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REVIEW

The molluscan HSP70s and their expression in hemocytes

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Abstract

The heat shock protein 70s (HSP70s) are a class of functionally related proteins involved in the folding and unfolding, translocation of other proteins, and stress responses in almost all organisms. As the most analyzed heat shock proteins, numerous HSP70s have been identified and characterized from bacteria, plants and animals. Molluscan HSP70 is one of the largest and most important groups in the invertebrate HSP70 family. Accumulating evidences have demonstrated the relevant physiological and ecological importance of HSP70 in response to pathogen infection and environmental stressors. This chapter reviews the interest arose around HSP70s in molluscan animals, mainly the recent research progress about the diversity of molluscan HSP70 family members, their sequence characters and expression profiles in hemocytes under various stressors.

Key Words: Mollusc; Heat shock protein 70; expression profile; immune challenge; environmental stressor

Introduction

Heat shock protein 70 (HSP70) is one of the most abundant HSP families involved in the folding and unfolding, translocation of other proteins, and stress responses in almost all organisms. They consist of a class of functionally related proteins including HSP68, HSP70, HSC70, HSP75 and HSP78 (GRP78), which are localized to distinct subcellular compartments including cytoplasm, mitochondria, and endoplasmic reticulum (ER) (Boorstein *et al.*, 1994; Mayer and Bukau, 2005).

The amino acid sequences of HSP70 family members are highly conservative from archaebacteria to humans, and there are two major functional domains, the N-terminal ATPase domain and the C-terminal peptide binding domain (Sung *et al.*, 2001; Mayer and Bukau, 2005). The HSP70s are involved in a variety of physiological processes and perform complex functions, such as serving as molecular chaperones (Gething & Sambrook, 1992), involved in the regulation of apoptosis (Böttger *et al.*, 2008), and playing important roles in response to bacterial challenge (Cellura *et al.*, 2006), oxidative stress (Golli-Bennour and Bacha, 2011) and various environmental stressors (Cellura *et al.*, 2006). Recent studies in land snails *Sphincterochila* species suggested that HSP70 was also involved in the natural annual cycle of activity and aestivation and the survival strategy during desiccation and heat stress, and the adaptation of land snails to different habitats engenders the development of distinct strategies of HSP70 expression in response to stress (Mizrahi *et al.*, 2012).

The mollusc phylum is one of the largest and most important groups in the animal kingdom, and around 130,000 extant species are described (Haszprunar and Wanninger, 2012). Most of them live in freshwater or seawater, and they have to survive environmental perturbation from homeostasis, a situation generically described as stress. The production of acute phase proteins, such as the HSPs, is regarded as a classical response against stressors. This chapter reviews the interest arose around molluscan HSP70s in the last 5 years, mainly in the diversity, sequence characters and their expression profiles in hemocytes under various stresses.

The HSP70 family members in mollusc

Due to the important roles of HSP70s in the response against environmental stressors and the maintenance of homeostasis in molluscs, they have been studied extensively and the amount of their nucleotide sequences has increased noticeably during the past decades. There are totally 213 nucleotide sequences of molluscan HSP70 so far available in the database of NCBI, including 124

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Table 1 The full length of cDNA sequences encoding HSP70 in mollusc species

