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นางสาวภรณี เพริศพรายวงศ์

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GENE CLONING, EXPRESSION AND CHARACTERIZATION OF CHITINASE FROM BLACK TIGER SHRIMP *Penaeus monodon*

Miss Porranee Proespraiwong

A Thesis Submitted in Partial Fulfillment of the Requirements

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Field of Study	Biotechnology
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ไคทิเนสเป็นเอนไซม์ที่จำเป็นต่อครัสเตเชียนและสัตว์ในกลุ่มใกล้เคียงกันในการลอกคราบและการย่อยอาหารที่มีไคทินเป็นองค์ประกอบ จากฐานข้อมูล EST ของกุ้งกุลาดำ พบ cDNA contig และ singleton ของไคทิเนส 3 ชนิด คือ *PmChi1*, *2* และ *3* ได้ทำการเพิ่มปริมาณยีนของ mature *PmChi1*, *3* และบางส่วนของ *PmChi2* และทำการโคลน กรอบอ่านรหัสของ *PmChi1* และ *3* แปลรหัสได้เป็น mature protein ที่มีกรดอะมิโน 644 และ 468 ตัว ซึ่งมีน้ำหนักโมเลกุล 72.4 และ 51.9 กิโลดาลตัน ตามลำดับ เมื่อเปรียบเทียบลำดับกรดอะมิโนของไคทิเนสในกุ้งสกุลพีเนียส พบว่า มีความคล้ายคลึงกันสูงมากถึงร้อยละ 90 โดย *PmChi1*, *2* และ *3* ถูกจัดกลุ่มร่วมกับไคทิเนสจากครัสเตเชียนอื่น ๆ และแมลงได้เป็น 3 กลุ่ม ซึ่งแยกออกจากไคทิเนสจากสัตว์เลี้ยงลูกด้วยนม *PmChi1* และ *3* มีการแสดงออกมากในตับ ในขณะที่ *PmChi2* มีการแสดงออกมากในเหงือก แต่ก็สามารถแสดงออกได้ในปริมาณเล็กน้อยในเนื้อเยื่ออื่น การแสดงออกของ *PmChi2* หลังการลอกคราบลดลง ในขณะที่การแสดงออกของ *PmChi1* และ *3* ไม่เปลี่ยนแปลง ผลเหล่านี้แสดงให้เห็นว่า *PmChi2* น่าจะเกี่ยวข้องกับการลอกคราบ ในขณะที่ *PmChi1* และ *3* อาจจะทำหน้าที่ในการย่อยอาหารที่มีไคทินเป็นองค์ประกอบ รีคอมบิแนนท์ *Pm*Chi1 (r*Pm*Chi1) ที่ผลิตได้ใน *Escherichia coli* มีค่าความเป็นกรด-ด่างที่เหมาะสมต่อการทำงานเป็น 5 แต่มีความเสถียรมากที่สุดที่ความเป็นกรด-ด่างที่เป็นกลาง ที่น่าสนใจ คือ อุณหภูมิที่เหมาะสมต่อการทำงานของเอนไซม์มีค่าสูงถึง 55 องศาเซลเซียส แม้ว่าจะมีความเสถียรที่อุณหภูมิต่ำกว่า 40 องศาเซลเซียส r*Pm*Chi1 ย่อยสารตั้งต้นที่ละลายได้ เช่น ไคทินที่ถูก *N*-acetylate บางส่วน (PNAC) และไคทินคอลลอยด์ลจากเปลือกกุ้งได้ดีกว่า β-ไคทินจากแกนหมึก

สาขาวิชา.....เทคโนโลยีชีวภาพ..... ลายมือชื่อนิสิต.....ภรณี เพริศพรายวงศ์.....

ปีการศึกษา..... 2552..... ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

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PORRANEE PROESPRAIWONG : GENE CLONING, EXPRESSION AND CHARACTERIZATION OF CHITINASE FROM BLACK TIGER SHRIMP *Penaeus monodon*. THESIS ADVISOR : ASSOC. PROF. VICHIEN RIMPHANITCHAYAKIT, Ph.D., THESIS CO-ADVISOR : PROF. ANCHALEE TASSANAKAJON, Ph.D., 124 pp.

Chitinases are essential enzymes for crustaceans and animal alike for their molting and digestion of foods containing chitin. From the *Penaeus monodon* EST database, cDNA contigs and singletons for three chitinases, namely *PmChi1*, *2* and *3*, were identified. The complete sequences of the mature *Pm*Chi1, 3 and partial *Pm*Chi2 were amplified and cloned. The reading frames of *PmChi1* and *3* encoded mature proteins of 644 and 468 amino acids with calculated molecular weights of 72.4 and 51.9 kDa, respectively. The amino acid sequence comparison among the penaeid chitinases revealed very high homology around 90%. They were, thus, grouped together along with those of other crustaceans and insects into three groups separated from those of mammals. The *PmChi1* and *3* were expressed mainly in hepatopancreas, whereas *PmChi2* were in gill. Small amount of them could be expressed in other tissues. After molting, only the expression of *PmChi2* was down-regulated while the expression of *PmChi1* and *3* was relatively unchanged. The results suggested that the *PmChi2* was likely involved in molting while the others might function in the digestion of chitinous foods. The recombinant *Pm*Chi1 (r*Pm*Chi1) over-produced from *Escherichia coli* had its optimal pH 5 but it was most stable at neutral pH. Interestingly, the optimal temperature was relatively high at 55 °C. Nevertheless, it was stable at lower temperature below 40 °C. The r*Pm*Chi1 preferentially hydrolyzed the more soluble substrates like partially *N*-acetylated chitin (PNAC) and colloidal chitin from shrimp shell as compared to the β-chitin from squid pen.

Field of Study : .....Biotechnology .... Student's Signature ...Porranee Proespraiwong.....
Academic Year : .....2009..... Advisor's Signature .....
Co-advisor's Signature .....

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LIST OF ABBREVIATIONS

bp	base pair
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
DEPC	diethylpyrocarbonate
dGTP	deoxyguanosine triphosphate
DNA	deoxyribonucleic acid
dTTP	deoxythymidine triphosphate
kb	kilobase
kDa	kilodalton
M	molar
mg	milligram
mL	milliliter
mM	millimolar
ng	nanogram
nm	nanometre
A	Absorbance
^{o}C	degree Celcius
PCR	polymerase chain reaction
ppt	parts per trimillion
RNA	ribonucleic acid
μg	microgram
μL	microliter
μM	micromolar

CHAPTER I

INTRODUCTION

1.1 Chitin

Chitin $(C_8H_{13}O_5N)_n$, is a long, unbranched insoluble polysaccharide of an amino sugar *N*-acetyl-β-D-glucosamine covalently bonded together by β-1,4 linkages or *O*-glycosidic bonds (Figure 1.1) (Muzzarelli and Peter, 1997). It is one of the most abundant polysaccharide in nature and found as structural constituent of fungal cell wall (Duo-Chuan, 2006) and cuticle and integument of some animals such as annelids (Talmont and Fournet, 1990), nematodes (Harris and Fuhrman, 2002; Foster et al., 2005), mollusks (Weiss et al., 2006; Weiss and Schönitzer, 2006) and particularly arthropods including crustaceans (Welinder, 1974; Acosta et al., 1993; Kurita, 2006) and insects (Vardanis, 1979; Merzendorfer and Zimoch, 2003; Arakane and Muthukrishnan, 2009).

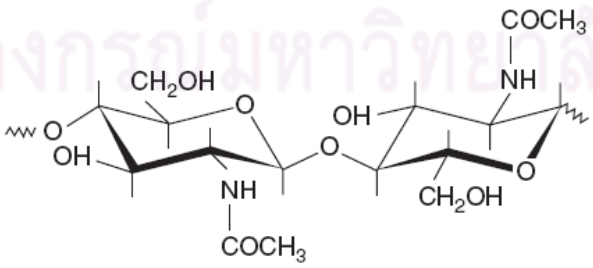

Figure 1.1 Schematic representation of completely acetylated chitin. (Muzzarelli and Peter, 1997)

The X-ray diffraction indicates three polymorphic configurations of chitin existed in nature, namely α-, β-, and γ-chitins which differ in the degree of hydration, size of the unit cell, the number of chitin chains per unit cell and the arrangement of molecular chains within the crystalline cell (Figure 1.2) (Rudall and Kenching, 1973). The α-chitin is the most stable thermodynamically, most crystalline, most compact and, hence, most abundant form. It can be found in the cuticle, integument and exoskeleton of the arthropods including crustaceans and insects. The unit cell of α-chitin consists of two antiparallel polymer chains and there is no water molecule within. Adjacent sheets along the *c* axis have the opposite direction (Carlsstrom, 1957; Minke and Blackwell, 1978). Meanwhile, the β-chitin, the rarer form, is found in squid pens, the spines of some diatoms, the tubes of pogonophores and vestimentiferans. The unit cell of β-chitin contains a parallel arrangement of the chains. Adjacent sheets along the *c* axis also have the same direction (Blackwel, 1969). For the γ-chitin, chains are in mixed character of either α- or β-chitins rather than a true third polymorphic form. Every third sheet has the opposite direction to the two preceding sheets, for example, two out of three chains are parallel with the third oriented in the opposite direction (Peberdy, 1985; Roberts, 1992a).

The distribution of the polymorphic configurations of chitin is not associated with taxonomy. One organism probably has chitin in many forms depending on the biological function of chitin in that organism. The α-chitin commonly tenders rigidity (Rudall and Kenching, 1973) whereas the β- and γ-chitins are for toughness, flexibility and motility (Muzzarelli, 1977). The chitin has strong intramolecular and intermolecular bonds among the acetamide and hydroxyl groups leading to close packing of the chains, sometimes with highly ordered and crystalline structure. These

result in insolubility in water and common solvents, high crystallinity and low reactivity (Urbanczyk et al., 1997). The β-chitin can swell in water but not the α-chitin since the α-chitin has more powerful intermolecular hydrogen bonding (Minke and Blackwell, 1978).

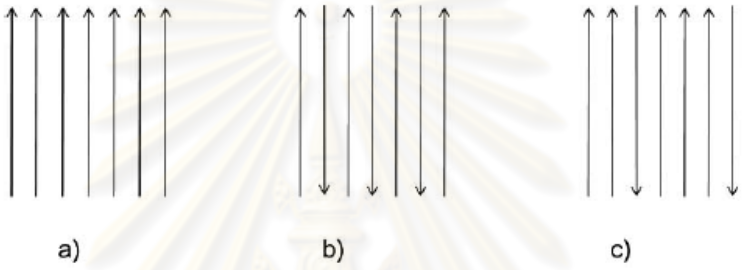

Figure 1.2 A schematic representation of arrangement of the polymer chains in the three polymorphic structures of chitin: a) β-chitin, b) α-chitin and c) γ-chitin.

1.2 Chitosan

Chitosan is a polycationic linear copolymer of supposedly alternating *N*-acetyl-D-glucosamine and D-glucosamine bonded together with β-1,4 glycosidic linkages (Figure 1.3). However, each preparation of chitosan is different owing to the degree of acetylation, size of the polymer and arrangement of different monomer units. Chitosan has two free hydroxyl groups and one primary amino group in each repeating hexosaminide residue (Sandfjord, 1989; Roberts, 1992b).

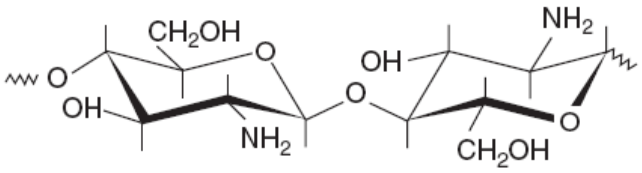

Figure 1.3 Schematic representation of completely deacetylated chitosan.

Chitosanases (EC 3.2.1.123) are hydrolytic enzymes that catalyze the random hydrolysis of the β-1,4 glycosidic bonds of chitosan (Figure 1.4) (Fukamizo, 2000). Several enzymes, comprising cellulases, hemicellulases, lysozymes, lipases and proteases have been used to deputize for chitosanases in chitosan hydrolysis (Yalpani and Pantaleone, 1994; Zhang et al., 1999; Kumar et al., 2005; Kumar, 2007; Roncal et al., 2007). Chitosan oligosaccharides can be produced by chemical or enzymatic hydrolysis of chitosan. Higher yield and friendlier environment are beneficial of enzymatic method (Ilyina et al., 1999; Kuroiwa et al., 2003; Sørbotten et al., 2005). Chitosan oligosaccharides have been wildly used in numerous applications with good attribution, such as antibacterial activities, antifungal activities, activation of pathogenesis-related proteins in higher plants, immunopotentiating effectors, antitumor property, anticancer property, antidiabetic effects, and specific inhibitors of family 18 chitinases (Walker-Simmons et al., 1983; Tokoro et al., 1988; Hadwiger et al., 1994; Jeon et al., 2001; Lee et al., 2003; Huang et al., 2006; Yoon et al., 2007; Moon et al., 2007; Palma-Guerrero et al., 2008; Cho et al., 2008; Rahman et al., 2008; Cederkvist et al., 2008).

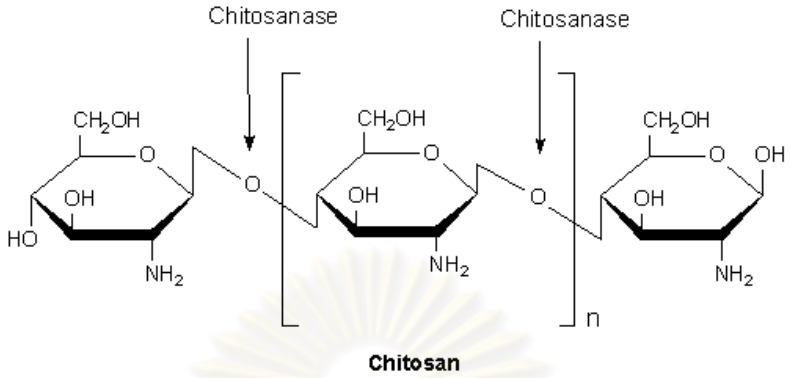

Figure 1.4 The hydrolysis locales of chitosanases on a chitosan molecule (Fukamizo, 2000).

Chitin can be converted into chitosan by partial deacetylation. This deacetylation can be achieved using the chemical or the enzymatic processes. Chitin deacetylase (EC 3.5.1.41) is the enzyme catalyzing the deacetylation of *N*-acetamido bonds in the chitin to generate chitosan transforming the GlcNAc (*N*-acetyl-β-D-glucosamine) to GlcN (2-amino-2-deoxy-β-D-glucose) (Figure 1.5) (Araki and Ito, 1975). It is categorized in carbohydrate esterase family 4 (Caufrier, 2003). Chitin deacetylases have been isolated from the fungi *Mucor rouxii* (Araki and Ito, 1975; Kafetzopoulos et al., 1993), *Colletotrichum lindemuthianum* (Tsigos and Bouriotis, 1995; Tokuyasu et al., 1996), *Absidia coerulea* (Gao et al., 1995), *Aspergillus nidulans* (Alfonso et al., 1995) and *Saccharomyces cerevisiae* (Christodoulidou et al., 1996).

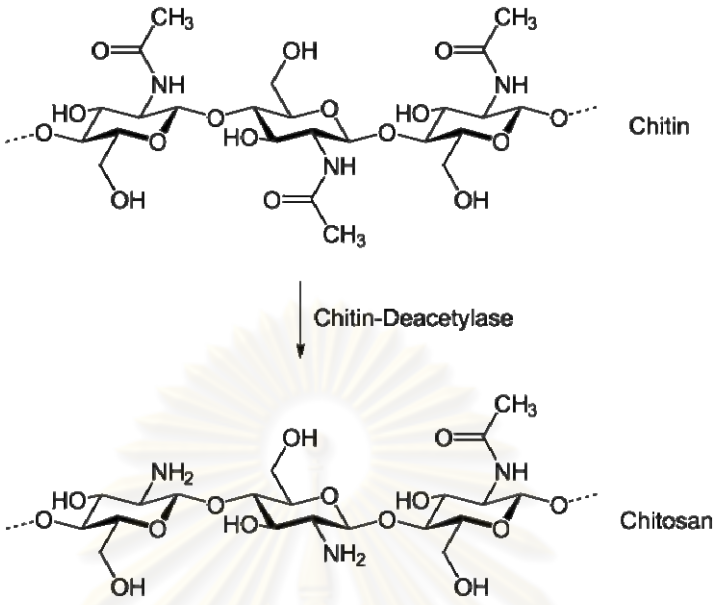

Figure 1.5 Deacetylation of chitin.

1.3 Applications of chitin and chitosan

Chitin and chitosan are utilized in numerous applications comprising pharmacy and medicine, food, cosmetics, agriculture, industry, textile, fishery, ecology and nanoparticles.

1.3.1 Pharmacy and medicine

Chitosan possesses low toxicity, biodegradability and biocompatibility leading to its considerable efficiently applied in pharmaceutical and biomedical utilization, for example, wound healing, gene delivery, drug delivery, immunoadjuvant strategies, tissue engineering, anticancerogen, antiviral, antimicrobial, bacteriostatic, fungistatic, hemostatic, and anticholesteremic properties. Although chitin is also biodegradable and biocompatible but insoluble in water and

common solvents due to high crystallinity and low reactivity, these result in less attention than chitosan (Singla and Chawla, 2001; Hejazi and Amiji, 2003; Jiang et al., 2007; Lee et al., 2006; 2009). Three areas of applications in pharmacy and medicine are discussed.

During wound healing, both chitin and chitosan not only encourage granulation with angiogenesis but also promote cell organization. They lead to reepithelization, tissue regeneration, and abatement of scar. Oxygen permeability, fungicidal and bactericidal activities are the main concerns to wound healing.

Since the water-soluble chitosan is capable of curing wound, and heparin is able to interact and stabilize growth factors dealing with the wound healing process, one can develop the water-soluble chitosan/heparin complex as an effective wound curing accelerator. Gross and histologic examination on rat skin indicate that the wound treated with water-soluble chitosan/heparin complex ointment shows nearly complete regeneration of appendage structure similar to normal in the dermis but not in untreated wound and wound treated with water-soluble chitosan ointment which show no and less number of skin appendages, respectively (Kweon et al., 2003).

The characteristic polycationic polymer of chitosan renders it complex to a negatively charged plasmid DNA. Thus, it is useful as non-viral gene delivery systems. Self-aggregates of hydrophobically modified chitosan by deoxycholic acid can generate charged complexes with the plasmid DNA. This is employed for the transfer of genes into the mammalian cells *in vitro* (Lee et al., 1998).

The drug delivery system also employs chitosan as vehicles in the form of granules and beads to move drugs to the target tissues, especially for oral

drugs (Ravi-Kumar, 2000). The nasal drugs and vaccines are delivered in chitosan nanoparticles, which assist macromolecules penetration through the nasal barrier (Hejazi, 2002). Antithrombotic activities of chitosan/polyethylene glycol/alginate miscrospheres could be applied in transference of low molecular weight heparin (Chandi et al., 2000). Meanwhile, chitosan/xanthan microspheres are suitable for the effective drug transportation in gastrointestinal tract (Chellet et al., 2000).

The chitosan and gelatin crosslinking with glutaraldehyde swells at low pH but de-swells at high pH. It is a good candidate for pH-sensitive drug transfer process since this matrix release drugs based on pH (Yao et al., 1995). Chitosan/carboxymethyl cellulose microcapsules are used as drug carriers for oral uptake with low pH endurance properties in the stomach (Bayoni, 2003).

1.3.2 Foods

Chitosan has an amphiphilic property, an air/solution surface activity, an electrical conductivity, a hydrophilic-lipophilic balance and an ability to increase the viscosity of the continuous aqueous phase. Hence, it is a stabilizer or emulsifier in food emulsions. Chitosan can be adsorbed at the oil/water interface, but not at the air/solution interface, increasing both mechanical and electrostatic stability to the droplets. Thier size can be controlled by the chitosan/oil ratio. Chitosan molecules are less deacetylated causing more hydrophobic environments, which stabilize the water droplets inside the oil drops. In contrast, if thier molecules are more deacetylated, they cause more hydrophilic environments, which stabilize the oil drops in the multiple emulsions (Schulz et al., 1998).

Chitosan probably influences the resorption of lipids in the intestinal tract (Sandfjord, 1989). Further, it leads to a combination of hydrophobic and

electrostatic interactions between the lipid droplets and polyelectrolytes in the intestine (Israelachvili and Adams, 1978; Onsøyen, 1991). For these reasons, a hypocholesterolemic agent is employed in the presence of chitosan for food additive (Furda, 1980).

Chitin and chitosan possess antiviral, fungicidal and bactericidal activities, particularly antibacterial property of low molecular weight chitosan (Sekiguchi et al., 1993). Polycationic chitosan strongly interacts with the negatively charged microbial cell membranes having the opposite charges, resulting in the leakage of proteinaceous and other intracellular constituents. Both biopolymers are used for food preservation in lieu of synthetic food preservatives (Ghaouth et al., 1992; 1997; Darmadji and Izumimoto, 1994; Ouattar et al., 2000; Coma et al., 2002; Tsai et al., 2002).

1.3.3 Cosmetics

Chitosan is a component in skin and hair supplements with the characteristics of polycationic polymer causing it to interact with the negatively charged biological surfaces (Juneau et al., 2001). It is incorporated in hair treatment to decrease the adhesion leading to smooth hair (Dee et al., 2001). Chitosan resists inflammation and activates the regeneration of damaged tissues (Wachter and Stenberg, 1997). Additionally, it has the property of water shade which is additive to the sun-block cream for skin (Horner et al., 1997). High molecular weight chitosan reduces transpiration, absorbs humidity and has antibacterial activity leading to the combination in the deodorants (Hohle and Griesbach, 1998). In addition, it also retains water in skin, increases flexibility and suppleness of skin, and decreases skin irritation (Juneau et al., 2001). While a low molecular weight chitosan is an anticavity

agent, incorporating it in chewing gums, oral rinsing solutions and toothpastes helps to fight the antibacterial activities such as *streptococci* in the mouth (Sano et al., 2003).

1.3.4 Agriculture

Defensive mechanisms in plants are enhanced by chitin and its derivatives (Yamaguchi et al., 2000). Chitosan with antimicrobial activity is utilized in the post-harvest preservation of vegetables and fruits (Galed et al., 2004). Chitin and chitosan have been employed against the parasitic nematodes in soils (Gooday, 1990), and induce symbiotic interactions between plant and microorganism in soil (Schisler et al., 2004). Antifungal properties of them are exploited for the control of many phytopathogenic fungi. Additionally, chitosan and its derivatives also have antiviral and bactericidal activity (Struszczyk et al., 1989). They increase germination and crop yields (Hirano et al., 1989; Hadwiger, 1992).

1.4 Chitinolytic enzymes

Chitinolytic enzymes are hydrolytic enzymes that catalyze the hydrolysis of the β-1,4 glycosidic bonds between the *N*-acetylglucosamine residues of chitin (Figure 1.6). They were found in many organisms such as virus, bacteria, fungi, higher plants, and animals. Their important roles are diverse among each type of organism. Viral chitinases are suggested to play roles in pathogenesis (Patil et al., 2000). Bacteria can consume chitin as a major carbon and energy source, and recycle chitin in nature (Tsujibo et al., 1993; Park et al., 1997; Wang and Chang, 1997; Svitil et al., 1997). Some bacterial chitinases also play an important role in the control of pathogenic fungi. In fungi, chitinases play the physiological and biological roles, such

as anti-parasitic, morphogenetic, nutritional and autolytic roles (Adams, 2004). Plants chitinases are pathogenesis-related proteins, which are involved in plant defense responses against pathogens (Graham and Sticklen, 1994). Known functions of chitinases in arthropods, insect and crustaceans are the degradation of the chitinous exoskeleton for molting (Merzendorfer and Zimoch, 2003). Chitinases encoded by human have a role in defense against chitinous human pathogens (Boot et al., 2001; 2005; Eijk et al., 2005).

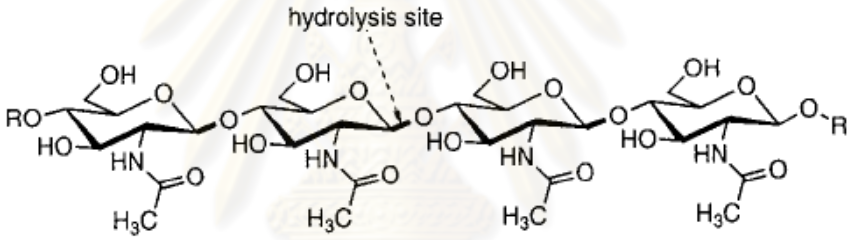

Figure 1.6 The hydrolysis locale of chitinases on a chitin molecule.

1.5 Types of chitinolytic enzymes according to substrate specificity and production yields

There are two broad groups of chitinases on the basis of substrate specificity and production yields. The first group, endochitinases (EC 3.2.1.14) are biocatalyte, which products of the reaction are soluble, low molecular mass oligomers of *N*-acetyl-β-D-glucosamine with various sizes such as chitobiose, chitotriose, and chititetrose, by randomly hydrolyze inside the chitin chain. The second group is exochitinases that

can be subcategorized into two minor groups, chitobiosidases and β-(1,4)-*N*-acetylglucosaminidases. Chitobiosidases or chitin-1,4-β-chitobiosidases (EC 3.2.1.29) generate sole chitobiose via digestion at nonreducing end of chitin chain. β-(1,4)-*N*-acetylglucosaminidases or chitobiases (EC 3.2.1.30) release monomers of *N*-acetyl-β-D-glucosamine from the yields occurring from endochitinases and chitobiosidases (Figure 1.7) (Dahiya et al., 2006).

Chitinases from crustaceans represented the characteristic glycosyl hydrolase catalytic domain, family 18, that has a three-dimensional structure fold as a TIM-barrel, $(\beta/\alpha)_8$-fold, with a tunnel-like active site (Scheltinga et al., 1996; Suzuki et al., 1999).

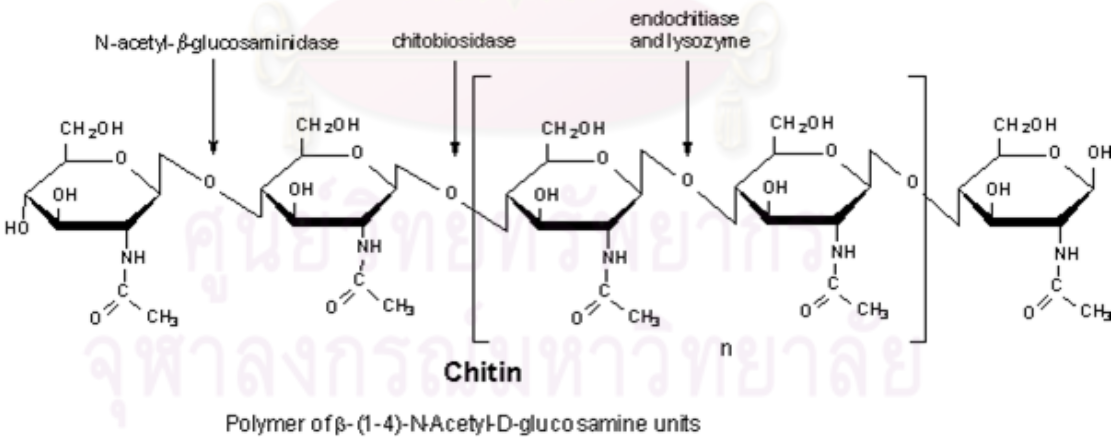

Figure 1.7 Chitinolytic enzymes specificity.

1.6 Classification of chitinolytic enzymes based on amino acid sequence similarities

Chitinolytic enzymes can be divided into 3 families of glycoside hydrolases in terms of amino acid sequence similarities, families 18, 19, and 20 (Henrissat and Bairoch, 1993) that differ in structure and mechanism. Chitinases in family 18 are diverse in evolutionary terms and widely express in a variety of organisms whereas family 19 chitinases are identified in *Streptomyces griseus* HUT6037 (Ohno et al., 1996), higher plants (Henrissat, 1999). Both families have significantly different amino acid sequences, three-dimensional structures and catalytic mechanisms. On the other hand, the β-*N*-acetylhexosaminidases, which catalyze the cleavage of terminal β-*N*-acetylglucosamine or β-*N*-acetylgalactosamine residues from the glycol-conjugates from *Streptomyces plicatus* (Mark et al., 1998), *Serratia marcescens* (Prag et al., 2000), *Aeromonas caviae* CB101 (Lin et al., 2006), and humans (Intra et al., 2008) are members of family 20. Their catalytic domain is an α/β TIM-barrel and the active site lies at the center of the barrel convex side (Gravel et al., 1995; Tews et al., 1996; Fernandes et al., 1997; Mark et al., 1998).

1.7 Hydrolysis mechanisms of chitinases

For the mechanism of glycosyl hydrolases, either retention or inversion of the anomeric configuration at C1′ is a step in acid-catalyzed hydrolysis. According to both theoretical models (Brameld and Goddard III, 1998) and crystallographic structural data (Scheltinga et al., 1995), the double-displacement retaining mechanism of family 18 chitinases involves anchimeric assistance by the C2′ *N*-acetyl group

(Scheltinga et al., 1995) and substrate distortion of the sugar residue to a boat conformation (Brameld and Goddard III, 1998). The hydrolysis yields a β-anomer (Figure 1.8a) (Armand et al., 1994; Scheltinga et al., 1995; Iseli et al., 1996). Meanwhile, the single-displacement inverting mechanism of family 19 chitinase results in the α-anomer (Figure 1.8b) (Fukamizo et al., 1995).

