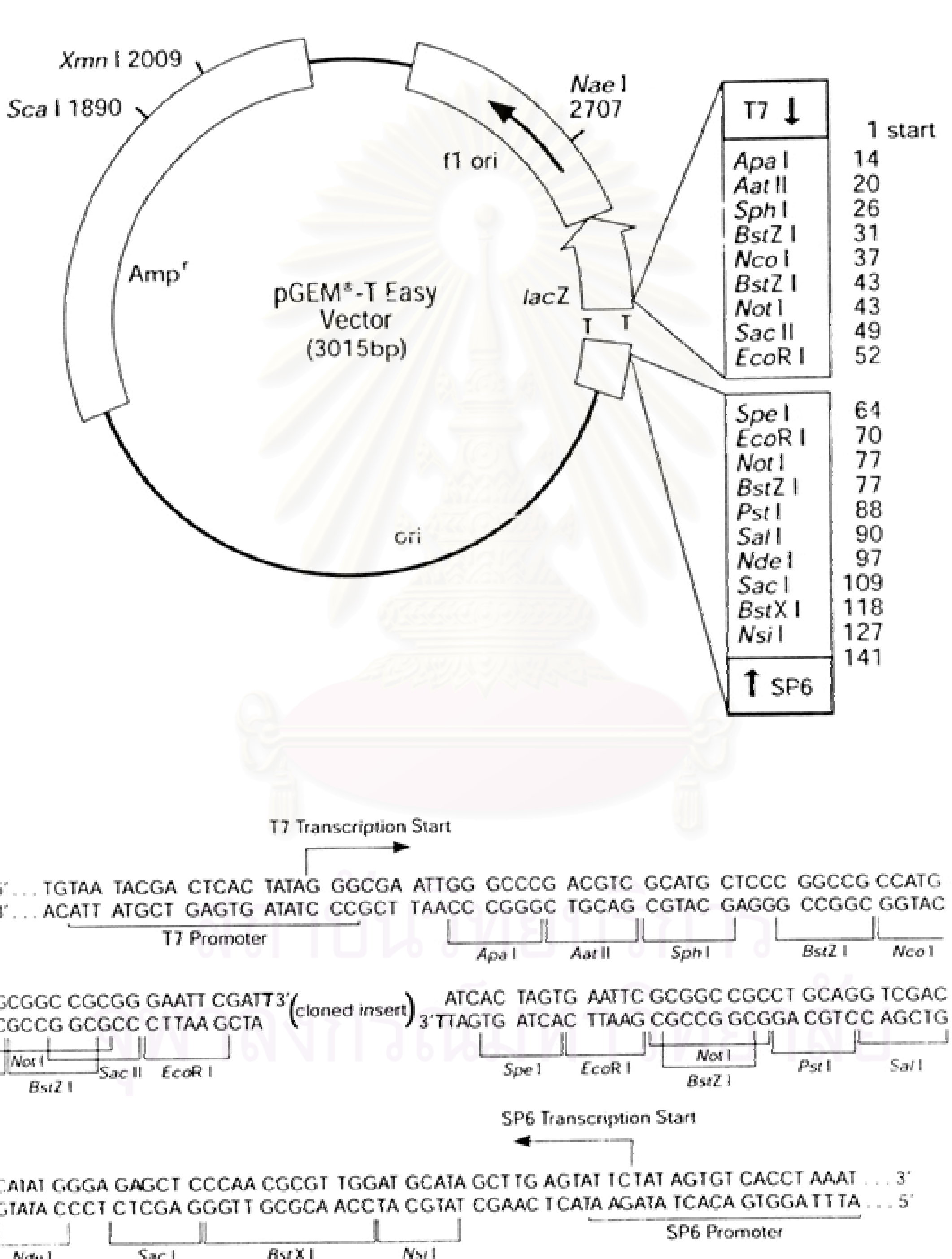


Figure B-1 Standard protein curve for determination protein concentration.

Appendix C

Retriction mapping of pGEMÒ T-easy Vector

pGEM® T-easy Vector



APPENDIX D

Nucleotide comparison of HSP 60 *P. monodon* using BLASTN

Sequences producing significant alignments:	Score (bits)	E Value
gi|5912573|emb|AJ249625.1|PLI249625 Paracentrotus lividus m...	143	2e-30
gi|33636452|gb|BT010206.1| Drosophila melanogaster SD06594 ...	141	6e-30
gi|21328459|gb|AC104510.6| Drosophila melanogaster X BAC RP...	141	6e-30
gi|24641192|ref|NM_167266.1| Drosophila melanogaster CG1210...	141	6e-30
gi|24641190|ref|NM_078560.2| Drosophila melanogaster CG1210...	141	6e-30
gi|22832075|gb|AE003485.3| Drosophila melanogaster chromoso...	141	6e-30
gi|3757827|emb|X99341.1|DMHSP60 D.melanogaster mRNA for hea...	141	6e-30
gi|28611161|gb|BC047350.1| Homo sapiens heat shock 60kDa pr...	107	8e-20
gi|49522864|gb|BC073746.1| Homo sapiens heat shock 60kDa pr...	107	8e-20
gi|45595680|gb|BC067082.1| Homo sapiens heat shock 60kDa pr...	107	8e-20
gi|12804340|gb|BC003030.1| Homo sapiens heat shock 60kDa pr...	107	8e-20
gi|6996445|emb|AJ250915.1|HSA250915 Homo sapiens p10 gene f...	107	8e-20
gi|190126|gb|M22382.1|HUMPMMPP1 Human mitochondrial matrix ...	107	8e-20
gi|41399284|ref|NM_199440.1| Homo sapiens heat shock 60kDa ...	100	2e-17
gi|41399283|ref|NM_002156.4| Homo sapiens heat shock 60kDa ...	100	2e-17
gi|38197215|gb|BC002676.2| Homo sapiens heat shock 60kDa pr...	100	2e-17
gi|50500495|emb|CR619688.1| full-length cDNA clone CS0DH002...	100	2e-17
gi|10047985|gb|AC020550.4|AC020550 Homo sapiens BAC clone R...	100	2e-17
gi|184411|gb|M34664.1|HUMHSP60A Human chaperonin (HSP60) mR..	100	2e-17
gi|51451|emb|X53584.1|MMHSP60A Mouse mRNA for HSP60 protein...	94	1e-15
gi|2738076|gb|U87959.1|CVU87959 Culicoides variipennis heat...	88	8e-14
gi|191148|gb|M22383.1|CRUP1P Chinese hamster P1 protein mRN...	88	8e-14
gi|46402512|ref|NM_181330.3| Danio rerio heat shock 60kD pr...	86	3e-13
gi|21805769|gb|AY112665.1| Danio rerio chaperonin Cpn60 (cp...	86	3e-13
gi|46329691|gb|BC068415.1| Danio rerio heat shock 60kD prot...	86	3e-13
gi|27881984|gb|BC044557.1| Danio rerio heat shock 60kD prot...	86	3e-13
gi|38091330|ref|XM_354605.1| Mus musculus similar to 60 kDa...	84	1e-12
gi|34875805|ref|XM_212759.2| Rattus norvegicus hypothetical...	84	1e-12
gi|31981678|ref|NM_010477.2| Mus musculus heat shock protei...	84	1e-12
gi|11560023|ref|NM_022229.1| Rattus norvegicus heat shock p...	84	1e-12
gi|16741092|gb|BC016400.1| Mus musculus heat shock protein ...	84	1e-12
gi|56382|emb|X54793.1|RNHSP60L Rat liver mRNA for heat shoc...	84	1e-12
gi|56380|emb|X53585.1|RNHSP60B Rat mRNA for HSP60 protein (...	84	1e-12
gi|51454|emb|X55023.1|MMHSP65R Mouse cDNA for heat shock pr...	84	1e-12
gi|21727379|emb|AL669943.9| Mouse DNA sequence from clone R...	84	1e-12
gi|26353953|dbj|AK088844.1| Mus musculus 2 days neonate thy...	84	1e-12
gi|51463945|ref|XM_047355.8| PREDICTED: Homo sapiens KIAA17...	82	5e-12
gi|21166209|gb|AC105749.2| Homo sapiens chromosome 3 clone ...	82	5e-12
gi|18958737|gb|AC097360.2| Homo sapiens chromosome 3 clone ...	82	5e-12
gi|37623946|gb|AF380943.2| Homo sapiens short heat shock pr...	76	3e-10
gi|29569255|gb|AC138940.3| Homo sapiens chromosome 5 clone ...	76	3e-10
gi|18854958|gb|AC091873.2| Homo sapiens chromosome 5 clone ...	76	3e-10
gi|40647590|gb|AY500892.1| Anemonia viridis mitochondrial 6...	76	3e-10
gi|1778211|gb|U68562.1|RNU68562 Rattus norvegicus chaperoni...	76	3e-10

Nucleotide comparison of HSP 70 *P. monodon* using BLASTN

Sequences producing significant alignments:	Score (bits)	E Value
gi|33319728|gb|AF474375.1| Penaeus monodon heat shock prote...	3844	0.0
gi|48766850|gb|AY645906.1| Litopenaeus vannamei heat shock ...	3479	0.0
gi|7363335|gb|AF182195.1|AF182195 Guancha lacunosa heat sho...	712	0.0
gi|28571720|ref|NM_176503.1| Drosophila melanogaster CG4264...	704	0.0
gi|28571718|ref|NM_176502.1| Drosophila melanogaster CG4264...	704	0.0
gi|24647037|ref|NM_169627.1| Drosophila melanogaster CG4264...	704	0.0
gi|24647035|ref|NM_169626.1| Drosophila melanogaster CG4264...	704	0.0
gi|24647033|ref|NM_169625.1| Drosophila melanogaster CG4264...	704	0.0
gi|28571722|ref|NM_079632.4| Drosophila melanogaster CG4264...	704	0.0
gi|15451478|gb|AC009904.7| Drosophila melanogaster, chromos...	704	0.0
gi|23171318|gb|AE003708.3| Drosophila melanogaster chromoso...	704	0.0
gi|13096034|gb|AC007648.6|AC007648 Drosophila melanogaster,...	704	0.0
gi|39979268|dbj|AB006814.1| Paralichthys olivaceus mRNA for...	700	0.0
gi|157660|gb|L01500.1|DROHSC4A Drosophila melanogaster heat...	696	0.0
gi|19527632|gb|AY084193.1| Drosophila melanogaster RH04426 ...	688	0.0
gi|3513539|gb|AF053059.1|AF053059 Paralichthys olivaceus he...	676	0.0
gi|33598989|gb|AY219845.1| Cyprinus carpio constitutive hea...	672	0.0
gi|157663|gb|M36114.1|DROHSC4A2 D.melanogaster heat shock c...	664	0.0
gi|28569549|gb|AY195744.1| Carassius auratus gibelio heat s...	654	0.0
gi|7715510|gb|AF252689.1|AF252689 Drosophila simulans strai...	634	e-178
gi|7715506|gb|AF252687.1|AF252687 Drosophila simulans strai...	634	e-178
gi|32813264|dbj|AB114672.1| Canis familiaris hsp70 mRNA for...	617	e-173
gi|32813270|dbj|AB114675.1| Canis familiaris hsp70 mRNA for...	617	e-173
gi|32813268|dbj|AB114674.1| Canis familiaris hsp70 mRNA for...	617	e-173
gi|32813266|dbj|AB114673.1| Canis familiaris hsp70 mRNA for...	617	e-173
gi|6457365|gb|AF194819.1|AF194819 Manduca sexta heat shock ...	613	e-172
gi|7716925|gb|AF255317.1|AF255317 Drosophila yakuba heat sh...	611	e-171
gi|17061838|dbj|AB062115.1| Xiphophorus maculatus HSC70 mRN...	603	e-169
gi|7715518|gb|AF252693.1|AF252693 Drosophila simulans strai...	595	e-166
gi|7715514|gb|AF252691.1|AF252691 Drosophila simulans strai...	595	e-166
gi|7715512|gb|AF252690.1|AF252690 Drosophila simulans strai...	595	e-166
gi|7715508|gb|AF252688.1|AF252688 Drosophila simulans strai...	595	e-166
gi|7715516|gb|AF252692.1|AF252692 Drosophila simulans strai...	587	e-164
gi|42542844|gb|BC066491.1| Danio rerio heat shock 70kDa pro...	577	e-161
gi|38649355|gb|BC063228.1| Danio rerio heat shock 70kDa pro...	577	e-161
gi|32967447|gb|AY226078.1| Monosiga brevicollis type Mb_C 7...	561	e-156
gi|51233018|emb|CR734665.1| Tetraodon nigroviridis full-len...	555	e-154
gi|51152824|emb|CR656379.1| Tetraodon nigroviridis full-len...	555	e-154
gi|51226363|emb|CR728102.1| Tetraodon nigroviridis full-len...	547	e-152
gi|51147696|emb|CR651251.1| Tetraodon nigroviridis full-len...	547	e-152
gi|51145266|emb|CR648821.1| Tetraodon nigroviridis full-len...	547	e-152
gi|37682086|gb|AY422994.1| Danio rerio heat shock 70kDa pro...	545	e-151
gi|51188966|emb|CR691059.1| Tetraodon nigroviridis full-len...	541	e-150
gi|51201855|emb|CR703946.1| Tetraodon nigroviridis full-len...	539	e-150
gi|51150554|emb|CR654109.1| Tetraodon nigroviridis full-len...	539	e-150
gi|51179464|emb|CR681557.1| Tetraodon nigroviridis full-len...	537	e-149
gi|29468049|gb|AY150182.1| Balanus amphitrite 70kDa heat sh...	527	e-146
gi|51179952|emb|CR682045.1| Tetraodon nigroviridis full-len...	527	e-146
gi|1408566|gb|L77146.1|ZEFHSC7R Danio rerio heat shock cogn...	525	e-145
gi|51161392|emb|CR664947.1| Tetraodon nigroviridis full-len...	519	e-144
gi|51179854|emb|CR681947.1| Tetraodon nigroviridis full-len...	515	e-142
gi|51153314|emb|CR656869.1| Tetraodon nigroviridis full-len...	515	e-142