Species	gene	Accession Number	Reference
snail			
Biomphalaria glabrataembryonic	HSP70.1	L44127	Laursen *et al.*, 1997
Pomacea canaliculata	HSC70	Not released	Zheng *et al.*, 2012
sea hare			
Aplysia californica	BiP/GRP78	NM_001204652	Kuhl *et al.*, 1992
oyster			
Crassostrea gigas	HSC70	AJ305315	Boutet *et al.*, 2003b
	HSP70	AJ318882	Boutet *et al.*, 2003b
	GRP78	BAD15288	Yokoyama *et al.*, 2006
	GRP94	AB262084	Kawabe and Yokoyama, 2009
	Other HSP70s	See the reference	Zhang *et al.*, 2012
Ostrea edulis	HSC70	AJ305316	Boutet *et al.*, 2003a
	HSP70	AF144646	Boutet *et al.*, 2003a
	Oedcl5	AF416608	Piano *et al.*, 2005
	OedclD2	AF416609	Piano *et al.*, 2005
Crassostrea hongkongensis	HSP70	FJ157365	Zhang and Zhang, 2012
mussel			
Mytilus galloprovincialis	HSP70	DQ178174, DQ178175	Franzellitti and Fabbri, 2005
	HSC70	DQ178176, DQ178177	Franzellitti and Fabbri, 2005
	HSP70	AY861684	Cellura *et al.*, 2006
	HSC70, HSC71	AJ783714, AJ783715	Kourtidis *et al.*, 2006
	HSP70-2, HSP70-3, HSP70-4	AJ783711, AJ783712, AJ783713	Kourtidis *et al.*, 2006
scallop			
Argopecten irradians	HSP70	AY485261	Song *et al.*, 2006
Chlamys farreri	HSP70	AY206871	Song *et al.*, 2006
Mizuhopecten. yessoensis	HSP70	AY485262	Song *et al.*, 2006
Pinctada fucata	HSP70	EU822509	Wang *et al.*, 2009
abalone			
Haliotis discus hannai	HSP70	DQ324856	Cheng *et al.*, 2007
Haliotis diversicolor	HSP70	ACO36048	unpublished
	HSC70	ACO36047	unpublished
clam			
Laternula elliptica	HSP70	EF198332.	Park *et al.*, 2007
Meretrix meretrix	HSC71	HQ256748	Yue *et al.*, 2011
Tegillarca granosa	HSP70	N936877	Zhou *et al.*, 2013

from bivalves, 77 from gastropods and 12 from cephalopods. The information about the full length cDNA sequences encoding HSC70 and HSP70 in mollusc is summarized in Table 1, and the species include snail (Laursen *et al.*, 1997; Zheng *et al.*, 2012), scallop (Song *et al.*, 2006; Wang *et al.*, 2009), oyster (Boutet *et al.*, 2003a and 2003b; Piano *et al.*, 2005; Zhang and Zhang, 2012; Zhang *et al.*, 2012), mussel (Franzellitti and Fabbri, 2005; Cellura *et al.*, 2006; Kourtidis *et al.*, 2006), abalone (Cheng *et al.*, 2007), and clam (Park *et al.*, 2007; Yue *et al.*, 2011). Other cDNA sequences encoding GRP78 and GRP94, the representatives of the GRP members in the molluscan HSP70 family, have also reported in sea hare (Kuhl *et al.*, 1992) and oyster (Yokoyama *et al.*, 2006; Kawabe and Yokoyama, 2009; Zhang *et al.*, 2012).

Though only one HSP70 has been reported in some molluscan species, all eukaryotes are believed to have more than one gene encoding HSP70 proteins in their genomes. For example, there are at least 11 unique HSP70 genes in human (Tavaria *et al.*, 1996), 39 putative HSP70s in sea urchin (Sodergren *et al.*, 2006), and 10 putative HSP70s in fungus *Blastocladiella emersonii* (Georg and Gomes, 2007). It is noteworthy that HSP70 gene family is remarkably expanded in *C. gigas* (Zhang *et al.*, 2012). A search of the genome sequence revealed that there were 88 members of HSP70 family in *C. gigas*, which were believed to play crucial roles in protecting cells against heat and other stressors (Zhang *et al.*, 2012).

Structural features of molluscan HSP70s

The molluscan HSP70s share common structural and evolutionary features with homologues from other species (Piano *et al.*, 2005; Kourtidis *et al.*, 2006), including the highly conserved N-terminal domain and the diverse C-terminal

domains (Demand *et al.*, 1998; Fuertes *et al.*, 2004; Kourtidis *et al.*, 2006). The highly conserved N-terminal domains of molluscan HSP70s usually shared three signature motifs (IDLGTTYS, IFDLGGGTFDVSIL, and IVLVGGSTRIPKIQK) and one ATP/GTP-binding motif (AEAYLGKT) (Wang *et al.*, 2009; Zhou *et al.*, 2013). In spite of high conservation, there are still some small variations in the N-terminal domains of molluscan HSP70s. For example, there is an extra NQSQ tetrapeptide in the ATPase domain of HSC70s from *O. edulis* (Boutet *et al.*, 2003a) and *C. gigas* (Boutet *et al.*, 2003b), and there are two nonsynonymous mutations, Y406I and G413E, in the ATP/GTP-binding motif of HSP70 from different geographical populations of *A. irradians* (Yang *et al.*, unpublished data).