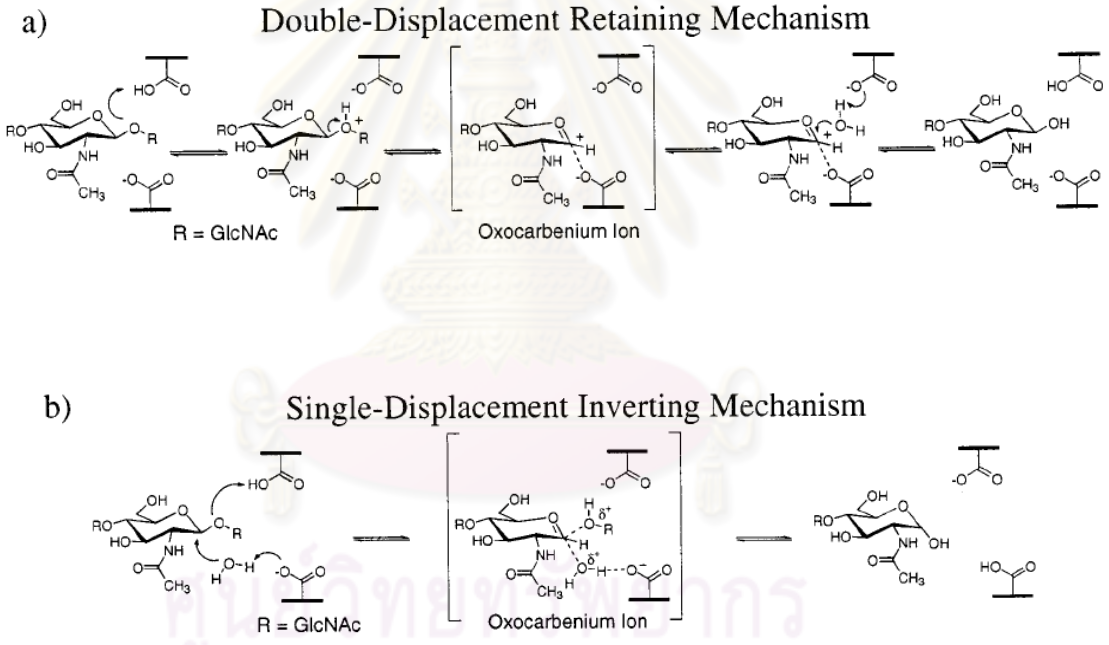

Figure 1.8 Hydrolysis mechanisms of chitinases. (a) Hydrolysis reaction of family 18 chitinases via the double-displacement retaining mechanism. A GlcNAc residue protonated in a boat conformation generating an oxazoline intermediate, which subsequence hydrolyzed so as to be produces a product with retention of the anomeric configuration. (b) Hydrolysis reaction of family 19 chitinases via the single-displacement inverting mechanism. Two acidic residues are required in the active site and the hydrolysis product has inversion of the anomeric configuration.

1.8 Chitinases in crustaceans

In crustaceans, chitin is a major component of the outer shell or exoskeleton covering the entire body of the animal providing protection for the animals. Under development, growth and certain environmental conditions, the crustaceans shed off their shells and regenerate the new ones in a molting process (Spindler-Barth et al., 1990; Kono et al., 1995). In the molting process, the old exoskeleton is digested by chitinolytic enzymes. To completely digest the chitinous shell, two chitinolytic enzymes are involved. The chitinase (EC 3.2.1.14) degrades chitin into chitooligosaccharides and then the β-*N*-acetylhexosaminidase (EC 3.2.1.52) hydrolyzes the chitooligosaccharides to *N*-acetylglucosamine monomer (Kramer and Muthukrishnan, 1997). Therefore, the chitinolytic enzymes are indispensable for crustaceans. Not only are they involved in the molting cycle, but the enzymes are also used for chitinous food digestion and probably defense against chitin-bearing pathogens.

There are some previous researches about chitinases in shrimps, which has more than one chitinase in one shrimp species. The activities of chitinolytic enzymes in the cuticle of shrimp increased before molting. This suggests that they are important for the break down of chitin-containing outer skeleton (Spindler-Barth et al., 1990; Watanabe and Kono, 1997; Buchholz, 1989). On the contrary, the activities of chitinolytic enzymes in the hepatopancreas over the molting cycle are quite constant. Their role may be involved in the degradation of chitinous foods (Buchholz, 1989; Spindler-Barth et al., 1990; Kono et al., 1995).

1.8.1 Chitinases in *Pandalus borealis*

For *Pandalus borealis*, chitinases (I, IIa, IIb, III, and IV) were isolated from the hepatopancreas with the molecular weights of 61, 69, 39, 57, and 54 kDa, respectively. They can not hydrolyse *N*,*N'*-diacetylchitobiose or *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide, but hydrolyse longer chitooligosaccharides or nitrophenylated chitooligosaccharides. The chitinases I and IIa randomly cleave the chitin for they are grouped as endochitinases whereas the chitinases III and IV generate chitobiose for they are chitobiosidases (Esaiassen et al., 1996).

1.8.2 Chitinases in *Marsupenaeus japonicus*

In penaeid shrimp, three genes coding for chitinases of family 18 glycosyl hydrolases are identified in *Marsupenaeus japonicus* (*Penaeus japonicus*), namely *Pjchi-1*, *Pjchi-2* and *Pjchi-3* (Watanabe et al., 1996; 1998; Watanabe and Kono, 1997). A cDNA encoding a chitinase, Pjchi-1, was isolated from the kuruma prawn *Penaeus japonicus* by PCR amplification of a hepatopancreas cDNA library using degenerate oligonucleotide primers derived from two conserved regions of known chitinases. The Pjchi-1 contains 572 amino acids, which is similar to other eukaryotic chitinases, most similar to a chitinase precursor from tobacco hornworm *Manduca sexta* (Watanabe et al., 1996). The *Pjchi-1* and *3* are expressed in the hepatopancreas but not in cuticular body parts. The expression of *Pjchi-3* transcripts was also relatively unchanged during the molting. They were probably involved in the digestion of chitinous food.

The *Pjchi-2* identified from the cDNA library of tail fan and blade is implicated to be involved in molting. The Pjchi-2 transcript is expressed in the tail fan and blade at late premolt states (states D3 and D4) but not in the intermolt state (state

C). The expression of *Pjchi-2* is up-regulated during the premolt stages in cuticular body parts prior to molting (Watanabe and Kono, 1997). Therefore, the possible role of Pjchi-2 is the digestion of chitinous exoskeleton and underlying epidermis in cuticular tissues upon molting (Watanabe and Kono, 1997). On the other hand, the Pjchi-3 from the hepatopancreas, 56.6 kDa, is expressed to certain quantity at the late premolt stage (stage D3 and D4) close to the intermolt stage (stage C) but the transcripts do not accumulate in the cuticular body parts (Watanabe et al., 1998).

1.8.3 Chitinases in *Penaeus monodon*

The fluctuation of both proteases and carbohydrases level including chitinases are observed during the developmental stages of *P. monodon*. The nauplius stage could detect a valuable chitinase activity. It reachs a maximum activity in zoea stage and is, subsequently, decreased to mysis and PL 10 stages, respectively. It, afterwards, remains constant in other stages: PL 10, juvenile and adult stages (Fang and Lee, 1992). A gene coding for *P. monodon* chitinase 1 (*PmChi-1*) was isolated from the cDNA library of *P. monodon* (Tan et al., 2000). Comparison of amino acid sequences indicates that the PmChi-1 of 621 amino acids was 80% homologous to Pjchi-1. RT-PCR analysis revealed that PmChi-1 was expressed in the hepatopancreas and the gut. The expression of *PmChi-1* was found variably at various molting stages but highest at premolt D_2 stage but none at stage D3. It was postulated, then, that the PmChi-1 might function in the digestion of endogenous chitin in the gut ahead of molting.

1.8.4 Chitinases in *Fenneropenaeus chinensis*

Most recently, a chitinase gene equivalent to *Pjchi-3* from *Fenneropenaeus chinensis*, *Fcchi-3*, was isolated (Zhang et al., 2010). At the cDNA

level, the Fcchi-3 has maximum identities of 92.0 and 91.4% to *L. vannamei* and *M. japonicus*, respectively. The predicted amino acid sequence was very similar to those of *L. vannamei* and *M. japonicus*. From the phylogenetic tree analysis, the Fcchi-3 was 96.8% most similar to Lvchi and 93.4% to Pjchi-3. The Fcchi-3 transcript was only detectable in the hepatopancreas. After injection of WSSV for 3 h, the Fcchi-3 mRNA in hepatopancreas was decreased and reached the minimum level at 5 h followed by returning to the original state at 37 h. The expression of *Fcchi-1* and *Fcchi-3* transcripts in the whole juvenile shrimp during the molt cycle of *F. chinensis* had been investigated. They were detected in all stages of molting with considerable fluctuations observed in the premolt D stages. The significance of this finding was not discussed (Priya et al., 2009).

1.9 Applications of chitinases

1.9.1 Antifungal activity

Fungi control currently relies on the use of inefficient chemical fungicides, given several disadvantages. Alternatively, many fungi are phytopathogens, which can be protected with some enzyme-secreted organisms. These are interesting biological control methods for they are not harmful to the environments. There are abundant researches pertaining to biofungicidal activity of chitinases that can be utilized in this application.

Plant chitinases are pathogenesis related proteins, which are involved in plant defense responses against pathogens. Rohini and Sankara reported that fertile transgenic plants of peanut (*Arachis hypogaea* L.) containing transgenically increased activity of expressing tobacco chitinase were resistant to attack by the fungal

pathogen *Cercospora arachidicola* (which causes leaf spot or Tikka disease of peanut) to different degrees which leads to increase ability of these plants to resist *C. arachidicola* (Rohini and Rao, 2001).

A recombinant chitinase (Chi3K) of Koshu grapes (*Vitis vinifera* cv.) restricted the spread of *Botrytis cinerea*, which caused grey mold disease in grapes (Ano et al., 2003). A 30.8 kDa chitinase from mung bean (*Phaseolus mungo*) seeds resisted against *Fusarium solani*, *Fusarium oxysporum*, *Mycosphaerella arachidicola*, *Pythium aphanidermatum*, and *Sclerotium rolfsii* (Yea and Ng, 2005).

A recombinant chitinase from wheat, a 33 kDa chitinase, exhibited a broad-spectrum inhibition activity against *Colletotrichum falcatum* (red rot of sugarcane), *Pestalotia theae* (leaf spot of tea), *Rhizoctonia solani* (sheath blight of rice), *Sarocladium oryzae* (sheath rot of rice), *Alternaria* sp. (grain discoloration of rice), and *Fusarium* sp. (scab of rye) (Singh et al., 2007). Similarly, 35 kDa recombinant chitinase from barley showed broad spectrum antifungal activity toward *Botrytis cinerea* (blight of tobacco), *Pestalotia theae*, *Bipolaris oryzae* (brown spot of rice), *Alternaria* sp., *Curvularia lunata* (leaf spot of clover) and *Rhizoctonia solani* (Kirubakaran and Sakthivel, 2007).

Some chitinases from microorganisms play an important role in the control of pathogenic fungi. For example, *Bacillus cereus* QQ308 secretes antifungal hydrolytic enzymes including chitinase, chitosanase and protease that hamper the spore germination and germ tube elongation of the plant-pathogenic fungi *Fusarium oxysporum*, *Fusarium solani*, and *Pythium ultimum* (Chang et al., 2007)**.** The chitinase produced from *Bacillus subtilis* CHU26, a 64 kDa chi18, displays antifungal activity toward *Rhizoctonia solani* (Yang et al., 2009).

1.9.2 Insecticidal activity

Nowadays, chemical insecticides are excessively consumed in agriculture resulting in more and more problems on the environment and health of organisms. Consequently, biopesticides are utilized for effective pest control. Entomopathogenic fungi, such as *Beauveria bassiana* and *Metarhizium anisopliae* were studied for uses instead of chemical pesticides. Their attack mechanism utilize secreted digestive enzymes such as chitinases, proteases, and lipases in order to degrade chitin, proteins, and lipids containing insect cuticle and use hyphal to direct penetrate (Charnley and St. Leger, 1991; Charnley, 2003).

1.9.3 Inhibition of nematodes

Silver birch (*Betula pendula*) leaves were genetically manipulated to produce chitinase IV from sugar beet and used in microcosms experiments to evaluate their impacts on growth and reproduction of decomposing soil fauna deriving nutrition from these leaves. The result exerted that transgenic leaves affected positively to population numbers of mesofauna collembolans (*Folsomia candida* and *Lepidocyrtus lignorum*) but negatively on the population numbers of microfauna nematodes. Growth or survival of macrofauna Woodlice (*Porcellio scaber*) juveniles were no differences between manipulated and control birches (Kotilainen et al., 2004). Researches about plants manipulated with chitinase and their effects on soil organisms were scarceness but the many results attest that plants producing chitinase cloud resistant against fungal pathogens and nematodes (Suslow, 1995; Grison and Grezes-Besset, 1996; Punja and Rahajaro, 1996).

1.9.4 Inhibition of phytopathogenic protozoa

Many diseases in different crop plant species are caused by the *Phytomonas* such as the phytopathogenic trypanosomatid *Phytomonas françai* which its cell surface was exposed with chitin. The plant chitinases *Urtica dioica* agglutinin (UDA) and *Arabidopsis thaliana Chia4* (ATCHIT4) proteins were overproduced in *Escherichia coli* and immunocytochemistry was operated for characterization of the interaction between these proteins with *P. françai* surface. The result indicated that UDA and ATCHIT4 proteins could specifically interact with the chitin present on the surface of *P.françai* and able to trim the surface-exposed chitin of *P. françai* (Rocha et al., 2003).

1.9.5 Enhancement of plant growth

Bacillus cereus QQ308 secreted hydrolytic enzymes, including chitinase, chitosanase and protease, supplemented growth (weight and height) of Chinese cabbage. This is the first report on the antifungal activity of *B. cereus*, which was unique characteristic among known strains of *B. cereus* (Chang et al., 2007).

1.9.6 Formation of fungal protoplasts

A new enzyme cocktail is defined to protoplast *Aspergillus niger*. The enzyme cocktail comprises lysing enzymes from *Trichoderma harzianum*, chitinase from *Streptomyces griseus* and β-glucuronidase from *Helix pomatia* (Bekker et al., 2009).

Exoenzymes secreted from *Trichoderma koningii* UC174 were used to lyse the cell wall for generation of protoplasts from *T. cutaneum* SL409. In contrast, these protoplasts could not produced by using neither cellulase Onozuka RS

nor snail gut enzymes from *Helix pomatia*, either alone or in combination. Nevertheless, when commercial chitinase was employed together with either of these two enzymes, protoplasts were able to be successfully produced (Liu and Zhu, 2000). The supplement of lytic enzyme systems with chitinase is usual protocol and essential for degradation of the cell walls of many organisms (Skujins et al., 1965; Kolar et al., 1985).

1.9.7 Leaf decomposition

Silver birch (*Betula pendula*) leaves were genetically manipulated to produce chitinase IV from sugar beet and used in microcosms experiments to compare decomposition rates of birch leaves. The result revealed that total decomposing rate and leaf mass loss per nematode were highest in chitinase leaves (Kotilainen et al., 2004).

1.9.8 Bioconversion of chitin waste

Majority of chitinases producing microorganisms can consume chitin as a major carbon source (Wang et al., 2006b). The marine wastes can be applied to decrease production costs of chitinases from microganisms and abate environmental problems (Liu et al., 2003; Wang et al., 2008b). Bioconversion of chitin containing substances offers alternative approach for shellfish wastes management (Wang and Yeh, 2006; Wang et al., 2006a; 2008b). However, strong acids or bases are still necessary for demineralization and deproteinization process of shellfish waste in the preparation of chitin (Synowiecki and Al-Khateeb, 2000; Wang et al., 2008b).

1.9.9 Production of *N*-acetyl-D-glucosamine, diacetylchitobiose and chitooligosaccharides

N-acetyl-D-glucosamine (GlcNAc) and N-acetylchitooligo saccharides is important to the food, agriculture, biotechnology and industrial applications such as plant-pathogenic biological controlling agents and plant growth promoters or biofertilizers. Oligosaccharides are water-soluble and have versatile functional properties such as antimicrobial activity and anti tumor activity leaded to production of oligosaccharides from chitin (Suzuki et al., 1986; Wang et al., 2006b; 2008a; Liang et al., 2007). Chemical processes of chitooligosaccharides production caused many problems including low yields of chitooligosaccharides, a large amount of short chain chitooligosaccharides, high cost in separation, and environmental pollution. In contrast, many usefulness of enzymatic method using chitinase are low cost in production, reproducibility, and friendly to environment (Kadokura et al., 2007).

Multi-chitinolytic enzyme complex from *Paenibacillus illinoisensis* KJA-424, showing strong chitinolytic activity, effectively convert colloidal chitin to *N*-acetyl-D-glucosamine, chitobiose, and *N*-acetylchitooligosaccharides (Jung et al., 2007). Furthermore, the production of *N*-acetyl-D-glucosamine could be accomplished by using enzymes from other microorganisms including *Serratia marcescens* (Haynes et al., 1999), *Bacillus thuringiensis* subsp. *Pakistani* (Thamthiankul et al., 2001), *Aeromonas hydrophila* H2330 (Sashiwa et al., 2002), *Trichoderma viride* and *Acremonium cellulolyticus* (Sashiwa et al., 2003) and *Aeromonas* sp. (Kuk et al., 2005a; 2005b).

1.10 Current research

Recently, we had searched the *Penaeus monodon* EST database and identified several contigs and singletons of chitinase clones. Mining of the EST clones gave rise to three different chitinases and named according to their counterparts in *M. japonicus*, *PmChi1*, *2* and *3*. In this research, we had the complete reading frames of *PmChi1* and *3* coding for mature chitinases and the partial reading frame of *Pm*Chi2 cloned. Their phylogenetic relationships to other chitinases were analyzed. Their tissue distribution in normal and early postmolted shrimp was investigated. The *Pm*Chi1 protein was over-expressed as fusion protein for preliminary elucidation of its enzymatic activity, and pH and temperature optima and stability.

CHAPTER II

MATERIALS AND METHODS

2.1 Materials

2.1.1 Equipments

Autoclave model # LS-2D (Rexall industries, Taiwan)

Automatic micropipettes P10, P100, P200 and P1000 (Gilson Medical Electrical S.A., France)

Balance Satorius 1702 (Scientific Promotion)

Bransonic 32 (Bandelin Sonopuls, Germany)

Gel documentation (Syngene)

Hyperfilm MP (Amersham International, England)

Incubator (Memmert)

Innova 4080 Incubator Shaker (New Brunswick Scientific)

LABO Autoclave (Sanyo)

Laminar Airflow Biological Safety Cabinets Class II Model NU-440-400E (NuAire, USA)

Magnetic stirrer model Fisherbrand (Fisher Scientific, USA)

Minicentrifuge (Costar, USA)

Orbital shaker SO3 (Stuart Scientific, Great Britain)

PCR Mastercycler (Eppendorf AG, Germany)

PCR Thermal Cycler: DNA Engine (MI Research, USA)

PCR Workstation model # P-036 (Scientific, USA)

pH Meter model # SA720 (Orion)

Power Supply Power PAC 3000 (Bio-RAD Laboratories, USA)

Refrigerated Microcentrifuge MIKRO 22R (Hettich Zentrifugen, Germany)

Spectrophotometer: Spectronic 2000 (Bausch & Lomb, USA)

Spectrophotometer DU 650 (Beckman, USA)

Transilluminator 2011 Macrovue (LKB)

Trans-Blot® SD (Bio-Rad)

Vacuum Blotter Model # 785 (Bio-Rad Laboratories, USA)

Vacuum pump (Bio-Rad Laboratories, USA)

Vertical Electrophoresis System (Hoefer™ miniVE)

Vortex model K-550-GE (Scientific Industries, USA)

Water Bath (Charles Hearson, England)

White/UV Transilluminator: UVP ImageStore 7500 (Mitsubishi Electric Corporation, Japan)

2.1.2 Chemicals and reagents

100 mM dATP, dCTP, dGTP, and dTTP (Fermentas)

2-Mercaptoethanol, C_2H_6OS (Fluka, Switzerland)

4-Nitro blue tetrazolium, NBT (Fermentas)

5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside, X-Gal (Fermentas)

5-Bromo-4-chloro-indolyl phosphate, *p*-toluidine salt, BCIP (Fermentas)

Absolute ethanol, C_2H_5OH (BDH, England)

Absolute methanol (J.T. Baker)

Acetic acid glacial, CH_3COOH (BDH, England)

Adenosine-5'-triphosphate potassium salt, ATP (Sigma, USA)

Agarose (Sekem)

Alkaline phosphatase-conjugated rabbit anti-mouse IgG (Jackson ImmunoResearch Laboratories)

Ammonium persulfate, $(NH_4)_2S_20_8$ (USB)

Anti-His antibody (GE Healthcare)

Bacto agar (Difco, USA)

Bacto tryptone (Scharlau, Spain)

Bacto yeast extract (Scharlau, Spain)

Boric acid, BH_3O_3 (Merck, Germany)

Bovine serum albumin (Fluka, Switzerland)

Bromophenol blue (Merck, Germany)

Calcium chloride, $CaCl_2$ (Merck, Germany)

Chloroform, $CHCl_3$ (Merck, Germany)

Coomassie brilliant blue G-250 (Fluka, Switzerland)

Coomassie brilliant blue R-250 (Sigma, USA)

Dialysis tube (Sigma, USA)

Diethyl pyrocarbonate (DEPC), $C_6H_{10}O_5$ (Sigma, USA)

Dimethyl formamide (Carlo Erba Reagenti, Italy)

di-Potassium hydrogen orthophosphate anhydrous, K_2HPO_4 (Univar Ajax)

di-Sodium hydrogen orthophosphate anhydrous, Na_2HPO_4 (Carlo Erba Reagenti, Italy)

Ethidium bromide (Sigma, USA)

Ethylene diamine tetraacetic acid (EDTA), disodium salt dihydrate (Fluka, Switzerland)

GeneRuler™ 100 bp Plus DNA Ladder (Fermentas)

GeneRuler™ 1 kb Plus DNA Ladder (Fermentas)

Glucose (Univar Ajax)

Glycerol, $C_3H_8O_3$ (BDH, England)

Glycine (Research Organic)

Hydrochloric acid, HCl (Merck, Germany)

Imidazole (Fluka, Switzerland)

Isopropanol (Merck, Germany)

Isopropyl-β-D-thiogalactoside, IPTG (Sigma, USA)

Magnesium chloride, $MgCl_2$ (Merck, Germany)

N, *N'*-methylene-bisacrylamide, $C_7H_{10}N_2O_2$ (USB)

Nickel-NTA agarose (GE Healthcare)

Nickel sufate, $NiSO_4$ (Sigma, USA)

Nytrans® super charge nylon membrane (Schleicher & Schuell)

PageRuler™ Prestained Protein Ladder (Fermentas)

PageRuler™ Unstained Protein Ladder (Fermentas)

Phenol crystals, C_6H_5OH (Carlo Erba Reagenti, Italy)

Phosphoric acid (Univar Ajax)

Potassium acetate, CH_3COOK (Merck, Germany)

Potassium ferricyanide (BDH, England)

Potassium dihydrogen phosphate, KH_2PO_4 (Merck, Germany)

Sodium acetate, CH_3COONa (Merck, Germany)

Sodium dihydrogen orthophosphate, NaH_2PO_4 (Univar Ajax)

Sodium carbonate (Carlo Erba Reagenti, Italy)

Sodium chloride, NaCl (BDH, England)

Sodium citrate, $Na_3C_6H_5O_7$ (Carlo Erba Reagenti, Italy)

Sodium dodecyl sulfate (Sigma, USA)

Sodium hydroxide, NaOH (Eka Nobel)

Tris-(hydroxymethyl)-aminomethane, $NH_2C(CH_2OH)_3$ (USB)

TritonX-100 (Merck, Germany)

TRI Reagent® (Gibco BRL, USA)

Tween™-20 (LabChem)

TEMED $(CH_3)_2NCH_2CH_2N(CH_3)_2$ (Amresco)

2.1.3 Kits

Advantage® 2 Polymerase Mixes and PCR kit (Clontech Laboratories)

ImProm-II™ Reverse Transcription system kit (Promega)

NucleoSpin® Extract II kit (Macherey-Nagel)

QIAprep Spin Miniprep kit (Qiagen)

RevertAid™ First Strand cDNA Synthesis kit (Fermentas)

SMART™ RACE cDNA Amplification kit (Clontech Laboratories)

T & A Cloning Vector kit (Real Biotech Corporation)

2.1.4 Enzymes

*BamH*I, *EcoR*I, *Hind*III, *Msc*I, *Nco*I, *Pst*I, *Sac*I, *SnaB*I, *Xho*I (New England Biolabs, USA)

Enterokinase, light chain (New England Biolabs, USA)

Ribonuclease A, RNase A (Sigma, USA)

RQ1 RNase-free DNase (Promega)

*Sma*I (Fermentas)

T_4 DNA ligase (Fermentas)

Taq™ DNA polymerase (Fermentas)

2.1.5 Substrates for chitinase activity assay

β-chitin from squid pen (Ta Ming Enterprises, Samutsakorn, Thailand)

Colloidal chitin from shrimp shell (Ta Ming Enterprises, Samutsakorn, Thailand)

Partially *N*-acetylated chitin (PNAC)

2.1.6 Antibiotics

Ampicillin (Sigma, USA)

Chloramphenicol

2.1.7 Bacterial strains

Escherichia coli XL-1 Blue MRF'

Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac [F' proAB lacI^{q}ZΔM15 Tn10 (Tet^r)]

Escherichia coli Rosetta(DE3)pLysS

F^- ompT hsdS_B(R_B^- m_B^-) gal dcm λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5]) pLysSRARE (Cam^R)

2.1.8 Software

Blastp (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi)

Clustal X (Thompson, 1997)

ExPASy ProtParam (http://au.expasy.org/tools/protparam.html)

GENETYX version 7.0 program (Software Development Inc.)

SECentral (Scientific & Educational Software)

SignalP 3.0 Server (http://www.cbs.dtu.dk/services/SignalP/)

SmartTM databases (http://smart.embl-heidelberg.de/)

Penaeus monodon EST database (http://pmonodon.biotec.or.th/)

2.1.9 Plasmid vectors

T&A cloning vector (Real Biotech Corporation)

pUC118 vector (Takara)

pET-32a(+) vector (Novagen)

2.2 Searching the *Penaeus monodon* EST database

The EST and contig pages in the *Penaeus monodon* EST database (http://pmonodon.biotec.or.th/home.jsp) were searched for the chitinases. The nucleotide sequences of the obtained contigs and singletons were analyzed for the open reading frames and the encoded amino acid sequences. The signal peptide was predicted using the online SignalP 3.0 program (http://www.cbs.dtu.dk/services/SignalP/) (Bendtsen et al., 2004). ClustalX was used

to compare the chitinase sequences from different contigs and EST clones (Chenna et al., 2003). The molecular weights were predicted from the amino acid sequences using the Expasy software (http://www.expasy.org/).

2.3 Primer designation

PCR-specific primer pairs were designed based on nucleotide sequences of the template DNA using the SECentral program (Scientific & Educational Software). The primer criteria are: the GC content is between 50-60%, Tm is ranging from 55-80 ºC, difference of GC content is less than 5%, difference of Tm is less than 10 ºC, stability value is more than 1.2 kcals, repetition of nucleotide pairs is minimum, self-priming and primer dimer formation are minimum.

2.4 Phylogenetic and amino acid sequence analyses

The amino acid sequences of *Pm*Chi1 (accession no. GU344706), *Pm*Chi2 (accession no. GU376734) and *Pm*Chi3 (accession no. GU344707) were submitted to protein BLAST (blastp) for the identity and similarity against the protein sequences in the GenBank (Altschul and Lipman, 1990). The amino acid sequences of chitinases from mammals, crustaceans and insects were down-loaded from the GenBank. The catalytic domains were defined using PROSITE and SmartTM databases (http://smart.embl-heidelberg.de/). The amino acid sequences of catalytic domain were aligned using the *ClustalX* **(**Chenna et al., 2003**).** Based on the alignment, a phylogenetic tree was constructed using the Phylip program. Bootstrap analysis was

performed for values representing 1,000 replicates using the SeqBoot. The mammalian chitinases were defined as an outgroup.

2.5 Shrimp preparation

Juvenile *P. monodon* shrimp weight approximately 20 g was acclimatized in aquaria at an ambient temperature of 28 ºC and a salinity of 15 ppt for a few days before used in the experiments. For postmolted samples, the shrimp were collected within 1 hour after the ecdysis, possibly in the postmolt stage A, for the new cuticle and setae were still very soft.

2.6 Total RNA isolation

Black tiger shrimp tissues: antennal gland, stomach, gill, intestine, lymphoid, eyestalk, hepatopancreas, heart, epipodite, pleopod and tail, were dissected separately and immediately snap-frozen in liquid nitrogen (-176 ºC). Hemolymph was collected from shrimps using a syringe pre-loaded with 200 µL of anticoagulant [10% (w/v) sodium citrate]. Hemocytes were isolated from the plasma by centrifugation at 8,000g for 10 minutes at 4 ºC. The hemocyte pellet was resuspended in 1 mL of TRI Reagent® (Molecular Research Center). Tissue samples were homogenized in 1 mL of TRI Reagent® per 50-100 mg of tissue using a glass-pestle. The homogenized samples were incubated for 5 minutes at room temperature to permit the complete dissociation of nucleoprotein complexes. They were centrifuged to remove cell debris and transfer the supernatant to new microcentrifuge tubes. Then, 0.2 mL of chloroform per 1 mL of TRI Reagent® was added and vortexed vigorously for 15 seconds. After incubation

at room temperature for 2-3 minutes, they were centrifuged at 12,000g for 15 minutes at 4 °C. The mixture was separated into lower red, phenol-chloroform phase, an interphase and a colorless upper aqueous phase, which RNA remains exclusively in the aqueous phase. The upper phase was transferred carefully into fresh 1.5 mL microcentrifuge tube.

The RNA was precipitated from the aqueous phase by mixing with 0.5 mL of isopropyl alcohol per 1 mL of TRI Reagent® used for the initial homogenization. Samples were afterwards incubated at 15-30 °C for 10 minutes and centrifuged at 12,000g for 10 minutes at 4 °C to remove the supernatant completely. The RNA pellet was washed with 1 mL of 75% (v/v) ethanol per 1 mL of TRI Reagent® and centrifuged 12,000g for 10 minutes at 4 °C. The pellet-washing step was repeated once. The RNA pellet was briefly air-dried for 5-10 minutes. Finally, the total RNA was dissolved in an appropriate amount of diethyl pyrocarbonate (DEPC)-treated water and stored at -80 °C until use.

2.7 DNase treatment of total RNA

The isolated total RNA was further treated with RQ1 RNase-free DNase (Promega) (1 unit/5 μg of total RNA) at 37 ºC for 30 minutes to remove the contaminating chromosomal DNA. Then, the RNA was purified by phenol/chloroform extraction following by isopropanol precipitation. Briefly, the reaction was adjusted the volume to 40 μL with DEPC-treated water, added 250 μl of TRI Reagent®, vortexed for 10 seconds, added 200 μL of chloroform and vigorously shaken for 15 seconds. The resulting mixture was kept at room temperature for 2-3

minutes and centrifuged at 12,000g for 15 minutes at 4 ºC. The RNA in the upper phase was separated, precipitated with isopropanol and washed with 70% (v/v) ethanol. The RNA pellet was briefly air-dried and dissolved with a proper amount of DEPC-treated water. The quantity and quality of DNA-free total RNA was determined.

