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Is HSP70 Involved in Acclimation to Cadmium in the Chinese Crab, *Eriocheir sinensis* ?

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Acclimation to a toxicant is defined as an increased tolerance to an elevated, usually lethal, concentration arising from chronic exposure to a sublethal concentration of that toxicant (McDonald and Wood, 1993). Acclimation has been observed for cadmium (Cd) in Chinese mitten crabs, *Eriocheir sinensis*, exposed to 50 μg Cd l^{-1} for 30 days in freshwater (chronic exposure), subsequently followed by an exposure to 500 μg Cd l^{-1} for three days (acute exposure) (Silvestre et al., 2005b). Biochemical and physiological data, strengthened by a high degree of structural alterations, suggest that after a direct acute exposure, anterior gills may have lost their permeability properties while posterior gills appear unaffected. A preliminary chronic exposure prevents most of these alterations.

Our understanding of cell defense mechanisms to overcome the toxic effects of metals and their relevance to the acclimation process is far from complete. In eukaryotes, cell sequestration of the metal by cysteine-rich proteins such as metallothioneins (MTs) is the most described mechanism (Klaassen et al., 1999). However, in Chinese crab anterior gills, we calculated that the cadmium binding potential of metallothioneins was largely exceeded whatever the type of exposure, suggesting that metallothionein induction cannot explain the observed acclimation alone (Silvestre et al., 2005a). A proteomic analysis on anterior gills after acute or chronic exposure to cadmium revealed some putative acclimation mechanisms, such as the overexpression of antioxidant enzymes, increase protein degradation potential, or a possible metabolism reshuffling through the pentose phosphate pathway (Silvestre et al., 2006). The overexpression of the molecular chaperone protein disulfide isomerase (PDI) has also been observed. However the proteomic methodology could not point out any induction of the well-known molecular chaperones stress proteins (HSPs).

Initially known as heat-shock proteins (HSPs), stress proteins include a number of families induced during different stress conditions. Members of the highly conserved HSP70 family are induced when challenging with a stress. They mainly prevent protein denaturation and help the cell to recover damaged proteins by chaperoning their refolding (Feder and Hofmann, 1999; Sanders, 1993). The toxicity of Cd is believed to originate mainly from its strong binding capacity to sensitive groups such as thiols and histidyls, resulting in the deterioration of biologically important molecules such as proteins (Vallee and Ulmer, 1972). Consequently, it is not surprising to observe higher HSP70 levels in organisms exposed to Cd (Radlowska and Pempkowiak, 2002).

Because the proteomic methodology used on Chinese crabs in a previous study was best suited when at least two-fold variations of protein expression were induced, it is likely that other protein over- or underexpressed by a factor lower than 2 were present in anterior gills submitted to cadmium exposure. The aim of the present study was to complete proteomic data by investigating the levels of HSP70 in anterior and posterior gills from Chinese crabs exposed to chronic or acute Cd, and to discuss a possible function of this chaperone in increased resistance to this metal.

Material and Methods

Chinese crabs were caught in freshwater lakes near Emden (Germany), kept in circulating freshwater (14.2 ± 0.5°C)

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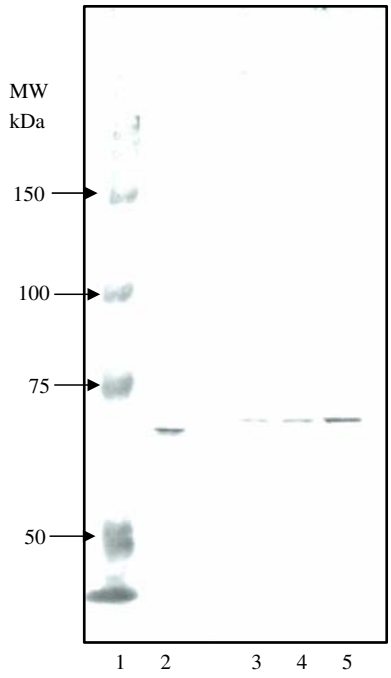

Fig. 1 Western blot showing one HSP70 isoform in gill tissue of the Chinese mitten crab, *Eriocheir sinensis*. Lane 1 shows molecular-weight markers (precision protein standard, Bio-rad). Lane 2 shows human HSP70 (StressGen SPP-755). Lanes 3 to 5 show crab HSP70. Detection was performed with anti-HSP70 monoclonal antibody (Affinity BioReagents MA3-006)

tanks and fed every other day. A first group of intermolt males (100 ± 29 g) was maintained in Cd-free tap water (C0) while a second group was exposed to Cd added as $CdCl_2$ (Sigma) at 50 µg Cd l^{-1} (C50) for 30 days. Afterwards, half of the organisms in each tank were sacrificed while the other half were challenged with a subsequent acute cadmium exposure (called CA0 and CA50, respectively), i.e., three days at 500 µg Cd l^{-1}. The three most posterior gills and the three anterior ones were cut free of their insertion, blotted on filter paper, and frozen separately to –80°C before analysis.