Nucleotide comparison of HSP 90 *P. monodon* using BLASTN

Sequences producing significant alignments:	Score (bits)	E Value
gi|42556385|gb|AY528900.1| Chiromantes haematocheir hsp-90 ...	1372	0.0
gi|31199702|ref|XM_308799.1| Anopheles gambiae ENSANGP00000...	389	e-104
gi|31199704|ref|XM_308800.1| Anopheles gambiae ENSANGP00000...	381	e-102
gi|31199698|ref|XM_308797.1| Anopheles gambiae ENSANGP00000...	375	e-100
gi|27564513|emb|BX015293.1|CNS08JYP Single read from an ext...	375	e-100
gi|2062376|gb|U75687.1|DAU75687 Drosophila auraria heat sho...	367	7e-98
gi|27626759|emb|BX053478.1|CNS09DFE Single read from an ext...	309	1e-80
gi|9124|emb|X03811.1|DSHSP82 Drosophila simulans gene fragm...	287	5e-74
gi|27558760|emb|BX009540.1|CNS08FIW Single read from an ext...	283	8e-73
gi|21483233|gb|AY122080.1| Drosophila melanogaster AT20544 ...	272	3e-69
gi|27558759|emb|BX009539.1|CNS08FIV Single read from an ext...	272	3e-69
gi|1832135|gb|U57471.1|DMU57471 Drosophila melanogaster hea...	272	3e-69
gi|1832133|gb|U57470.1|DMU57470 Drosophila melanogaster hea...	272	3e-69
gi|1832131|gb|U57469.1|DMU57469 Drosophila melanogaster hea...	272	3e-69
gi|1832129|gb|U57468.1|DMU57468 Drosophila melanogaster hea...	272	3e-69
gi|1832127|gb|U57467.1|DMU57467 Drosophila melanogaster hea...	272	3e-69
gi|1832119|gb|U57463.1|DMU57463 Drosophila melanogaster hea...	272	3e-69
gi|1832117|gb|U57462.1|DMU57462 Drosophila melanogaster hea...	272	3e-69
gi|1832113|gb|U57460.1|DMU57460 Drosophila melanogaster hea...	272	3e-69
gi|1832111|gb|U57459.1|DMU57459 Drosophila melanogaster hea...	272	3e-69
gi|8125|emb|X03810.1|DMHSP82 Drosophila melanogaster gene f...	272	3e-69
gi|21397249|gb|AC097725.2| Drosophila melanogaster 3L BAC R...	268	5e-68
gi|24656565|ref|NM_079175.2| Drosophila melanogaster CG1242...	268	5e-68
gi|12005808|gb|AF254880.1|AF254880 Spodoptera frugiperda 90...	268	5e-68
gi|23092855|gb|AE003477.3| Drosophila melanogaster chromoso...	268	5e-68
gi|51142784|emb|CR646339.1| Tetraodon nigroviridis full-len...	266	2e-67
gi|1832139|gb|U57473.1|DMU57473 Drosophila melanogaster hea...	264	8e-67
gi|1832137|gb|U57472.1|DMU57472 Drosophila melanogaster hea...	264	8e-67
gi|1832125|gb|U57466.1|DMU57466 Drosophila melanogaster hea...	264	8e-67
gi|1832123|gb|U57465.1|DMU57465 Drosophila melanogaster hea...	264	8e-67
gi|1832121|gb|U57464.1|DMU57464 Drosophila melanogaster hea...	264	8e-67
gi|1832115|gb|U57461.1|DMU57461 Drosophila melanogaster hea...	264	8e-67
gi|8101|emb|X00065.1|DMHS83 Drosophila melanogaster 5'end o...	264	8e-67
gi|1008866|gb|L47285.1|MSQHSP82G Anopheles albimanus heat s...	262	3e-66
gi|51210764|emb|CR712547.1| Tetraodon nigroviridis full-len...	242	3e-60
gi|2352614|gb|AF006561.1| Drosophila miranda strain miranda...	234	7e-58
gi|2352612|gb|AF006560.1| Drosophila miranda strain miranda...	234	7e-58
gi|37696947|gb|AY394438.1| Sphoeroides annulatus Hsp90-like...	232	3e-57
gi|29826098|gb|AF006562.2| Drosophila miranda strain mirand...	220	1e-53
gi|29826092|gb|AF006551.2| Drosophila persimilis strain per...	218	4e-53
gi|29826085|gb|AF006543.2| Drosophila pseudoobscura bogotan...	218	4e-53
gi|29826084|gb|AF006542.2| Drosophila pseudoobscura bogotan...	218	4e-53
gi|29826083|gb|AF006541.2| Drosophila pseudoobscura bogotan...	218	4e-53
gi|29826082|gb|AF006539.2| Drosophila pseudoobscura strain ...	218	4e-53
gi|2352596|gb|AF006552.1| Drosophila persimilis strain pers...	218	4e-53
gi|2352590|gb|AF006549.1| Drosophila persimilis strain pers...	218	4e-53
gi|2352566|gb|AF006536.1| Drosophila pseudoobscura strain p...	218	4e-53
gi|2352562|gb|AF006534.1| Drosophila pseudoobscura strain p...	218	4e-53
gi|2352558|gb|AF006532.1| Drosophila pseudoobscura strain p...	218	4e-53