2.8 Determination of the quantity and quality of RNA samples

The quantity and quality of total RNA was spectrophotometrically measured at 260 nm and analyzed by agarose gel electrophoresis, respectively. The total RNA concentration can be determined via UV absorption at 260 nm (A_{260}). 1 µL of RNA was diluted in DEPC-treated water and measured the A_{260}. The concentration could be determined in ng/µL using the following formular: ng/µL of RNA = A_{260} × dilution factor × 40 for absorption at 260 nm corresponds to 40 ng/µL of RNA (Sambrook et al., 1989).

The relative purity of RNA samples was examined by measuring the ratio of $A_{260/280}$ and $A_{260/230}$. The maximum absorption of organic solvent, nucleic acid, and protein is at 230, 260, and 280 nm, respectively. The ratio of absorbance at 260 nm and 280 nm is used to assess the purity of RNA. An approximately ratio above 2.0 is generally accepted as pure RNA. If the ratio is appreciably lower, it may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm. The quality was further investigated through an agarose gel electrophoresis.

2.9 First-strand cDNA synthesis

The first strand cDNA was synthesized from 1 μg of total RNA using the RevertAid™ First Strand cDNA Synthesis kits (Fermentas). The reaction mixture consist of total RNA 1 μg, oligo$(dT)_{18}$ primer 1 μL, and DECP-treated water in a total volume of 12 μL. After annealed at 70 °C for 5 minutes, it was chilled on ice for 5 minutes, spun down and placed back on ice. 5× reaction buffer 4 μL, RiboLock™ RNase Inhibitor (20 U/μL) 1 μL, 10 mM dNTP mix 2 μL, RevertAid™ M-MuLV Reverse Transcriptase (200U/μL) 1 μL were then added, mixed gently and spun down. After incubated at 42 °C for 60 minutes, the reaction was terminated by heating at 70 °C for 10 minutes. The synthesized cDNA was stored at -20 ºC until use.

Occasionally, an ImProm-II™ Reverse Transcription System kit (Promega) was used instead. Total RNA was combined with 0.5 μg of oligo$(dT)_{15}$ primer and DECP-treated water in a volume of 8 μL, annealed at 70 ºC for 5 minutes and immediately placed on ice for 5 minutes. Then, 4 μL of 5× reaction buffer, 2.6 μL of 25 mM $MgCl_2$, 1 μL of dNTP mix (10 mM each), 20 units of ribonuclease inhibitor and 1 μL of ImProm-II reverse transcriptase were added and gently mixed. The reaction mixture was incubated at 25 ºC for 5 minutes followed by 42 ºC for 60 minutes. Then, the reaction was terminated as described above.

2.10 Tissue specific expression of chitinases

Tissue distribution was examined by semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR). To determine the expression of chitinase in various shrimp tissues, cDNAs isolated from various shrimp tissues: antennal gland,

stomach, hemocyte, gill, intestine, lymphoid, eyestalk, hepatopancreas, heart, epipodite, pleopod and tail, were used for chitinase gene-specific amplification using primers specific to the chitinase genes (Table 2.1). The *β-actin* gene was generally used as an internal control using the gene-specific primers (Table 2.1) designed according to the shrimp *β-actin* cDNA sequence (GenBank accession no. DW042525).

One microliter of the first-strand cDNA was subjected to PCR in a 25 μL reaction volume containing 750 mM Tris-HCl pH 8.8, 200 mM $(NH_4)_2SO_4$, 0.1% (v/v) Tween 20, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 μM of each specific primers and 0.5 units of Taq™ DNA polymerase (Fermentas). The reaction was pre-denatured at 94 ºC for 1-2 minutes followed by 30 cycles of denaturation at 94 ºC for 30 seconds, annealing at 50 or 55 ºC for 30 seconds and extension at 72 ºC for 1-2 minutes, and final extension at 72 ºC for 7-10 minutes. The amplification reactions were analyzed by agarose gel electrophoresis.

Table 2.1 Primer pairs used for the amplification.

Gene	Primer	Sequence *	PCR product	Task
PmChi1	FChi1_1	5' GCCCATGGACCCGAGATTCGAGCAAGAAGG 3'	1,937 bp	Cloning
	RChi1_1	5' CCCTCGAGGTTCTTTACCATCTTCATTAGC 3'		
	FChi1_2	5' GTGTGGAACCAGGCTATCAA 3'	319 bp	RT-PCR
	RChi1_2	5' GTGCTGGCTTAACACGTACT 3'		
PmChi2	FChi2	5' CAACTGGGCGTGGTA 3'	1,117 bp	Cloning and RT-PCR
	RChi2	5' GGTTACATGTTGCAC 3'		
	GSPout1	5' AGAAGCCGAGTGCCGTCCAGAACAG 3'	-	5' RACE
	GSPout2	5' CCTTCTTGCAGTTCACCTGCCAGCA 3'		
	GSPin	5' TCTTCGGGTCTGTATTTGCCGGCGC 3'		
	UPM	5' CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT 3' 5' CTAATACGACTCACTATAGGGC 3'		
	NUP	5' AAGCAGTGGTATCAACGCAGAGT 3'		
PmChi3	FChi3	5' ATGGATCCGGTGATGGTGTGCTACTTC 3'	1,416 bp	Cloning and RT-PCR
	RChi3	5' GCCTCGAGGCAGTCATTCGGGCAAACG 3'		
β-Actin	actinF	5' GCTTGCTGATCCACATCTGCT 3'	320 bp	Internal control for RT-RCR
	actinR	5' ATCACCATCGGCAACGAGA 3'		

* The restriction sites are underlined.

2.11 Cloning of the *Pm*Chi1 gene

Total RNA was extracted from hepatopancreas of black tiger shrimp using TRI Reagent® (Gibco BRL), treated with DNase (Promega) and used to synthesize the first-strand cDNAs. The *PmChi1* encoding the mature protein was amplified with FChi1_1 and RChi1_1 (Table 2.1). The primers were designed with extended *Nco*I and *Xho*I sites at their 5' ends, respectively.

The reaction mixture of 50 µL contained 0.5 µg cDNA, 0.2 mM dNTP, 0.2 µM FChi1_1 and RChi1_1, 1× Taq™ buffer, 0.02 unit/µL Taq™ DNA polymerase. The condition was first denaturation at 95 ºC for 3 minutes followed by 30 cycles of

denaturation at 95 ºC for 30 seconds, annealing at 55 ºC for 30 seconds, extension 72 ºC for 3 minutes, and then final extension at 72 ºC for 10 minutes.

The amplified product with expected size of 1937 bp was analyzed using 1% agarose gel electrophoresis and eluted using NucleoSpin® Extract II kit (Macherey-Nagel). The DNA fragment band was excised from an agarose gel and transferred to a clean 1.5 microcentrifuge tube. Buffer NT 200 μL was added per each 100 mg of agarose gel and incubated at 50 °C until the gel pieces were dissolved. A NucleoSpin® Extract II column was placed into a Collection Tube and loaded with the sample. After centrifuged for 1 minute at 11,000g, the flow-through was discarded. The silica membrane was washed with 600 μL of Buffer NT3 and centrifuged. The column was centrifuged for 2 minutes at 11,000g for completely removal of NT3 buffer and was placed into a clean 1.5 mL microcentrifuge tube. The DNA was eluted by adding 15-50 μl of Elution Buffer NE (5 mM Tris-Cl, pH 8.5) into the center of each column, leaving at room temperature for 1 minute and centrifugation for 1 minute at 11,000g.

The purified DNA fragment was tailed with an adenine nucleotide for ligation into the T&A cloning vector (Real Biotech Corporation). The insert DNA:vector molar ratio was about 3:1. DNA ligation mixture was composed of T&A cloning vector 1 μL (25 ng), Buffer A 1 μL, Buffer B 1 μL, T_4 DNA ligase 1 μL (3 Weiss units/μL), suitable amount of the PCR product, and sterile deionized water to 10 μL. This reaction was mixed and incubated overnight at 4 °C.

The plasmids were transformed into an *E. coli* XL1-Blue using the $CaCl_2$ method. The $CaCl_2$ competent cells of 100 μL were mixed with the DNA (0.5 μL of plasmid DNA or 10 μL of ligation mixture) and incubated on ice for 30 minutes. Then,

the mixture was immediately heat-shocked at 42 °C for 1 minute and instantly added with 1 mL of LB medium. After shakiness at 37 °C for 1 hour, appropriate amount of cell suspension was spreaded onto the LB agar plate with proper selection and incubated overnight at 37 °C. In this case, the LB agar plates contained 50 μg/mL ampicillin, 100 μL of 100 mM IPTG and 20 μL of 50 mg/mL X-gal. The individual colonies were picked and grown in 1.5 mL LB medium containing appropriate antibiotic at 37 ºC overnight with shaking for plasmid DNA preparation.

The recombinant plasmid clones were screened by digestion with *Nco*I and *Xho*I and analyzed by agarose gel electrophoresis. The expected size of *PmChi1* DNA fragment was about 2 kb. The resulting clone was pT-*Pm*Chi1. The correct clone was subjected to DNA sequencing.

2.12 Plasmid DNA preparation

LB 1.5 ml, which contains the antibiotic selective for the bacterial culture, with a single isolated colony picked from an LB agar plate containing the selective antibiotic was grew overnight at 37 °C with shaking. The cells were collected by centrifugation for 1 minute at 12,000 rpm. The bacterial pellet was resuspended in 100 μL of Solution I (25mM Tris-HCl pH 8.0, 50 mM glucose, 10 mM EDTA) by vortexing, added with 200 μL of Solution II (0.2 N NaOH, 1% SDS), mixed by inverting the tube gently. Then, 150 μL of Solution III (3 M potassium acetate, pH 4.8) was added, mixed by inverting the tube gently and centrifuged at 12,000 rpm for 10 minutes. The supernatant was transferred to a fresh tube and added 5 μL of 10 mg/mL ribonuclease A. After incubation at 37 °C for 30 minutes, phenol-chloroform 400 μL

was added, mixed by vortexing and then centrifuged for 1 minutes at 12,000 rpm. The supernatant was transferred to a fresh tube and added 900 μL of absolute ethanol, frozen at -20 °C for 30 minutes. After centrifugation at 12,000 rpm for 5 minutes, the DNA pellet was washed with 500 μL of ice cold 75% (v/v) ethanol and centrifuged for 5 minutes. The pellet was air dried 5-20 minutes and resuspended in 50 μL of sterile deionized water or TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA). The extracted plasmid was stored at -20 ºC until use.

For efficacious plasmid DNA extraction, a QIAprep® Miniprep kit (QIAGEN, Germany) was employed. The overnight culture of bacteria was centrifuged at 12,000 rpm for 1 minute. The supernatant was removed. Bacterial cells were resuspended in 250 μL Buffer P1 containing RNaseA. The 250 μL Buffer P2 was added and mixed thoroughly by inverting the tube 4–6 times to lyse the cells. The cell lysate was neutralized by adding 350 μL Buffer N3. After maximum speed centrifugation for 10 minutes, the supernatant was applied to a QIAprep spin column by pipetting. The column was centrifuged at 12,000 rpm for 1 minute. The flow-through was discarded. The column was washed twice with 0.5 mL Buffer PB and 0.75 mL Buffer PE, respectively, and then centrifuged at 12,000 rpm for 1 minute to remove residual ethanol from Buffer PE. The plasmid DNA was eluted by adding 30-50 μL Buffer EB to the center of column, incubating at room temperature for 1 minute and centrifugation at 12,000 rpm for 1 minute. The flow-through containing the plasmid was then stored at -20 ºC until use.

2.13 Construction of *Pm*Chi1-expression clone pET-32a(+)-*Pm*Chi1

The *PmChi1* in T&A cloning vector, pT-*Pm*Chi1, was digested with *Nco*I and *Xho*I. The *PmChi1* gene fragment encoding the mature *Pm*Chi1 was isolated and cloned into the *Nco*I-*Xho*I digested pET-32a(+) expression vector (Novagen) (Fig. 2.1). The resulting expression plasmid clone, pET-32a(+)-*Pm*Chi1, was transformed into an *E. coli* host strain Rosetta(DE3)pLysS for recombinant protein expression.

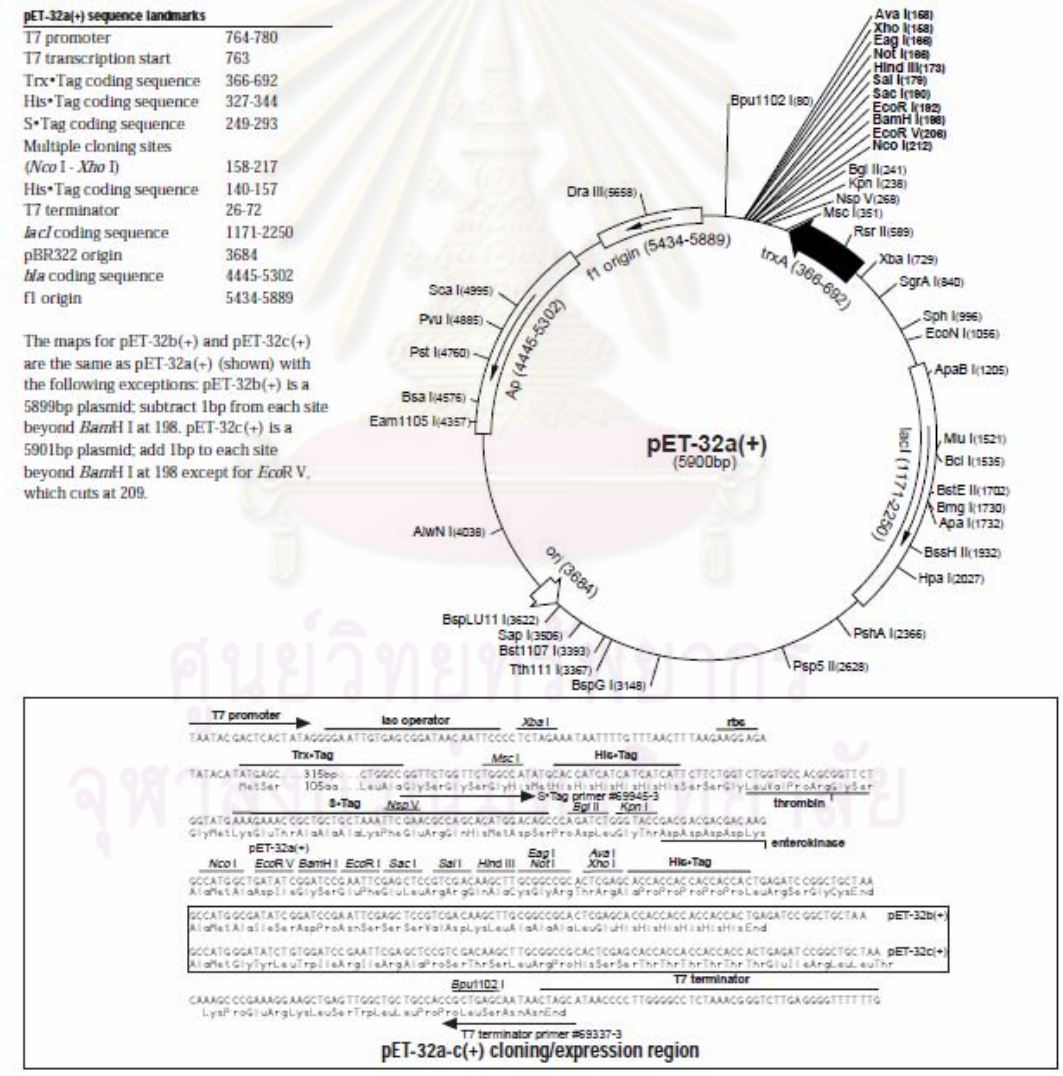

Figure 2.1 The pET-32a(+) vector map (Novagen®, Germany).

2.14 Cloning of the *Pm*Chi2 gene

Total RNA was isolated from eyestalk of black tiger shrimp using TRI Reagent® (Gibco BRL) according to the manufacturer's instruction. The total RNA was treated with DNase (Promega) to remove the DNA contamination. Then, first-strand cDNAs were synthesized from 1 µg of total RNA samples using the RevertAid™ First Strand cDNA Synthesis kit (Fermentas) according to the manufacturer's protocol for use as PCR template. The partial *Pm*Chi2 gene fragment was amplified with FChi2 (5' CAACTGGGCGTGGTA 3') and RChi2 (5' GGTTACATGTTGCAC 3') (Table 2.1). The reaction mixture of 50 µL contained 1 µg cDNA, 0.2 mM dNTP, 0.5 µM FChi2 and RChi2, 1× Taq™ buffer, 0.02 unit/µL Taq™ DNA polymerase. The condition was first denaturation at 95 ºC for 2 minutes followed by 30 cycles of denaturation at 95 ºC for 30 seconds, annealing at 50 ºC for 30 seconds, extension 72 ºC for 2 minutes, and then final extension at 72 ºC for 7 minutes. The PCR product of 1,117 bp for partial *PmChi2* was analyzed using 1% agarose gel electrophoresis, isolated using NucleoSpin® Extract II kit, cloned into the T&A cloning vector (RBC) and sequenced.

Since the DNA sequence of *PmChi2* was only partial, lacking the 5' sequence portion, the 5' amplification of cDNA end or 5' RACE was carried out using the SMART™ RACE cDNA Amplification Kit (Clontech Laboratories) following the manufacturer's protocol. Briefly, the 5′ RACE libraries were prepared from the cDNAs from eyestalk and gill such that a short sequence containing universal annealing sites for 5′ RACE universal primers was added at the 5′ end. Three gene-specific primers, GSPout1, GSPout2 and GSPin, were designed as listed in Table 2.1. One of the outer gene-specific primers (GSPout) along with the 5′ RACE universal

primer were used for primary PCR of the 5′ RACE library. Then, the inner primer (inner GSPout or GSPin) and the 5′ RACE nested universal primer were used for secondary PCR. The amplified DNA was purified through 1% agarose gel electrophoresis.

The purified DNA fragments were tailed with an adenine nucleotide for ligation into the T&A cloning vector (Real Biotech Corporation). The ligated plasmids were transformed into *E. coli* XL1blue strain and selected with a Luria-Bertani (LB) agar containing 100 μg/mL of ampicillin, 40 μg/mL X-gal, 0.4 mM IPTG (Blue-white screening). The recombinant plasmids were subjected to nucleotide sequencing with T7 promoter and M13reverse promoter so as to look for sequence of *Pm*Chi2 gene.

2.15 5' Rapid Amplification of cDNA Ends (5'RACE)

5' Rapid Amplification of cDNA Ends was carried out using SMART™ RACE cDNA Amplification Kit (Clontech Laboratories) to determine the 5'-upstream sequence of the *Pm*Chi2. The total RNA from either eye stalk or gill was used. RNA sample of 50 ng-1 μg was mixed with 1 μL of 5'-CDS primer A, 1 μL of SMART II A oligo and sterile ultrapure water to a final volume 5 μL. After incubation at 70 °C for 5 minutes, the mixture was cooled on ice for 5 minutes and added with 2 μL of 5× First-Strand Buffer, 1 μL of 20 mM DTT, 1 μL of 10 mM dNTP Mix and 1 μL of MMLV Reverse Transcriptase. It was incubated at 42 °C for 1.5 hours follow by 72 °C for 10 minutes and stored at -20 °C until use.

For primary amplification, 1 µL of 5'-RACE-Ready cDNA was amplified with Universal Primer A Mix (UPM) (Table 2.1) and GSPout1 or GSPout2 under the condition: first denaturation 94 °C for 2 minutes; 25 cycles of denaturation 94 °C for 30 seconds, annealing 68 °C for 30 seconds, extension 72°C for 1 minute; then final extension 72 °C for 7 minutes. The reaction mixture of 50 µL contained 1 µl of 5'-RACE-Ready cDNA from either eye stalk or gill, 0.2 mM dNTP, 1×UPM, 0.2 µM either GSPout1 or GSPout2, 1× Advantage 2 PCR buffer, and 1× Advantage 2 Polymerase Mix.

For secondary amplification, primary PCR product was diluted 50 fold and 5 µL of diluted primary PCR product was nested amplified with Nested Universal Primer A (NUP) (Table 2.1) and GSPout1 or GSPin under the above condition. The reaction mixture of 50 µL consisted of 5 µL of 50 fold diluted primary PCR product, 0.2 mM dNTP, 0.2 µM NUP, 0.2 µM either GSPout1 or GSPin, 1× Advantage 2 PCR buffer, and 1× Advantage 2 Polymerase Mix. Secondary PCR product was purified, cloned into the T&A cloning vector (RBC) and sequenced.

2.16 Cloning of the *Pm*Chi3 gene

Total RNA was extracted from hepatopancreas of black tiger shrimp using TRI Reagent® (Gibco BRL) according to the manufacturer's instruction and treated with DNase (Promega) to remove the DNA contamination. Then, first-strand cDNAs were synthesized from 1 µg of total RNA samples using the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas) according to the manufacturer’s protocol for use as PCR template. The *Pm*Chi3 gene fragment encoding the mature proteins without

signal peptides was amplified with FChi3 (5' ATGGATCCGGTGATGGTGTGCTA CTTC 3') and RChi3 (5' GCCTCGAGGCAGTCATTCGGGCAAACG 3') (Table 2.1). The primers were designed with extended *Bam*HI and *Xho*I sites at their 5' ends, respectively. The amplified fragment was 1416 bp in size**.**

The reaction mixture of 50 µL contained 0.7 µg cDNA, 0.2 mM dNTP, 0.3 µM FChi3 and RChi3, 1× Taq™ buffer, 0.02 unit/µL Taq™ DNA polymerase. The condition was first denatured at 95 ºC for 3 minutes followed by 30 cycles of denaturation at 95 ºC for 30 seconds, annealing at 55 ºC for 30 seconds, extension 72 ºC for 2 minutes, and then final extension at 72 ºC for 10 minutes. The PCR product of 1416 bp for *PmChi3* was isolated, cloned into the T&A cloning vector (RBC) and sequenced.

The amplified product was analyzed using 1% agarose gel electrophoresis and purified by means of NucleoSpin® Extract II kit (Macherey-Nagel). The purified DNA fragment was tailed with an adenine nucleotide for ligation into the T&A cloning vector (Real Biotech Corporation). The ligated plasmids were transformed into *E. coli* XL1blue strain and selected with a Luria-Bertani (LB) agar containing 100 µg/mL of ampicillin, 40 µg/mL X-gal, 0.4 mM IPTG (Blue-white screening). Recombinant plasmid was, then, subjected to nucleotide sequencing with T7 promoter and M13reverse promoter for mature sequence of *Pm*Chi3 gene.

2.17 Recombinant expression of *Pm*Chi1 gene in the *Escherichia coli* expression system

A single colony of *E. coli* Rosetta(DE3)pLysS containing pET-32a(+)-*Pm*Chi1 was cultured with shaking at 37 ºC overnight in 1 mL terrific broth containing 100 μg/mL of ampicillin and 34 μg/mL of chloramphenicol. The overnight culture was diluted 1:100-fold in 25 mL terrific broth containing 100 μg/mL of ampicillin and 34 μg/mL of chloramphenicol. The culture was grown at 37 ºC with shaking at 250 rpm until the optical density at 600 nm of the culture reached 0.5-0.6. The expression of recombinant *Pm*Chi1 (r*Pm*Chi1) was optimized by varying the condition, such as temperature, final concentration of IPTG and periods of induction.

The culture was continuously incubated at 17 ºC or 37 ºC further with shaking. One mL of cell suspension was aliquot at each time point after induction, the pellets harvested by centrifugation at 12,000 rpm for 1 minute at 4 ºC, and the protein expression was analyzed by 10% SDS-PAGE and western blotting.

The *E.coli* Rosetta(DE3)pLysS containing pET-32a(+)-*Pm*Chi1 were finally induced with 0.2 mM IPTG for 6 hours, cells from 1 ml of cell suspension were harvested and resuspended with 1× PBS. They were freeze-thawed 3 times followed by the addition of 1 μL of 10 mg/mL DNase and 2 μL of 10 mg/mL RNase. After gently shaken for 20 minutes at room temperature, they were centrifuged to separate the supernatant soluble proteins and the inclusion body protein pellet. Both supernate and pellet were analyzed by 10% SDS-PAGE and western blot.

2.18 Western blot detection of the His-tagged protein

After the SDS-PAGE, the protein gel can be partly stained with Coomassie brilliant blue R-250 or analyzed by wastern blotting. The part to be used for electroblotting was soaked in transfer buffer (25 mM Tris base, 150 mM glycine and 20% methanol) for 15-30 minutes together with Whatman® 3MM chromatography paper and nitrocellulose membrane which were cut to the size of the gel. The Whatman® 3MM paper was placed onto the Trans-Blot® SD (Bio-Rad). This is then wet with transfer buffer. The prewetted nitrocellulose membrane was put onto the filter paper and a gel is put on top of the membrane followed by filter paper again. Great care should be taken to ensure that there are no air bubbles. Proteins in transfer buffer are negative in charge and move from negative to positive poles. Western blotting was performed for 30-90 minutes at approximately constant 90 mA.

After finishing the transfer, the membrane was incubated in blocking buffer (1× PBS buffer [10 mM phosphate buffer pH 7.4, 150 mM NaCl] containing 0.05% (v/v) Tween™-20 and 5% (w/v) non-fat dry milk) at room temperature for an overnight with gently shaking. Then, it was washed 3 times for 10 minutes each in washing buffer, PBS-Tween buffer [1× PBS buffer containing 0.025% (v/v) Tween™-20]. It was incubated in an anti-His antibody solution, 1:3000 dilution in washing buffer containing 1% (w/v) non-fat dry milk with volume enough to cover it and allow it to float freely when agitated. After gently mixing at ambient temperature for 3 hours, it was washed 3 times for 10 minutes each in washing buffer and subsequently incubated in a secondary antibody solution, the alkaline phosphatase-conjugated rabbit anti-mouse IgG, 1:2500 dilution in washing buffer with 1% (w/v) non-fat dry milk, with agitation at room temperature for 1 hour. Next, it was washed 3

times as above and detected in darkness by color development using 10 mL of detection buffer (100 mM Tris-HCl, 100 mM NaCl and 50 mM $MgCl_2$, pH 9.5) containing 33 µL each of 4-nitroblue tetrazolium, (NBT) and 5-bromo-4-chloro-indolyl phosphate (BCIP) as substrate. The BCIP was prepared 50 mg/mL in water or dimethyl formamide and NBT also 50 mg/mL in water. Reaction product is purple and appears in a few minutes. Incubation can be up to an hour if the signal is weak. Finally, the development was stopped by washing with deionized water.

2.19 Production of r*Pm*Chi1

A single colony of *E. coli* Rosetta(DE3)pLysS containing pET-32a(+)-*Pm*Chi1 was cultured with shaking at 37 ºC overnight in terrific broth 25 mL containing 100 µg/mL of ampicillin and 34 µg/mL of chloramphenicol. The overnight culture was diluted 1:100-fold in 250 mL terrific broth containing 100 µg/mL of ampicillin and 34 µg/mL of chloramphenicol. The 2 L cell culture was grown at 37 ºC until the optical density at 600 nm reached 0.5-0.6. The expression of r*Pm*Chi1 was induced by adding IPTG to the final concentration of 0.2 mM. The culture was continued at 17 ºC with shaking for 6 hours. Cells from 2 L culture were collected by centrifugation at 8,000g for 10 minutes at 4 ºC and the cell pellet was resuspended in 240 mL of 1× PBS buffer, pH 7.4 by vortexing and then disrupted by freeze-thawed at least 3 rounds followed by sonication using a Bransonic 32 (BANDELIN SONOPULS, Germany) on ice with 60% amplitude pulse 10 times for 2 minutes each time.

Since the r*Pm*Chi1 existed as inclusion bodies, the pellet was collected and the supernatant liquid discarded. The pellet was washed with 0.5 M NaCl containing 2%

tritonX-100 twice followed by 0.5 M NaCl twice and sterile deionized water twice. The cells lysate were centrifuged at 10,000g for 10 minutes at 4 ºC to remove supernatant. The inclusion bodies were dissolved with 50 mM sodium phosphate buffer pH 12 by gently shaking overnight at 10 °C. Finally, the remaining precipitate was removed by centrifugation and the supernatant liquid containing r*Pm*Chi1 was dialyzed against 20 mM sodium carbonate buffer pH 10.

2.20 Purification of r*Pm*Chi1

The r*Pm*Chi1 was expressed as a thioredoxin fusion protein with His-tags and could be purified under non-denaturing condition using a Ni-NTA affinity chromatography (GE Healthcare) column. Protein solution in 20 mM sodium carbonate buffer pH 10 was centrifuged at 10,000g for 10 minutes at 4 ºC to clarify the protein solution. This protein solution was added with 2 M imidazole to the final concentration of 20 mM.

Slurry 2-4 mL of the Ni-NTA agarose in 20% ethanol was packed into the PD-10 column and washed with 10 mL of sterile deionized water 3 times followed by 10 mL of 20 mM sodium carbonate buffer pH 10 containing 20 mM imidazole. The protein solution was loaded into the column and fractions were collected immediately by a gravity flow. Subsequently, the column was washed with 10 mL of 20 mM sodium carbonate buffer pH 10 containing 20 mM imidazole to remove unbound proteins. After washing, proteins were eluted with 20 mM sodium carbonate buffer pH 10 containing 50, 100, 200, 300, 500 mM imidazole, respectively. The column was finally washed with 10 mL of 20 mM sodium carbonate buffer pH 10 containing

500 mM imidazole followed by 10 mL of sterile deionized water 3 times. The Ni-NTA agarose was stored in 20% ethanol at 4 °C.

The presence and purity of the purified protein was evaluated by 10% SDS-PAGE. The imidazole was removed by dialysis against 20 mM sodium carbonate buffer pH 10 for overnight at 4 ºC.

2.21 Determination of protein concentration

The protein concentration was determined according to the Bradford's dye-binding method (Bradford, 1976) using bovine serum albumin (Fluka) as a standard. This method is base on the binding of Coomassie brilliant blue G250 dye to the proteins converting the red dye color to blue. A sample solution of 100 μL was mixed with 1 mL Bradford working buffer and left for 2 minutes before the absorbance at 595 nm was measured. The Bradford working buffer (100 mL) was a mixture of 6 mL Bradford stock solution (350 g Coomassie blue G250, 100 mL 95% ethanol and 200 mL 85% phosphoric acid), 3 mL 95% ethanol, 6 mL 85% phosphoric acid and 85 mL distilled water.

2.22 Characterization of rChi*Pm*1

2.22.1 Colloidal chitin agar diffusion

The qualitative determination of chitin hydrolytic activity of r*Pm*Chi1 was carried out using colloidal chitin agar diffusion method. A plate of 1% (w/v) agarose gel in 30 mL of 1× PBS, containing 0.05% (w/v) colloidal chitin from shrimp shell and 100 μg/mL of ampicillin was prepared. Wells of 0.8-cm diameter

were cut into the solidified gel and added with 50 μg r*Pm*Chi1 in 20 mM sodium carbonate buffer pH 10. Chitinase from *Bacillus licheniformis* SK-1 (Kudan and Pichyangkura, 2009) and 20 mM sodium carbonate buffer pH 10 were used as positive and negative controls, respectively. The plate was incubated overnight at 37 ºC. Digestion halo zones around the wells were observed for chitinase samples.