Congruous with the difference in their subcellular localizations and functions, the C-terminal domains of different HSP70s usually display low sequence homology with each other (Demand *et al.*, 1998; Fuertes *et al.*, 2004; Piano *et al.*, 2005), especially between HSP70 and HSC70 (Fabbri *et al.*, 2008). The tetrapeptide motif GGMP is an important element mediating cofactor binding to the HSP molecule by forming a structural entity together with the helical subdomain and the EEVD motif (Demand *et al.*, 1998), and it has been once regarded as the peculiar sequence of HSC70s (Fuertes *et al.*, 2004; Piano *et al.*, 2005; Fabbri et al., 2008). However, in some species, both of HSP70 and HSC70 contain GGMP tetrapeptide with variable numbers. For example, there are one, two, three and five GGMP tetrapeptides in the HSP70s from pearl oyster *Pinctada fucata*, blood clam *Tegillarca granosa*, Pacific abalone *Haliotis discus hannai* and *Argopecten irradians* respectively (Wang *et al.*, 2009; Zhou *et al.*, 2013; Cheng *et al.*, 2007; Song *et al.*, 2006). Therefore, it is necessary to investigate the effects of such structural variations on the expression profiles of HSP70 and HSC70 (Fuertes *et al.*, 2004), and these information could also provide insights into functional specificities of HSP70s (Wang *et al.*, 2009). Moreover, there is a large amino acid deletion about 60 residues encompassing the end of the peptide-binding domain and a part of the C-terminal domain of HSC70 from *O. edulis* (Kourtidis *et al.*, 2006; Fabbri *et al.*, 2008).

The molluscan HSP70s located in cytosolism, ER, nuclear and mitochondrion always have the specific localization motifs GP(T/K)(V/I)EE(V/M)D, KDEL, NUCDISC and MITDISC, respectively. Multiple alignments revealed that most of molluscan HSP70s localized in the cytosolism sharing the cytosolic localization motif GP(T/K)(V/I)EE(V/M)D (Boorstein *et al.*, 1994; Demand *et al.*, 1998; Zhang and Zhang, 2012; Zhou *et al.*, 2013). For example, 76 out of 88 HSP70s from *C. gigas* shared the motifs of GP(T/K)(V/I)EE(V/M)D, and they were predicted locating in the cytoplasm (Yang *et al.*, unpublished data). Though EEVD and EEMD are both regarded as the cytosolic localization motifs, the effect of their sequence difference on structure and function still need further confirmation (Zhang and Zhang, 2012). Besides, GRP78 (Yokoyama *et al.,* 2006) and other seven HSP70s from *C. gigas* (Yang *et al.*, unpublished data) located in the ER also contain the motif KDEL. It is noteworthy that one HSP70 in oyster possessed a mitochondrial localization motif MITDISC, and this is the first mitochondrial HSP70 found in mollusc (Yang *et al.*, unpublished data).

Molluscan HSP70s are also classified into two groups of inducible HSP70s and cognate HSC70s at the present time, and they are closely matched to the corresponding HSP groups of other phylum in the phylogenetic analysis (Fabbri *et al.*, 2008). However, it is not always accurate to assign a HSP70 into a specific group according to the phylogeny relationship. For example, several HSP70s from oysters (Boutet *et al*., 2003a and 2003b; Kourtidis *et al.*, 2006) and scallops (Song *et al.*, 2006) identified as inducible HSP70 proteins were clustered into HSC70 according to the phylogenetic analysis (Fabbri *et al.*, 2008). Since there is limited information about the functions or activities of molluscan HSP70s, their classification is still not available currently. It has been reported that divergent evolution usually predominates when the members within one gene family acquire different functions (Ohta and Nei, 1994), and this is confirmed by inducible and cognate HSP70s, which belong to one family but display different expression patterns and functions. The phylogenetic reconstruction of molluscan HSP70s also indicates the occurrence of multiple duplication events in the evolution of HSP70 family, which is in agreement with the presence of multiple copies of the heat-inducible gene in molluscs. A phylogenetic analysis of 169 molluscan HSP70 proteins, including 88 from *C. gigas*, 12 from *L. gigantean* and 68 from other molluscs showed that 71 out of 88 *C. gigas* HSP70s were clustered together (Zhang *et al.*, 2012). It suggested that these genes were likely received significant positive selection and derived from oyster-specific expansions, and they might play major roles in oyster's adaptation to heat and other stressors (Zhang *et al.*, 2012).