APPENDIX E

Putative amino acid comparison of full length HSP60 *P. monodon* using BLASTX

<table>
<tr><th>Sequences producing significant alignments:</th><th>Score (bits)</th><th>E Value</th></tr>
<tr><td>gi|31231072|ref|XP_318461.1| ENSANGP00000014839 [Anopheles ...</td><td>820</td><td>0.0</td></tr>
<tr><td>gi|24641193|ref|NP_727489.1| CG12101-PB [Drosophila melanog...</td><td>801</td><td>0.0</td></tr>
<tr><td>gi|33636453|gb|AAQ23524.1| SD06594p [Drosophila melanogaster]</td><td>801</td><td>0.0</td></tr>
<tr><td>gi|2738077|gb|AAB94640.1| heat shock protein 60 [Culicoides...</td><td>790</td><td>0.0</td></tr>
<tr><td>gi|3757828|emb|CAA67720.1| heat shock protein 60 [Drosophil...</td><td>784</td><td>0.0</td></tr>
<tr><td>gi|5912574|emb|CAB56199.1| Chaperonin [Paracentrotus lividus]</td><td>781</td><td>0.0</td></tr>
<tr><td>gi|47938737|gb|AAH72058.1| Hspd1 protein [Xenopus laevis]</td><td>771</td><td>0.0</td></tr>
<tr><td>gi|90207|pir||A34173 mitochondrial protein P1 precursor - C...</td><td>765</td><td>0.0</td></tr>
<tr><td>gi|41399285|ref|NP_955472.1| chaperonin; mitochondrial matr...</td><td>763</td><td>0.0</td></tr>
<tr><td>gi|31044489|ref|NP_851847.1| heat shock 60 kD protein 1 [Da...</td><td>762</td><td>0.0</td></tr>
<tr><td>gi|16741093|gb|AAH16400.1| Heat shock protein 1 (chaperonin...</td><td>762</td><td>0.0</td></tr>
<tr><td>gi|31981679|ref|NP_034607.2| heat shock protein 1 (chaperon...</td><td>762</td><td>0.0</td></tr>
<tr><td>gi|72957|pir||HHMS60 chaperonin groEL precursor - mouse >gi...</td><td>761</td><td>0.0</td></tr>
<tr><td>gi|306890|gb|AAA36022.1| chaperonin (HSP60)</td><td>761</td><td>0.0</td></tr>
<tr><td>gi|6066606|emb|CAB58441.1| Hsp60 protein [Myzus persicae]</td><td>761</td><td>0.0</td></tr>
<tr><td>gi|11560024|ref|NP_071565.1| heat shock protein 60 (liver);...</td><td>760</td><td>0.0</td></tr>
<tr><td>gi|247242|gb|AAB21806.1| heat shock protein hsp60, hsp60=ch...</td><td>760</td><td>0.0</td></tr>
<tr><td>gi|1778213|gb|AAC53362.1| chaperonin 60 [Rattus norvegicus]</td><td>759</td><td>0.0</td></tr>
<tr><td>gi|34875806|ref|XP_212759.2| hypothetical protein XP_212759...</td><td>759</td><td>0.0</td></tr>
<tr><td>gi|51452|emb|CAA37653.1| unnamed protein product [Mus muscu...</td><td>757</td><td>0.0</td></tr>
<tr><td>gi|1334284|emb|CAA37654.1| unnamed protein product [Rattus ...</td><td>752</td><td>0.0</td></tr>
<tr><td>gi|40647591|gb|AAR88509.1| mitochondrial 60 kDa heat shock ...</td><td>745</td><td>0.0</td></tr>
<tr><td>gi|21105712|gb|AAM34755.1| heat shock protein 60 [Trichinel...</td><td>738</td><td>0.0</td></tr>
<tr><td>gi|45550936|ref|NP_723105.2| CG7235-PB [Drosophila melanoga...</td><td>737</td><td>0.0</td></tr>
<tr><td>gi|21064097|gb|AAM29278.1| AT16985p [Drosophila melanogaster]</td><td>737</td><td>0.0</td></tr>
<tr><td>gi|27735378|gb|AAH41192.1| Hspd1 protein [Xenopus laevis]</td><td>736</td><td>0.0</td></tr>
<tr><td>gi|3928008|emb|CAA10230.1| heat shock protein 60 (HSP60) [P...</td><td>729</td><td>0.0</td></tr>
<tr><td>gi|39584025|emb|CAE66431.1| Hypothetical protein CBG11701 [...</td><td>728</td><td>0.0</td></tr>
<tr><td>gi|17555558|ref|NP_497429.1| heat shock protein (60.1 kD) ...</td><td>718</td><td>0.0</td></tr>
<tr><td>gi|4680247|gb|AAD27589.1| chaperonine protein HSP60 [Onchoc...</td><td>716</td><td>0.0</td></tr>
<tr><td>gi|533167|gb|AAA28077.1| homologous to chaperonin protein</td><td>706</td><td>0.0</td></tr>
<tr><td>gi|21634531|gb|AAM69406.1| heat shock protein HSP60 [Schist...</td><td>706</td><td>0.0</td></tr>
<tr><td>gi|116253|sp|P25420|CH63_HELVI 63 kDa chaperonin, mitochond...</td><td>662</td><td>0.0</td></tr>
<tr><td>gi|34877409|ref|XP_212745.2| similar to heat shock protein ...</td><td>647</td><td>0.0</td></tr>
<tr><td>gi|34878823|ref|XP_229566.2| similar to heat shock protein ...</td><td>645</td><td>0.0</td></tr>
<tr><td>gi|51463946|ref|XP_047355.4| PREDICTED: KIAA1765 protein [H...</td><td>637</td><td>0.0</td></tr>
<tr><td>gi|17864606|ref|NP_524925.1| CG2830-PA [Drosophila melanoga...</td><td>624</td><td>e-177</td></tr>
<tr><td>gi|34860098|ref|XP_219278.2| similar to 60 kDa heat shock p...</td><td>623</td><td>e-177</td></tr>
<tr><td>gi|28436902|gb|AAH46687.1| MGC53106 protein [Xenopus laevis]</td><td>614</td><td>e-174</td></tr>
<tr><td>gi|15010456|gb|AAK77276.1| GH05807p [Drosophila melanogaster]</td><td>612</td><td>e-173</td></tr>
<tr><td>gi|48103847|ref|XP_392899.1| similar to ENSANGP00000014839 ...</td><td>607</td><td>e-172</td></tr>
<tr><td>gi|34856232|ref|XP_218673.2| similar to chaperonin 60 [Ratt...</td><td>606</td><td>e-172</td></tr>
<tr><td>gi|23197790|gb|AAN15422.1| mitochondrial chaperonin HSP60 [...</td><td>601</td><td>e-170</td></tr>
<tr><td>gi|49079648|ref|XP_403446.1| hypothetical protein UM05831.1...</td><td>598</td><td>e-169</td></tr>
<tr><td>gi|38105103|gb|EAA51570.1| hypothetical protein MG03165.4 [...</td><td>592</td><td>e-168</td></tr>
<tr><td>gi|4099014|gb|AAD00521.1| heat-shock protein [Coccidioides ...</td><td>588</td><td>e-166</td></tr>
<tr><td>gi|2506275|sp|P29185|CH61_MAIZE Chaperonin CPN60-1, mitocho...</td><td>587</td><td>e-166</td></tr>
<tr><td>gi|2493646|sp|Q43298|CH62_MAIZE CHAPERONIN CPN60-2, MITOCHO...</td><td>586</td><td>e-166</td></tr>
<tr><td>gi|309557|gb|AAA33450.1| chaperonin 60</td><td>586</td><td>e-166</td></tr>
</table>

Putative amino acid comparison of full length HSP7 *P. monodon* using BLASTX

<table>
<tr><th>Sequences producing significant alignments:</th><th>Score (bits)</th><th>E Value</th></tr>
<tr><td>gi|48766851|gb|AAT46566.1| heat shock protein 70 [Litopenae...</td><td>1293</td><td>0.0</td></tr>
<tr><td>gi|33319729|gb|AAQ05768.1| heat shock protein 70 [Penaeus m...</td><td>1290</td><td>0.0</td></tr>
<tr><td>gi|42794532|gb|AAS45710.1| heat shock protein 70 [Macrobrac...</td><td>1215</td><td>0.0</td></tr>
<tr><td>gi|48104285|ref|XP_392933.1| similar to heat shock cognate ...</td><td>1163</td><td>0.0</td></tr>
<tr><td>gi|246719|gb|AAB21658.1| HSC71 [Oncorhynchus mykiss] >gi|10...</td><td>1156</td><td>0.0</td></tr>
<tr><td>gi|47223819|emb|CAF98589.1| unnamed protein product [Tetrao...</td><td>1152</td><td>0.0</td></tr>
<tr><td>gi|6457366|gb|AAF09496.1| heat shock cognate 70 protein [Ma...</td><td>1147</td><td>0.0</td></tr>
<tr><td>gi|27371247|gb|AAH41201.1| Hsc70-prov protein [Xenopus laevis]</td><td>1146</td><td>0.0</td></tr>
<tr><td>gi|39979269|dbj|BAD05136.1| hsc71 [Paralichthys olivaceus]</td><td>1145</td><td>0.0</td></tr>
<tr><td>gi|18031682|gb|AAK31583.1| heat shock protein 70 [Ambystoma...</td><td>1145</td><td>0.0</td></tr>
<tr><td>gi|20563125|dbj|BAB92074.1| heat shock cognate protein [Bom...</td><td>1145</td><td>0.0</td></tr>
<tr><td>gi|28569550|gb|AAO43731.1| heat shock cognate 70 kDa protei...</td><td>1145</td><td>0.0</td></tr>
<tr><td>gi|42542845|gb|AAH66491.1| Hspa8 protein [Danio rerio] >gi|...</td><td>1144</td><td>0.0</td></tr>
<tr><td>gi|43439894|gb|AAS46619.1| heat shock cognate 70 kDa protei...</td><td>1144</td><td>0.0</td></tr>
<tr><td>gi|27805925|ref|NP_776770.1| heat shock 70 kDa protein 8; h...</td><td>1144</td><td>0.0</td></tr>
<tr><td>gi|50759965|ref|XP_429266.1| PREDICTED: hypothetical protei...</td><td>1144</td><td>0.0</td></tr>
<tr><td>gi|37993866|gb|AAP57537.3| heat shock protein 70 [Locusta m...</td><td>1142</td><td>0.0</td></tr>
<tr><td>gi|50603788|gb|AAH77998.1| Unknown (protein for MGC:82390) ...</td><td>1142</td><td>0.0</td></tr>
<tr><td>gi|27802643|gb|AAO21473.1| hsp70 family member [Locusta mig...</td><td>1142</td><td>0.0</td></tr>
<tr><td>gi|37682087|gb|AAQ97970.1| heat shock 70kDa protein 8 [Dani...</td><td>1140</td><td>0.0</td></tr>
<tr><td>gi|28279108|gb|AAH45841.1| Hspa8 protein [Danio rerio]</td><td>1140</td><td>0.0</td></tr>
<tr><td>gi|5729877|ref|NP_006588.1| heat shock 70kDa protein 8 isof...</td><td>1139</td><td>0.0</td></tr>
<tr><td>gi|31981690|ref|NP_112442.2| heat shock protein 8; heat sho...</td><td>1139</td><td>0.0</td></tr>
<tr><td>gi|42542422|gb|AAH66191.1| Heat shock protein 8 [Mus musculus]</td><td>1138</td><td>0.0</td></tr>
<tr><td>gi|1661134|gb|AAB18391.1| heat shock 70 protein [Mus muscul...</td><td>1137</td><td>0.0</td></tr>
<tr><td>gi|123647|sp|P19378|HS7C_CRIGR Heat shock cognate 71 kDa pr...</td><td>1135</td><td>0.0</td></tr>
<tr><td>gi|28374367|gb|AAH46262.1| MGC53952 protein [Xenopus laevis]</td><td>1134</td><td>0.0</td></tr>
<tr><td>gi|3513540|gb|AAC33859.1| heat shock protein 70 [Paralichth...</td><td>1134</td><td>0.0</td></tr>
<tr><td>gi|56385|emb|CAA49670.1| Hsc70-ps1 [Rattus norvegicus] >gi|...</td><td>1132</td><td>0.0</td></tr>
<tr><td>gi|4838561|gb|AAD31042.1| heat shock protein 70 [Crassostre...</td><td>1132</td><td>0.0</td></tr>
<tr><td>gi|25527326|gb|AAN73310.1| heat-shock protein 70 [Cotesia r...</td><td>1130</td><td>0.0</td></tr>
<tr><td>gi|38882982|gb|AAR01102.2| HSP70 [Dicentrarchus labrax]</td><td>1130</td><td>0.0</td></tr>
<tr><td>gi|45384370|ref|NP_990334.1| heat shock cognate 70 [Gallus ...</td><td>1129</td><td>0.0</td></tr>
<tr><td>gi|1235933|gb|AAB03704.1| heat shock cognate [Danio rerio] ...</td><td>1129</td><td>0.0</td></tr>
<tr><td>gi|31322197|gb|AAO41703.1| heat shock protein 70 [Crassostr...</td><td>1127</td><td>0.0</td></tr>
<tr><td>gi|942594|gb|AAA74394.1| heat shock cognate protein</td><td>1127</td><td>0.0</td></tr>
<tr><td>gi|1495233|gb|AAB06239.1| HSC70</td><td>1126</td><td>0.0</td></tr>
<tr><td>gi|33598990|gb|AAP51388.1| constitutive heat shock protein ...</td><td>1125</td><td>0.0</td></tr>
<tr><td>gi|7960186|gb|AAF71255.1| HSC71 [Rivulus marmoratus]</td><td>1123</td><td>0.0</td></tr>
<tr><td>gi|38683403|gb|AAO38780.1| heat shock protein 70 [Chlamys f...</td><td>1120</td><td>0.0</td></tr>
<tr><td>gi|31241095|ref|XP_320971.1| ENSANGP00000019887 [Anopheles ...</td><td>1119</td><td>0.0</td></tr>
<tr><td>gi|47225582|emb|CAG12065.1| unnamed protein product [Tetrao...</td><td>1115</td><td>0.0</td></tr>
<tr><td>gi|34785094|gb|AAH56797.1| Hypothetical protein MGC63663 [D...</td><td>1115</td><td>0.0</td></tr>
<tr><td>gi|23193450|gb|AAN14525.1| heat shock cognate 70 [Chironomu...</td><td>1113</td><td>0.0</td></tr>
<tr><td>gi|42494887|gb|AAS17723.1| heat shock protein 70 [Argopecte...</td><td>1113</td><td>0.0</td></tr>
<tr><td>gi|662802|gb|AAC23392.1| heat shock-like protein, similar t...</td><td>1112</td><td>0.0</td></tr>
<tr><td>gi|29468050|gb|AAN74984.1| 70kDa heat shock protein [Balanu...</td><td>1112</td><td>0.0</td></tr>
<tr><td>gi|761725|gb|AAA64872.1| heat shock protein 70 >gi|1346318|...</td><td>1110</td><td>0.0</td></tr>
<tr><td>gi|23193452|gb|AAN14526.1| heat shock cognate 70 [Chironomu...</td><td>1109</td><td>0.0</td></tr>
<tr><td>gi|17061839|dbj|BAB72169.1| stress protein HSC70 [Xiphophor...</td><td>1109</td><td>0.0</td></tr>
<tr><td>gi|27684119|ref|XP_214603.1| similar to Heat shock cognate ...</td><td>1108</td><td>0.0</td></tr>
<tr><td>gi|42494889|gb|AAS17724.1| heat shock protein 70 [Mizuhopec...</td><td>1106</td><td>0.0</td></tr>
<tr><td>gi|1326171|gb|AAB41583.1| heat shock cognate 70.II protein ...</td><td>1103</td><td>0.0</td></tr>
</table>