2.22.2 Chitinase activity assay

For quantitative measurement of chitinase activity, a colorimetric assay was employed. The reaction mixtures consisted of 1 mg/mL colloidal chitin, 0.1 M sodium phosphate buffer pH 7 and various quantities of r*Pm*Chi1 in a total volume of 750 μL. The reactions were incubated at 37 ºC for 1 hour, added 1 mL color reagent (0.5 g/L potassium ferricyanide in 0.5 M sodium carbonate), boiled for 15 minutes and swiftly cooled on ice. The reactions were centrifuged at 5,000 rpm for 15 minutes to clarify the solutions of which the supernatant liquids were measured visible absorption at wavelength 420 nm (A_{420}). The experiments were repeated five times. Pre-boiled r*Pm*Chi1 for 20 minutes was used as a negative control. A recombinant thioredoxin-SPI*Pm*4 fusion protein (Visetnan et al., 2009) was used as a control for the chitinase assay to see if thioredoxin possessed any hydrolytic activity.

2.22.3 Effect of pH on r*Pm*Chi1 activity (optimal pH)

To determine the pH optimum of the chitinase reactions, three buffer systems were used, namely, sodium acetate buffer pH 3-7, sodium phosphate buffer pH 7-9 and sodium glycine buffer pH 9-12. The reactions were carried out by incubating 50 μg of the r*Pm*Chi1 with 1 mg/mL colloidal chitin in a

total volume of 750 μL under various pHs for 1 hour at 37 ºC before the color reaction was developed.

2.22.4 Effect of temperature on r*Pm*Chi1 activity (optimal temperature)

The r*Pm*Chi1 was tested for its optimum working temperature by incubating 50 μg of the enzyme with 1 mg/mL colloidal chitin in a total volume of 750 μL for 1 hour at pH 7 and various temperatures of 20-80 ºC before the color reaction was developed.

2.22.5 Effect of pH on r*Pm*Chi1 stability (pH stability)

To test its stability under various pHs, 50 μg of r*Pm*Chi1 was incubated at different pHs of 3-12 and 37 ºC for 5 hours. Then, the chitinase activity was assayed at pH 5 and 37 ºC for 1 hour.

2.22.6 Effect of temperature on r*Pm*Chi1 stability (temperature stability)

The temperature stability of the enzyme was tested by incubating 50 μg of the r*Pm*Chi1 at various temperatures of 5-80 ºC for 5 hours before the chitinase activity was assayed. The assay was carried out in acetate buffer pH 5 at 37 ºC for 1 hour. The color reaction was developed as mentioned above.

2.22.7 Substrate preference of r*Pm*Chi1

The r*Pm*Chi1 was tested for its substrate preference by incubating 50 μg of the recombinant enzyme in sodium acetate buffer, pH 5, for 1 hour at 37 ºC with diverse substrates: 0.1 mg/mL partially *N*-acetylated chitin (PNAC), 1 mg/mL

colloidal chitin from shrimp shell, 1 mg/mL β-chitin from squid pen. The reactions were, then, assayed as mentioned above. The test was repeated five times. The data were expressed as mean ± standard deviation. The statistical significance of the data was evaluated using one-way analysis of variance (ANOVA) followed by post hoc test (Duncan's new multiple range test). Significant differences were accepted at $p < 0.05$.

CHAPTER III

RESULTS

3.1 Searching the *Penaeus monodon* EST database

From a total of 10,536 unique contigs and singletons in the *Penaeus monodon* database (http://pmonodon.biotec.or.th/database.jsp) (Tassanakajon et al., 2006), three contigs and two singletons representing three chitinase genes were identified and named *PmChi1*, *2* and *3* according to amino acid sequence homology to the chitinases from *Marsupenaeus japonicus* (Watanabe et al., 1996; 1998; Watanabe and Kono, 1997). Two contigs, CT508 and CT585, respectively, represented the *PmChi1* and its variant; one singleton, SG3857, was the *PmChi2*; one contig, CT226, and one singleton, SG8242, were the *PmChi3*. The three chitinase genes were truncated and needed mending. PCR and RACE were employed to complete the gene sequences. Primers were designed from the available DNA sequences.

3.2 Phylogenetic analyses of *Pm*Chi1, 2 and 3

The phylogenetic relationships of the *P. monodon* chitinases to other chitinases were analyzed. The chitinases from various organisms; mammals, insects and crustaceans including the penaeid shrimp were subjected to amino acid sequence comparison. The amino acid sequences were analyzed online for the typical glycoside hydrolase catalytic (Glyco 18) domain at http://smart.embl-heidelberg.de/. The catalytic domains were compared using the ClustalX and a phylogenetic tree was constructed using the Phylip program. The mammalian chitinases were treated as an outgroup.

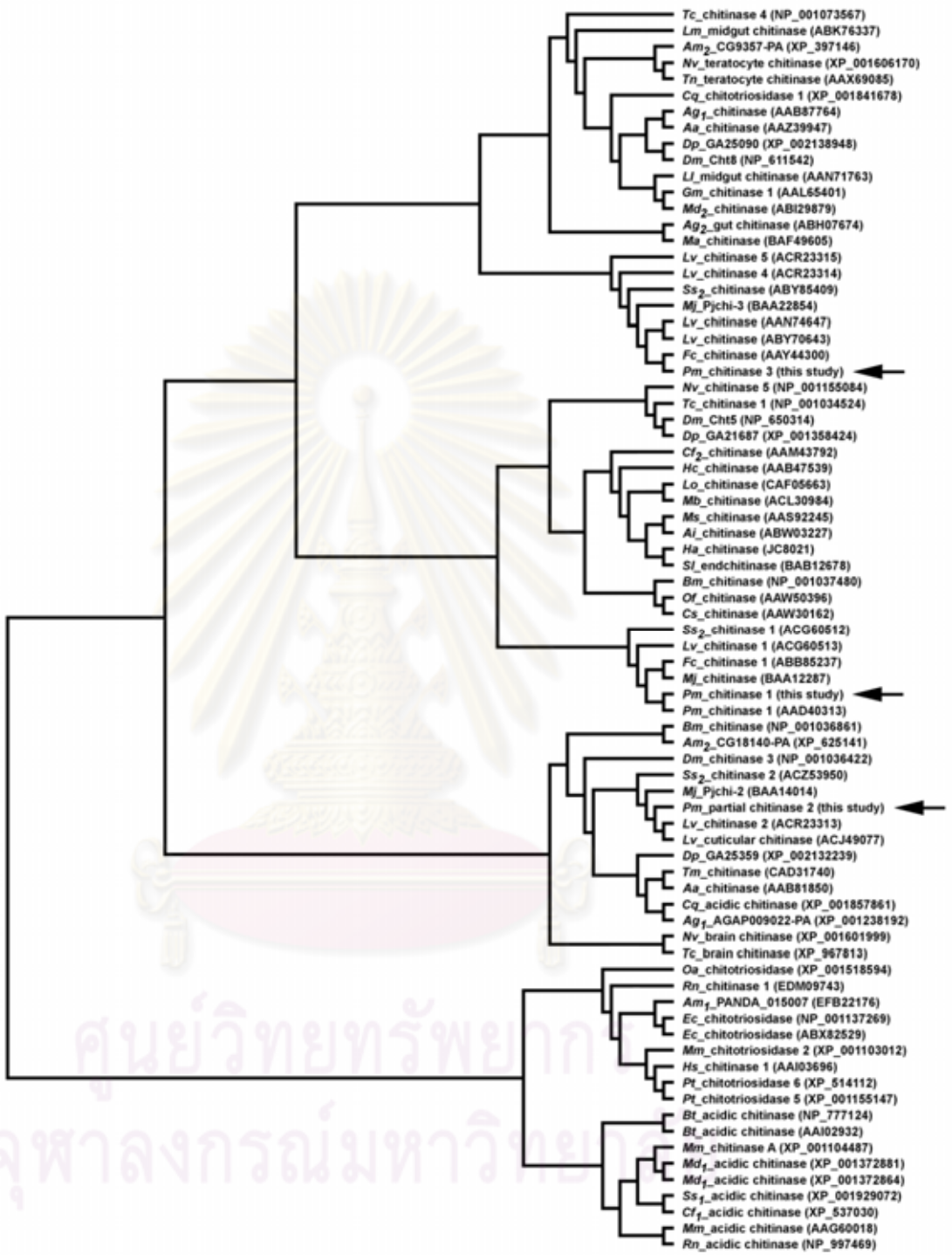

Figure 3.1 Phylogenetic trees based on the alignment of amino acid sequences of the catalytic domains of chitinases using ClustalX and Phylip programs. Bootstrap analysis was performed for values representing 1000 replicates by the SeqBoot. The amino acid sequences of mammalian chitinases were defined as an outgroup. The amino acid sequences are from the GenBank. *Aa*, *Aedes aegypti*; Ag_1, *Anopheles gambiae*, Ag_2, *Apriona germari*; *Ai*, *Agrotis ipsilon*; Am_1, *Ailuropoda melanoleuca*; Am_2,

Apis mellifera; *Bm*, *Bombyx mori*; *Bt*, *Bos taurus*; *Cf_1*, *Canis familiaris*; *Cf_2*, *Choristoneura fumiferana*; *Cq*, *Culex quinquefasciatus*; *Cs*, *Chilo suppressalis*; *Dm*, *Drosophila melanogaster*; *Dp*, *Drosophila pseudoobscura pseudoobscura*; *Ec*, *Equus caballus*; *Fc*, *Fenneropenaeus chinensis*; *Gm*, *Glossina morsitans morsitans*; *Ha*, *Helicoverpa armigera*; *Hc*, *Hyphantria cunea*; *Hs*, *Homo sapiens*; *Ll*, *Lutzomyia longipalpis*; *Lm*, *Locusta migratoria manilensis*; *Lo*, *Lacanobia oleracea*; *Lv*, *Litopenaeus vannamei*; *Ma*, *Monochamus alternates*; *Mb*, *Mamestra brassicae*; *Md_1*, *Monodelphis domestica*; *Md_2*, *Musca domestica*; *Mj*, *Marsupenaeus japonicus*; *Mm*, *Macaca mulatta*; *Ms*, *Mythimna separate*; *Nv*, *Nasonia vitripennis*; *Oa*, *Ornithorhynchus anatinus*; *Of*, *Ostrinia furnacalis*; *Pm*, *Penaeus monodon*; *Pt*, *Pan troglodytes*; *Rn*, *Rattus norvegicus*; *Sl*, *Spodoptera litura*; *Ss_1*, *Sus scrofa*; *Ss_2*, *Scylla serrata*; *Tc*, *Tribolium castaneum*; *Tm*, *Tenebrio molitor*; *Tn*, *Toxoneuron nigriceps*. The numbers in parentheses are the GenBank accession numbers.

The amino acid sequence of Glyco 18 catalytic domain of *Pm*Chi1, 2 and 3 were A20-D371, Y28-D373 and V1-D348, respectively. That of *Pm*Chi1 shared similarities of 65-93% to crustacean chitinases, 48-60% to insect chitinases and 35-37% to mammalian chitinases. That of *Pm*Chi2 shared similarities of 77-97% to crustacean chitinases, 39-42% to insect chitinases and 39-74% to mammalian chitinases. For *Pm*Chi3, its similarities to the crustacean, insect and mammalian chitinases were 49-97%, 38-46% and 35-40%, respectively. The phylogenetic relationship among the chitinases is shown in Figure 3.1. The chitinases from crustaceans and insects were clustered into three lineages. Within each lineage, the chitinases from crustaceans were also separately grouped together. The groups of *Pm*Chi1 and 3 were closely related to each other than to the group of *Pm*Chi2.

3.3 Tissue specific expression of chitinases

For the clues to their functions, tissue localization of the three chitinases was investigated in normal and early postmolted shrimp. Equivalent amounts of cDNAs from antennal gland, stomach, hemocyte, gill, intestine, lymphoid, eyestalk,

hepatopancreas, heart, epipodite, pleopod and tail were analyzed by RT-PCR using the *β-actin* gene as an internal control. The results shown in Figure 3.2 revealed that the *PmChi1* was expressed mainly in hepatopancreas but relatively small amount was found in antennal gland, stomach and intestine. The *PmChi2* was expressed mainly in gill and smaller amount in eyestalk and epipodite. The *PmChi3* was expressed mainly in hepatopancreas. At early postmolt stage A, only the amount of *PmChi2* messenger was down-regulated in gill, eyestalk and epipodite but could be seen faintly in all tissues. The expression of *PmChi1* was more or less the same except an up-regulated in lymphoid and down-regulated in antennal gland and intestine. The significant changes in antennal gland, intestine and lymphoid were under study. The expression of *PmChi3* remained unchanged at early postmolt stage A.

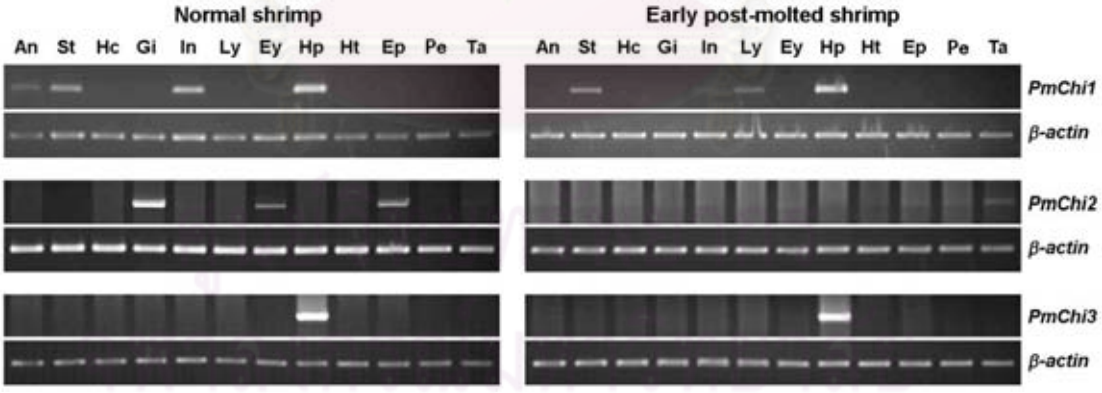

Figure 3.2 Tissue distribution of *PmChi1*, *2* and *3* in the black tiger shrimp. Tissues tested are antennal gland (An), stomach (St), hemocyte (Hc), gill (Gi), intestine (In), lymphoid (Ly), eyestalk (Ey), hepatopancreas (Hp), heart (Ht), epipodite (Ep), pleopod (Pe) and tail (Ta). The *β-actin* was used as an internal control.

3.4 Cloning of the *PmChi1* gene

3.4.1 Primer design and PCR amplification for *PmChi1* cloning

As compared to that of the Pjchi-1, the amino acid sequence of *Pm*Chi1 from the CT508 contig covered two-third of the chitinase 1 open reading frame with the 5′ sequence truncated. The 3′ untranslated sequence went as far as the poly(A) tail. When compared to the amino acid sequence of the *P. monodon* chitinase 1 reported by Tan et al. (2000), the *Pm*Chi1 in our study was 46 amino acids longer at its C-terminus (see Figure 3.6). To complete the *PmChi1* gene coding for mature chitinase 1, the 5′ forward primer (FChi1_1) was designed from the cDNA sequence described by Tan et al. (2000) whereas the 3′ reverse primer (RChi1_1) was from the contig sequence (Table 1). The nucleotide sequence coding for the signal peptide predicted by the SignalP 3.0 was omitted in the amplification for subsequent cloning into the expression vector.

PmChi1 gene was amplified from the hepatopancreas cDNA with FChi1_1 and RChi1_1 and isolated by agarose gel electrophoresis (Figure 3.3). PCR product of 1,937 bp was purify using NucleoSpin® Extract II kit and cloned into the T&A cloning vector (RBC) giving rise to a recombinant clone pT-*Pm*Chi1.

3.4.2 Screening of recombinant pT-*Pm*Chi1 and sequencing result

Recombinant plasmids from the positive transformants were prepared, cleaved with restriction enzymes *Nco*I and *Xho*I and analyzed by agarose gel electrophoresis to identify the correct insert. The approximately 2 kb of *PmChi1* gene fragment and 2.7 kb of T&A cloning vector were present in lanes 9, 10 and 11 (Figure 3.4). They were the pT-*Pm*Chi1. Only one of them was subjected to sequencing with T7 promoter primer and M13reverse primer to ensure the correct mature sequence of *ChiPm*1 gene (Figure 3.5).

Figure 3.3 An agarose gel electrophoresis of amplified *PmChi1* gene. Lane M is lambda DNA/HindIII marker and lane 1 is the amplified *PmChi1* gene fragment from hepatopancreas cDNA of the black tiger shrimp with FChi1_1 and RChi1_1.

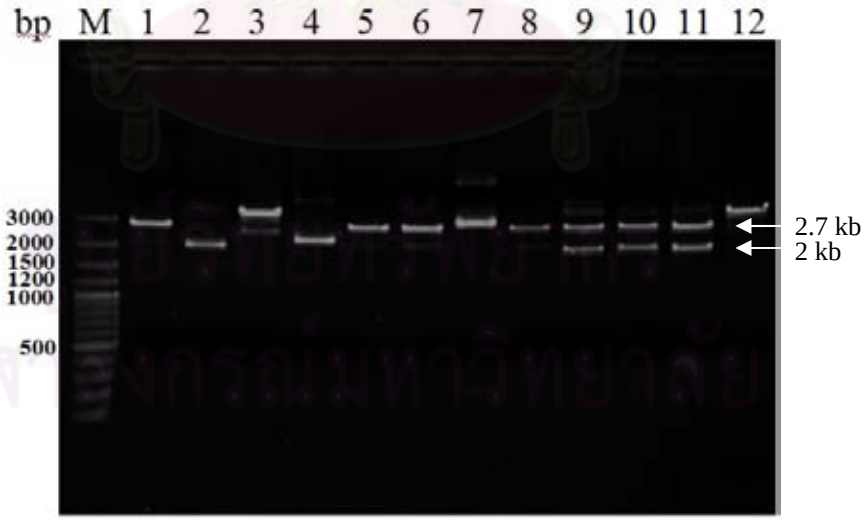

Figure 3.4 An agarose gel electrophoresis of recombinant plasmids cleaved with *Nco*I and *Xho*I. Lane M is a GeneRuler™ 100 bp DNA Ladder (Fermentas) and lanes 1-12 are recombinant plasmids cleaved with *Nco*I and *Xho*I. DNA band of 1937 bp indicates the existence of *PmChi1* gene fragment.

```
   1  GACCCGAGATTCGAGCAAGAAGGAGCCCAGCGGCGGTGGGTGCGGCCCGAGGGCCAGGCG   60
      D  P  R  F  E  Q  E  G  A  Q  R  R  W  V  R  P  E  G  Q  A
  61  CGCCGCGTGTGCTACTACGAGGCGTGGGCCATCTACCGGCCCGGCGACGGCTTCTACGAC  120
      R  R  V  C  Y  Y  E  A  W  A  I  Y  R  P  G  D  G  F  Y  D
 121  ATCGAGGACATCCCCGCCAACCTGTGCACGGACCTCATCTACTCGTTCATTGGCCTCTCC  180
      I  E  D  I  P  A  N  L  C  T  D  L  I  Y  S  F  I  G  L  S
 181  AACGTCACGTGGGAAGTGCTCATTCTCGACCCTGAGTACGACATTAACCTGAACGGTTTC  240
      N  V  T  W  E  V  L  I  L  D  P  E  Y  D  I  N  L  N  G  F
 241  CGGCGGTTCGTGGCGCTGAAGGACAAGTACCCTGACATGAAGACAAACATCGCCGTGGGC  300
      R  R  F  V  A  L  K  D  K  Y  P  D  M  K  T  N  I  A  V  G
 301  GGCTGGGCCGAGGGCGGAAGGAAGTACTCGCAGATGGTGATGGTGCCCGAAAGGAGGGCG  360
      G  W  A  E  G  G  R  K  Y  S  Q  M  V  M  V  P  E  R  R  A
 361  TCTTTCATCAGGAGCGTCGTCCAGCTGCTCACCGACTACGGCTTCGACGGGTTGGACTTG  420
      S  F  I  R  S  V  V  Q  L  L  T  D  Y  G  F  D  G  L  D  L
 421  GACTGGGAATACCCTGGCGCCACAGACCGAGGAGGCCAATATGCCGATAAGGACAACTTC  480
      D  W  E  Y  P  G  A  T  D  R  G  G  Q  Y  A  D  K  D  N  F
 481  CTTAAACTGGTGCAGGAGCTGCGCGAAGCCTTCGACACCGTGGAGCTGGGCTGGGAAATC  540
      L  K  L  V  Q  E  L  R  E  A  F  D  T  V  E  L  G  W  E  I
 541  ACGTGCGCCGTGCCCGTGGCCAAGTTCCGCCTGCAGGAGGGCTACCATGTGCCTCAGCTC  600
      T  C  A  V  P  V  A  K  F  R  L  Q  E  G  Y  H  V  P  Q  L
 601  TGCAGCCTGCTGGACGCCATCCACCTGATGACGTACGACCTGCGGGGCAACTGGGTCGGC  660
      C  S  L  L  D  A  I  H  L  M  T  Y  D  L  R  G  N  W  V  G
 661  TTCGCGGACGTGCACTCCATGCTGTACACTCGGCCCGGACCGGACGAGTGGGCCTACGAG  720
      F  A  D  V  H  S  M  L  Y  T  R  P  G  P  D  E  W  A  Y  E
 721  AAGCTGAACGTGAACGACGGCGCTCTCCTGTGGGTGGAATTCGGGTGTCCGCGTGATAAG  780
      K  L  N  V  N  D  G  A  L  L  W  V  E  F  G  C  P  R  D  K
 781  TTGGTGGTCGGGACGCCATTCTACGGGCGCACCTACACGCTGGGTGACCCCACCAACAAC  840
      L  V  V  G  T  P  F  Y  G  R  T  Y  T  L  G  D  P  T  N  N
 841  GACCTGCACGCGCCCATCAAGAAGTGGGAGGGAGGTGGCAAGCCCGGCCCTTATACCAAC  900
      D  L  H  A  P  I  K  K  W  E  G  G  G  K  P  G  P  Y  T  N
 901  GCCACCGGCACTATGGCTTACTTCGAGATCTGCCTCATGATGAAGGAGGACTCCGAGTGG  960
      A  T  G  T  M  A  Y  F  E  I  C  L  M  M  K  E  D  S  E  W
 961  GTCGATTGCTACGATGACGTCGGCCTCGTCCCATTCACGCACAAAGGCGACCAGTGGGTG 1020
      V  D  C  Y  D  D  V  G  L  V  P  F  T  H  K  G  D  Q  W  V
1021  GGCTACGAGGACCCTGACAGTCTCAAGATCAAGATGGACTTCATCCGCGAGCAGGGCTAC 1080
      G  Y  E  D  P  D  S  L  K  I  K  M  D  F  I  R  E  Q  G  Y
1081  CTCGGCGCTATGACCTGGGCCATCGACCAGGACGACTTCCGGAACTGGTGTGGAAGGGGA 1140
      L  G  A  M  T  W  A  I  D  Q  D  D  F  R  N  W  C  G  R  G
1141  CAGAACCCGATGATGAACACCATTTACGATGGCATGAAGGACTACGTAGTGCCTGTTGCT 1200
      Q  N  P  M  M  N  T  I  Y  D  G  M  K  D  Y  V  V  P  V  A
1201  CCCACTCTTCCTCCAACCACAACAAGCCCCTGGTGGACCCCACCAACTACTACTACCACA 1260
      P  T  L  P  P  T  T  T  S  P  W  W  T  P  P  T  T  T  T  T
1261  ACACGGGACCCCAGCATCACCACGACCACGAGAGATCCCAACTTGCCGACCACAACTATG 1320
      T  R  D  P  S  I  T  T  T  T  R  D  P  N  L  P  T  T  T  M
1321  GGGCCTATTGACTGTACTGCGCAAGAATACTGGCCGCATCCGGACTGTGACAAGTACTAC 1380
      G  P  I  D  C  T  A  Q  E  Y  W  P  H  P  D  C  D  K  Y  Y
1381  TGGTGCTTCGAAGGCATACCCCACCTGGAGTACTGCCCCGCTGGCACCGTGTGGAACCAG 1440
      W  C  F  E  G  I  P  H  L  E  Y  C  P  A  G  T  V  W  N  Q
1441  GCTATCAAGGCGTGCGACTGGCCGGCCAACGTGGACACCTCCGGCTGCAACATGCCCTCG 1500
      A  I  K  A  C  D  W  P  A  N  V  D  T  S  G  C  N  M  P  S
1501  CTCTCGAAAGACGCCAGTCAGCGGCCCCTCCACAACACTATCCCTCTCAACGTCCGAACC 1560
      L  S  K  D  A  S  Q  R  P  L  H  N  T  I  P  L  N  V  R  T
1561  AAGGGGACCCCGCACTCGGGCAAGGCGCCAAAGGTTCCTCTTAACTTGATTTCCAAGAAG 1620
      K  G  T  P  H  S  G  K  A  P  K  V  P  L  N  L  I  S  K  K
1621  CCAGCCCCGGCGAAGTCTTTACCTGCGAAGTCAGTTGATGCTAAATTAGTTCACAATAAT 1680
      P  A  P  A  K  S  L  P  A  K  S  V  D  A  K  L  V  H  N  N
1681  GCACCACCTGCTAAACCAGCACATGCTAAGCCTCTTCATGCTAAACCAGTACGTGTTAAG 1740
      A  P  P  A  K  P  A  H  A  K  P  L  H  A  K  P  V  R  V  K
1741  CCAGCACCGGTAAAGCCACTTCATACTAAAGCAATTCATGCTAAGCCAGCACAGGTAAAG 1800
      P  A  P  V  K  P  L  H  T  K  A  I  H  A  K  P  A  Q  V  K
1801  TCAGCTCATACTAAACCACAAGCGGCTAACCTAGAACCTTCCACACCAGAACCGTCGAAC 1860
      S  A  H  T  K  P  Q  A  A  N  L  E  P  S  T  P  E  P  S  N
1861  CTAAAACTTTCCAAGTCAGAGCCTGCTAAGTCAGTACCACCACTGCTAATGAAGATGGTA 1920
      L  K  L  S  K  S  E  P  A  K  S  V  P  P  L  L  M  K  M  V
1921  AAGAACTAA  1929
      K  N  *
```

Figure 3.5 Nucleotide and amino acid sequences of mature *Pm*Chi1.

3.4.3 Amino acid sequence analyses of PmChi1

The reading frame for mature *Pm*Chi1 was amplified from the cDNA pool of hepatopancreas, cloned into the T&A vector and sequenced. Examining the open reading frame of the amplified *PmChi1* showed that the amino acid sequence at the C-terminus was still longer than the *P. monodon Chi1* by Tan et al. (2000). Besides the longer C-terminal end, a few amino acid variations were observed (Figure 3.6) possibly owing to variation in shrimp stock. Amino acid sequence comparison reveals that, except at the C-terminal end, the *Pm*Chi1 amino acid sequence was very similar to those of *M. japonicus*, *F. chinensis* and *L. vannamei* (Figure 3.6); the homology was about 88-91%. The reading frame for mature *Pm*Chi1 coded for a polypeptide chain of 644 amino acid residues with a calculated molecular weight of 72.4 kDa. The Glyco 18 catalytic domain of *Pm*Chi1 was defined using PROSITE and SmartTM databases to be A20-D371. The cysteine-rich chitin-binding domain resided at the C-terminus I443-M498.

3.5 Construction of *Pm*Chi1-expression clone

To express the recombinant *Pm*Chi1, the gene specific primers FChi1_1 and RChi1_1 were designed with extended *Nco*I and *Xho*I sites at their 5' ends, respectively. The included *Nco*I and *Xho*I sites, respectively, at the 5' and 3' ends of the *PmChi1* gene fragments were for further cloning into the expression vector. Both pT-*Pm*Chi1 and pET-32a(+) vector were cleaved with *Nco*I and *Xho*I and were separated using agarose gel electrophoresis (Figure 3.7). The 2 and 5.9 kb of the *Nco*I-*Xho*I *PmChi1* fragments and linearized pET-32a(+) vector, respectively, were purified and ligated together.

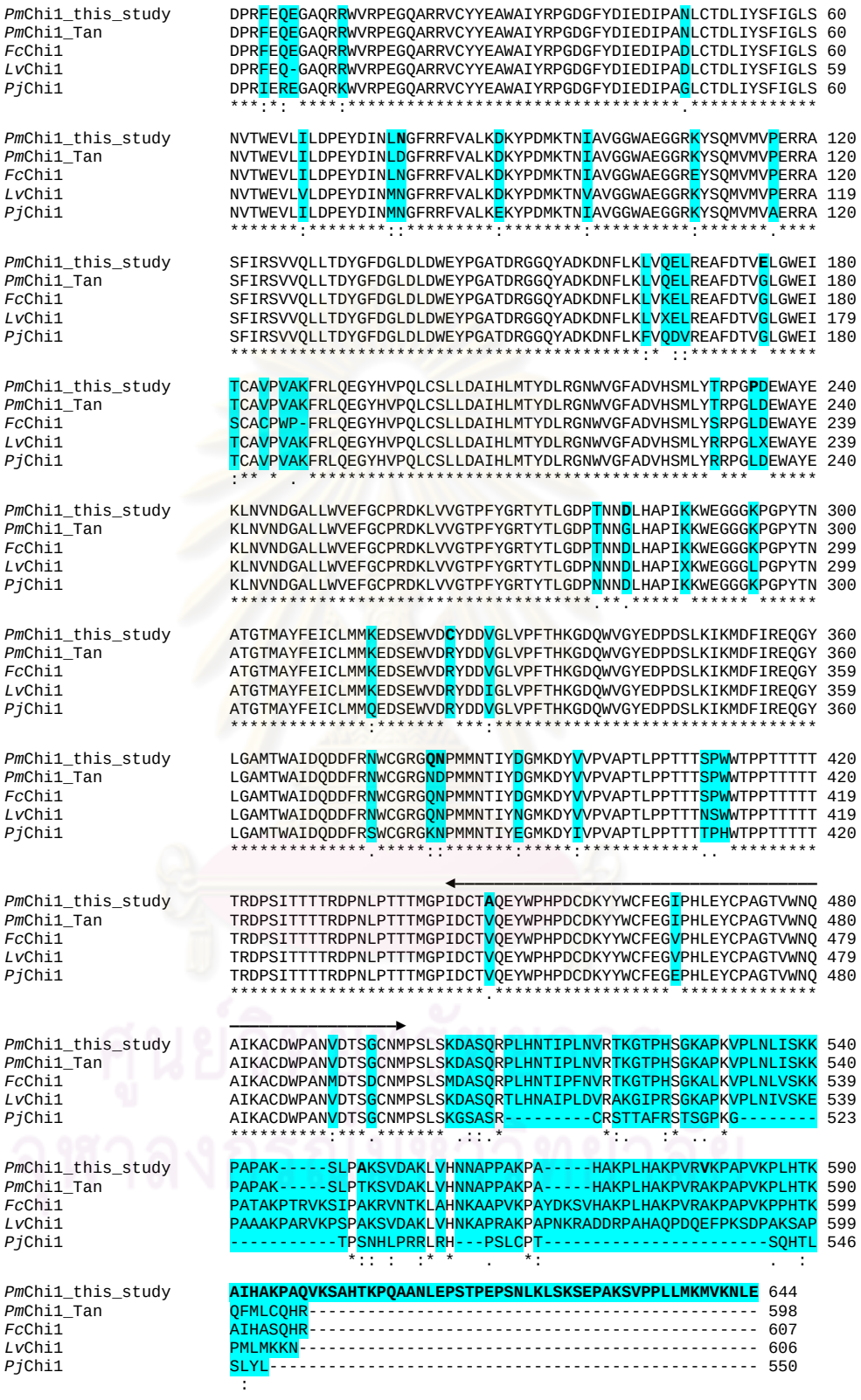

Figure 3.6 Amino acid sequence comparison among the mature chitinases 1 from the penaeid shrimp. The positions of variable amino acid residues are shaded. The amino acid residues of *Pm*Chi1 from this

study that are different from those of *Pm*Chi1 described by Tan et al. (2000) are in bold. The Glyco 18 catalytic domain is from A20 to D371. The arrow above the sequences indicates the cysteine-rich chitin binding domain from I443 to M498. *Pm*, *Fc*, *Lv* and *Pj* are for *Penaeus monodon*, *Fenneropenaeus chinensis*, *Litopenaeus vannamei* and *Peneaus* (*Marsupenaeus*) *japonicus*, respectively.