Expression of molluscan HSP70s in hemocytes under various stressors

As the most abundant and well studied HSPs, HSP70s are considered to play important roles in various physiological processes and protect organisms against various stressors. There are numerous studies to recognize the relevant physiological and ecological importance of molluscan HSP70s expression in response to the stresses resulted from changes of season and other environmental factors, such as temperature (Cellura *et al.*, 2006), heavy metal (Boutet *et al.*, 2003b; Thompson *et al*., 2012; Taylor *et al*., 2013), hypoxia (Clark and Peck, 2009; Clark *et al*., 2013), pH (Cummings *et al.*, 2011), pollutants of PAHs (Song *et al.*, 2006) and toxins (Mello *et al.*, 2012; Mello *et al.*, 2013), pharmaceuticals (Gust *et al.*, 2013) and bacteria challenge (Cellura *et al.*, 2006; Song *et al.*, 2006; Cheng *et al.*, 2007; Xu and Faisal, 2009). Most of the information on HSP70 expression in molluscs was mainly obtained from five tissues including gill, digestive gland, muscle, mantle and hemocytes. In the gill of *C. gigas,* the expression pattern of HSP70s altered significantly at different temperatures. There were some HSP70 genes highly expressed at

normal temperature, and some genes were highly expressed at low temperature, while some other genes were highly expressed at high temperature (Zhang *et al.*, 2012). Regardless of the expression in other tissues, the research progress about the expression of molluscan HSP70s in hemocytes under various stressors is summarized in this chapter based on the reports in the past 5 years.

Most molluscs have an open circulatory system composing of heart, blood vessels, sinusoids and hemolymph. As the major part of the hemolymph, hemocytes comprise the major component of the non-specific defense mechanisms, and they are involved in a series of cellular immune reactions (Song *et al.*, 2010; Mello *et al.*, 2012). The circulating hemocytes are able to migrate from the hemolymph to connective tissues, promote localized responses following injury or microorganism invasion (Mello *et al.*, 2012), and discriminate pathogenic and non-pathogenic bacteria. For example, the expression of HSP70 gene in mussel hemocytes increased significantly after *V. anguillarum* challenge, while *V. splendidus* and *M. lysodeikticus* could not induce the expression of HSP70 (Cellura *et al.*, 2006). Recently, the expression of HSP70s in molluscan hemocytes have been investigated extensively against several environment stressors, such as high temperature (Yang *et al.*, unpublished data), heavy metal (Taylor *et al*., 2013), pollutants of toxins (Mello *et al.*, 2012; Mello *et al.*, 2013), pharmaceuticals (Gust *et al.*, 2013), bacterial infections (Wang *et al.*, 2009) and seasonal changes (Li *et al.*, 2009), and their expression profiles are generally divided into three cases, up-regulated, invariable and down-regulated.

When exposed to different stressors, up-regulated expression of HSP70s mRNA was the general case observed in molluscan hemocytes. For example, the mRNA expression of HSP70 in pond snail *Lymnaea stagnalis* increased (2.6-fold) after they were exposure to the mixtures of four pharmaceuticals (Gust *et al.*, 2013). After incubation with the purified paralytic toxin of dinoflagellate *Alexandrium minutum*, saxitoxin (STX), the mRNA level of HSP70 in oyster hemocytes increased 2-fold (Mello *et al.*, 2013). Moreover, the up-regulation of HSP70s expression in molluscan hemocytes usually displays a clearly time-dependent and dose-dependent pattern. The mRNA level of HSP70 in hemocytes of *C. gigas* increased at 4 h after the hemocytes were incubated with 1000 μg/L of PbTx-2 (Mello *et al.*, 2012). At 6 h, 12 h and 24 h post heat stress treatment, the expression of HSP68 in *C. gigas* was up-regulated and relative mRNA level was 3.78-, 16.11- and 112.16- fold of that in the control group, respectively (Yang *et al.*, unpublished data). After challenged by *V. alginolyticus*, the mRNA expression of HSP70 in hemocytes of pearl oyster *P. fucata* increased to the maximum level at 4 h, and returned to control level at 32 h (Wang *et al.*, 2009). The mRNA expression of HSP70 in hemocytes of zebra mussel *Dreissena polymorpha* reached the highest level (2.8-fold) at 1 h post LPS stimulation, and decreased at 2 h, and then increased again from 3 h to 6 h post-stimulation (Xu and Faisal, 2009). The mRNA expression of HSP70 in hemocytes of blood clam *T. granosa* were all significantly up-regulated at 6 h after Pb^{2+}, Cd^{2+} and Cu^{2+} treatments, and peaked at 12 h after treatments (Zhou *et al.*, 2013).