Putative amino acid comparison of full length HSP90 *P. monodon* using BLASTX

<table>
<tr><th>Sequences producing significant alignments:</th><th>Score (bits)</th><th>E Value</th></tr>
<tr><td>gi|42556386|gb|AAS19788.1| hsp-90 [Chiromantes haematocheir]</td><td>1238</td><td>0.0</td></tr>
<tr><td>gi|72221|pir||HHCH90 heat shock protein 90 - chicken</td><td>1173</td><td>0.0</td></tr>
<tr><td>gi|28467005|ref|NP_786937.1| heat shock protein 1, alpha; h...</td><td>1172</td><td>0.0</td></tr>
<tr><td>gi|47522774|ref|NP_999138.1| 90-kDa heat shock protein [Sus...</td><td>1172</td><td>0.0</td></tr>
<tr><td>gi|34392343|dbj|BAC82487.1| 90-kDa heat shock protein alpha...</td><td>1171</td><td>0.0</td></tr>
<tr><td>gi|6754254|ref|NP_034610.1| heat shock protein 1, alpha; he...</td><td>1170</td><td>0.0</td></tr>
<tr><td>gi|29145077|gb|AAH49124.1| Hspca protein [Mus musculus]</td><td>1170</td><td>0.0</td></tr>
<tr><td>gi|63516|emb|CAA30251.1| unnamed protein product [Gallus ga...</td><td>1170</td><td>0.0</td></tr>
<tr><td>gi|72219|pir||HHHU86 heat shock protein 90-alpha - human >g...</td><td>1170</td><td>0.0</td></tr>
<tr><td>gi|40254816|ref|NP_005339.2| heat shock 90kDa protein 1, al...</td><td>1169</td><td>0.0</td></tr>
<tr><td>gi|1170383|sp|P46633|HS9A_CRIGR Heat shock protein HSP 90-a...</td><td>1163</td><td>0.0</td></tr>
<tr><td>gi|17865490|sp|Q9GKX7|HS9A_HORSE Heat shock protein HSP 90-...</td><td>1162</td><td>0.0</td></tr>
<tr><td>gi|12005809|gb|AAG44630.1| 90-kDa heat shock protein HSP83 ...</td><td>1162</td><td>0.0</td></tr>
<tr><td>gi|46358051|dbj|BAD15163.1| heat shock protein [Antheraea y...</td><td>1153</td><td>0.0</td></tr>
<tr><td>gi|13699184|dbj|BAB41209.1| 90-kDa heat shock protein [Bomb...</td><td>1152</td><td>0.0</td></tr>
<tr><td>gi|50603918|gb|AAH77195.1| Unknown (protein for MGC:78910) ...</td><td>1145</td><td>0.0</td></tr>
<tr><td>gi|27681923|ref|XP_217339.1| similar to heat shock protein ...</td><td>1139</td><td>0.0</td></tr>
<tr><td>gi|34392345|dbj|BAC82488.1| 90-kDa heat shock protein beta ...</td><td>1138</td><td>0.0</td></tr>
<tr><td>gi|37142918|gb|AAQ88393.1| heat shock protein 90 [Equus cab...</td><td>1137</td><td>0.0</td></tr>
<tr><td>gi|34304590|gb|AAQ63401.1| heat shock 90kDa protein 1 beta ...</td><td>1137</td><td>0.0</td></tr>
<tr><td>gi|49118048|gb|AAH72998.1| MGC82579 protein [Xenopus laevis]</td><td>1137</td><td>0.0</td></tr>
<tr><td>gi|37623887|gb|AAQ95586.1| HSP-90 [Dicentrarchus labrax]</td><td>1134</td><td>0.0</td></tr>
<tr><td>gi|123681|sp|P11499|HS9B_MOUSE Heat shock protein HSP 90-be...</td><td>1134</td><td>0.0</td></tr>
<tr><td>gi|40807203|gb|AAH65359.1| Hsp90b protein [Danio rerio]</td><td>1134</td><td>0.0</td></tr>
<tr><td>gi|72222|pir||HHHU84 heat shock protein 90-beta [validated]...</td><td>1134</td><td>0.0</td></tr>
<tr><td>gi|194027|gb|AAA37866.1| heat-shock protein hsp84</td><td>1134</td><td>0.0</td></tr>
<tr><td>gi|50740540|ref|XP_444655.1| PREDICTED: heat shock protein ...</td><td>1133</td><td>0.0</td></tr>
<tr><td>gi|34879302|ref|XP_216334.2| similar to heat shock protein ...</td><td>1132</td><td>0.0</td></tr>
<tr><td>gi|20177936|sp|Q9GKX8|HS9B_HORSE Heat shock protein HSP 90-...</td><td>1132</td><td>0.0</td></tr>
<tr><td>gi|47604960|ref|NP_996842.1| heat shock protein 90 beta [Ga...</td><td>1130</td><td>0.0</td></tr>
<tr><td>gi|3212009|gb|AAC21566.1| heat shock protein hsp90beta [Dan...</td><td>1130</td><td>0.0</td></tr>
<tr><td>gi|18858875|ref|NP_571385.1| heat shock protein 90-beta [Da...</td><td>1129</td><td>0.0</td></tr>
<tr><td>gi|2062377|gb|AAB58358.1| heat shock protein 83 [Drosophila...</td><td>1129</td><td>0.0</td></tr>
<tr><td>gi|21483234|gb|AAM52592.1| AT20544p [Drosophila melanogaste...</td><td>1127</td><td>0.0</td></tr>
<tr><td>gi|309317|gb|AAA37865.1| 84 kD heat shock protein</td><td>1123</td><td>0.0</td></tr>
<tr><td>gi|37787287|gb|AAO92751.1| heat shock protein 90 beta [Para...</td><td>1122</td><td>0.0</td></tr>
<tr><td>gi|4835864|gb|AAD30275.1| heat shock protein hsp90 beta [Sa...</td><td>1120</td><td>0.0</td></tr>
<tr><td>gi|1346320|sp|P34058|HS9B_RAT Heat shock protein HSP 90-bet...</td><td>1115</td><td>0.0</td></tr>
<tr><td>gi|6807647|emb|CAB66478.1| hypothetical protein [Homo sapie...</td><td>1113</td><td>0.0</td></tr>
<tr><td>gi|38146757|gb|AAR11781.1| heat shock protein 90 [Chlamys f...</td><td>1107</td><td>0.0</td></tr>
<tr><td>gi|51470849|ref|XP_084514.6| PREDICTED: heat shock 90kDa pr...</td><td>1105</td><td>0.0</td></tr>
<tr><td>gi|18858873|ref|NP_571403.1| heat shock protein 90-alpha [D...</td><td>1104</td><td>0.0</td></tr>
<tr><td>gi|49899168|gb|AAH75757.1| Unknown (protein for MGC:86652) ...</td><td>1104</td><td>0.0</td></tr>
<tr><td>gi|19855062|sp|O61998|HS90_BRUPA Heat shock protein 90 >gi|...</td><td>1104</td><td>0.0</td></tr>
<tr><td>gi|30313869|gb|AAO52675.1| heat shock protein 90 alpha; hea...</td><td>1103</td><td>0.0</td></tr>
<tr><td>gi|3096951|emb|CAA06694.1| heat shock protein 90 [Brugia pa...</td><td>1103</td><td>0.0</td></tr>
<tr><td>gi|1066808|gb|AAB05639.1| heat shock protein 82 [Anopheles ...</td><td>1101</td><td>0.0</td></tr>
<tr><td>gi|1899173|gb|AAB49983.1| heat shock protein hsp90 [Oncorhy...</td><td>1081</td><td>0.0</td></tr>
<tr><td>gi|40956306|gb|AAO14563.2| Hsp90 [Heterodera glycines]</td><td>1076</td><td>0.0</td></tr>
<tr><td>gi|14041148|emb|CAC38753.1| heat shock protein 90 [Dendrone...</td><td>1070</td><td>0.0</td></tr>
<tr><td>gi|39589853|emb|CAE60851.1| Hypothetical protein CBG04560 [...</td><td>1060</td><td>0.0</td></tr>
<tr><td>gi|17559162|ref|NP_506626.1| heat shock protein, abnormal D...</td><td>1041</td><td>0.0</td></tr>
<tr><td>gi|47224556|emb|CAG03540.1| unnamed protein product [Tetrao...</td><td>1030</td><td>0.0</td></tr>
<tr><td>gi|39644662|gb|AAH09206.2| HSPCB protein [Homo sapiens]</td><td>1025</td><td>0.0</td></tr>
</table>

APPENDIX F

Nucleotide and amino acid sequences of full length of HSP60, HSP70 and HSP90 *P. monodon*.

HSP60

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77 $77 &*7 7*& &77 &&7 *&7 77$ *$7 $&7 &7& $&7 &&$ $*& $$& *$$ *$& &$* *$* *77
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*$ *&7 *&7 $&$ **$ $&& 77& *77 $$& &77 *7* *$$ *&$ **$ $7& $77 *$7 &&& $&& $$*
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*77 *7& &*7 $&$ *&& &7$ $&7 *$7 *&7 *&$ **$ *7* *&7 7&& &7& &7& $&& $&$ *&7 *$*
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$*7 *7& $77 $&$ *$* $7& &&& $$* *$$ *$$ &&$ *&7 **7 $7* **$ **& $7* **7 **7 $7*
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** **$ $7* **7 **& $7* **& **$ $7* **$ **& $7* **& **& $7* $7* 7$$
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     7* $&$ $$* 77$ &77 7** 77& 7$& $$* $** 7$& **$ *$* 7$& $7$ *$7 *&& $&$ *$$ &7$
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HSP70

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HSP90

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APPENDIX G

Nucleotide sequences of immune related gene screened from cDNA library and partial of HSP gene were constructed the full length of HSP genes.