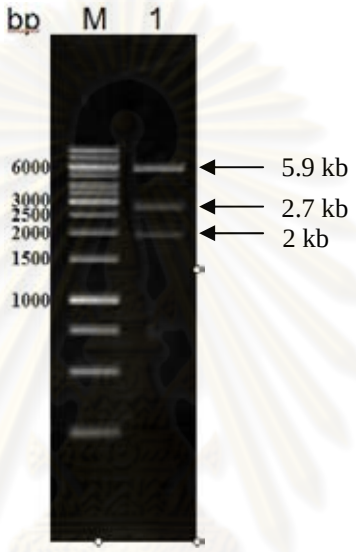

Figure 3.7 An agarose gel electrophoresis of the pT-*Pm*Chi1 and pET-32a(+) vector cut with *Nco*I and *Xho*I**.** Lane M a GeneRuler™ 1 kb DNA Ladder (Fermentas).

In the screening procedure, the recombinant plasmids were digested with *Nco*I and *Xho*I, and analyzed by agarose gel electrophoresis (data not shown). A transformant was shown to be the correct expression plasmid by re-digestion with *Pst*I (Figure 3.8). DNA bands of 5.1 and 2.6 kb indicate the existence of *PmChi1* gene in pET-32a(+) vector. The resulting expression plasmid clone, pET-32a(+)-*Pm*Chi1, was transformed into an *E. coli* host strain Rosetta(DE3)pLysS for recombinant protein expression.

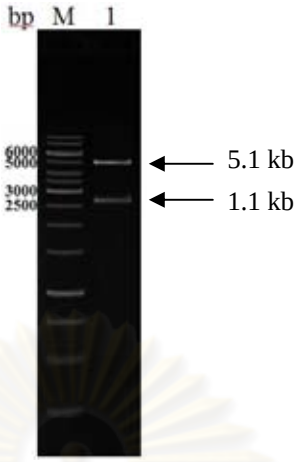

Figure 3.8 An agarose gel electrophoresis of a recombinant plasmid digested with restriction enzyme *Pst*I. Lane M is a GeneRuler™ 1 kb DNA Ladder (Fermentas). DNA bands of 5.1 and 2.6 kb indicate the existence of *Pm*Chi1 gene in pET-32a(+) vector.

3.6 Cloning of the *PmChi2* gene

3.6.1 Primer design and PCR amplification for partial *PmChi2*

For *PmChi2*, a singleton SG3857 represented the enzyme. The cDNA sequence covered a third of the open reading frame in the middle of the gene lacking both the 5′ upstream and 3′ downstream sequences coding for the N- and C-terminal sequences of the chitinase, respectively. Thanks to the high nucleotide sequence homology among the chitinases 2 from the penaeid shrimp, *L. vannamei* and *M. japonicus*, a reverse RChi2 primer was designed from the homologous sequence. The forward primer designed from the *Pjchi-2* sequence at the start of open reading frame failed to give any PCR products. The forward FChi2 primer was, then, designed from the *PmChi2* singleton in which the gene segment amplified covered only two-third of the chitinase 2 open reading frame (Figure 3.12). The amplified sequence was sequenced and used to design primers for 5′ RACE.

Partial *Pm*Chi2 gene was amplified from eyestalk cDNA with FChi2 and RChi2 and analyzed through agarose gel electrophoresis (Figure 3.9)**.** The DNA fragment of 1,117 bp was eluted using NucleoSpin® Extract II kit for the cloning into the T&A cloning vector (RBC).

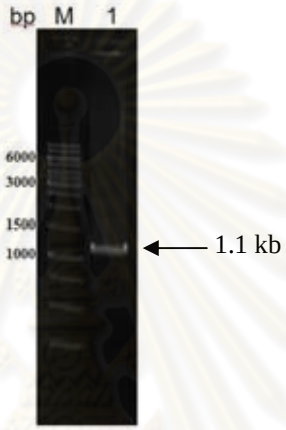

Figure 3.9 An agarose gel electrophoresis of partial *PmChi2* gene. Lane M is a GeneRuler™ 1 kb DNA Ladder (Fermentas) and lane 1 is the partial amplified *PmChi2* gene from eyestalk cDNA of black tiger shrimp with FChi2 and RChi2.

Figure 3.10 An agarose gel electrophoresis of nested PCR product of *Pm*Chi2 gene. Lane M is a GeneRuler™ 100 bp DNA Ladder (Fermentas) and lane 1 is secondary amplified *Pm*Chi2 gene with GSPin and NUP from 5' RACE libraries prepared from the eyestalk and gill cDNAs.

3.6.2 Screening of recombinant plasmid

Recombinant plasmids were isolated from individual white bacterial colonies, cleaved with restriction enzyme *Hind*III, analyzed with agarose gel electrophoresis (data not shown) and sequenced for the correct sequence of Chi*Pm*2 gene (Figure 3.11).

3.6.3 5' Rapid Amplification of cDNA Ends (5' RACE)

Three primers, GSPout1, GSPout2 and GSPin (Table 1), were designed for the 5' RACE libraries constructed from the eyestalk and gill. All possible combinations of the gene-specific primers and 5′ RACE universal primers for primary and secondary PCRs were tested. Nested PCR product was analyzed using agarose gel electrophoresis (Figure 3.10). Secondary amplified DNA fragment of 300 bp was purified and cloned into the T&A cloning vector (RBC) and DNA sequenced. Only 35 more N-terminal amino acid residues were obtained by using the GSPout1 and GSPin for primary and secondary PCRs, respectively (Figure 3.12).

3.6.4 Amino acid sequence analyses of *Pm*Chi2

As a consequence, we were able to define the Glyco18 catalytic domain of *Pm*Chi2 to be Y28-D373. The chitin binding domain could be defined only from the Pjchi-2 at the N-terminus. The partial *PmChi2* in this study was, then, 1223 bp long coding for a polypeptide chain of 406 amino acid residues**.** Comparison of the available amino acid sequences showed that there was very high homology among the chitinases 2 from the penaeid shrimp (Figure 3.12). The homology was about 90-97%.

```
   1  ACGCGGGGAGACCGACCACTTCCCCCAAGCCGATTTCCACCACCATCAGCACCCACATTG   60
        A   G   R   P   T   T   S   P   K   P   I   S   T   T   I   S   T   H   I   E

  61  AGACGATCATCCCAGACACAGGATACAAGGTGGTATGTTACTTCACCAACTGGGCGTGGT  120
        T   I   I   P   D   T   G   Y   K   V   V   C   Y   F   T   N   W   A   W   Y

 121  ATCGACAAGGCGCCGGCAAATACAGACCCGAAGACATTGATCCCAACCTCTGCACTCATA  180
        R   Q   G   A   G   K   Y   R   P   E   D   I   D   P   N   L   C   T   H   I

 181  TTGTATATGGTTTTGCTGTTCTGGACGGCACTCGGCTTCTTATCAAACCCCACGACACTT  240
        V   Y   G   F   A   V   L   D   G   T   R   L   L   I   K   P   H   D   T   W

 241  GGGCTGATTATGATAACAAGTTCTACGAAAAGGTAGCCGCACTGAGGGCGAGAGGCATCA  300
        A   D   Y   D   N   K   F   Y   E   K   V   A   A   L   R   A   R   G   I   K

 301  AGGTCACCATCGCTATCGGTGGCTGGAACGACTCTGCCGGAGACAAGTACAGTCGCCTAG  360
        V   T   I   A   I   G   G   W   N   D   S   A   G   D   K   Y   S   R   L   V

 361  TGAACAATCCCGAAGCTCGTAGGAAGTTCAACGAGCATGTAATTGAGTTCATCCAGAGAC  420
        N   N   P   E   A   R   R   K   F   N   E   H   V   I   E   F   I   Q   R   H

 421  ACAACTTCGACGGTCTCGATTTGGATTGGGAATATCCCGTCTGCTGGCAGGTGAACTGCA  480
        N   F   D   G   L   D   L   D   W   E   Y   P   V   C   W   Q   V   N   C   K

 481  AGAAGGGACCTGCCTCTGACAAAGCTGCTTTTGCCGAATGGATCAAGGAACTCCACTACG  540
        K   G   P   A   S   D   K   A   A   F   A   E   W   I   K   E   L   H   Y   A

 541  CTTTCAAGCCTCATGGTCTGCTCCTCTCCGCCGCCGTTTCTCCGAGCAACAAGGTCATCG  600
        F   K   P   H   G   L   L   L   S   A   A   V   S   P   S   N   K   V   I   D

 601  ACGCTGGGTACGACGTCCCTGCTCTGAACCGGTACCTGGACTGGATCGCCGTCATGACCT  660
        A   G   Y   D   V   P   A   L   N   R   Y   L   D   W   I   A   V   M   T   Y

 661  ATGATTATCACGGTCACTGGGACAAGAAGACAGGTCACGTCGCCCCCATGTATCTTCACC  720
        D   Y   H   G   H   W   D   K   K   T   G   H   V   A   P   M   Y   L   H   P

 721  CTGAGGACGAGGACATATACTTCAACTCTAACTTCACCATCCACTACTGGATGGAGAAGG  780
        E   D   E   D   I   Y   F   N   S   N   F   T   I   H   Y   W   M   E   K   G

 781  GCGCCGACCGCAAGAAGCTGATCATGGGCATGCCACTGTACGGCCAGTCCTTCTCCCTGG  840
        A   D   R   K   K   L   I   M   G   M   P   L   Y   G   Q   S   F   S   L   A

 841  CCTCGGCTTCCGACAACGGACTCAACCAGAAGGCGTACGGACGGGGCACTGCAGGAGAGT  900
        S   A   S   D   N   G   L   N   Q   K   A   Y   G   R   G   T   A   G   E   F

 901  TCACGAGAGCTGGCGGATTCTTGGCTTACTATGAGATCTGTGACCGCGTCCTGAACCGTG  960
        T   R   A   G   G   F   L   A   Y   Y   E   I   C   D   R   V   L   N   R   G

 961  GCTTCACCGTAGTCAAGGATCCCGAAGGCAGAATGGGTCCTTATGCCTATAATGGAAATC 1020
        F   T   V   V   K   D   P   E   G   R   M   G   P   Y   A   Y   N   G   N   Q

1021  AGTGGTTCGGATACGATGACATCGCTATGATCAGATACAAGTCTGAATGGGTCAAGAAGA 1080
        W   F   G   Y   D   D   I   A   M   I   R   Y   K   S   E   W   V   K   K   M

1081  TGGGTCTGGGCGGCGGCATGATCTGGGCCCTCGACCTCGACGACTTCAGTAACCGCTGCG 1140
        G   L   G   G   G   M   I   W   A   L   D   L   D   D   F   S   N   R   C   G

1141  GCTGCGAACCACACCCTCTCCTCCGCACCATCAACAGAGTCCTGAGGAGCCATCCCGACC 1200
        C   E   P   H   P   L   L   R   T   I   N   R   V   L   R   S   H   P   D   P

1201  CAGATCCTAAGTGCAACATGTAA  1223
        D   P   K   C   N   M   *
```

Figure 3.11 Nucleotide and amino acid sequence of partial *Pm*Chi2.

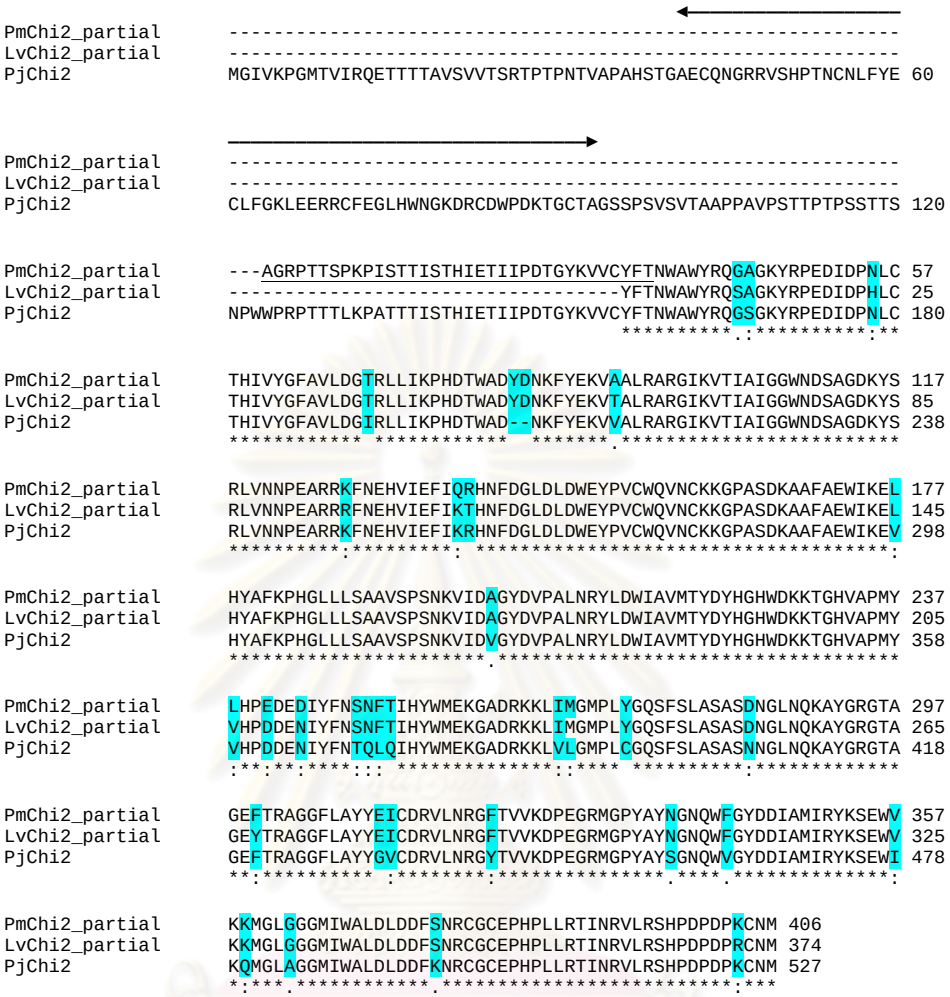

Figure 3.12 Amino acid sequence comparison of chitinases 2 among the penaeid shrimp. The positions of variable amino acid residues are shaded. Underlined amino acid sequence of *Pm*Chi2 was obtained from 5' RACE. The Glyco18 catalytic domain of *Pm*Chi2 is from Y28 to D373. The chitin binding domain is found in Pjchi-2. The arrow above the sequences indicates the cysteine-rich chitin binding domain. *Pm*, *Lv* and *Pj* are for *Penaeus monodon*, *Litopenaeus vannamei* and *Peneaus* (*Marsupenaeus*) *japonicus*, respectively.

3.7 Cloning of the *Pm*Chi3 gene

3.7.1 Primer design and PCR amplification for *Pm*Chi3 cloning

One contig, CT226, and one singleton, SG8242, represented the *PmChi3*. Using the *PjChi3* gene sequence as a guideline, the singleton covered the N-

terminal part of the mature enzyme including a portion of the signal peptide. The contig covered the C-terminal portion of the enzyme with the 3′ untranslated nucleotide sequence went down to the poly(A) tail. To retrieve the middle sequence portion, primers were designed from the DNA sequences of the contig and singleton to amplify the *PmChi3* gene coding for mature protein (Table 1). The size of mature *Pm*Chi3 was 468 amino acids with calculated molecular weight of 51.9 kDa.

*Pm*Chi3 gene was amplified from hepatopancreas cDNA with FChi3 and RChi3 (Figure 3.13). The PCR product was analyzed with agarose gel electrophoresis, purified and cloned into the T & A cloning vector (RBC).

3.7.2 Screening of recombinant plasmid and sequencing result

The recombinant plasmids were digested with restriction enzyme *Hind*III and analyzed by agarose gel electrophoresis (data not shown). The correct clone was subjected to DNA sequencing and shown in Figure 3.14.

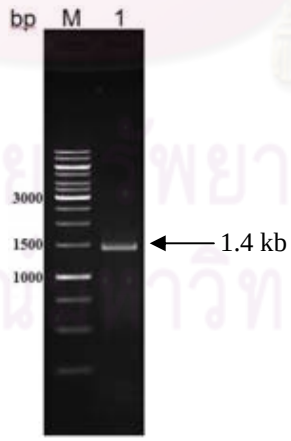

Figure 3.13 An agarose gel electrophoresis of amplified *Pm*Chi3 gene. Lane M is a GeneRuler™ 1 kb DNA Ladder (Fermentas) and lane 1 is amplified *Pm*Chi3 gene from hepatopancreas cDNA of black tiger shrimp with FChi3 and RChi3.

3.7.3 Amino acid sequence analyses of *Pm*Chi3

As illustrated in Figure 3.15, the *Pm*Chi3 showed very high homology to those chitinases 3 of *F. chinensis*, *L. vannamei* and *M. japonicus*. The homology was about 93-97%. The Glyco 18 catalytic domain of *Pm*Chi3 was defined to be V1-D348. Like the *Pm*Chi1, the chitin binding domain resided at the C-terminus from V402 to L459.

3.8 Recombinant expression of *Pm*Chi1 in the *Escherichia coli* expression system

The *Pm*Chi1 was chosen for protein expression and characterization for it was believed to be involved in the digestion of chitin in food and degradation of endogenous chitin in the gut, and its activity might be interesting for further application. The DNA fragment coding for mature *Pm*Chi1 was cloned into a pET32a(+)-expression vector and expressed in an *E. coli* strain Rosetta(DE3)pLysS. The recombinant *Pm*Chi1 (r*Pm*Chi1) was expressed as a fusion protein to the thioredoxin with the approximately calculated molecular mass of 86 kDa since molecular mass of the r*Pm*Chi1 and thioredoxin proteins were approximately 72 and 14 kDa, respectively, according to the deduced amino acid sequences.

The culture of an *E.coli* Rosetta(DE3)pLysS transformant in terrific broth was induced at 17 ºC or 37 ºC for protein expression by adding IPTG to a final concentration of 0.125, 0.2, 0.25, or 0.5 mM, harvested at each time point after induction and analyzed by SDS-PAGE and western blotting (data not shown). The condition of 0.2 mM IPTG at 17 °C was the best. When the protein expression was induced at this condition, the amount of expressed r*Pm*Chi1 was increased with time up to 6 h (Figure 3.16). It should be noted that the r*Pm*Chi1 was not over-expressed when not fused with thioredoxin.

```
   1  GTGATGGTGTGCTACTTCGGCTCGTGGGCCGTGTACCGCCAGGGCCTCGGCAAGTTCGAC   60
      V  M  V  C  Y  F  G  S  W  A  V  Y  R  Q  G  L  G  K  F  D

  61  GTGGAGGACATCGATCCCAAGATCTGCACCCACATCATCTTCGGCTTCGCTGGTCTGGCA  120
      V  E  D  I  D  P  K  I  C  T  H  I  I  F  G  F  A  G  L  A

 121  CACGATTCTTCGATCAGAGTCCTGGACCCTTGGAACGAACTGTGCGACAACTACGGCAAG  180
      H  D  S  S  I  R  V  L  D  P  W  N  E  L  C  D  N  Y  G  K

 181  TGCGCCTACGATAGGTTCACGGCCCTCAAGCAGCACAACGCTAACCTGAAGGCTATCTTG  240
      C  A  Y  D  R  F  T  A  L  K  Q  H  N  A  N  L  K  A  I  L

 241  GCCGTCGGTGGCTGGAACGAGGGATCCCCTAAGTATTCCAAGATGGCAGCCGACCCAGTA  300
      A  V  G  G  W  N  E  G  S  P  K  Y  S  K  M  A  A  D  P  V

 301  TTGAGGGACCGATTCATCACCTCCTCAATCGAGCTCCTTAAGAAACACGGCTTCGACGGC  360
      L  R  D  R  F  I  T  S  S  I  E  L  L  K  K  H  G  F  D  G

 361  CTCGACATGGACTGGGAGTACCCGACTCAGCGAGGCGGGGCCCCTGAGGACTATGATAAC  420
      L  D  M  D  W  E  Y  P  T  Q  R  G  G  A  P  E  D  Y  D  N

 421  TTCGCCATCCTCATGGCTGAACTCAACCAAGCCCTGCACGCAGAGGGGATGCTGCTGACG  480
      F  A  I  L  M  A  E  L  N  Q  A  L  H  A  E  G  M  L  L  T

 481  GCCGCCGTGTCAGCAGGCAAGGCCACCATTGATCCGGCCTACAACGTGCCAGAGATATCG  540
      A  A  V  S  A  G  K  A  T  I  D  P  A  Y  N  V  P  E  I  S

 541  AAGTCCCTCGACTTGATTAACGTAATGACCTACGATCTGCACGGCGCCTGGGACGACTAC  600
      K  S  L  D  L  I  N  V  M  T  Y  D  L  H  G  A  W  D  D  Y

 601  ACTCACCACCAGTCTGGCCTCTATGCTCATCCTCTCGACGAGGGAGATAATACCTTCTTG  660
      T  H  H  Q  S  G  L  Y  A  H  P  L  D  E  G  D  N  T  F  L

 661  AACGTGGACTTTGCAATCAGTTACTGGATCGAGAAGGGAGCTCGCCCCGGCCAGATCGCC  720
      N  V  D  F  A  I  S  Y  W  I  E  K  G  A  R  P  G  Q  I  A

 721  CTGGGTATCCCGCTGTACGGCCGCTGCTGGACCCTCGCCAGCCAGCAGGAAACCGGGTAT  780
      L  G  I  P  L  Y  G  R  C  W  T  L  A  S  Q  Q  E  T  G  Y

 781  TACGCCCCCGCGCACCAGCCCGGCGCCGCTGGCGACTGGACTAAGAGCCCTGGCATGCTT  840
      Y  A  P  A  H  Q  P  G  A  A  G  D  W  T  K  S  P  G  M  L

 841  GGCTATAATGAGATCTGTTACATGCAGACTACTCAGGATTGGACCGTAGTGAATGACCCT  900
      G  Y  N  E  I  C  Y  M  Q  T  T  Q  D  W  T  V  V  N  D  P

 901  GCCATGAACGAGCCCTATGCCTACTACTTCCCCATGAACAACATCTGGTGCTCGTACGAC  960
      A  M  N  E  P  Y  A  Y  Y  F  P  M  N  N  I  W  C  S  Y  D

 961  CACGCAGCCTCCGTCGCTACGAAGGCAGAGTATGCGAAGTCTAAAGGCCTGGCTGGCACG 1020
      H  A  A  S  V  A  T  K  A  E  Y  A  K  S  K  G  L  A  G  T

1021  ATGGCCTGGAGTGTGGAGACCGACGACTTCCGTGGATTATGCCACAACCGCAAGTACCAT 1080
      M  A  W  S  V  E  T  D  D  F  R  G  L  C  H  N  R  K  Y  H

1081  CTGATAAAGACCATGGTTGAGGTGTTTGGTGGTGGCAGCATCACCGAACCCCCACCTCTC 1140
      L  I  K  T  M  V  E  V  F  G  G  G  S  I  T  E  P  P  P  L

1141  CCCACAACCACCAGGGATCCCAACGAGCCAACCACCACGACCAGAGCGCCTCCCCCACCT 1200
      P  T  T  T  R  D  P  N  E  P  T  T  T  T  R  A  P  P  P  P

1201  GGTGTCCACTGCAGCCAACCAGGCCTCAACCCGGACCCGCTGGACTGCACGCACTACTAC 1260
      G  V  H  C  S  Q  P  G  L  N  P  D  P  L  D  C  T  H  Y  Y

1261  CTGTGCTCTCTCAACACCTCAGGCGGTTACAACGAGAAGGAGGAGGTGTGCCCTGAGGGA 1320
      L  C  S  L  N  T  S  G  G  Y  N  E  K  E  E  V  C  P  E  G

1321  ACGCTCTACAATCCCCAGAGCTACTACTGCGACTGGGCTTCTTCCGTGTGTCATCTCGGC 1380
      T  L  Y  N  P  Q  S  Y  Y  C  D  W  A  S  S  V  C  H  L  G

1381  GAGGACGTTTGCCCGAATGACTGCTAA  1407
      E  D  V  C  P  N  D  C  *
```

Figure 3.14 Nucleotide and amino acid sequence of mature *Pm*Chi3.

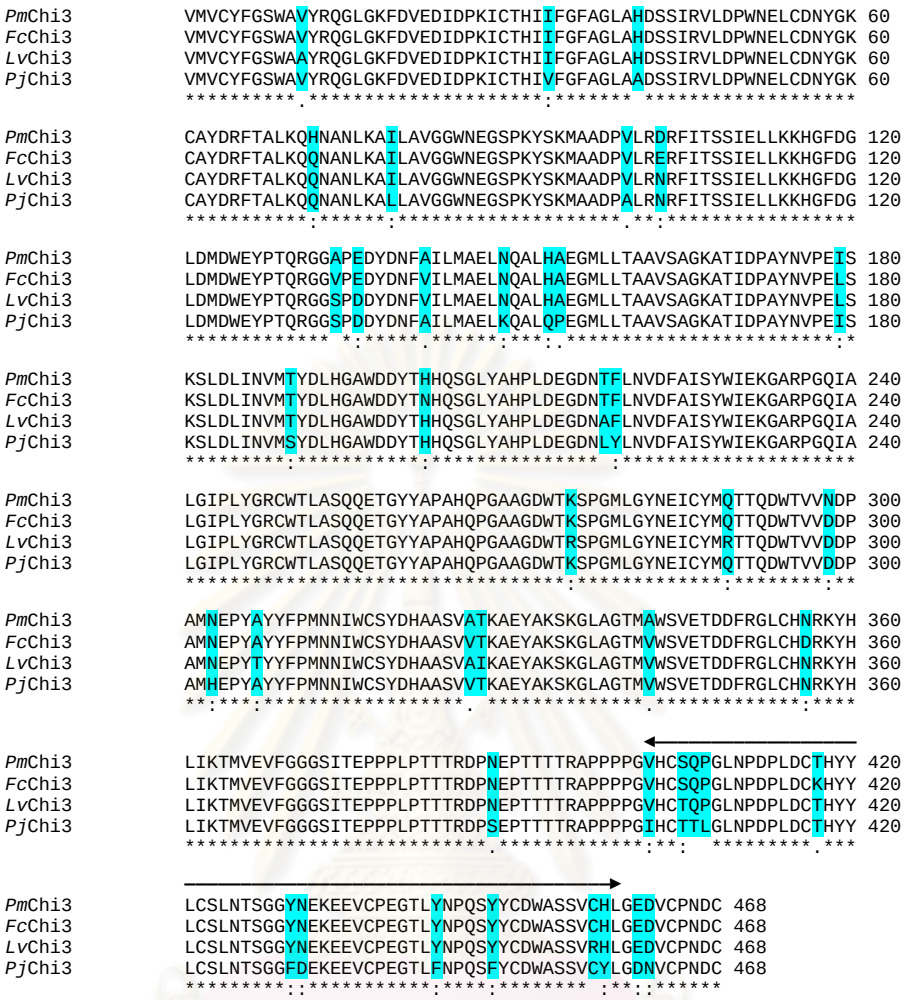

Figure 3.15 Amino acid sequence comparison of mature chitinases 3 among the penaeid shrimp. The Glyco 18 catalytic domain of *Pm*Chi3 is from V1 to D348. The arrow above the sequences, V402-L459, indicates the cysteine-rich chitin binding domain. *Pm*, *Fc*, *Lv* and *Pj* are for *Penaeus monodon*, *Fenneropenaeus chinensis*, *Litopenaeus vannamei* and *Peneaus* (*Marsupenaeus*) *japonicus*, respectively. The positions of variable amino acids are shaded.

When induced cells of *E.coli* Rosetta(DE3)pLysS containing pET-32a(+)-*Pm*Chi1 were lyzed, centrifuged to separate the supernatant soluble proteins and the inclusion body protein pellet and analyzed by 10% SDS-PAGE and western blotting. The r*Pm*Chi1 was found in the inclusion bodies (Figure 3.17). The inclusion bodies could be solubilized with 50 mM sodium phosphate buffer pH12.