Except for the frequently up-regulation of HSP70s, it is interesting that the expression of HSP70 could also be down-regulated under some stressors. For example, the expression of HSP70 was significantly down-regulated when the Sydney Rock oyster *Saccostrea glomerata* was exposed to some heavy mental, such as zinc and copper (Taylor *et al*., 2013), cadmium and lead (Thompson *et al.*, 2012). The excretory-secretory products (ESPs) from the larva of parasite *Schistosoma mansoni* could reduce the HSP70 protein levels in hemocytes of its snail intermediate host *Biomphalaria glabrata*, and the reduction in hemocytes of *S. mansoni*-resistant strain was less marked, while that in hemocytes of *S. mansoni*-susceptible snails was remarkable (approximately 70%) after infected by *S. mansoni* for 35 days (Zahoor *et al.*, 2010).

Regulation of molluscan HSP70 expression

The up-regulation and down-regulation, as well as the dose-dependent and time-dependent expression pattern of HSP70 in the hemocytes of mollusks exposed to various stressors strongly suggested that the regulation mechanism of HSP70 expression was indeed complicated. Generally, the expression of HSP70 genes is mainly regulated at the transcription level (Park *et al.*, 2007), and the regulation is mediated by direct interaction of heat shock transcription factors (HSFs) and their corresponding heat shock elements (HSEs) in the promoters of HSP70s (Wu, 1995; Buckley *et al.*, 2001), and other indirect signaling pathways (Buckley et al., 2001; Park and Liu, 2001; Gourgou et al., 2010; Zahoor *et al.*, 2010).

The interaction of HSFs and HSEs in the promoters of HSP70s is the prime strategy to regulate HSP70 expression. Though molluscan HSF1s have been identified in the genome of *M. trossulus*, *C. gigas* and *Haliotis asinina*, the relevant study on the regulation mechanism of molluscan HSP70 expression is at the very beginning. It has been reported that HSF1 of intertidal mussels (genus *Mytilus*) releases from HSP70 and translocates into the nucleus in response to small increase of temperature, and remains inactive on the promoter until the mussels encounter a higher temperature (Buckley *et al.*, 2001).

The regulation of HSP70 expression also involves other cell proteins and signaling pathways after HSF1 has been bound to the promoter, including the mitogen-activated protein kinases (MAPK) signaling cascade (Buckley *et al.*, 2001; Park and Liu, 2001; Gourgou *et al.*, 2010) and the extracellular signal-regulated kinase (ERK) signaling pathway (Zahoor *et al.*, 2010). In *M. galloprovincialis*, the increased phosphorylation of p38-MAPK and c-Jun N-terminal kinase (JNK) paralleled with the increased expression of HSP70, strongly supporting the involvement of MAPK signaling cascade in the induction of HSP70 genes under various stressors (Malagoli *et al.*, 2004; Kefaloyianni *et al.*, 2005; Anestis *et al.*, 2007; Gourgou *et al.*, 2010). After *M. galloprovincialis* was exposed to 30 °C acute thermal

stress, the activation profile of p38-MAPK phosphorylation was sustained and significant, while that of JNKs was transient and relatively moderate (Gourgou *et al.*, 2010). This direct evidence demonstrated the principal roles of p38-MAPK and JNKs in transducing the stress signal via mobilization of specific transcription factors and the transcriptional up-regulation of HSP70 genes (Gourgou *et al.*, 2010). The ERK signaling pathway has also been reported to regulate HSP70 expression in ESP-challenged hemocytes of *B. glabrata*, in which the mitogen-activated protein-ERK kinase 1/2 (MEK1/2) inhibitor could significantly reduce HSP70 protein levels, and this might be a strategy employed by the parasite to manipulate the immune response of the intermediate snail host (Zahoor *et al.*, 2010).

Conclusion

Molluscan HSP70 is one of the largest and most important groups in the invertebrate HSP70 family, with consequential specializations in member diversity and sequence characteristics. The expanded family of oyster HSP70 offers an explanation for extensive repertoire of HSPs as well as the sophisticated strategies in response to stresses. Accumulating evidences have demonstrated the relevant similar expression profiles of molluscan HSP70s responding against pathogen infection and environmental stressor, which could be mainly regulated at the transcription level and be mediated by the interaction of HSFs and corresponding HSEs in the promoters of HSP70s.

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