1. ES-N-S02-0060-W

GGCACGAGGCAGCAAGAGGGTGACTGCAAACAACTCAAGCTAAGATTTGACGTCAGCCAGTA
CAAGCCCGAGGAGATTGTTGTCAAGACAGTTGATAATAAGCTTCTGGTCCACGCCAAGCACG
AGGAGAAGTCTGACAACCGCTCCGTCTACCGCGAGTACAACAGAGAGTTCCTGCTTCCCAAG
GGCACCAACCCCGAGCTCATCAAGTCCTCGCTCTCCAAAGACGGCGTCCTGACGGTGGAAGC
TCCCCTGCCGGCCATAGCTGGGAACGAAGAGAAGGTCATCCCCATCGCACAGAACTGATTGA
CTGATTTCATCGCTTCTGTCGTTACGAATTTTCGCCACAGTGTTGAAGGAAGGAAGCCACGT
GTAAGAGGATTGGAGGAATCATCTCGCCAGCATCAGTGTTTTTTTTATATATATATATCAAA
TCGATAGAGTAAAATTTGTGTTATTGTATGAAGAAAGGGAAAGTANAAAGGAAAGTCTCTTT
TTTTTAATAAATAATAAATGTGATTTTTTTTCTTATGTGTGAAACACCTTCATTATGCCTTT
GAAATTGCATGGGAAATATTTTTGTTCTAAGGTGTTATTTCA

2. HC-H-S01-0055

GGCACGAGGATTCAAGGATCAACTCCCTGCTGAAGAGACTCAAAAGTTGAAGGAGCAGCTGA
CTACTGTGAAGGACCTCTTGGCAAATAAAGACTCTGCAGACCCAGAGGAAATCAAGCAAAAG
GTCAGCGAGCTCCAGCAAGCCTCGCTCAAGCTCTTCGAGATGGCCTATAAGAAGATGGCATC
TGAGAGAGAGTCATC

3. HC-H-S01-0225

GGCACGAGGCCTCGTGCCGAATTCGGCACGAGGGAGAAATGTCAGTTTCCTTCGGGATATAT
CTCGGGAGTAGTTCGTGCTGCATTGCCGTCAACAAGGATGGAAAATTTGAAGTTGTGGCCAA
TGCCTCTGGAGACAGAGTTACTCCTGCAGTGGTGGCATATCATGAGATGGAAGTGGTAACAG
GACTTGCAGCTAAGCAGGGAATGATCCGACATGCGGCAAATACCATTCAGAATGTGCTACGC
AGTGCCAGCTGCAGTGAAGATGAAGGAACCTGTCAGTCTAATGTATCATGTTGTCCTAAATG
GGAAAATAACCAAGTGTGCTACACAGTGCAACGAGGAGAGAAAACTGCAACTGTTACCGCAG
AGGAGGTTTTAACTCACATTTTTACTCTGCTCAAAGGTATTGCCATGAATCAGACAAGTGAA
TCAGAGCTCCCTCTTGTTATAAGTGTTCCAGCTTGGACCAGTGAAGCAGCTGTTGAGGTTAT
CAAGAAAGCTGCCAAGAAAGCTTCTTTTAATTTGATGTCAACTATCAGTCAACCAGTTGCTG
CAGTCTTAGCATATGGCCTTGTAAATGACAACAAAAAGAATT

4. HC-W-S01-0030

GGCACGAGGGTGACAAGTCCGAGGCTGTGCAGGACCTGTTGCTGTTGGACGTGACCCCCTTG
TCCCTGGGTATCGAGACTGCCGGCGGTGTGATGACTGCGCTCATCAAGCGTAACACCACCAT
CCCCACCAAGCAGACCCAGACCTTCACCACCTACTCTGACAACCAGCCAGGTGTGCTCATCC
AGGTGTACGAGGGAGAGCGTGCCATGACCAAGGACAACAACCTCCTGGGTAAGTTCGAGCTG
AGTGGCATCCCACCTGCTCCCCGTGGCGTGCCTCAGATCGAGGTCACCTTCGACATCGACGC
CAACGGCATCCTGAACGTATCTGCCGTGGACAAGTCTACTGGTAAGGAGAACAAGATTACCA
TCACCAACGACAAGGGTCGCCTCTCCAAGGAGGAGATCGAGCGCATGGTGCAGGACGCCGAG
AAGTACAAGGCTGACGATGAGAAGCAGAGGGACCGTATTTCTGCCAAGAACTCCCTCGAGTC
TTACTGCTTCAACATGAAGTCGACAGTTGAGGACGAGAAGTTCAAGGAGAAGATTTCTGAGG
AGGACCGCAACAAGATTTTGGAGACCTGCAACGAGACTATCA

5. HC-W—S01-0037

GGCACGAGGAGAAGGTCGGCGGTGAGCGCAATGTCTTGATCTTCGATCTTGGCGGTGGTACC
TTCGATGTGTCCATCCTTACCATCGAGGATGGTATCTTCGAGGTCAAGTCAACAGCTGGTGA
CACTCACTTGGGCGGTGAAGACTTCGACAACCGCATGGTGAACCACTTCATCCAGGAATTCA
AGCGCAAGTACAAGAAGGACCCAAGTGAGAACAAGCGCTCCCTGCGTCGCCTGCGTACGGCC
TGTGAGCGTGCGAAGCGCACCCTGTCTTCCTCGACACAGGCCAGTGTGGAGATCGATTCCCT
CTTCGAAGGTATCGACTTCTACACCTCTATCACTCGTGCTCGCTTCGAGGAGCTGTGCGCCG
ATCTGTTCCGTGGCACCTTGGAGCCCGTGGAGAAGTCACTCCGT

6. HC-W-S01-0046

GGCACGAGGCCGTGCCGTGCAGAAGCTCCGTCGTGAAGTGGAAAAGGCTAAGCGCTCCCTCT
CCTCTAGCCACCAGGTCAGGATCGAGATCGAGTCCTTCTTCGAGGGTGATGACTTCTCGGAG
ACCCTCACCCGTGCCAAGTTCGAGGAATTGAACATGGATCTCTTCAGGTCCACCATGAAGCC
CGTACAGAAGGTGCTGGAAGATTCTGACCTCCAGAAGAAGGAAATTGACGAGATTGTACTTG
TTGGTGGTTCCACTCGTATCCCTAAGATCCAGCAGCTGGTGAAAGAGTTCTTCGGTGGCAAG
GAGCCATCCCGAGGCATTAACCCGGACGAGGCTGTAGCTTACGGCGCTGCTGTCCAGGCTGG
CGTGCTCTCGGGTGAGGACGACACCAACGACCTCGTGCTGCTCGACGTGAACCCCTTGACCC
TGGGTATTGAGACTGTGGGAGGTGTCATGACAAAGCTGATCCCTCGCAACACTGTCATCCCC
ACCAAGAAGTCCCAGATCTTCTCCACTGCCTCTGACAACCAGCACACTGTCACCATCCAAGT
ATTCGAAGGTGAACGTCCCATGACCAAGGATAACCACATCCT

7. HC-W-S01-0230

GGCACGAGGGGAGATCGAGCGCATGGTGCAGGACGCCGAGAAGTACAAGGCTGACGATGAGA
AGCAGAGGGACCGTATTTCTGCCAAGAACTCCCTCGAGTCTTACTGCTTCAACATGAAGTCG
ACAGTTGAGGACGAGAAGTTCAAGGAGAAGATTTCTGAGGAGGACCGCAACAAGATTTTGGA
GACCTGCAACGAGACTATCAAGTGGCTGGACATGAACCAGCTGGGCGAGAAGGAAGAGTATG
AGCACAAGCAGAAGGAGATCGAACAGGTGTGCAACCCCATCATTACCAAGATGTACGCTGCT
GCTGGTGGTGCTCCTCCAGGCGGCATGCCCGGCGGCTTCCCAGGTGGTGCCCCAGGTGCTGG
CGGTGCTGCTCCCGGTGCTGGTGGTTCCTCCGGACCCACCATCGAGGAAGTCGATTAAACGA
TTCCTCCGCGTCTACTAGTCTCATTGTGAATTGTCCATGCAAATCGACCCATCGTAGATCAT
TCCGCATTTTATTTATGATGTTGGTGGCTTGTGCCATTGGCAGACTTCACATTGCAAGTTTT
CAGTAAACCATTCCAGAAATC

8. HC-W-S01-0248

AAAGTATACTTATACGCCAGCTCGAAATTACCCTCACTAAAGGGAACAAAAGCTGGAGCTCG
CGCGCCTGCAGGTCGACACTAGTGGATCCAAAGATTCGGCACGAGGGTTGGAACCCGCCCGG
CGTGACAGACAGACGAACTCGTGCCTCCGCAATGGCTGCAATTAAGACGCTAAATCCCAAAG
CCGAAGTGGCGAGGGCGCAGCAGGCCCTTGCTATCAATATTTCAGGCGCGAGGGGCATCCAG
GATGTCCTCAGGACGAACCTGGGACCCAAAGGAACCATGAAAATGTTGGTATCTGGGGCTGG
AGACATTAAGATCACAAAAGATGGAAACATTCTACTTCATGAGATGCAAATCCAGCACCCAA
CTGCCAGCATGATTGCAAAGGCCTGCACTGCTCAGGATGACATCATTGGAGATGGCACCACC
TCAACTGTTCTCCTCATTGGGGAGATGCTCA

9. HC-W-S01-0257

GGCACGAGGCCTCGATGGTGCTCATCAAAATGAAGGAGACCGCCGAGGCTTACCTGGGATCC
ACAGTGAAGGATGCTGTAGTCACTGTACCTGCTTACTTCAACGATTCTCAGCGCCAGGCCAC
CAAGGACGCTGGAACCATCTCGGGTCTTAATGTGCTGCGTATCATCAACGAACCCACCGCTG
CTGCCATCGCCTACGGCCTCGACAAGAAGGTCGGCGGTGAGCGCAATGTCTTGATCTTCGAT
CTTGGCGGTGGTACCTTCGATGTGTCCATCCTTACCATCGAGGATGGTATCTTCGAGGTCAA
GTCAACAGCTGGTGACACTCACTTGGGCGGTGAAGACTTCGACAACCGCATGGTGAACCACT
TCATCCAGGAATTCAAGCGCAAGTACAAGAAGGACCCAAGTGAGAATAAGCGCTCCCTGCGT
CGCCTGCGTACGGCCTGTGAGCGTGCGAAGCGCACCCTGTCTTCCTCGACACAGGCCAGTGT
GGAGATCGACTCCCTCTTCGAAGGTATCGACTTCTACACCTCTATCACTCGTGCTCGCTTCG
AGGAGCTGTGCGCCGATCTGTTCCGTGGCACCTTGGAGCCCG

10. HC-W-S01-0309

GGCACGAGGAAATGGCAAAGGCACCTGCTGTCGGTATTGATCTGGGAACCACCTACTCCTGC
GTGGGTGTGTTCCAGCATGGCAAGGTGGAGATCATCGCCAACGACCAGGGGCAACCGCACCA
CGCCCTCCTACGTCGCCTTCACAGACACAGAGCGTCTGATTGGTGACGCCGCCAAGAACCAG
GTGGCGATGAACCCCAACAACACTGTATTCGACGCCAAGCGACTCATCGGCCGCAAATTCGA
AGACCACACAGTCCAGAGCGACATGAAGCATTGGCCCTTCACCATCATCAACGAGAGCACAA
AGCCAAAGATCCAGGTAGAGTACAAGGGAGACAAGAAGACCTTCTACCCAGAAGAGATCTCC
TCGATGGTGCTCATCAAAATGAAGAGAGACCGCCGAGGCTTACCTGGGATCCACAGTGAAGG
ATGCTGTAGTCACTGTACCTGCTTACTTCAACGATTCTCAGCGCCAGGCCACCAAGGACGCT
GGAACCATCTCGAGGTCTTAATGTGCTGCGTATCATCAACGAACCCACCGACTGCTGCCATC
GCCTACGGCCTCGACAAGAAGGTCGGCGGTGAGCGCAATGTC

11. HC-W-S01-0349

ACGCTATACTTGATACGCACAGCTCGAAATTACCCTCACTAAAGGGAACAAAGCTGGAGCTC
GCGCGCCTGCAGGTCGACACTAGTTGGATCCAAAGAATTCGGCACGAGGCGGACGTGTAACA
ATTAGCTCTTAGGACTATTTAAAAATATCTAAAATAAGATAAAATGGCAAAGGCACCTGCTG
TCGGTATTGATCTGGGAACCACCTACTCCTGCGTGGGTGTGTTCCAGCATGGCAAGGTGGAG
ATCATCGCCAACGACCAGGGCAACCGCACCACGCCCTCCTACGTCGCCTTCACAGACACAGA
GCGTCTGATTGGTGAC