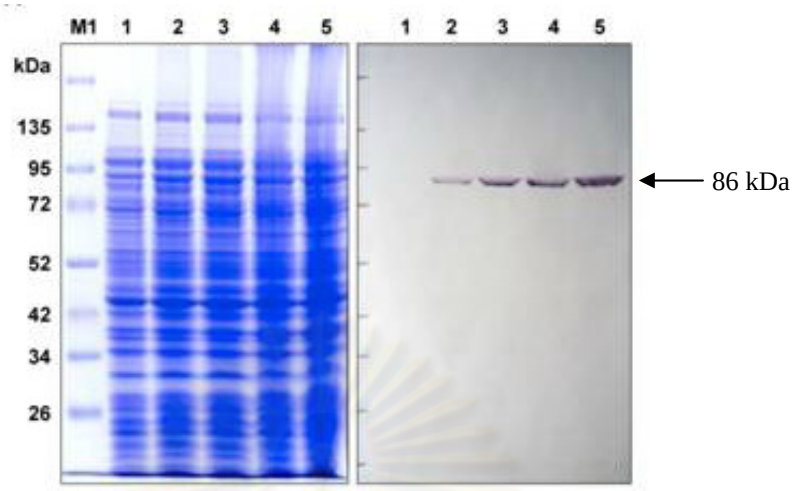

Figure 3.16 SDS-PAGE and western blot analysis of the r*Pm*Chi1 expression. The induction of r*Pm*Chi1 expression by SDS-PAGE (left) and western blot (right). Lane M1 is a molecular weight marker and lanes 1-5 are total protein of un-induced and induced cells after 0.2 mM IPTG induction at 17 ºC for 3, 4, 5 and 6 h, respectively.

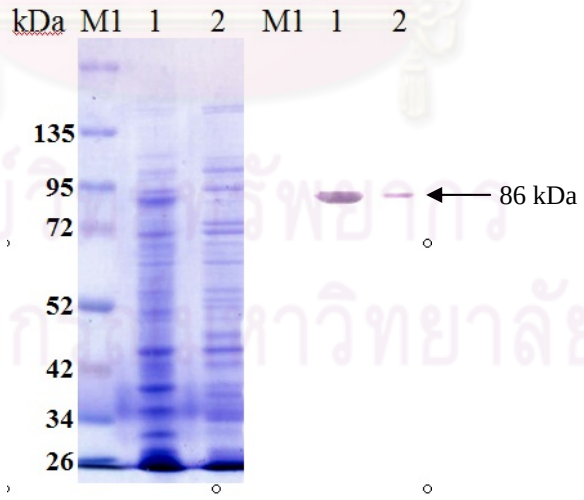

Figure 3.17 SDS-PAGE and western blot analysis of proteins in inclusion body and soluble. Lane M1 is a molecular weight marker, lane 1 are inclusion bodies, and lane 2 are soluble proteins.

3.9 Production of r*Pm*Chi1

After induction with 0.2 mM IPTG for 6 h at 17 ºC**,** cells from 2-L culture were collected by centrifugation. The cell pellet was resuspended in 240 mL of 1× PBS buffer pH 7.4 and freeze-thawed 3 times followed by sonication on ice. Since the r*Pm*Chi1 existed as inclusion bodies, the pellet was collected and washed. The inclusion bodies were dissolved with 50 mM sodium phosphate buffer pH 12 by gently shaking overnight at 10 ºC. The remaining precipitate was removed by centrifugation. The recombinant protein was traced by 10% SDS-PAGE (Figure 3.18), dialyzed against 20 mM sodium carbonate buffer pH 10, and purified using a nickel-NTA column.

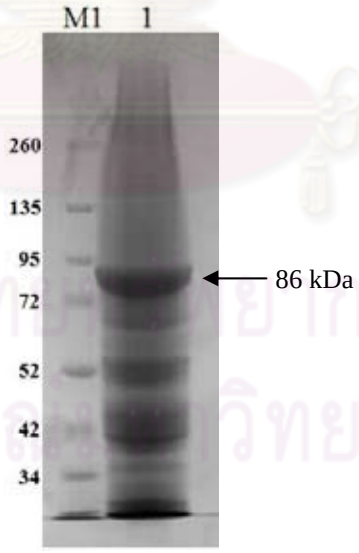

Figure 3.18 The inclusion bodies dissolved with 50 mM sodium phosphate buffer pH12. Lane M1 is a molecular weight marker.

3.10 Purification of r*Pm*Chi1

The r*Pm*Chi1 in the carbonate buffer pH 10 was purified using a nickel-NTA column. The protein solution was loaded into the column, the contaminated protein were washed and eluted from the column by 20 mM sodium carbonate buffer pH 10 containing 20-100 mM imidazole**.** The rChi*Pm*1 was eluted using 20 mM sodium carbonate buffer pH 10 containing 200, 300, 500 mM imidazole. The suitable purification procedure was to elute the contaminated protein with 20 mM sodium carbonate buffer pH 10 containing 100 mM imidazole and then the rChi*Pm*1 with 20 mM sodium carbonate buffer pH 10 containing 500 mM imidazole (Figure 3.19). The purified r*Pm*Chi1was analyzed by 10% SDS-PAGE (Figure 3.20).

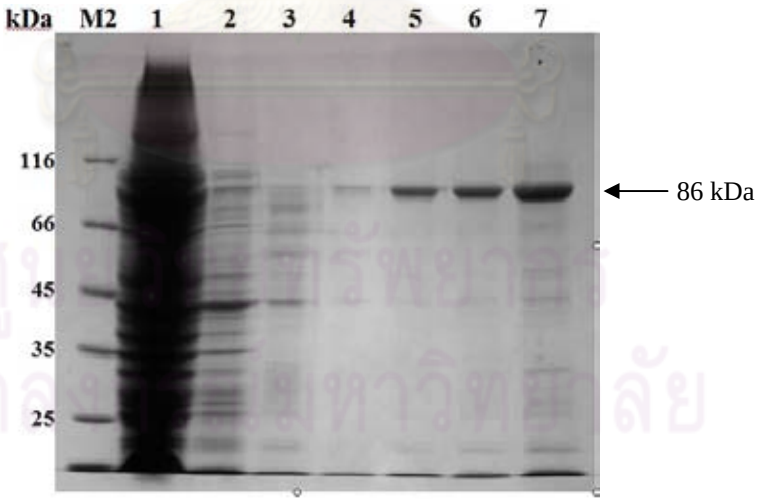

Figure 3.19 SDS-PAGE analysis of rChi*Pm*1 purification. Lane M2 is a molecular weight marker, lane 1 is the flow-through of the expressed proteins and lanes 2-7 are the eluted proteins with 20 mM sodium carbonate buffer pH 10 containing 20, 50, 100, 200, 300 and 500 mM imidazole, respectively.

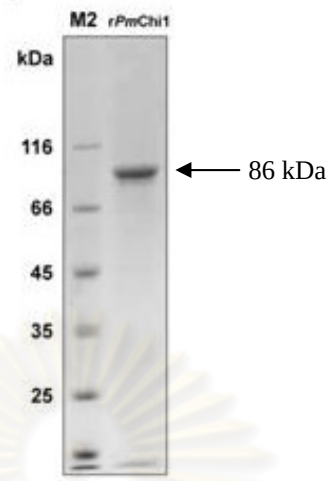

Figure 3.20 SDS-PAGE of purified r*Pm*Chi1. Lane M2 is a molecular weight marker.

Attempt to separate the thioredoxin moiety by enterokinase and thrombin failed because the proteinases also cleaved the r*Pm*Chi1. Since the fusion protein possessed the chitinase activity, we, therefore, continued our characterization of the r*Pm*Chi1 albeit the fusion protein. We had also tested another similar fusion protein, the thioredoxin-SPI*Pm*4 (Visetnan et al., 2009), for chitinase activity and found none. Therefore, the chitinase activity was actually from the *Pm*Chi1 itself.

3.11 Characterization of rChi*Pm*1

3.11.1 Colloidal chitin agar diffusion

The chitinolytic activity of r*Pm*Chi1 was tested on a colloidal chitin agar plate. After incubation overnight at 37 ºC, clear zones were clearly seen around the wells added with 50 μg of r*Pm*Chi1 and chitinase from *Bacillus licheniformis* SK-1 but not the buffer control (Figure 3.21). This result clearly indicated that the r*Pm*Chi1 was active in chitin hydrolysis.

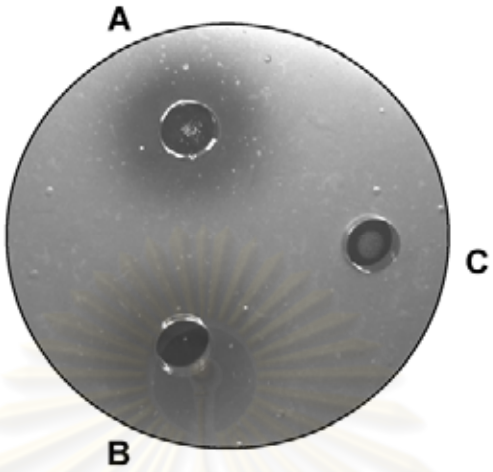

Figure 3.21 The chitinolytic activity of r*Pm*Chi1 on colloidal chitin agar. The activity was tested by adding 50 μg of r*Pm*Chi1 (A), unknown amount of chitinase from *Bacillus licheniformis* SK-1 as a positive control (B) and 20 mM sodium carbonate buffer pH 10 as a negative control (C). Clear zones around the wells indicate positive result.

3.11.2 Chitinase activity assay

To further characterize the biochemical properties of the r*Pm*Chi1, the colloidal chitin hydrolytic reaction was used for the assay. The r*Pm*Chi1 was assayed to determine the range of activity that was linearly proportional to the r*Pm*Chi1 concentrations. The amount of r*Pm*Chi1 used in the future assays should be within this range. The reaction mixtures consisted of excess colloidal chitin. With the r*Pm*Chi1 increased in the reaction mixture, chitinolytic activity was proportionally increased and reached the early plateau when the r*Pm*Chi1 was more than 100 μg (Figure 3.22)**.**

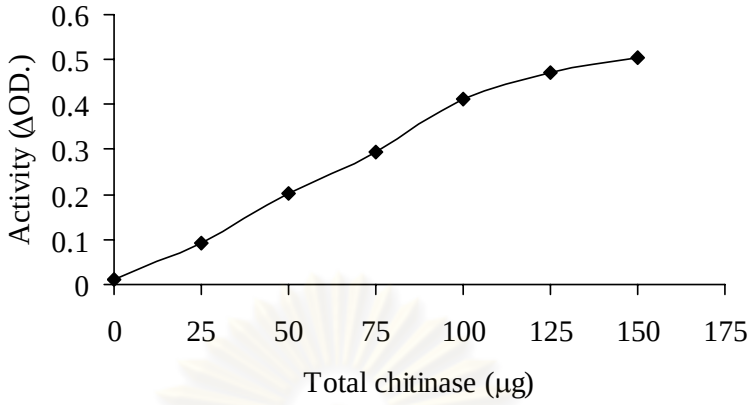

Figure 3.22 Relationship between chitinolytic activity and the quantity of r*Pm*Chi1.

3.11.3 Effect of pH on r*Pm*Chi1 activity (optimal pH)

Using three standard buffer systems to cover a pH range 3-12, the chitinolytic activity of r*Pm*Chi1 was assayed at various pHs. It was found that the r*Pm*Chi1 had a maximal chitinolytic activity or optimal pH at pH 5 and 37 ºC (Figure 3.23).

3.11.4 Effect of pH on r*Pm*Chi1 stability (pH stability)

To determine its stability, the r*Pm*Chi1 was incubated at 37 ºC at various pHs for five hours before its activity was assayed at pH 5 and 37 ºC. The r*Pm*Chi1 was most stable at neutral pH (Figure 3.24). When incubated at pH lower than 6 or higher than 9, its stability was considerably decreased.

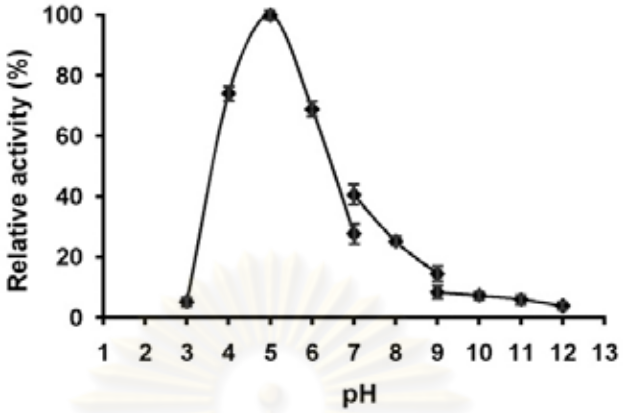

Figure 3.23 Effect of pH on the activity of r*Pm*Chi1. To determine the optimal pH, the r*Pm*Chi1 was assayed at various pHs using three buffer systems. The results are the mean values of five replicates with ± standard deviations.

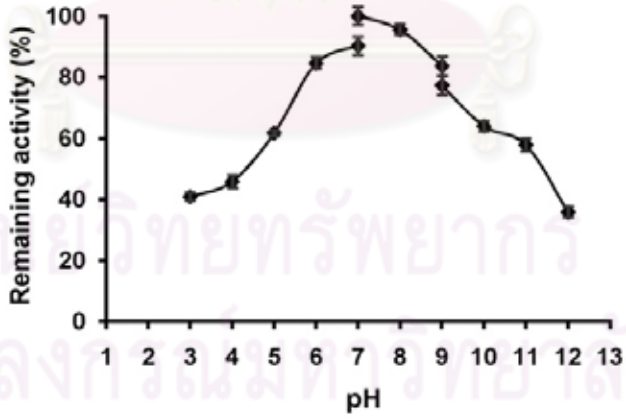

Figure 3.24 Effect of pH on the stability of r*Pm*Chi1. The r*Pm*Chi1 was incubated at various pHs for five hours to test its pH stability before it was assayed at pH 5. The results are the mean values of five replicates with ± standard deviations.

3.11.5 Effect of temperature on r*Pm*Chi1 activity (optimal temperature)

The r*Pm*Chi1 activity was determined at various temperatures for its optimal temperature. The maximal chitinolytic activity was observed at 55 ºC (Figure 3.25).

3.11.6 Effect of temperature on r*Pm*Chi1 stability (temperature stability)

When its temperature stability was determined, the r*Pm*Chi1 was incubated at 5-80 ºC for five hours before its chitinase activity was measured at 37 ºC and pH 5. The r*Pm*Chi1 was stable at temperature lower than 40 ºC (Figure 3.26). Above 40 ºC, the enzyme was not stable and the chitinolytic activity decreased rapidly.

3.11.7 Substrate preference of r*Pm*Chi1

The soluble PNAC was a good substrate for the r*Pm*Chi1 for it was the easiest to be hydrolyzed. Relative to PNAC, the colloidal chitin from shrimp shell and β-chitin from squid pen were tested. The colloidal chitin was the second best substrate for r*Pm*Chi1 followed by β-chitin as they are hydrolyzed about 90 and 50%, respectively (Figure 3.27). These revealed that it had substrate digestion capability depend on chitin solubility. Analysis of variance was used and compared means difference by Duncan's new multiple range test at 95% of confidence. The hydrolytic activity of r*Pm*Chi1 with each substrate had significantly different ($P < 0.05$) at 95% confidence level (Table 3.1).

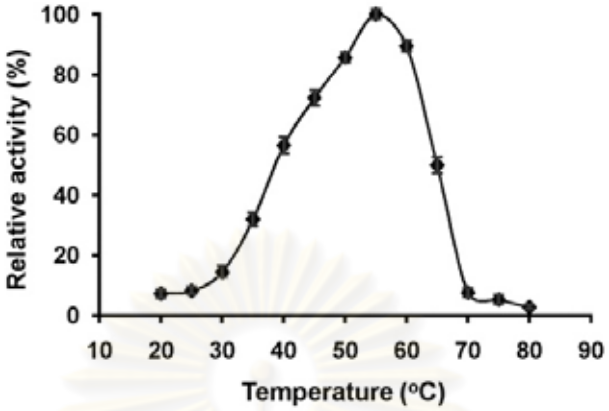

Figure 3.25 Effect of temperature on r*Pm*Chi1 activity. The activity of r*Pm*Chi1 was measured at various temperatures to determine the optimal temperature. The results are the mean values of five replicates with ± standard deviations.

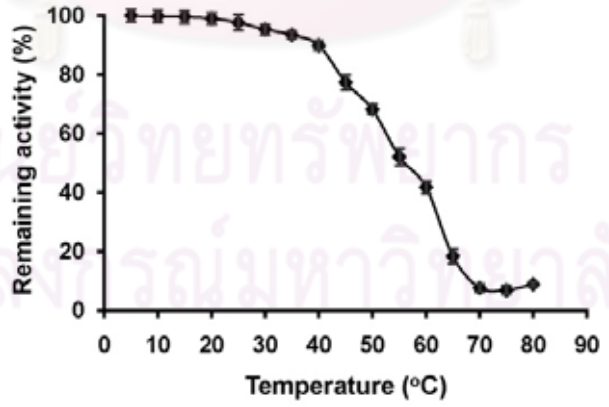

Figure 3.26 Effect of temperature on r*Pm*Chi1 stability. The enzyme was incubated at various temperatures for five hours before it was assayed at 37 ºC and pH 5 to determine its stability. The results are the mean values of five replicates with ± standard deviations.

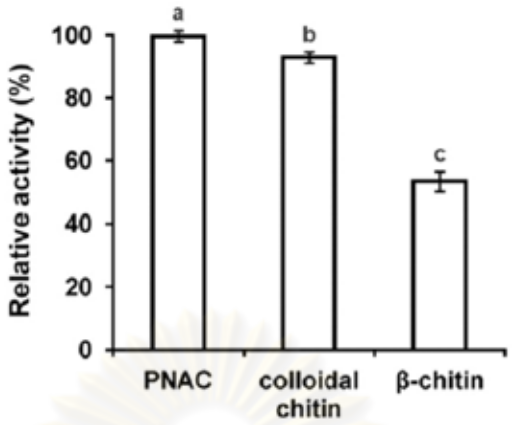

Figure 3.27 Substrate preference of r*Pm*Chi1. The r*Pm*Chi1 was assayed in the presence of PNAC, colloidal chitin from shrimp shell and β-chitin from squid pen. The reaction using PNAC was set as 100%. The results are the mean values with ± standard deviations. Different letters (a, b and c) indicate significant difference ($p < 0.05$) in the mean hydrolytic activity of r*Pm*Chi1.

Table 3.1 The hydrolytic activity of r*Pm*Chi1 with various substrates.

I. Anova test of r*Pm*Chi1 activity

		Sum of Squares	df	Mean Square	F	Sig.
VAR00002	Between Groups	.167	2	8.331E-02	164.067	.000
	Within Groups	6.093E-03	12	5.078E-04		
	Total	.173	14			

II. Duncan test of r*Pm*Chi1 activity

		Subset for alpha = .05		
VAR00001	N	1	2	3
3.00	5	.2744		
2.00	5		.4776	
1.00	5			.5139
Sig.		1.000	1.000	1.000

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

CHAPTER IV

DISCUSSION

Upon searching the *Penaeus monodon* EST database, three different chitinase genes, *PmChi1*, *2* and *3*, were identified from a total of 10,536 unique contigs and singletons. Even with such huge numbers of unique genes being searched, it was still too early to conclude that there were only three different chitinases in *P. monodon*. It is already known that multiple genes encoding for different chitinases do exist in organisms especially in insects. For example, there are at least three chitinases (ChiA, ChiB and ChiC) in *Serratia marcescens*, one of the most effective bacteria for chitin degradation (Suzuki et al., 2002). In insects, a variety of chitinases are identified and found to be encoded by as many as 16 genes depending upon the species of interest. The involvement of these individual proteins in growth and development is largely unknown (Zhu et al., 2008a; Arakane and Muthukrishnan, 2009). In mud crab *Scylla serrata*, there are tentatively four chitinases identified (accession no. ACG60512, ACZ53950, ABY85409 and ACZ53951). In penaeid shrimp, chitinases 1-3 are identified from *M. japonicus* (Watanabe et al., 1996; 1998; Watanabe and Kono, 1997) and *P. monodon* in this study. Chitinases 1 (accession no. ABB85237) and 3 (accession no. AAY44300) are from *F. chinensis*. At least six chitinases, chitinases 1-6, has been identified from *L. vannamei* (accession no. ACG60513, ACR23313, ABY70643, ACR23314, ACR23315 and ACX68556).

Amino acid sequence comparison of the Glyco 18 catalytic domains of the chitinases from the penaied shrimp, insects and mammals revealed that there was a

quite high homology among those from the shrimp such that they were clustered closely together in the phylogenetic trees. Treating the mammalian chitinases as an outgroup, the insect chitinases were separated into three clusters along with the three shrimp chitinases 1, 2 and 3. The groups of chitinases 1 and 3 were more closely related to each other than to the group of chitinase 2 suggesting their difference in activities as well as their biological function. The chitinase 2 was, indeed, implicated as chitinase involved in molting (Watanabe and Kono, 1997) where the other two chitinases were involved in chitinous food digestion and degradation of endogenous chitin prior to molting (Tan et al., 2000)

The *Pm*Chi1 in this study was the longest chitinase 1 among the penaeid shrimp and longer than the *Pm*Chi1 reported by Tan et al. (2000). At its C-terminus were the extended 46 amino acids. Examining the nucleotide sequences at this end revealed a shift in the reading frame in *Pm*Chi1. We had confirmed the existence of this extended amino acid sequence in three different occasions. In short, three *PmChi1* had been PCR amplified and cloned from three different cDNA preparations from different lots of shrimp, possibly from different aquacultures in Thailand, at different periods of time over 2 years and their sequences were almost the same. Considering the high variability of the C-terminal amino acid sequences of chitinases 1 as in Fig.1, it might be possible that the difference between the *Pm*Chi1 in this study and that of Tan et al. (2000) arose from the variation in shrimp stock.

The expression of *PmChi1* was mainly in hepatopancreas, less in intestine, stomach and antenna. In early postmolt shrimp stage A, the pattern of tissue distribution remained more or less similar albeit up- and down-regulation in lymphoid and intestine. Tan et al. (2000) had shown that the expression of *Pm*Chi1 was highest

during the premolt stage and suggested that the *Pm*Chi1 was for the degradation of endogenous chitin in the gut peritrophic membrane before molting. In *F. chinensis*, the expression of *Fcchi-1* was highest during the premolt stage too but fluctuated rather widely (Priya et al., 2009).

In this study, we had not been able to acquire a full length *PmChi2* gene albeit using different gene-specific primers and different 5′ RACE cDNA libraries from eyestalk and gill. By comparing the *Pm*Chi1, 2 and 3 with the insect chitinases, we observed that the *Pm*Chi1 and 3 were comparable in size to the insect chitinases in the same groups. For example, the chitinase 5 from *Drosophila melanogaster* (accession no. NP_650314) (Hoskins et al., 2007) and the chitinase from *Helicoverpa armigera* (JC8021) (Ahmad et al., 2003) contain 595 and 588 amino acids, respectively, as compared to 644 amino acids of mature *Pm*Chi1. The midgut chitinase from *Lutzomyia longipalpis* (AAN71763) (Ramalho-Ortigão and Traub-Csekö, 2003) and chitinase 4 from *Tribolium castaneum* (NP_001073567) (Zhu et al., 2008b) contain 474 and 475 amino acids, respectively, as compared to 468 amino acids of mature *Pm*Chi3.

If the *Pm*Chi2 were of similar size to the insect chitinases in the same group, we anticipated that the *Pm*Chi2 we obtained was only about one-sixth as big as those of insects. The chitinase 3 from *D. melanogaster* (NP_001036422) (Hoskins et al., 2007) and the chitinase 10 from *T. castaneum* (NP_001036067) (Zhu et al., 2008b) are 2286 and 2700 amino acids in length while the partial *Pm*Chi2 in this study is only 406 amino acids. Sequence comparison indicated that the partial *Pm*Chi2 was at the C-terminus and a lot more sequence at the N-terminus remained to be uncovered (data not shown).

The expression of *Pm*Chi2 was mainly in gill, eyestalk and epipodite of the intermolt stage C shrimp. The messenger was detectable faintly in all tissues at postmolt stage A. Watanabe and Kono (1997) observed in *M. japonicus* that the Pjchi-2 was up-regulated in cuticular tissues late in premolt stage. The transcription was down-regulated at postmolt stage as observed with *Pm*Chi2. Therefore, the *Pm*Chi2 very likely played a direct role in molting of the exoskeleton.

The amino acid sequence comparison of the chitinases 3 revealed high homology among the penaeid shrimp. The *Pm*Chi3 was expressed mainly in hepatopancreas and remained unchanged at postmolt stage. This result agreed well with that of *Fcchi-3* (Priya et al., 2009; Zhang et al., 2010). Like that of *Fcchi-1*, the expression of *Fcchi-3* occurred at all stages in molting cycle but was highest during the premolt stage (Priya et al., 2009). Like the *Pm*Chi1, the *Pm*Chi3 may function in the digestion of chitinous food and degradation of endogenous chitin prior to molting.

Thus far, chitinases in crustacean has been found to have one catalytic domain and one chitin-binding domain. On the contrary, insect chitinases have one or more catalytic domains and chitin-binding domains, which are the result of domain duplication and shuffling, especially the insect chitinase group II (Tellam, 1996; Henrissat, 1999; Zhu et al., 2008). For example, the chitinase from the beetle, *Tenebrio molitor*, has multi-domain structure containing five catalytic domains and four chitin binding domains (Royer et al., 2002). The *Pm*Chi1 and 3 shared the same domain pattern having an N-terminal catalytic domain and a C-terminal chitin binding domain, while the chitin binding domain of *Pm*Chi2 is at the N-terminus of catalytic domain. The difference in domain organization of chitinases may lead to the difference in substrate preference and, hence, their biological functions.

Chitin binding domain in crustacean chitinases is cysteine-rich and has several highly conserved aromatic residues, especially in chitinase1 (Shen and Jacobs-Lorena, 1999). The six conserved cysteine residues help to maintain the structural stability of the enzyme by forming three internal disulfide linkages. Chitin binding domain binds the insoluble chitin via highly conserved aromatic residues interacting with saccharide units in the ligand-binding pocket (Tjoelker et al., 2000; Boraston et al., 2004).

Since the *Pm*Chi1 was possibly involved in both food digestion and degradation of endogenous chitin, it was chosen for over-production in *E. coli*. The mature *Pm*Chi1 was produced as fusion protein to the thioredoxin. The thioredoxin that could not be removed by specific protease digestion for the whole protein was rapidly digested upon treatment. Since the protein was active in chitinolysis as it readily hydrolyzed the colloidal chitin, we, therefore, characterized the *Pm*Chi1 for its optimum pH and temperature, its pH and temperature stability and its substrate preference.

The *Pm*Chi1 worked well at pH 5 and lost its activity rapidly above pH 6 and below pH 4 but it was most stable at neutral pH 7. Its optimal temperature for chitinase activity was 55 ºC, higher than that we could expect from the aquatic animal like shrimp. Above 60 ºC and below 50 ºC, the activity subsided rather rapidly. The enzyme was stable if stored at low temperature below 40 ºC; the lower the temperature, the better the stability. Compared to those of insects, a chitinase from *Manduca sexta* (Zhu et al., 2001) has wider range of optimum pH 5-8 while the two chitinases, 65 and 88 kDa proteins, from *Bombyx mori* (Koga et al., 1997) work well at pH 5.5 and 6.5. Their optimum temperatures were 50 ºC for the *M. sexta* chitinase and 60 ºC for the two *Bombyx mori* chitinases. Like the r*Pm*Chi1, the 65 and 88 kDa

chitinases from *Bombyx mori* are stable at lower temperature 30 and 40 ºC, respectively.

At its optimum pH, the *Pm*Chi1 readily hydrolyzed the more soluble substrates like PNAC and colloidal chitin. With its rather high temperature optimum and low pH optimum, it might be useful for shrimp waste treatment and the production of chitooligosaccharides and glucosamine (Dahiya et al., 2006).

The partially *N*-acetylated chitin (PNAC) was the most preferred substrate for the r*Pm*Chi1 because the chitin chain was partially deacetylated making it polycation due to D-glucosamine moieties and more soluble. The colloidal chitin (α-chitin) was the second best substrate for r*Pm*Chi1 followed by β-chitin. Colloidal chitin was prepared form the shrimp shell chitin hydrolyzed with concentrated HCl. It is amorphous, without definite character, resulting in its easier hydrolysis than the squid pen β-chitin, either crystalline or amorphous, which is prepared by swelling the pen in water. The abilities of r*Pm*Chi1 to degrade both α-chitin of shrimp shell and β-chitin of squid pen coincide with the feeding habit of the black tiger shrimp, which feeds on various chitinous foods. The chitinase, therefore, possesses wide substrate specificity for degrading shrimp shell, crab shell and squid pen (Ikeda et al., 2009).

To apply the r*Pm*Chi1 in marine waste management, the shrimp shell or crab shell should be partial hydrolyzed by concentrated HCl to colloidal or amorphous form before treated with r*Pm*Chi1. Alternatively, chitinous waste can be treated with 40% (w/w) NaOH or chitin deacetylase because partial deacetylation can enhance the efficiency of chitin degradation with r*Pm*Chi1.

CHAPTER V

CONCLUSIONS

1. Three different chitinase genes, namely *PmChi1*, *2* and *3*, were identified from the *Penaeus monodon* EST database (http://pmonodon.biotec.or.th/database.jsp).
2. For each chitinase, the amino acid sequence comparison among the penaeid chitinases revealed very high homology about 90%.
3. The phylogenetic analysis indicated that the *Pm*Chi1, 2 and 3 along with those of other crustaceans and insects were grouped into three clusters separated from those of mammals. Within each group, the chitinases from crustaceans were also separately grouped together. The groups of *Pm*Chi1 and 3 were closely related to each other than to the group of *Pm*Chi2.
4. The Glyco 18 catalytic domain of *Pm*Chi1 and *Pm*Chi3 resided at the N-terminus while that of *Pm*Chi2 was found at the C-terminus.
5. The semi-quantitative RT-PCR showed that in normal shrimp, the *PmChi1* and *3* were expressed mainly in hepatopancreas whereas *PmChi2* were in gill. Small amount of them could be expressed in other tissues. At early postmolt stage A, only the amount of *PmChi2* messenger was significantly down-regulated while the mRNA expression levels of *PmChi1* and *3* were relatively unchanged.
6. The reading frames of *PmChi1* and *3* encoded mature proteins of 644 and 468 amino acids with calculated molecular weights of 72.4 and 51.9 kDa, respectively. While, the partial *PmChi2* was 1223 bp long coding for a polypeptide chain of 406 amino acid residues.

7. Recombinant *Pm*Chi1 was successfully over-expressed as a fusion protein to the thioredoxin moiety with the calculated molecular mass of 86 kDa and, with its His-tags, purified under a non-denaturing condition using a nickel-NTA affinity chromatography column.
8. The r*Pm*Chi1 clearly showed chitinolytic activity on a colloidal chitin agar plate. It had an optimal pH of 5 but it was most stable at neutral pH. The optimal temperature was relatively high at 55 ºC. Nevertheless, it was stable at temperature lower than 40 ºC.
9. The partially *N*-acetylated chitin was a best substrate for the r*Pm*Chi1 followed by the colloidal chitin from shrimp shell and β-chitin from squid pen, respectively.