12. HC-W-S01-0669

GGGNNACTTATTACGCCCACGCTCGANAATTACGCCTCACTAAAGGGAACACAAAGCTGGAG
CTCGCGCGCCTGCAGGTCGACACTAGTGGATCCAAAGCTACGGCGCCGCTGTCCAGGCCGCC
ATTCTGTGCGGTGACAAGTCCGAGGCTGTGCAGGACCTGTTGCTGTTGGACGTGACCCCCTT
GTCCCTGGGTATCGAGACTGCCGGCGGTGTGATGACTGCGCTCATCAAGCGTAACACCACCA
TCCCCACCAAGCAGACCCAGACCTTCACCACCTACTCTGACAACCAGCCAGGTGTGCTCATC
CAGGTGTACGAGGGAGAGCGTGCCATGACCAAGGACAACAACCTCCTGGGTAAGTTCGAGCT
GAGTGGCATCCCACCTGCTCCCCGTGGCGTGCCTCAGATCGAGGTCACCTTCGACATCGACG
CCAACGGCATCCTGAACGTATCCGCCGTGGACAAGTCTACTGGTAAGGAGAACAAGATTACC
ATCACCAACGACAAGGGTCGCCTCTCCAAGGAGGAGATCGAGCGCATGGTGCAGGACGCCGA
GAAGTACAAGGCTGACGATGAGAAGCAGAGGGACCGTATTTCTGCCAAGAACTCCCTCGAAG
TCTTACTGCTTCAACATGAAGTCGACAGTTGAGGACGAGAAGTTCAAGGAGAAGATTTCTGA
GGAGGACCGCAAC

13. Hpa-N-S01-0166

GGCACGAGGCCACAGTGAAGGATGCTGTAGTCACTGTACCTGCTTACTTCAACGATTCTCAG
CGCCAGGCCACCAAGGACGCTGGAACCATCTCGGGTCTTAATGTGCTGCGTATCATCAACGA
ACCCACCGCTGCTGCCATCGCCTACGGCCTCGACAAGAAGGTCGGCGGTGAGCGCAATGTCT
TGATCTTCGATCTTGGCGGTGGTACCTTCGATGTGTCCATCCTTACCATCGAGGATGGTATC
TTCGAGGTCAAGTCAACAGCTGGTGACACTCACTTGGGCGGTGAAGACTTCGACAACCGCAT
GGTGAACCACTTCATCCAGGAATTCAAGCGCAAGTACAAGAAGGACCCAAGTGAGAACAAGC
GCTCCCTGCGTCGCCTGCGTACGGCCTGTGAGCGTGCGAAGCGCACCCTGTCTTCCTCGACA
CAGGCCAGTGTGGAGATCGACTCCCTCTTCGAAGGTATCGACTTCTACACCTCTATCACTCG
TGCTCGCTTCGAGGAGCTGTGCGCCGATCTGTTCCGTGGCACCTTGGAGCCCGTGGAGAAGT
CACTCCGTGATGCCAAGATGGACAAGGCCCAGATCCACGACA

14. LP-N-S01-0011

CCGTGGCGTGCCTCAGATCGAGGTCACCTTCGACATCGACGCCAACGGCATCCGTGAACGTA
TCCGCCGTGGACAAGTCTACTGGTAAGGAGAACAAGATTACCATCACCAACGACAAGGGTCG
CCTCTCCAAGGAGGAGATCGAGCGCATGGTGCAGGACGCCGAGAAGTACAAGGCTGACGATG
AGAAGCAGAGGGACCGTATTTCTGCCAAGAACTCCCTCGAGTCTTACTGCTTCAACATGAAG
TCGACAGTTGAGGACGAGAAGTTCAAGGAGAAGATTTCTGAGGAGGACCGCAACAAGATTTT
GGAGACCTGCAACGAGACTATCAAGTGGCTGGACATGAACCAGCTGGGCGAGAAGGAAGAGT
ATGAGCACAAGCAGAAGGAGATCGAACAGGTGTGCAACCCCATCATTACCAAGATGTACGCT
GCTGCTGGTGGTGCTCCTCCAGGCGGCATGCCCGGCGGCTTCCCAGGTGGTGCCCCAGGTGC
TGGCGGTGCTGCTCCCGGTGCTGGTGGTTCCTCCGGACCCACCATCGAGGAAGTCGATTAAA
CGATTCCTCCGCGTCTACTAGTCTCATTGTGAATTGTCCATGCAAATCGACCCATCGTAGAT
CATTCCGCATTTTATTTATGATGTTGGTGGCTTGTGCCATTGGCAGACTTCACATTGCAAGT
TTTCAGTAAACCATTCCAGAAATCTGTAAAACGAATAAAAAAAAACAGGAAACAAAAAAAAA
AAAAAAAGGGGGGGCCCGTACCCAATTCGCCCTATAAGTGAGTGTATACATCATGC

15. LP-N-S01-0061

GGCACGAGGCACAAGGGAGGAGAGACGTGCTTTCGCGAGGTCCAAGTTCTAACACCAAAATG
GCTGTCGCACTCAATCCAGTACAGATTATGAAATCTGAGGCTGAGGAGGAGCGCTCGGAAAC
TGCTCGGCTCTCATCCTTCATTGGTGCCATTGCACTCGGAGAACTGGTGCGCTCCACACTTG
GTCCCAGGGGCATGGACAAAATTCTTGTAGCCATGGGCAGAAGTGAAGGACAAATCGAAGTC
ACAAATGACGGTGCTACCATCTTGAGGAACATTGGTGTGGACAATCCAGCAGCCAAGATTTT
AGTTGACATCAGCAAGACACAGGATGATGAGGTTGGAGATGGAACGACATCTGTGGTTGTCT
TGGCATCAGAGTTGCTACGAGAGGCTGAGAAGTTGGTAGCTATGAAGATTCATCCCCAGACC
ATTATTGCTGGTTACCGCAGGGCCACAGATGTTGCTCGTGAGGCACTAACAAAGTCGGCTCA
AGATAATTCGGCCAATCCCGAAAAATTTAGGGAAGACCTCCTGAAGATTGCCAAGACCACAC
TGAGTTCCAAGATTTTGGCTCAACACAAAGATTTCTTCTCCA

16. LP-N-S01-0255

GGCACGAGGACCACCATCCCCACCAAGCAGACCCAGACCTTCACCACCTACTCTGACAACCA
GCCAGGTGTGCTCATCCAGGTGTACGAGGGAGAGCGTGCCATGACCAAGGACAACAACCTCC
TGGGTAAGTTCGAGCTGAGTGGCATCCCACCTGCTCCCCGTGGCGTGCCTCAGATCGAGGTC
ACCTTCGACATCGACGCCAACGGCATCCTGAACGTATCTGCCGTGGACAAGTCTACTAGTCT
CATTGTGAATTGTCCATGCAAATCGACCCATCGTAGATCATTCCGCATTTTATTTATGATGT
TGGTGGCTTGTGCCATTGGCAGACTTCACATTGCAAGTTTTCAGTAAACCATTCCAGAAATC
TGTAAAACGAATAAAAAAACAGGAAACAAGAAAAAAAAAAAAAAAAAAAAA

17. OV-N-S01-0513

GCACGAGGCTCGAGTTTTATTGTAACAAAGTTGTTACAAATTATTTACATAAACGAAAGAAT
AAATCCTTATAACAGAATATATCTTTGTCTATAACTAAAACTAATGCTTGTTTAAATCTTAT
GGAATGTTGGGCGTGATCGGAATGATGAAAGCCAGAGAAGCCAAAATATACCAACATTAGAG
AAGTATTTTGGTTCCCGAATGAATGACAATGATTAGCTTTGGTATATAATGGGTGAATATTT
TAAGTTGTGACGAATATTTAATCGACTTCTTCCATGCGAGAGGCGTCCTCGTCATCACCTTC
GAGGGGGGGCATATCCTCCTCCAAGGTCTCGGCCTCCTCCATCGGGGCGTCCTCCTCGTCAA
TACCCAGGCCAAGCTTGATCATTCTGTAGATGCGGCTGGCGTGGACACCTGGGTCCTCCAAG
CTGAAGCCAGACGACAGAAGGGAGCTCTCGAACAGCAGCATCACCAGATCCTTCACAGACTT
GTCGTTCTTGTCGGCATCCGCCTTTTGTCTCAGGGTTTCGATGATGCTGTGGTCGGGGTTGA
TCTCAGGTGCTTCTTGGCCGGCCATGTAGCCCATGGTCGAG

18. OV-N-S01-0968-W

GGCACGAGGCACGAACACGATCCTCGCCAGTCGTCTACAGCGTCCCCCTTGGTCTGGGCTTG
CTCGCGAAACTCCAAAAATGCTCCAGTTACGATACCGATTCCTATGTCTTCTCGTGGGATTA
CTCCTTTTAGGAGGAACAAGAGCCGAGGATGTGGAGAGTCCAGGCACTGTTGAAGCTGATCT
TGGGGCTGATGTGGAAGGTTCGCGTACTGATGATAATGTAGTGGCCCGGGAAGAGGAGGCTA
TCAAGCTGGACGGACTCAATGTAGCACAGATCAAAGAAATGCGGGAGAAAGCAGAAAAGCAT
GCATTCCAAGCAGAGGTCAACCGGATGATGAAGCTTATTATCAATTCTCTGTACAGGAACAA
AGAGATCTTTTTGAGGGAGTTGATCAGCAATGCCTCTGATGCACTTGACAAAATCCGTCTGC
TGTCACTGACTGACAAGGATCAGCTGAGTACTAACCCAGAGTTGGCCATCAGAATAAAAGCA
GACAAGGACAACCACATACTTCACATCACTGACAGTGGTATTGGCATGACAAAAGCAGATTT
GGTCAATAACCTTGGAACAATTGCAAAATCTGGAACTTCAGA

19. OV-N-S01-0981-W

GGCACGAGGGGAGGTCAGGGCACTGATGCTGCAGGGCGTCGACGTCCTCACCGACGCCGTGG
CTGTCACCATGGGCCCCAAGGGTCGAAATGTAATCATTGAGCAGAGCTGGGGCAGTCCCAAG
ATCACAAAGGATGGTGTTACAGTTGCAAAGGCTGTTGAACTGAAAGACAAGTTCCAGAACAT
TGGAGCTAAGTTGGTCCAAGATGTTGCCAACAACACCAATGAAGAGGCTGGTGATGGAACCA
CCACAGCCACAGTCCTGGCTCGTACTATTGCAAAGGAAGGGTTTGACAGGATTAGCAAAGGT
GCCAACCCTGTGGAGATCAGGCGTGGAGTTATGTTGGCCGTGGATGCCATTGTTGCTCACCT
GAAGACCCTGTCAAAGCCTGTGACCACTCCTGCTGAGATTGCTCAGGTTGCAACCATCTCTG
CTAATGGAGACATTGAAGTAGGCAGTCTTATCTCGGCAGCCATGGAAAAGGTTGGTCGTGAG
GGTGTCATCACTGTGAAAGATGGCAAGACCTTGAAGGATGAGTTGGAGGTCATTGAAGGCAT
GAAGTTCGATCGTGGTTACATTTCTCCTTACTTCATAAACTC

20. OV-N-S01-0988-W

GGCACGAGGGGCTTGCTCGCGAAACTCCAAAAATGCTCCAGTTACGATACCGATTCCTATGT
CTTCTCGTGGGATTACTCCTTTTAGGAGGAACAAGAGCCGAGGATGTGGAGAGTCCAGGCAC
TGTTGAAGCTGATCTCGGGGCTGATGTGGAAGGTTCGCGTACTGATGATAATGTAGTGGCCC
GGGAAGAGGAGGCTATCAAGCTGGACGGACTCAATGTAGCACAGATCAAAGAAATGCGGGAG
AAAGCAGAAAAGCATGCATTCCAAGCAGAGGTCAACCGGATGATGAAGCTTATTATCAATTC
TCTGTACAGGAACAAGGAGATCTTTTTCGAGGGAGTTGATCAGCAATGCCTCTGATGCACTT
GACAAAATCCGTCTGCTGTCACTGACTGACAAGGATCAGCTGAGTACTAACCCAGAGTTGGC
CATCAGAATAAAAGCAGACAAGGACAACCACATACTTCACATCACTGACAGTGGTATTGGCA
TGACAAAAGCAGATTTGGTCAATAACCTTGGAACAATTGCAAAATCTGGAACTTCAGAATTT
TTCCTCTAAACTGCCAGGAGTCGGAGAATGCAGAGGAGACAA

21. HSP70-P1C7 (clone 742 bp)

TATGACATGATACGCCACNCTCGAAANTTACCTCACTAAAGGGAACAAAAGCTGGAGCTCCA
CCGCGGTGGCGGCCGCTCTAGAACTAGTGGATCCCCCGGGCTGCAGGAATTCGGCACGAGGG
AAGAGCGGACGTGTTACAATTAGCTCTTAGGACTATTTAAAAATATCTAAAATAAGATAAAA
TGGCAAAGGCACCTGCTGTCGGTATTGATCTGGGAACCACCTACTCCTGCGTGGGTGTGTTC
CAGCATGGCAAGGTGGAGATCATCGCCAACGACCAGGGCAACCGCACCACGCCCTCCTACGT
CGCCTTCACAGACACAGAGCGTCTGATTGGTGACGCCGCCAAGAACCAGGTGGCGATGAACC
CCAACAACACTGTATTCGACGCCAAGCGACTCATCGGCCGCAAATTCGAAGACCACACAGTC
CAGAGCGACATGAAGCATTGGCCCTTCACCATCATCAACGAGAGCACAAAGCCAAAGATCCA
GGTAGAGTACAAGGGAGACAAGAAGACCTTCTACCCAGAAGAGATCTCCTCGATGGTGCTCA
TCAAAATGAAGGAGACCGCCGAGGCTTACCTGGGATCCACAGTGAAGGATGCTGTAGTCACT
GTACCTGCTTACTTCAACGATTCTCAGCGCCAGGCCACCAAGGACGCTGGAACCATCTCGGG
TCTTAATGTGCTGCGTATCATCAACGAACCCACCGCTGCTGCCATCGCCTACGGCCTCGA

22. HSP70 P1/2C1 (clone 933 bp)

AATNGACATGATACGCACGNCTCGAAATTACCTCACTAAAGGGAACAAAAGCTGGAGCTCCA
CCGCGGTGGCGGCCGCTCTAGAACTAGTGGATCCCCCGGGCTGCAGGAATTCGGCACGAGGC
TGAGTGGCATCCCACCTGCTCCCCGTGGCGTGCCTCAGATCGAGGTCACCTTCGACATCGAC
GCCAACGGCATCCTGAACGTATCTGCCGTGGACAAGTCTACTGGTAAGGAGAACAAGATTAC
CATCACCAACGACAAGGGTCGCCTCTCCAAGGAGGAGATCGAGCGCATGGTGCAGGACGCCG
AGAAGTACAAGGCTGACGATGAGAAGCAGAGGGACCGTATTTCTGCCAAGAACTCCCTCGAG
TCTTACTGCTTCAACATGAAGTCGACAGTTGAGGACGAGAAGTTCAAGGAGAAGATTTCTGA
GGAGGACCGCAACAAGATTTTGGAGACCTGCAACGAGACTATCAAGTGGCTGGACATGAACC
AGCTGGGCGAGAAGGAAGAGTATGAGCACAAGCAGAAGGAGATCGAACAGGTGTGCAACCCC
ATCATTACCAAGATGTACGCTGCTGCTGGTGGTGCTCCTCCAGGCGGCATGCCCGGCGGCTT
CCCAGGTGGTGCCCCAGGTGCTGGCGGTGCTGCTCCCGGTGCTGGTGGTTCCTCCGGACCCA
CCATCGAGGAAGTCGATTAAACGATTCCTCCGCGTCTACTAGTCTCATTGTGAATTGTCCAT
GCAAATCGACCCATCGTAGATCATTCCGCATTTTATTTATGATGTTGGTGGCTTGTGCCATT
GGCAGACTTCACATTGCAAGNTTTTCAGTAAACCATTCCAAAATCTGTAAAACGAATANAAA
ACCAGCGAAACAANAACAAAACACGGGGGGGCCCGGTAGCCAATTCGCCTATATGATCTATT
ACA

23. HSP60 primer F112/R861

TTTGGCACGGAGGTCAGGGCACTGATGCTGCAGGGCGTCGACGTCCTCACCGACGCCGTGGC
TGTCACCATGGGCCCCAAGGGTCGAAATGTAATCATTGAGCAGAGCTGGGGCAGTCCCAAGA
TCACAAAGGATGGTGTTACAGTTGCAAAGGCTTTGAACTGAAAGACAAGTTCCAGAACATTG
GAGCTAAGTTGGTCCAAGATGTTGCCAACAACACCAATGAAGAGGCTGGTGATGGAACCACC
ACGGCCACAGTCCTGGCTCGCACTATTGCAAAGGAAGGTTTTGACAGGATTAGCAAAGGTGC
CAACCCTGTGGAGATCAGGCGTGGAGTTATGTTGGCCGTGGATGCCATTGTTGCTCACCTGA
AGACCCTGTCAAAGCCTGTGACCACTCCTGCTGAGATTGCTCAGGTTGCAACCATCTCTGCT
AATGGAGATATTGAAGTAGGCAGTCTTATCTCGGCAGCCATGGAGAAGGTTGGTCGTGAGGG
TGTCATCACTGTTAAAGATGGCAAGACCTTGAAGGATGAGTTGGAGGTCATTGAAGGCATGA
AGTTCGATCGCGGCTACATTTCTCCTTACTTCATAAACTCCAAGCAAGGGAGCTAAGGTTGA
ATACCCAGACTGCCTTGTTTTGCTCTCGGAGAAGAAAATTTCTTCTATCCAGTCCCATTATC
CCCAGTGCTAGAACTGGCCAATGCCCCAAAGGAAACCCTCTATTGATCATTGCTGAGGACGT
CGATGGCGAG

24. HSP70 primer F243/R545

ATAAACGAACCGACAGCTGCCGCCATTGCTTATGGCCTAGACAAGAAGAACGTAGGAATGGC
TGAGCAAAACGTGTTGATCTTCGACCTAGGAGGCGGTACCTTCGACGTGTCCATCCTCAGTA
TCGACGACGGAGTGTTCGAGGTGAAGGCAACAGCCGGCGACACGCATTTGGGAGGCGAAGAC
TTCGATAACAGGATGGTTAGTCACTTCACACAAGAGTTTCACAGGAAATACAAGAAGGATCT
CACCACCAATAAACGCGCACTTCGACGTCTTCGAACTGCTTGTGAACGAGCCAAGCGAACTC
TCTCTTCCTCCACACAAGCCAGTCTGGAAATTGATTCTCTCTTCGAAGGCATTGATTTTTAC
ACTTCCATCACCCGTGCAAGATTTGAAGAGCTTTGTTCTGACCTTTTCAGAGGAACTCTACA
CCCGGTGGAGAAAGCTCTACGAGATGCTAAGTTAGACAAGACAAGCATCCACGAAATCGTCT
TGGTAGGTGGGTCCACACGCATCCCCAAAGTGCAAAAACTACTTCAAGATTTCTTCAGTGGG
AAAGAACTGAACAAGTCCATTAACCCAGATGAAGCTGTTGCTTACGGTGCTGCAGTTCAAGC
AGCCATTTTACGTGGTGATCAGTCCGACACTGTGAAGGGCATGTTACTTCTTGATGTGCTCC
CACTTTCCATGGGTCTTGAGACAGCTGGAGGAGTCATGACAGTGCTTATTAAGCGCAATACC
ACAATTCCCACAAAGCAATCTCAGATCTTCACTACATATTCGGACAATCAACCAGGCGTTCT
CATTCAGGTATACGAAGGCGAACGAGCCATGACCAAGGATAATAATTTACTGGGCAAGTTTG
ATCTAAGTGGAATTCCTCCTGCTCCTCGTGGAGTGCCACAGATCGAAGTCACCTTCGATATT
GACGCGAATGG

25.HSP70 primer F215/R447

CAAGCGACAAAAGATGCAGGGACTATTGCAGGGTTCAAGGTTGAACGAATCATTAATGAGCC
GACGGCGGCGGCCGTCGCCTATGGTTTAAATGCAAAAAATAACAGCGAAGAAAAAAATATAT
TGGTCTTCGACTTCGGCGGTGGCACGTTTGATGTATCCGTCTTGACTATGGCCGAAGGGGTG
ATTGAGGTTAAGGCCACCGCTGGAAATACGCATTTGGGAGGGGAAGACATCGACGATAAGAT
GGTGGAACATTTTGTGAGAGAAATCAAGAGGAAATACAAAAAAGACATAAGGGACAACAAGC
GAGCGCTGAGACGCTTAAAAACCGCATGCGAACAAGCCAAGAGAACACTGTCATCGTCTACT
CAGGCTGAAATCACACTAGAGTCTCTCTGCGATGGCATCGATATAAACTTCGTCATGACCCG
TGCTAGATTCGACGAGATTTGCACGAATCTATTTCAGAGCACTATAGATCTCGTAAAAAANG
CTTTAGAAGACGCCAAGATGGACAAGAGTTCTATAACGACATCGTGGTTGGGCCGGAGGATC
TACCCGCATACCGAAGGTCCAAAACTGGTCCCGGGCTATTTTTGAAAAAGACCTGACAAATC
TATCAACCCCCGACGAAACTGTAATCACCTATG

26.HSP70 primerF215/R545

CAAGCGACAAAAGATGCTGGACAGATTGCAGGATTGAATGTTCTTCGTGTAATTAATGAGCC
AACTGCTGCTGCTCTTGCCTATGGCATGGACAAAACTGATGATAAAATCATTGCTGTGTATG
ACTTGGGTGGTGGAACTTTTGATATCTCTGTCCTGGAAATCCAGAAGGGTGTCTTCGAAGTG
AAGTCTACCAATGGTGATACTTTCCTTGGTGGAGAAGATTTTGACAATCACCTTGTGAATTT
CCTCGCTTCAGAATTTAAGCGAGAGCAAGGTGTTGATGTCACAAAGGACAACATGGCAATGC
AACGTCTGAAGGAGGCTTCAGAAAAGGCGAAGATTGAACTGTCTTCCTCCACACAGACTGAC
ATTAACCTGCCTTACCTTACCATGGACGCTTCAGGGCCAAAGCACATGATGTACAAGTTGAC
CCGATCCAAGTTTGAGAGCATCGTGGACAAGCTAGTGAAGCGCACTGTGGACCCCTGCCTCA
AGGCAATCAAAGATCTGAATGTGGCAAAGTCTGANATTGAGAAATCATNCNTGTGGGTGGCA
TGACCAAGATGCCCAAGGTGGGTACTACTGTGCAAGACATCTTTGGGCCGGGCCCCAAGTAA
GTCGGNGAATCCTGATGAAACTGNGCTGGGGGACCTGCCATNCACGGNGGAGTGCTTGCTNG
GGATGTAACAAANGTGCCGCTTCTTGATGTACCNCCCTTTTTNTTGGGATTGAGGCCCTNGG
GAGGATCTTGACTTANCTTATCAACNGGAACNCCCCCTTTCCCNCCCCCNAATNACAAGGNT
TNTTCCCAC