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Appendices

Appendix A: Submitted chitinase sequences

1. *Penaeus monodon* chitinase 1

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LOCUS       bankit1300425          1929 bp    mRNA    linear   INV 18-DEC-2009
DEFINITION  Penaeus monodon chitinase1 mRNA, mature protein.
ACCESSION   1300425
VERSION
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            Eumalacostraca; Eucarida; Decapoda; Dendrobranchiata; Penaeoidea;
            Penaeidae; Penaeus.
REFERENCE   1  (bases 1 to 1929)
  AUTHORS   Proespraiwong,P., Tassanakajon,A. and Rimphanitchayakit,V.
  TITLE     Activities, phylogenetics and tissue expression of chitinases from
            the black tiger shrimp Penaeus monodon
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1929)
  AUTHORS   Proespraiwong,P., Tassanakajon,A. and Rimphanitchayakit,V.
  TITLE     Direct Submission
  JOURNAL   Submitted (18-DEC-2009) Biochemistry, Chulalongkorn University,
            Phayathai, Bangkok, Bangkok 10330, Thailand
COMMENT     Bankit Comment: cookie_andcream@live.com.
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2. *Penaeus monodon* chitinase 2

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            Penaeidae; Penaeus.
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  AUTHORS   Proespraiwong,P., Tassanakajon,A. and Rimphanitchayakit,V.
  TITLE     Activities, phylogenetics and tissue expression of chitinases from
            the black tiger shrimp Penaeus monodon##
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1223)
  AUTHORS   Proespraiwong,P., Tassanakajon,A. and Rimphanitchayakit,V.
  TITLE     Direct Submission
  JOURNAL   Submitted (02-JAN-2010) Biochemistry, Chulalongkorn University,
            Phayathai, Bangkok, Bangkok 10330, Thailand
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  TITLE     Activities, phylogenetics and tissue expression of chitinases from
            the black tiger shrimp Penaeus monodon##
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1407)
  AUTHORS   Proespraiwong,P., Tassanakajon,A. and Rimphanitchayakit,V.
  TITLE     Direct Submission
  JOURNAL   Submitted (04-MAR-2010) Biochemistry, Chulalongkorn University,
            Phayathai, Bangkok, Bangkok 10330, Thailand
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Appendix B: SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

1. Preparation for polyacrylamide gel electrophoresis

- **30% (w/v) acrylamide, 0.8% (w/v) bis-acrylamide, 100 mL**

acrylamide	29.2	g
bis-acrylamide	0.8	g

Adjust volume to 100 mL with distilled water.

- **1.5 M Tris-HCl pH 8.8**

Tris (hydroxymethyl)-aminomethen	18.17	g

Adjust pH to 8.8 with 1 M HCl and adjust volume to 100 mL with distilled water.

- **2.0 M Tris-HCl (pH 8.8)**

Tris (hydroxymethyl)-aminomethen	24.2	g

Adjust pH to 8.8 with 1 M HCl and adjust volume to 100 mL with distilled water.

- **0.5 M Tris-HCl pH 6.8**

Tris (hydroxymethyl)-aminomethen	6.06	g

Adjust pH to 6.8 with 1 M HCl and adjust volume to 100 mL with distilled water.

- **1.0 M Tris-HCl pH 6.8**

Tris (hydroxymethyl)-aminomethen	12.1	g

Adjust pH to 6.8 with 1 M HCl and adjust volume to 100 mL with distilled water.

2. SDS-PAGE

- **10% Separating gel**

H_2O	4.0	mL
30% (w/v) Acrylamide solution	3.3	mL
1.5 M Tris (pH 8.8)	2.5	mL
10% SDS	100	µL
10% Ammonium persulfate	100	µL
TEMED	4	µL

- **5% Stacking gel**

H_2O	2.7	mL
30% (w/v) Acrylamide solution	0.67	mL
1.0 M Tris (pH 6.8)	0.5	mL
10% SDS	0.04	mL
10% Ammonium persulfate	0.04	mL
TEMED	5	µL

- **5× Sample buffer**

1 M Tris-HCl pH 6.8	0.6	mL
50% (w/v) Glycerol	5.0	mL
10% SDS	2.0	mL
2-mercaptoethanol	0.5	mL
1% Bromophenol blue	1.0	mL
Distilled water	0.9	mL

One part of sample buffer was added to four parts of sample. The mixture was heated 5 min. in boiling water before loading to the gel.

3. Electrophoresis buffer, 1 litre

(25 mM Tris, 192 mM glycine)

Tris (hydroxymethyl)-aminomethane	3.03	g
Glycine	14.40	g
SDS	1.0	g

Dissolve in distilled water to 1 litre. Do not adjust pH with acid or base (final pH should be 8.3).

Appendix C: Substrate preparations

1. Preparation of β-crystalline chitin

The β-crystalline chitin was prepared from squid pen chitin. A 5 g flake squid pen chitin was grinded in a high-speed blender at 10,000 rpm with water until squid pen chitin swell in water. The solution was added 0.05% NaN_3 and kept at 4 ºC.

2. Preparation of colloidal chitin

Colloidal chitin was prepared form the flake shrimp shell chitin by the methods described by Jeuniaux, (1996) and Yamada and Imoto, (1981) with some modification. A 40 g of shrimp shell chitin was hydrolyzed by adding 400 mL of concentrated HCl (12 M) and stirred for 4-6 h on ice with magnetic stirrer. The solution was stirred for a while at 37 ºC. The hydrolyzed chitin was filtrated into 4,000 mL of chill distilled water. The milk-like mixture was kept for overnight at 4 ºC. The colloidal chitin was collected by centrifugation at 8,000 g for 15 min and then resuspended with distilled water to wash the pellet. The pellet was washed until the pH was 6-7.

The colloidal chitin was resuspended in distilled water and kept at 4 ºC. The solution was determined as the percent dry weight. The colloidal chitin was added 0.05% NaN_3 and kept at 4 ºC.

3. Preparation of partially *N*-acetylated chitin (PNAC)

Partially *N*-acetylated chitin (PNAC) was prepared form squid pen chitin. A 10 g flake squid pen chitin in 250 mL 40% (w/w) NaOH was vacuumed for 4 h. Then, the 750 g of crushed ice was added and vigorously shaked. In this step, the layer of ice was appeared and covered around the flask. The mixture was stirred for 4-6 h at 4 ºC. Next, the pH of the solution was adjusted until the pH was 7.0 with concentrated

HCl. Two volume of cold acetone was added to precipitate the PNAC. The PNAC was collected by filtration and dialyzed against water to remove salt. Finally, the PNAC was lyophilized.

Appendix D: Publication

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Chitinases from the black tiger shrimp *Penaeus monodon*: Phylogenetics, expression and activities

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ABSTRACT

Chitinases are essential enzymes for crustaceans and animal alike for their molting and digestion of foods containing chitin. From the *Penaeus monodon* EST database, cDNA contigs and singletons for three chitinases, namely *PmChi1*, *2* and *3*, were identified. The complete sequences for the mature *Pm*Chi1, 3 and partial *Pm*Chi2 were amplified and cloned. The reading frames of *PmChi1* and 3 encoded mature proteins of 644 and 468 amino acids with calculated molecular masses of 72.4 and 51.9 kDa, respectively. The amino acid sequence comparison among the penaeid chitinases revealed homology around 90%. Therefore, they were grouped together along with those of other crustaceans and insects into three groups separated from those of mammals. *PmChi1*, *2* and *3* were expressed mainly in hepatopancreas, gill and hepatopancreas, respectively, though small amounts were expressed in other tissues. After molting, only the expression of *PmChi2* was down-regulated, while the expression of *PmChi1* and 3 was relatively unchanged. The results suggested that the *PmChi2* was likely involved in molting while the others might function in the digestion of chitinous foods. The recombinant *Pm*Chi1 (r*Pm*Chi1) over-produced from *Escherichia coli* had its optimal pH 5 but it was most stable at neutral pH. Interestingly, the optimal temperature was relatively high at 55 °C. Nevertheless, it was stable at lower temperature below 40 °C. The r*Pm*Chi1 preferentially hydrolyzed the more soluble substrates like partially *N*-acetylated chitin (PNAC) and colloidal chitin from shrimp shell as compared to the β-chitin from squid pen.

1. Introduction

Chitin is a long, unbranched insoluble polysaccharide of an amino sugar *N*-acetyl-β-D-glucosamine linked together by β-1,4-glycosidic linkages. It is one of the most abundant polysaccharides in nature found as structural constituent of fungal cell wall (Duo-Chuan, 2006), and cuticle and integument of some animals, particularly the arthropods including crustaceans (Kurita, 2006) and insects (Merzendorfer and Zimoch, 2003; Arakane and Muthukrishnan, 2009). The chitin is degraded by chitinolytic enzymes or chitinases which are found widely in all sorts of organism from viruses to animals. The chitinase participates in several biological processes, such as nutrient digestion, morphogenesis, pathogenesis and pathogen defense (Spindler-Barth et al., 1990; Kono et al., 1995; Duo-Chuan, 2006; Mali et al., 2004; Dahiya et al., 2006).

In crustaceans, chitin is a major component of the outer shell or exoskeleton covering the entire body of the animal providing protection for the animals. Under developmental, growth and certain environmental conditions, the crustaceans shed off their shells and regenerate the new ones in a molting process (Spindler-Barth et al., 1990; Kono et al., 1995). In the molting process, the old exoskeleton is digested by chitinolytic enzymes. To completely digest the chitinous shell, two chitinolytic enzymes are involved. The chitinase (EC 3.2.1.14) degrades chitin into chitooligosaccharides and then the β-*N*-acetylhexosaminidase (EC 3.2.1.52) hydrolyzes the chitooligosaccharides to *N*-acetylglucosamine monomer (Kramer and Muthukrishnan, 1997). Therefore, the chitinolytic enzymes are indispensable for crustaceans. Not only are they involved in the molting cycle, but the enzymes are also used for chitinous food digestion and probably defense against chitin-bearing pathogens.

In penaeid shrimp, three genes coding for chitinases of family 18 glycosyl hydrolases were identified in *Marsupenaeus japonicus* (*Penaeus japonicus*), namely *Pjchi-1*, *Pjchi-2* and *Pjchi-3* (Watanabe et al., 1996, 1998; Watanabe and Kono, 1997). The *Pjchi-1* and 3 were found to express in hepatopancreas but not in cuticular body parts. The expression of *Pjchi-3* transcripts was also relatively unchanged during the molting. They were probably involved in the digestion of chitinous food. The *Pjchi-2* was identified from the cDNA library of tail fan and blade, and was implicated to be involved in molting. The expression of *Pjchi-2* was up-regulated in the tail fan or blade during the premolt stages. The *Pjchi-2* was not detectable in hepatopancreas.

A gene coding for *Penaeus monodon* chitinase 1 (*PmChi-1*) was isolated from the cDNA library of *P. monodon* (Tan et al., 2000). A

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PmChi-1 of 621 amino acids was 80% homologous to Pjchi-1 of *M. japonicus*. The expression of *PmChi-1* was found variably at various molting stages but highest at premolt D_2 stage. It was postulated, then, that the PmChi-1 may function in the digestion of endogenous chitin in the gut ahead of molting.

Most recently, a chitinase gene equivalent to *Pjchi-3* from *Fenneropenaeus chinensis*, *Fcchi-3*, was isolated (Zhang et al., 2010). The predicted amino acid sequence was very similar to those of *Litopenaeus vannamei* and *M. japonicus*. The expression was only detectable in the hepatopancreas and down-regulated upon WSSV infection. The expression of *Fcchi-1* and *Fcchi-3* transcripts in the whole juvenile shrimp during the molt cycle of *F. chinensis* had been investigated (Priya et al., 2009). They were detected in all stages of molting with considerable fluctuations observed in the premolt D stages. The significance of this finding was not discussed.

Recently, we had searched the *P. monodon* EST database and identified several contigs and singletons of chitinase clones. Mining of the EST clones gave rise to three different chitinases and named according to their counterparts in *M. japonicus*, *PmChi1*, *2* and *3*. In this research, we had the complete reading frames of *PmChi1* and *3* coding for mature chitinases and the partial reading frame of *Pm*Chi2 cloned. Their phylogenetic relationships to other chitinases were analyzed. Their tissue distribution in normal and early postmolted shrimp was investigated. The *Pm*Chi1 protein was over-expressed as fusion protein for preliminary elucidation of its enzymatic activity, and pH and temperature optima and stability.

2. Materials and methods

2.1. Searching the P. monodon EST database

The EST and contig pages in the *P. monodon* EST database (http://pmonodon.biotec.or.th/home.jsp) were searched for the chitinases. The nucleotide sequences of the obtained contigs and singletons were analyzed for the open reading frames and the encoded amino acid sequences. The signal peptide was predicted using the online SignalP 3.0 program (http://www.cbs.dtu.dk/services/SignalP/) (Bendtsen et al., 2004). ClustalX was used to compare the different chitinase sequences (Chenna et al., 2003). The molecular weights were predicted from the amino acid sequences using the Expasy software (http://www.expasy.org/).

2.2. Shrimp and cDNA preparation

Juvenile *P. monodon* shrimp weight approximately 20 g were acclimatized in aquaria at an ambient temperature of 28 °C and a salinity of 15 ppt for a few days before use in the experiments. For postmolted samples, the shrimp were collected within 1 h after the ecdysis, possibly in the postmolt stage A, for the new cuticle and setae were still very soft. Shrimp tissues: antennal gland, stomach, gill, intestine, lymphoid, eyestalk, hepatopancreas, heart, epipodite, leg and tail. were dissected and snap-frozen in liquid nitrogen. Hemocytes were isolated by centrifugation at 800 *g* for 10 min at 4 °C. The hemocyte pellet was resuspended in 1 mL of TRI Reagent® (Molecular Research Center).

The tissue sample was homogenized in TRI Reagent® and total RNA was extracted according to the manufacturer's instruction. The total RNA was treated with DNase (Promega) to remove the DNA contamination. Then, first-strand cDNAs were synthesized from 1 μg of total RNA samples using the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas) according to the manufacturer's protocol. The synthesized cDNA was stored at −20 °C.

2.3. Cloning of the chitinase genes

The reading frames of *PmChi1*, partial *PmChi2* and *PmChi3* were amplified from the cDNA samples prepared from hepatopancreas, eyestalk and hepatopancreas, respectively, using the gene-specific primers listed in Table 1. The FChi1_1, RChi1_1, FChi3 and RChi3 were designed with extended NcoI, XhoI, BamHI and XhoI sites at their 5′ ends, respectively, for further cloning into the expression vectors.

The reaction mixture of 50 μL contained 0.3–1 μg cDNA, 0.2 mM dNTP, 0.2–0.5 μM forward and reverse primers, 1× Taq™ buffer, 0.02 unit/μL Taq™ DNA polymerase. The condition was first denatured at 95 °C for 2–4 min followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 50–55 °C for 30 s, extension 72 °C for 2–3 min, and then final extension at 72 °C for 7–10 min. The PCR products of 1937, 1117 and 1416 bp for *PmChi1*, *PmChi2* and *PmChi3*, respectively, were isolated, cloned into the T&A cloning vector (RBC) and sequenced.

Since the DNA sequence of *PmChi2* was only partial, lacking the 5′ sequence portion, the 5′ amplification of cDNA end or 5′ RACE was carried out using the SMART™ RACE cDNA Amplification Kit (Clontech) following the manufacturer's protocol. Briefly, the 5′ RACE libraries were prepared from the cDNAs from eyestalk and gill such that a short sequence containing universal annealing sites for 5′ RACE universal primers was added at the 5′ end. Three gene-specific primers, GSPout1, GSPout2 and GSPin, were designed as listed in Table 1. One of the outer gene-specific primers (GSPout) along with the 5′ RACE universal primer were used for primary PCR of the 5′ RACE library. Then, the inner primer (inner GSPout or GSPin) and the 5′ RACE nested universal primer were used for secondary PCR. The PCR fragments were cloned and their sequences determined.

2.4. Tissue specific expression of chitinases

To determine the expression of chitinase in various shrimp tissues, cDNAs isolated from various shrimp tissues were used for chitinase gene-specific amplification using primers specific to the chitinase genes (Table 1). The *β-actin* gene was used as an internal control using the gene-specific primers designed according to the shrimp *β-actin* cDNA sequence (accession no. DW042525).

One microliter of the first-strand cDNA was subjected to PCR in a 25 μL reaction volume containing 750 mM Tris–HCl, pH 8.8, 200 mM $(NH_4)_2SO_4$, 0.1% (v/v) Tween 20, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 μM of each specific primers and 1.25 units of *Taq* DNA polymerase (Fermentas). The reaction was pre-denatured at 94 °C for 1 min followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 50 or 55 °C for 30 s and extension at 72 °C for 1–2 min, and final extension

Table 1
Primer pairs used for the amplification of chitinase genes.

Gene	Primer	Sequence (5′-3′)[a]	PCR product	Task
PmChi1	FChi1_1	GCCCATGGACCCGAGATTCGAGCAAGAAGG	1,937 bp	Cloning
	RChi1_1	CCCTCGAGGTTCTTTACCATCTTCATTAGC		
	FChi1_2	GTGTGGAACCAGGCTATCAA	319 bp	RT-PCR
	RChi1_2	GTGCTGGCTTAACACGTACT		
PmChi2	FChi2	CAACTGGGCGTGGTA	1,117 bp	Cloning and RT-PCR
	RChi2	GGTTACATGTTGCAC		
	GSPout1	AGAAGCCGAGTGCCGTCCAGAACAG	–	5′ RACE
	GSPout2	CCTTCTTGCAGTTCACCTGCCAGCA		
	GSPin	TCTTCGGGTCTGTATTTGCCGGCGC		
PmChi3	FChi3	ATGGATCCGGTGATGGTGTGCTACTTC	1,416 bp	Cloning and RT-PCR
	RChi3	GCCTCGAGGCAGTCATTCGGGCAAACG		
β-actin	actinF	GCTTGCTGATCCACATCTGCT	320 bp	Internal control for RT-RCR
	actinR	ATCACCATCGGCAACGAGA		

[a] The restriction sites are underlined.

at 72 °C for 7–10 min. Ten microliters of the amplification reactions were analyzed by agarose gel electrophoresis.

2.5. Phylogenetic and amino acid sequence analyses of PmChi1, 2 and 3

The amino acid sequences of *Pm*Chi1 (accession no. GU344706), *Pm*Chi2 (GU376734) and *Pm*Chi3 (GU344707) were submitted to protein BLAST (blastp) for the identity and similarity against the protein sequences in the GenBank (Altschul and Lipman, 1990). The amino acid sequences of chitinases from mammals, crustaceans and insects were down-loaded from the GenBank. The catalytic domains were defined using PROSITE and SmartTM databases (http://smart.embl-heidelberg.de/). The amino acid sequences of catalytic domain were aligned using the ClustalX (Chenna et al., 2003). Based on the alignment, a phylogenetic tree was constructed using the Phylip program. Bootstrap analysis was performed for values representing 1000 replicates using the SeqBoot. The mammalian chitinases were defined as an outgroup.

2.6. Recombinant expression of PmChi1

The *PmChi1* in T&A vector was digested with NcoI and XhoI. The *PmChi1* gene fragment was isolated and cloned into the pET-32a(+) expression vector. The resulting expression plasmid clone, pET32a(+)-*Pm*Chi1, was transformed into an *Escherichia coli* host strain Rosetta (DE3)pLysS for recombinant protein expression.

A single colony of *E. coli* Rosetta(DE3)pLysS containing pET32a(+)-*Pm*Chi1 was cultured with shaking at 37 °C overnight in a few milliliters of terrific broth containing 100 μg/mL ampicillin and 34 μg/mL chloramphenicol. The overnight culture was diluted 1:100-fold in 250 mL terrific broth containing 100 μg/mL of ampicillin and 34 μg/mL of chloramphenicol. The culture was grown at 37 °C until the optical density at 600 nm reached 0.5–0.6. The expression of recombinant *Pm*Chi1 (r*Pm*Chi1) was induced by adding isopropyl-β-D-thiogalacto-pyranoside (IPTG) to the final concentration of 0.2 mM. The culture was continued at 17 °C, and 1 mL cell suspension was collected at 3, 4, 5 and 6 h after induction. The protein expression was analyzed by 10% SDS-PAGE and western blotting. The protein concentration was determined using Bradford's dye-binding method (Bradford, 1976).

2.7. Purification of recombinant PmChi1

After IPTG induction for 6 h, cells from 2-L culture were collected by centrifugation. The cell pellet was resuspended in 240 mL of 1× PBS and freeze-thawed 3 times followed by sonication on ice with 60% amplitude pulse for 20 min. Since the r*Pm*Chi1 existed as inclusion bodies, the pellet was collected and the supernatant liquid discarded. The pellet was washed with 0.5 M NaCl containing 2% Triton X-100 twice followed by 0.5 M NaCl twice and sterile deionized water twice. The inclusion bodies were dissolved with 50 mM sodium phosphate buffer pH 12 by gently shaking overnight at 10 °C. The remaining precipitate was removed by centrifugation and the supernatant liquid dialyzed against 20 mM sodium carbonate buffer pH 10.

The r*Pm*Chi1 in 20 mM sodium carbonate buffer pH 10 was purified using a nickel-NTA column. The purification of chitinase protein was traced by 10% SDS-PAGE.

2.8. Characterization of rPmChi1

2.8.1. Chitinase activity assays

The qualitative determination of chitin hydrolytic activity of r*Pm*Chi1 was carried out using colloidal chitin agar diffusion method. A plate of 1% (w/v) agarose gel in 30 mL of 1× PBS, containing 0.05% (w/v) colloidal chitin from shrimp shell and 100 μg/mL of ampicillin was prepared. Wells of 0.8-cm diameter were cut into the solidified gel and added with 50 μg r*Pm*Chi1 in 20 mM sodium carbonate buffer pH 10. Chitinase from *Bacillus licheniformis* SK-1 (Kudan and Pichyangkura, 2009) and 20 mM sodium carbonate buffer pH 10 were used as positive and negative controls, respectively. The plate was incubated overnight at 37 °C. Digestion halo zones around the wells were observed for chitinase samples.

For quantitative measurement of chitinase activity, a colorimetric assay was employed. The reaction mixtures consisted of 1 mg/mL colloidal chitin, 0.1 M sodium phosphate buffer pH 7 and various quantities of r*Pm*Chi1 in a total volume of 750 μL. The reactions were incubated at 37 °C for 1 h, added 1 mL color reagent (0.5 g/L potassium ferricyanide in 0.5 M sodium carbonate), boiled for 15 min and swiftly cooled on ice. The reactions were centrifuged at 5000 rpm for 15 min to clarify the solutions of which the supernatant liquids were measured at 420 nm. The experiments were repeated five times. Pre-boiled r*Pm*Chi1 for 20 min was used as a negative control. A recombinant thioredoxin-SPI*Pm*4 fusion protein (Visetnan et al., 2009) was used as a control for the chitinase assay to see if thioredoxin possessed any hydrolytic activity. One unit of chitinase activity was defined as the amount of chitinase that generated 1 μmol of *N*-acetylglucosamine or D-glucosamine per 1 h.

2.8.2. Substrate preference of rPmChi1

The r*Pm*Chi1 was tested for its substrate preference by incubating 50 μg of the recombinant enzyme in sodium acetate buffer, pH 5, for 1 h at 37 °C with 0.1 mg/mL PNAC, 1 mg/mL colloidal chitin from shrimp shell, 1 mg/mL β-chitin from squid pen. The reactions were, then, assayed as mentioned above. The test was repeated five times. The data were expressed as mean ± standard deviation. The statistical significance of the data was evaluated using one-way analysis of variance (ANOVA) followed by post hoc test (Duncan's new multiple range test). Significant differences were accepted at $p<0.05$.

2.8.3. Effect of pH on rPmChi1 activity and stability

To determine the pH optimum of the chitinase reactions, three buffer systems were used, namely, sodium acetate buffer pH 3–7, sodium phosphate buffer pH 7–9 and sodium glycine buffer pH 9–12. The reactions were carried out by incubating 50 μg of the r*Pm*Chi1 with 1 mg/mL colloidal chitin in a total volume of 750 μL under various pHs for 1 h at 37 °C before the color reaction was developed.

To test its stability under various pHs, 50 μg of r*Pm*Chi1 was incubated at different pHs of 3–12 and 37 °C for 5 h. Then, the chitinase activity was assayed at pH 5 and 37 °C.

2.8.4. Effect of temperature on rPmChi1 activity and stability

The r*Pm*Chi1 was tested for its optimum working temperature by incubating 50 μg of the enzyme with 1 mg/mL colloidal chitin in a total volume of 750 μL for 1 h at pH 7 and various temperatures of 20–80 °C before the color reaction was developed.

The temperature stability of the enzyme was tested by incubating 50 μg of the r*Pm*Chi1 at various temperatures of 5–80 °C for 5 h before the chitinase activity was assayed. The assay was carried out in acetate buffer pH 5 at 37 °C for 1 h. The color reaction was developed as mentioned above.

3. Results

From a total of 10,536 unique contigs and singletons in the *P. monodon* database (http://pmonodon.biotec.or.th/database.jsp) (Tassanakajon et al., 2006), three contigs and two singletons representing three chitinase genes were identified and named *PmChi1*, *2* and *3* according to amino acid sequence homology to the chitinases from *M. japonicus* (Watanabe et al., 1996, 1998; Watanabe and Kono, 1997). Two contigs, CT508 and CT585, respectively, represented the *PmChi1* and its variant; one singleton, SG3857, was the *PmChi2*; one contig, CT226, and one singleton, SG8242, were the *PmChi3*. The three chitinase genes were truncated and needed mending. PCR and RACE were employed to complete the gene sequences. Primers were designed from the available DNA sequences.

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Fig. 1. Amino acid sequence comparison among the mature chitinases 1 from the penaeid shrimp. The positions of variable amino acid residues are shaded. The amino acid residues of *Pm*Chi1 from this study that are different from those of *Pm*Chi1 described by Tan et al. (2000) are in bold. The Glyco 18 catalytic domain is from A20 to D371. The arrow above the sequences indicates the cysteine-rich chitin binding domain from I443 to M498. *Pm*, *Fc*, *Lv* and *Pj* are for *Penaeus monodon*, *Fenneropenaeus chinensis*, *Litopenaeus vannamei* and *Penaeus* (*Marsupenaeus*) *japonicus*, respectively. Asterisks indicate identity. Colons and full-stops indicate similarity.

3.1. *PmChi1*

As compared to that of the Pjchi-1, the amino acid sequence of *Pm*Chi1 from the CT508 contig covered two-third of the chitinase 1 open reading frame with the 5′ sequence truncated. The 3′ untranslated sequence went as far as the poly(A) tail. When compared to the amino acid sequence of the *P. monodon* chitinase 1 reported by Tan et al. (2000), the *Pm*Chi1 in our study was 46 amino acids longer at its C-terminus (see Fig. 1). To complete

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the *PmChi1* gene coding for mature chitinase 1, the 5′ forward primer (FChi1_1) was designed from the cDNA sequence described by Tan et al. (2000) whereas the 3′ reverse primer (RChi1_1) was from the contig sequence (Table 1). The nucleotide sequence coding for the signal peptide predicted by the SignalP 3.0 was omitted in the amplification.

The reading frame for mature *Pm*Chi1 was amplified from the cDNA pool of hepatopancreas, cloned into the T&A vector and sequenced. Examining the open reading frame of the amplified *PmChi1* showed that the amino acid sequence at the C-terminus was still longer than the *P. monodon Chi1* by Tan et al. (2000). Besides the longer C-terminal end, a few amino acid variations were observed (Fig. 1) possibly owing to variation in shrimp stock. Amino acid sequence comparison reveals that, except at the C-terminal end, the *Pm*Chi1 amino acid sequence was very similar to those of *M. japonicus*, *F. chinensis* and *L. vannamei* (Fig. 1); the homology was about 88–91%. The reading frame for mature *Pm*Chi1 coded for a polypeptide chain of 644 amino acid residues with a calculated molecular weight of 72.4 kDa. The Glyco 18 catalytic domain of *Pm*Chi1 was defined using PROSITE and SmartTM databases to be A20-D371. The cysteine-rich chitin binding domain resided at the C-terminus I443-M498.

3.2. PmChi2

For *Pm*Chi2, a singleton SG3857 represented the enzyme. The cDNA sequence covered a third of the open reading frame in the middle of the gene lacking both the 5′ upstream and 3′ downstream sequences coding for the N- and C-terminal sequences of the chitinase, respectively. Thanks to the high nucleotide sequence homology among the chitinases 2 from the penaeid shrimp, *L. vannamei* and *M. japonicus*, a reverse RChi2 primer was designed from the homologous sequence. The forward primer designed from the *Pjchi-2* sequence at the start of open reading frame failed to give any PCR products. The forward FChi2 primer was, then, designed from the *PmChi2* singleton in which the gene segment amplified covered only two-third of the chitinase 2 open reading frame (Fig. 2). The amplified sequence was sequenced and used to design primers for 5′ RACE.

Three primers, GSPout1, GSPout2 and GSPin (Table 1), were designed for the 5′ RACE libraries constructed from the eyestalk and gill. All possible combinations of the gene-specific primers and 5′ RACE universal primers for primary and secondary PCRs were tested. Only 35 more N-terminal amino acid residues were obtained by using the GSPout1 and GSPin for primary and secondary PCRs, respectively (Fig. 2). As a consequence, we were able to define the Glyco18 catalytic domain of *Pm*Chi2 to be Y28-D373. The chitin binding domain could be defined only from the Pjchi-2 at the N-terminus.

The partial *PmChi2* in this study was, then, 1223 bp long coding for a polypeptide chain of 406 amino acid residues. Comparison of the available amino acid sequences showed that there was very high homology among the chitinases 2 from the penaeid shrimp (Fig. 2). The homology was about 90–97%.

3.3. PmChi3

One contig, CT226, and one singleton, SG8242, represented the *PmChi3*. Using the *PjChi3* gene sequence as a guideline, the singleton covered the N-terminal part of the mature enzyme including a portion

Fig. 2. Amino acid sequence comparison of chitinases 2 among the penaeid shrimp. The positions of variable amino acid residues are shaded. Underlined amino acid sequence of *Pm*Chi2 was obtained from 5′ RACE. The Glyco18 catalytic domain of *Pm*Chi2 is from Y28 to D373. The chitin binding domain is found in Pjchi-2. The arrow above the sequences indicates the cysteine-rich chitin binding domain. *Pm*, *Lv* and *Pj* are for *Penaeus monodon*, *Litopenaeus vannamei* and *Penaeus* (*Marsupenaeus*) *japonicus*, respectively. Asterisks indicate identity. Colons and full-stops indicate similarity.