27.HSP90 primer F370/R1631

GGGGAGTTCAAGGCCTTCCTTCGTCACCGAGACAAGCTGCTTCCCGTCGTATTCCTTCAGCT
GCTGAACGCAGTATTCGTCGATGGGTTCGGTCATGTAGATGACCTCGAAGCCGCGCTTCTTC
ACCCTCTCCACGAAGGCAGAGTTCTGCACCTGTTCGCGAGTCTCGCCAGTGATGAAGTAGAT
GTGCTTCTGGTTCTCCTTCATGCGGGACACGTACTCCTTGAGGGAGGACATTTCGTCGCCAG
AGGCAGAAGTGTGGTACCTCAGGAATTCGGCAAGCTTCTTGCGGTTGGTGGAATCCTCGTGG
ATTCCGAGTTTGAGGTTCTTGGAGAAGTTTTCGTAGAACTTCTTGTAGCTTTCCTTGTCGTC
AACAATTTCTTCAAAAAGTTCGAGGGTCTTCTTGACGAGATTCTTTCCTGATAACTTTCAGG
ATCTTGTTCTGTTGCAGCATCTCACGAGAGATGTTGAGAGGCAGATCCTCGGAGTCGACGAC
ACCGTTGATGAAGTTCAGGACTCGGGATCAGTCCTCGCAGTCTCATGATGAACACGCGACGC
ACGTACAGCTTGATCTTGTTCTTCTGCTTGCGGTCTCGACAGTCAAGGGCGCGGCGCGGCAG
AACAGGAGGGCGCGGAACTCAAGCTGGCCCTCCACGCTGAAGTGCTTCACGGCCAGGTGGTC

CTCCCAGTCGTTGGTCAGCGACTTGTAGAACTCGCCGTACTCCTCCTTCGAGATGTCGTCGG
GGTTGCGCGTCCACAGGGGCTTCGTCTTGTTCAGCTCTTCGTCCTCCGTGTACTTCTCCTTC
ACCGTCTTCTTTTTCTTCTTGTCTTCGCCCTTCTCTTTGTCGGCTTCTTCGTCCTCGCCTAC
ATCTTCGATTTTGGGCTTGTCCTCCTCTGCTTCCTCTTCCTTCTCTTCTTTCTCCTCTTCCT
CATCGTCAGACACTTCCTTGTCCCTCTCCTTCTCGACGAGGAGCTTGATGGGATAGCCAATG
AATTGCGAGTGCTTCTTCACGATCTCCTTCACGCGACGCTCCTCGAGGTACTCTGTCTGGTC
CTCCTTCAGGTGGAGGGTGATCTTTGTACCACGGCCGATGGGTTCGCCGGTGTCGTGGCGCA
CCGTGAACGACCCGCCCGCCGACGACTCCCAGATGTACTGCTCGTCGTCGTTGTTCCTCGAC
ACTACGGTCACCTTGTCGGCCACCAGGTACGCGGAGTAGAAGCCCACG

28.HSP90 primer F445/R1631 (M13 Reverse)

CGAGCAGTACATCTGGGAGTCGTCGGCGGGCGGGTCGTTCACGGTGCGCCACGACACCGGTG
AACCCATCGGCCGTGGTACAAAGATCACCCTCCACCTGAAGGAGGACCAGACAGAGTACCTC
GAGGAGCGTCGCGTGAAGGAGATCGTGAAGAAGCACTCGCAATTCATTGGCTATCCCATCAA
GCTCCTCGTCGAGAAGGAGAGGGACAAGGAAGTGTCTGACGATGAGGAAGAGGAGAAAGAAG
AGAAGGAAGAGGAAGCAGAGGAGGACAAGCCCAAAATCGAAGATGTAGGCGAGGACGAAGAA
GCCGACAAAGAGAAGGGCGAAGACAAGAAGAAAAAGAAGACGGTGAAGGAGAAGTACACGGA
GGACGAGGAGCTGAACAAGACGAAGCCCCTGTGGACGCGCAACCCCGACGACATCTCGAAGG
AGGAGTACGGCGAGTTCTACAAGTCGCTGACCAACGACTGGGAGGACCACCTGGCCGTGAAG
CACTTCAGCGTGGAGGGCCAGCTTGAGTTCCGCGCCCTCCTGTTCCTGCCGCGCCGCGCCCC
CTTCGACCTGTTCGAGAACCGCAAGCAGAAGAACAAGATCAAGCTGTACGTGCGTCGCGTGT
TCATCATGGAG

29. HC-H-S01-0904-LF HSP70

GGCACGAGGGAGCGTGCCATGACCAAGGACAACAACCTCCTGGGTAAGTTCGAGCTGAGTGG
CATCCCACCTGCTCCCCGTGGCGTGCCTCAGATCGAGGTCACCTTCGACATCGACGCCAACG
GCATCCTGAACGTATCCGCCGTGGACAAGTCTACTGGTAAGGAGAACAAGATTACCATCACC
AACGACAAGGGTCGCCTCTCCAAGGAGGAGATCGAGCGCATGGTGCAGGACGCCGAGAAGTA
CAAGGCTGACGATGAGAAGCAGAGGGACCGTATTTCTGCCAAGAACTCCCTCGA

30.OV-N-S01-0834-W HSP75

GTTATAGCTATACGCACCCGTCGAAATTTACCCTCAGCTGAAAGGGAACNAAGGCTGGAGCT
CCACCGCGGTGGCGGNCGCTCTATGGAACTAGTGGATGCCCGCGGGGCTGCAGGAATTCGGC
ACGAGGGCTCCGCGTCGTCTGGCCAACAACAACAACAAGACATGGCTCGTGCGGCGTGGGTT
CGGCTGCGCTGGCGAGGAGGCTTTTCCTCGGCTCCTCGCTGAAATTCTCCGCGGGGGCCTTG
TCAGCTCTGTCAGGGAGGTCGAGGAGATATGGCTTAAGCAATTATGGACACTCAGGACCCCT
ACACAGGAACTTCCATTTAACTACTCAGAGGCTGCAAGAGGCAGTCAAAGCAGATGACGACA
AAACCTACGAGTTCCAAGCCGAGACCCGCATGCTGTTGGATATTGTGGCAAAGTCTCTCTAT
TCAGAAAAAGAGGTCTTTGTTAGAGAGCTGATTTCCAATGCCTCCGATGCTATTGAGAAGGC
CCGATACCTGGCTATTCAGGGGACGGAGTTGGACAGCTCGGAAGCCACCATGAAGATCCACA
TCACAACGGACAAGTACAGCAAAACGCTGACCATACAGGATTCAGGCATTGGAATGACCAAA
GAGGAGCTCATGGATAATTTGGGAACCATTGCACGATCGGGGTCAAAGGCCTTCATCCAGCA
GCTTCAGGAGGGAGGTGGTGCAGACCCCCAGAGTATTATCGGTCAGTTTGGTGTACGGTTTT
TACTCTGCTTTCATGGTTGCCGACAAGGTTGAAGTGTACACGAAGTCCCGTCTTCC

31.HC-H-S01-0553-LF

GGCACGAGGGGTTTGTGGACTGGTTGAGGTTTACTAACAAAATACAAAGCAAAGTACCATTG
TGTGTGTTGACTAGATTTCTTGTCTAGATCATGAAAGCTGAAGTTGGAGAGAGGCTGTTGGA
AACACCATGTGCCCTTGTTGCTTCCAAATTTGGTTGGACAGGAAACATGCAACGTATTGTAA
CCTCGCAGACGCATTCCAAGGCAAATGATGTGCAGAGGGAGTAAGTAGAAAGTTTTTCACAT
TGTGGAAAACTTGATTATTTTTTTTATGAATCTTGCTTTTTTTTGTCTTTTTTATGAAGTTT
CAAATATCTTTATTGAAAGGTATCTGCTAGTAACTCATGGAATTGTTACTGTTTTTATGCAT
GTTAATTTGCACAACTTGAAGAGGCATTGTGCCTATGCATAAATACAATTACTTAAGATTTA
TACCTCTTAATCCTTATTTGAACTTGTTTAGACTGTTAAGAGAAGGTACAGTTTTATCAGCA
GTATCGGTTTGTAAGTGTTCAACTTTTGAGTTTGGTGCTGTGCATTGTTGCATGAATAAGCA
AGCATATAGTTTTACACAGTGCAATTTGAAGTAACTATCATT

32.HPO-N-S01-0423-LF

GGCACGAGGAAGACAGTTGATAATAAGCTTCTGGTCCACGCCAAGCACGAGGAGAAGTCTGA
CAACCGCTCCGTCTACCGCGAGTACAACAGAGAGTTCCTGCTTCCCAAGGGCACCAACCCCG
AGCTCATCAAGTCCTCGCTCTCCAAAGACGGCGTCCTGACGGTGGAAGCTCCCCTGCCGGCC
ATAGCTGGGAACGAAGAGAAGGTCATCCCCATCGCACAGAACTGATTGACTGATTTCATCGC
TTCTGTCGTTACGAATTTTCGCCACAGTGTTGAAGGAAGGAAGCCACGTGTAAGAGGATTGG
AGGAATCATCTCGCCAGCATCAGTGTTTTTTTTATATATATATATCAAATCGATAGAGTAAA
ATTTGTGTTATTGTATGAAGAAAGGGAAAGTAAAAGGAAAGTCTCTTTTTTTTTAATAAATA
ATAAATGTGATTTTTTTTCTTATGTGTGAAACACCTTCATTATGCCTTTGGAAATTGCCTGG
GAACAATATTTTTTGTTCTAAGCGTGTTATTTTCAACATAGATTAAGAGATAAAACCAAATT
ATGTCCATGATCATATGTTAAATGTACAACTGCCAGTAATTT

33.HC-W-S01-0349-LF

ACGCTATACTTGATACGCACAGCTCGAAATTACCCTCACTAAAGGGAACAAAGCTGGAGCTC
GCGCGCCTGCAGGTCGACACTAGTTGGATCCAAAGAATTCGGCACGAGGCGGACGTGTAACA
ATTAGCTCTTAGGACTATTTAAAAATATCTAAAATAAGATAAAATGGCAAAGGCACCTGCTG
TCGGTATTGATCTGGGAACCACCTACTCCTGCGTGGGTGTGTTCCAGCATGGCAAGGTGGAG
ATCATCGCCAACGACCAGGGCAACCGCACCACGCCCTCCTACGTCGCCTTCACAGACACAGA
GCGTCTGATTGGTGAC

BIOGRAPHY

Miss Atchariya Buethong was born on January 13, 1970 in Ubonratchathani, Thailand. She graduated with the degree of Bachelor of Science in Biology from Ramkhamhang University in 1993. She has studied for a degree of Master degree of Science at the Department of Biotechnology, Chulalongkorn University.