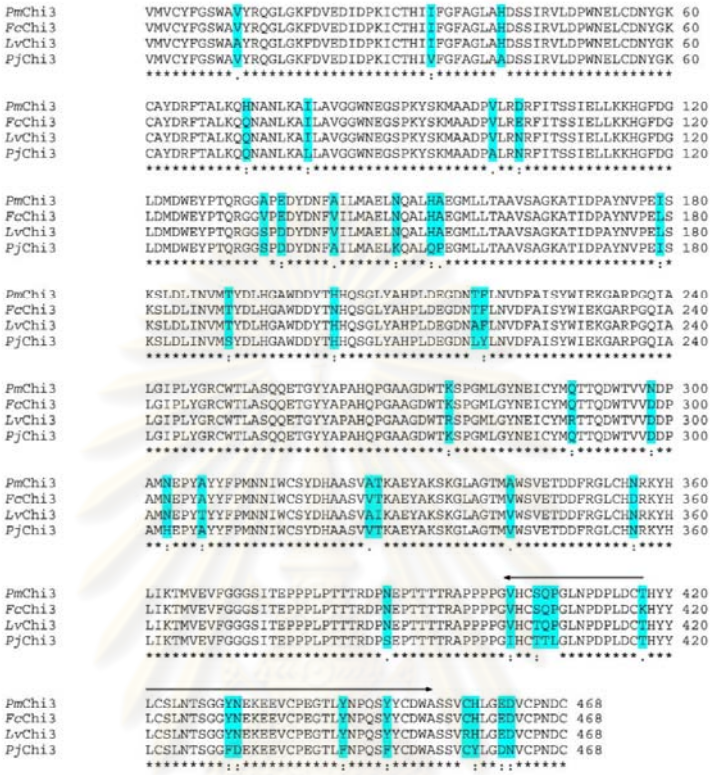

Fig. 3. Amino acid sequence comparison of mature chitinases 3 among the penaeid shrimp. The Glyco 18 catalytic domain of *Pm*Chi3 is from V1 to D348. The arrow above the sequences, V402-L459, indicates the cysteine-rich chitin binding domain. *Pm*, *Fc*, *Lv* and *Pj* are for *Penaeus monodon*, *Fenneropenaeus chinensis*, *Litopenaeus vannamei* and *Penaeus* (*Marsupenaeus*) *japonicus*, respectively. The positions of variable amino acids are shaded. Asterisks indicate identity. Colons and full-stops indicate similarity.

of the signal peptide. The contig covered the C-terminal portion of the enzyme with the 3′ untranslated nucleotide sequence went down to the poly(A) tail. To retrieve the middle sequence portion, primers were designed from the DNA sequences of the contig and singleton to amplify the *PmChi3* gene coding for mature protein (Table 1). The size of mature *Pm*Chi3 was 468 amino acids with calculated molecular weight of 51.9 kDa.

As illustrated in Fig. 3, the *Pm*Chi3 showed very high homology to those chitinases 3 of *F. chinensis*, *L. vannamei* and *M. japonicus*. The homology was about 93–97%. The Glyco 18 catalytic domain of *Pm*Chi3 was defined to be V1-D348. Like the *Pm*Chi1, the chitin binding domain resided at the C-terminus from V402 to L459.

3.4. Phylogenetic studies

The phylogenetic relationships of the *P. monodon* chitinases to other chitinases were analyzed. The chitinases from various organisms; mammals, insects and crustaceans including the penaeid shrimp were subjected to amino acid sequence comparison. The amino acid sequences were analyzed online for the typical glycoside hydrolase catalytic (Glyco 18) domain at http://smart.embl-heidelberg.de/. The catalytic domains were compared using the ClustalX and a phylogenetic tree was constructed using the Phylip program. The mammalian chitinases were treated as an outgroup.

The amino acid sequences of Glyco 18 catalytic domain of *Pm*Chi1, 2 and 3 were A20-D371, Y28-D373 and V1-D348, respectively. That of *Pm*Chi1 shared similarities of 65–93% to crustacean chitinases, 48–60% to insect chitinases and 35–37% to mammalian chitinases. That of *Pm*Chi2 shared similarities of 77–97% to crustacean chitinases, 39–42% to insect chitinases and 39–74% to mammalian chitinases. For *Pm*Chi3, its similarities to the crustacean, insect and mammalian chitinases were 49–97%, 38–46% and 35–40%, respectively. The phylogenetic relationship among the chitinases is shown in Fig. 4. The chitinases from crustaceans and insects were clustered into three lineages. Within each

Fig. 4. Phylogenetic trees based on the alignment of amino acid sequences of the catalytic domains of chitinases using ClustalX and Phylip programs. Bootstrap analysis was performed for values representing 1000 replicates by the SeqBoot. The amino acid sequences of mammalian chitinases were defined as an outgroup. The amino acid sequences are from the GenBank. *Aa*, *Aedes aegypti*; Ag_1, *Anopheles gambiae*, Ag_2, *Apriona germari*; *Ai*, *Agrotis ipsilon*; Am_1, *Ailuropoda melanoleuca*; Am_2, *Apis mellifera*; *Bm*, *Bombyx mori*; *Bt*, *Bos taurus*; Cf_1, *Canis familiaris*; Cf_2, *Choristoneura fumiferana*; *Cq*, *Culex quinquefasciatus*; *Cs*, *Chilo suppressalis*; *Dm*, *Drosophila melanogaster*; *Dp*, *Drosophila pseudoobscura pseudoobscura*; *Ec*, *Equus caballus*; *Fc*, *Fenneropenaeus chinensis*; *Gm*, *Glossina morsitans morsitans*; *Ha*, *Helicoverpa armigera*; *Hc*, *Hyphantria cunea*; *Hs*, *Homo sapiens*; *Ll*, *Lutzomyia longipalpis*; *Lm*, *Locusta migratoria manilensis*; *Lo*, *Lacanobia oleracea*; *Lv*, *Litopenaeus vannamei*; *Ma*, *Monochamus alternates*; *Mb*, *Mamestra brassicae*; Md_1, *Monodelphis domestica*; Md_2, *Musca domestica*; *Mj*, *Marsupenaeus japonicus*; *Mm*, *Macaca mulatta*; *Ms*, *Mythimna separate*; *Nv*, *Nasonia vitripennis*; *Oa*, *Ornithorhynchus anatinus*; *Of*, *Ostrinia furnacalis*; *Pm*, *Penaeus monodon*; *Pt*, *Pan troglodytes*; *Rn*, *Rattus norvegicus*; *Sl*, *Spodoptera litura*; Ss_1, *Sus scrofa*; Ss_2, *Scylla serrata*; *Tc*, *Tribolium castaneum*; *Tm*, *Tenebrio molitor*; *Tn*, *Toxoneuron nigriceps*. The numbers in parentheses are the GenBank accession numbers. Arrows indicate the *P. monodon* chitinases in this study.

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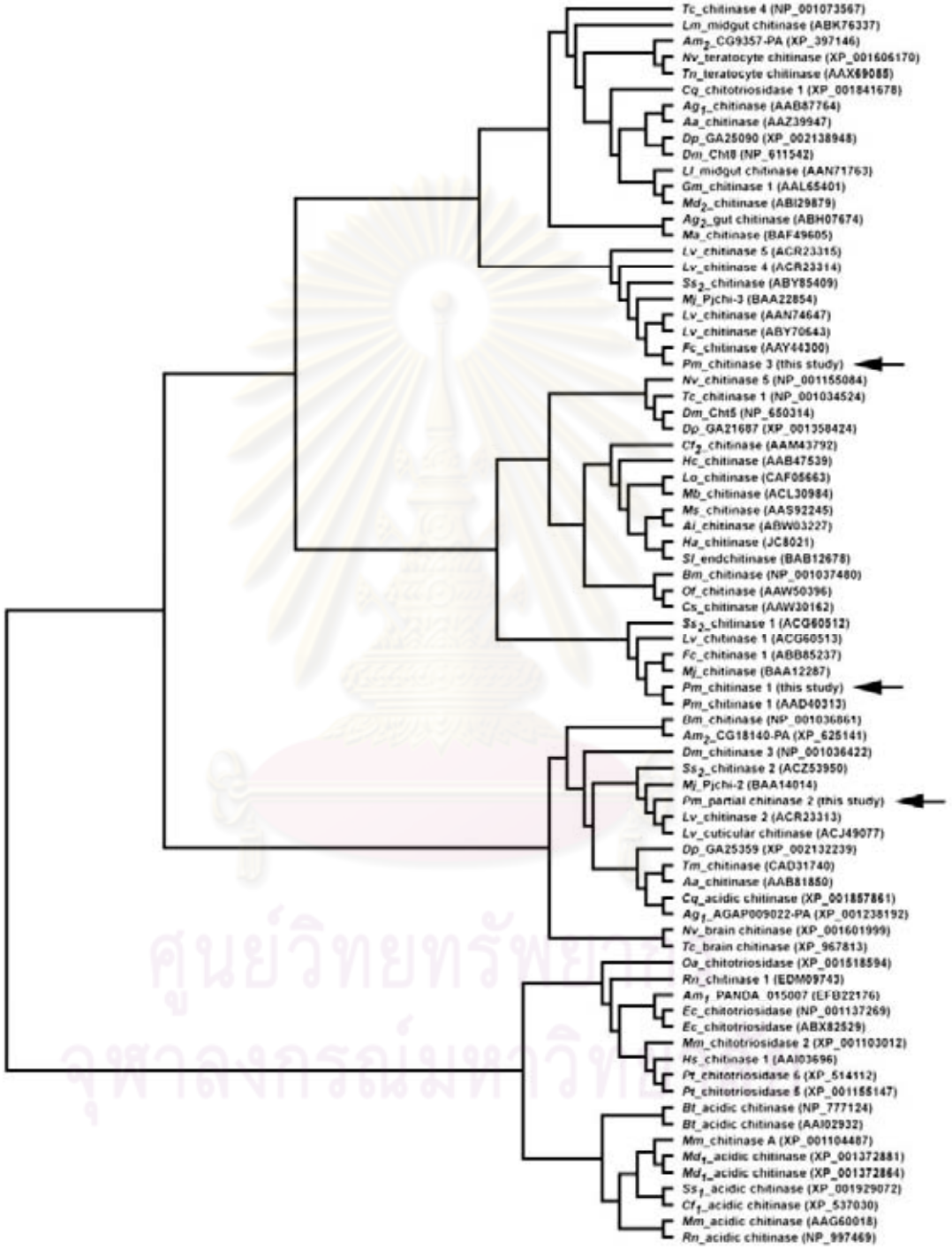

lineage, the chitinases from crustaceans were also separately grouped together. The groups of *Pm*Chi1 and 3 were closely related to each other than to the group of *Pm*Chi2.

3.5. Tissue distribution of chitinases in normal and postmolted shrimp

For the clues to their functions, tissue localization of the three chitinases was investigated in normal and early postmolted shrimp. Equivalent amounts of cDNAs from antennal gland, stomach, hemocyte, gill, intestine, lymphoid, eyestalk, hepatopancreas, heart, epipodite, leg and tail were analyzed by RT-PCR using the β-*actin* gene as an internal control. The results shown in Fig. 5 revealed that the *PmChi1* was expressed mainly in hepatopancreas but relatively small amount was found in antennal gland, stomach and intestine. The *PmChi2* was expressed mainly in gill and smaller amount in eyestalk and epipodite. The *PmChi3* was expressed mainly in hepatopancreas. At early postmolt stage A, only the amount of *PmChi2* messenger was down-regulated in gill, eyestalk and epipodite but could be seen faintly in all tissues. The expression of *Pm*Chi1 was more or less the same except an up-regulated in lymphoid and down-regulated in intestine. The significance of changes in intestine and lymphoid was not known. The expression of *PmChi3* remained unchanged at early postmolt stage A.

3.6. Recombinant PmChi1 expression

The *Pm*Chi1 was chosen for protein expression and characterization for it was believed to be involved in the digestion of chitin in food and degradation of endogenous chitin in the gut, and its activity might be interesting for further application. The DNA fragment coding for mature *Pm*Chi1 was cloned into a pET32a(+)-expression vector and expressed in an *E. coli* strain Rosetta(DE3)pLysS. The recombinant *Pm*Chi1 (r*Pm*Chi1) was expressed as a fusion protein to thioredoxin with the calculated mass of 86 kDa. The bacterial cell culture was induced by adding IPTG and harvested at 6 h after induction (Fig. 6A). The r*Pm*Chi1 found in the inclusion bodies was solubilized with phosphate buffer pH 12, dialyzed against the carbonate buffer pH 10, and purified using a nickel-NTA column. The protein was traced by SDS-PAGE (Fig. 6B). It should be noted that the r*Pm*Chi1 was not over-expressed when not fused with thioredoxin sequence in other pET plasmids.

Attempt to separate the thioredoxin moiety by enterokinase and thrombin failed because the proteinases also cleaved the r*Pm*Chi1. Since the fusion protein possessed the chitinase activity, we, therefore, continued our characterization of the r*Pm*Chi1 albeit the fusion protein. We had also tested another similar fusion protein, the thioredoxin-SP*Pm*4 (Visetnan et al., 2009), for chitinase activity and found none. Therefore, the chitinase activity was actually from the *Pm*Chi1 itself.

3.7. Characterization of chitinase

3.7.1. Assay of chitinase activity

The chitinolytic activity of r*Pm*Chi1 was tested on a colloidal chitin agar plate. After incubation overnight at 37 °C, clear zones were clearly seen around the wells added with 50 μg of r*Pm*Chi1 and chitinase from *B. licheniformis* SK-1 but not the buffer control (Fig. 7). This result clearly indicated that the r*Pm*Chi1 was active in chitin hydrolysis.

To further characterize the biochemical properties of the r*Pm*Chi1, the colloidal chitin hydrolytic reaction was used for the assay. The r*Pm*Chi1 in the reaction mixture was assayed to determine the range of activity that was linearly proportional to the r*Pm*Chi1 concentration (data not shown). The amount of r*Pm*Chi1 used in the reactions was within this range.

3.7.2. Effect of pH on rPmChi1 activity and stability

Using three standard buffer systems to cover a pH range 3–12, the chitinolytic activity of r*Pm*Chi1 was assayed at various pHs. It was found that the r*Pm*Chi1 had a maximal chitinolytic activity or optimal pH at pH 5 and 37 °C (Fig. 8A). To determine its stability, the r*Pm*Chi1 was incubated at 37 °C at various pHs for 5 h before its activity was assayed at pH 5 and 37 °C. The r*Pm*Chi1 was most stable at neutral pH (Fig. 8B).

3.7.3. Effect of temperature on rPmChi1 activity and stability

The r*Pm*Chi1 activity was determined at various temperatures for its optimal temperature. The maximal chitinolytic activity was observed at 55 °C (Fig. 9A). When its temperature stability was determined, the r*Pm*Chi1 was incubated at 5–80 °C for 5 h before its chitinase activity was measured at 37 °C and pH 5. The r*Pm*Chi1 was stable at temperature lower than 40 °C (Fig. 9B). Above 40 °C, the enzyme was not stable and the chitinolytic activity decreased rapidly.

3.7.4. Substrate preference of rPmChi1

The soluble PNAC was a good substrate for the r*Pm*Chi1 for it was the easiest to be hydrolyzed. Relative to PNAC, the colloidal chitin from shrimp shell and β-chitin from squid pen were tested. The colloidal chitin was the second best substrate for r*Pm*Chi1 followed by β-chitin as they are hydrolyzed about 90 and 50%, respectively (Fig. 10).

4. Discussion

Upon searching the *P. monodon* EST database, three different chitinase genes, *PmChi1, 2* and *3*, were identified from a total of 10,536 unique contigs and singletons. Even with such huge numbers of unique genes being searched, it was still too early to conclude that there were only three different chitinases in *P. monodon*. It is already known that multiple genes encoding for different chitinases do exist in organisms especially in insects. For example, there are at least three

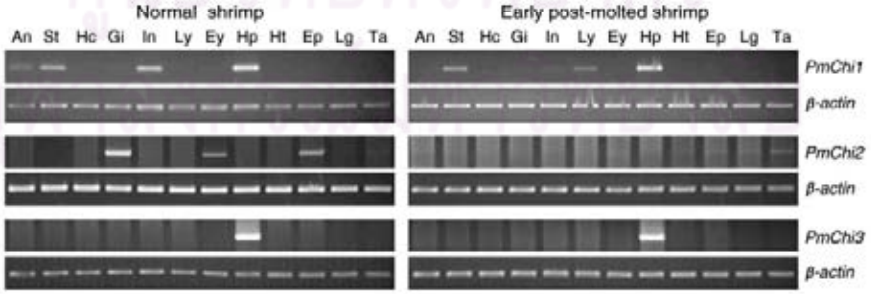

Fig. 5. Tissue distribution of *PmChi1, 2* and *3* in the black tiger shrimp. Tissues tested are antennal gland (An), stomach (St), hemocyte (Hc), gill (Gi), intestine (In), lymphoid (Ly), eyestalk (Ey), hepatopancreas (Hp), heart (Ht), epipodite (Ep), leg (Lg) and tail (Ta). The β-*actin* was used as an internal control.

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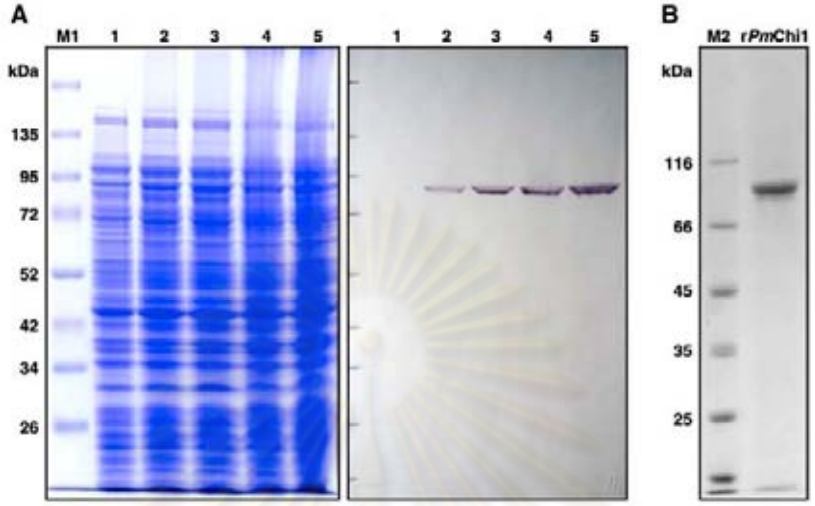

Fig. 6. SDS-PAGE and western blot analysis of the rPmChi1 expression and purification. (A) The induction of rPmChi1 expression by SDS-PAGE (left) and western blot (right). Lanes 1–5 are total protein of un-induced and induced cells after IPTG induction for 3, 4, 5 and 6 h, respectively. (B) SDS-PAGE of purified rPmChi1. Lanes M1 and M2 are two different molecular weight markers.

chitinases (ChiA, ChiB and ChiC) in *Serratia marcescens*, one of the most effective bacteria for chitin degradation (Suzuki et al., 2002). In insects, a variety of chitinases are identified and found to be encoded by as many as 16 genes depending upon the species of interest. The involvement of these individual proteins in growth and development is largely unknown (Zhu et al., 2008a,b; Arakane and Muthukrishnan, 2009). In mud crab *Scylla serrata*, there are tentatively four chitinases identified (accession no. ACG60512, ACZ53950, ABY85409 and ACZ53951). In penaeid shrimp, chitinases 1–3 are identified from *M. japonicus* (Watanabe et al., 1996, 1998; Watanabe and Kono, 1997) and *P. monodon* in this study. Chitinases 1 (accession no. ABB85237) and 3 (AAY44300) are from *F. chinensis*. At least six chitinases, chitinases 1–6, have been identified from *L. vannamei* (ACG60513, ACR23313, ABY70643, ACR23314, ACR23315 and ACX68556).

Amino acid sequence comparison of the Glyco 18 catalytic domains of the chitinases from the penaied shrimp, insects and mammals revealed that there was a quite high homology among those from the shrimp such that they were clustered closely together in the

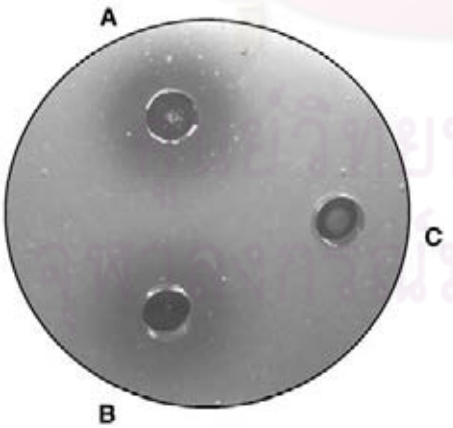

Fig. 7. The chitinolytic activity of rPmChi1 on colloidal chitin agar. The activity was tested by adding 50 μg of rPmChi1 (A), unknown amount of chitinase from *Bacillus licheniformis* SK-1 as a positive control (B) and 20 mM sodium carbonate buffer pH 10 as a negative control (C). Clear zones around the wells indicate positive result.

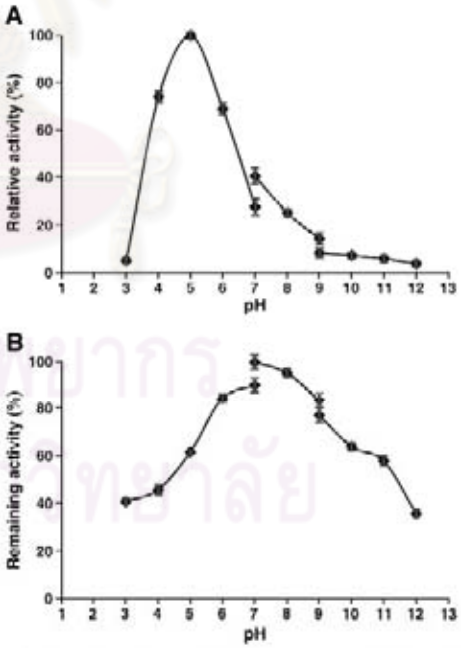

Fig. 8. Effect of pH on the activity and stability of rPmChi1. To determine the optimal pH, the rPmChi1 was assayed at various pHs using three buffer systems (A). The rPmChi1 was incubated at various pHs for 5 h to test its pH stability before it was assayed at pH 5 (B). The results are the mean values of five replicates with ± standard deviations.

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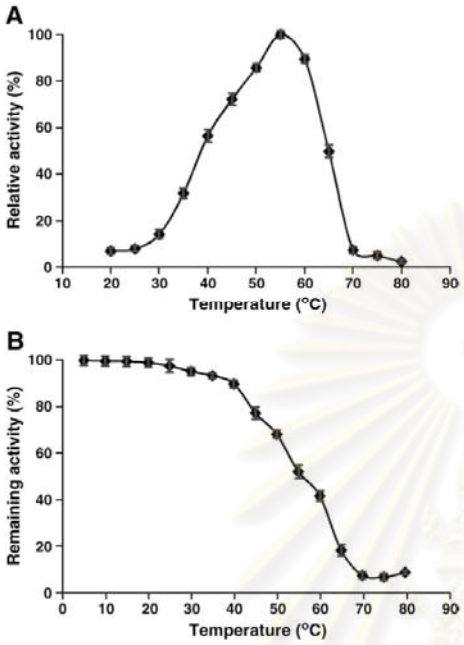

Fig. 9. Effect of temperature on r*Pm*Chi1 activity and stability. The activity of r*Pm*Chi1 was measured at various temperatures to determine the optimal temperature (A). The enzyme was incubated at various temperatures for 5 h before it was assayed at 37 °C and pH 5 to determine its stability (B). The results are the mean values of five replicates with ± standard deviations.

phylogenetic trees. Treating the mammalian chitinases as an outgroup, the insect chitinases were separated into three clusters along with the three shrimp chitinases 1, 2 and 3. The groups of chitinases 1 and 3 were more closely related to each other than to the group of chitinase 2 suggesting their difference in activities as well as their biological function. The chitinase 2 was, indeed, implicated as chitinase involved in molting (Watanabe and Kono, 1997) where the other two chitinases were involved in chitinous food digestion and degradation of endogenous chitin prior to molting (Tan et al., 2000).

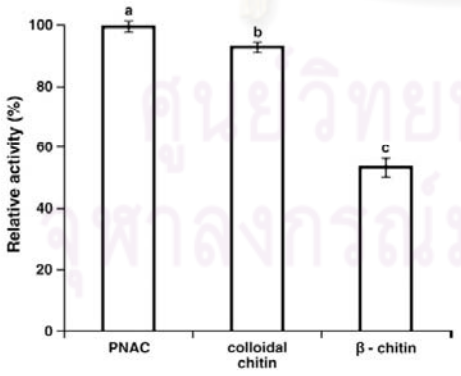

Fig. 10. Substrate preference of r*Pm*Chi1. The r*Pm*Chi1 was assayed in the presence of PNAC, colloidal chitin from shrimp shell and β-chitin from squid pen. The reaction using PNAC was set as 100%. The results are the mean values with ± standard deviations. Different letters (a, b and c) indicate significant difference ($p<0.05$) in the mean hydrolytic activity of r*Pm*Chi1.

The *Pm*Chi1 in this study was the longest chitinase 1 among the penaeid shrimp and longer than the *Pm*Chi1 reported by Tan et al. (2000). At its C-terminus were the extended 46 amino acids. Examining the nucleotide sequences at this end revealed a shift in the reading frame in *Pm*Chi1. We had confirmed the existence of this extended amino acid sequence in three different occasions. In short, three *PmChi1* had been PCR amplified and cloned from three different cDNA preparations from different lots of shrimp, possibly from different aquacultures in Thailand, at different periods of time over 2 years and their sequences were almost the same. Considering the high variability of the C-terminal amino acid sequences of chitinases 1 as in Fig. 1, it might be possible that the difference between the *Pm*Chi1 in this study and that of Tan et al. (2000) arose from the variation in shrimp stock.

The expression of *PmChi1* was mainly in hepatopancreas, less in intestine, stomach and antenna. In early postmolt shrimp stage A, the pattern of tissue distribution remained more or less similar albeit up- and down-regulation in lymphoid and intestine. Tan et al. (2000) had shown that the expression of *Pm*Chi1 was highest during the premolt stage and suggested that the *Pm*Chi1 was for the degradation of endogenous chitin in the gut peritrophic membrane before molting. In *F. chinensis*, the expression of *Fcchi-1* was highest during the premolt stage too but fluctuated rather widely (Priya et al., 2009).

In this study, we had not been able to acquire a full length *PmChi2* gene albeit using different gene-specific primers and different 5′ RACE cDNA libraries from eyestalk and gill. By comparing the *Pm*Chi1, 2 and 3 with the insect chitinases, we observed that the *Pm*Chi1 and 3 were comparable in size to the insect chitinases in the same groups. For example, the chitinase 5 from *Drosophila melanogaster* (accession no. NP_650314) (Hoskins et al., 2007) and the chitinase from *Helicoverpa armigera* (JC8021) (Ahmad et al., 2003) contain 595 and 588 amino acids, respectively, as compared to 644 amino acids of mature *Pm*Chi1. The midgut chitinase from *Lutzomyia longipalpis* (AAN71763) (Ramalho-Ortigão and Traub-Csekö, 2003) and chitinase 4 from *Tribolium castaneum* (NP_001073567) (Zhu et al., 2008a,b) contain 474 and 475 amino acids, respectively, as compared to 468 amino acids of mature *Pm*Chi3.

If the *Pm*Chi2 were of similar size to the insect chitinases in the same group, we anticipated that the *Pm*Chi2 we obtained was only about one-sixth as big as those of insects. The chitinase 3 from *D. melanogaster* (NP_001036422) (Hoskins et al., 2007) and the chitinase 10 from *T. castaneum* (NP_001036067) (Zhu et al., 2008a,b) are 2286 and 2700 amino acids in length while the partial *Pm*Chi2 in this study is only 406 amino acids. Sequence comparison indicated that the partial *Pm*Chi2 was at the C-terminus and a lot more sequence at the N-terminus remained to be uncovered (data not shown).

The expression of *Pm*Chi2 was mainly in gill, eyestalk and epipodite of the intermolt stage C shrimp. The messenger was detectable faintly in all tissues at postmolt stage A. Watanabe and Kono (1997) observed in *M. japonicus* that the Pjchi-2 was up-regulated in cuticular tissues late in premolt stage. The transcription was down-regulated at postmolt stage as observed with *PmChi2*. Therefore, the *PmChi2* very likely played a direct role in molting of the exoskeleton.

The amino acid sequence comparison of the chitinases 3 revealed high homology among the penaeid shrimp. The *PmChi3* was expressed mainly in hepatopancreas and remained unchanged at postmolt stage. This result agreed well with that of *Fcchi-3* (Priya et al., 2009; Zhang et al., 2010). Like that of *Fcchi-1*, the expression of *Fcchi-3* occurred at all stages in molting cycle but was highest during the premolt stage (Priya et al., 2009). Like the *Pm*Chi1, the *Pm*Chi3 may function in the digestion of chitinous food and degradation of endogenous chitin prior to molting.

Since the *Pm*Chi1 was possibly involved in both food digestion and degradation of endogenous chitin, it was chosen for over-production

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in *E. coli*. The mature *Pm*Chi1 was produced as fusion protein to the thioredoxin. The thioredoxin that could not be removed by specific protease digestion for the whole protein was rapidly digested upon treatment. Since the protein was active in chitinolysis as it readily hydrolyzed the colloidal chitin, we, therefore, characterized the *Pm*Chi1 for its optimum pH and temperature, its pH and temperature stability and its substrate preference.

The *Pm*Chi1 worked well at pH 5 and lost its activity rapidly above pH 6 and below pH 4 but it was most stable at neutral pH 7. Its optimal temperature for chitinase activity was 55 °C, higher than that we could expect from the aquatic animal like shrimp. Above 60 °C and below 50 °C, the activity subsided rather rapidly. The enzyme was stable if stored at low temperature below 40 °C; the lower the , the better the stability. Compared to those of insects, a chitinase from *Manduca sexta* (Zhu et al., 2001) has wider range of optimum pH 5–8 while the two chitinases, 65 and 88 kDa proteins, from *Bombyx mori* (Koga et al., 1997) work well at pH 5.5 and 6.5. Their optimum temperatures were 50 °C for the *M. sexta* chitinase and 60 °C for the two *B. mori* chitinases. Like the r*Pm*Chi1, the 65 and 88 kDa chitinases from *B. mori* are stable at lower temperature 30 and 40 °C, respectively.

At its optimum pH, the *Pm*Chi1 readily hydrolyzed the more soluble substrates like PNAC and colloidal chitin. With its rather high temperature optimum and low pH optimum, it might be useful for shrimp waste treatment and the production of chitooligosaccharides and glucosamine (Dahiya et al., 2006).

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