Samples were homogenized in 25:1 v/w buffer containing 50 mM Trizma-HCl pH 7.6, 1% Nonidet and 0.25% protease inhibitor cocktail (Sigma P8340). Two consecutive centrifugations at 100,000 x g (at 4°C) for 60 min concentrated all soluble HSP70 in the supernatant. Total soluble protein concentration was determined using a Bio-Rad protein assay with bovine serum albumin as the standard.

HSP70 analysis using slot blot technique has been shown to be simple and quick, allowing the assessment of a large number of samples (Duffy et al., 1999; Scofield et al., 1999). Before using slot blot on Chinese crab gills, the specificity of the primary antibody was tested using 7% SDS polymerase gel electrophoresis (PAGE) and Western blot. The primary monoclonal antibody (Affinity BioReagents MA3-006) was also raised against human purified HSP70 as a positive control (StressGen SPP–755). In the Chinese crab gill soluble protein fraction, the primary antibody recognizes one HSP70 isoform and does not cross react with other protein (Fig. 1).

Afterwards, samples were diluted in a buffer containing Tris buffer saline (TBS) (10 mM Tris-HCl, 150 mM NaCl, pH 7.5), 0.1% sodium dodecyl sulfate (SDS) and 5 mM dithiothreitol (DTT), and the solution was boiled for 5 min at 100°C. Three micrograms of proteins were directly loaded onto a nitrocellulose membrane (0.45 µm) with a slot blotter (Bio-Dot SF microfiltration apparatus from BioRad). Human purified HSP70 were used as standards at increasing quantities (0.5, 1, 2.5, and 5 ng) with 3 µg of milk proteins added. Membranes were blocked for 1 hour in 5% dry milk in TTBS buffer (TBS plus 0.05% Tween 20, Merck), rinsed twice in TTBS and once in TBS for 5 min, and then incubated for 1 hour with the primary antibody 1:1000 in TTBS plus 2% dry milk. The blots were rinsed twice in TTBS and once in TBS for 5 min and incubated for 1 h with the secondary antibody 1:2000 (horseradish peroxidase anti-mouse Ig, NA931 Amersham). Finally, blots were rinsed thrice in TTBS and once in TBS and developed with the Amersham ECL™ (enhanced chemiluminescence) kit as recommended by the manufacturer. Films were scanned with a densitometer (ImageScanner™). The optical density of each band was plotted versus purified human HSP70. Since the species-specific HSP70 proteins are not available as positive controls, the data are reported as µg equivalents HSP70 per mg of total soluble proteins (µg equiv. HSP70 mg^{-1} prot.) and expressed as mean ± standard deviation (SD). We carried out nonparametric Kruskal-Wallis one-way analysis of variations (ANOVA) on ranks followed by the Mann-Whithney *U* test. The significance level was defined as $p < 0.05$. Intrablot variability was estimated to be 2.8% while interblot variability was estimated to be 17.3%. The detection limit was 0.05 µg equiv. HSP70 mg^{-1} prot.

Results and Discussion

Even if HSP70 are well-known chaperone proteins, few studies have investigated their induction by pollutants in decapod crustaceans. Data are only available for the shore crab *Carcinus maenas*, challenged with copper exposure (Pedersen et al., 1997; Vedel and Depledge, 1995), and for *Homarus americanus* larvae exposed to heptachlor (Snyder and Mulder, 2001). In the Chinese crab *Eriocheir sinensis*, we observed a constitutive HSP70 level of 1.1 ± 0.1 µg equiv. mg^{-1} prot. in anterior gills and of 1.0 ± 0.2 µg equiv. mg^{-1} prot. in posterior gills (Fig. 2). The three most posterior located pairs of gills and the anterior ones are

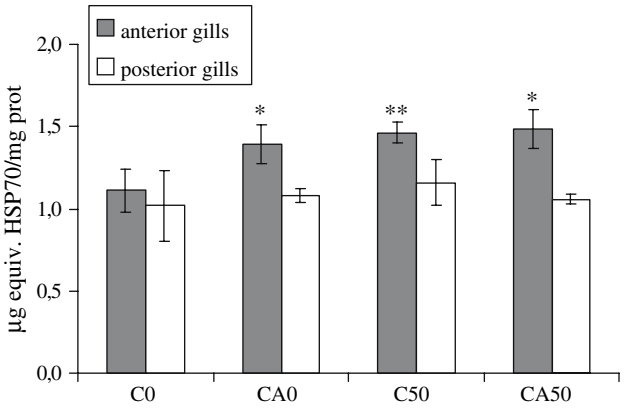

Fig. 2 HSP70 level (µg equiv. mg^{-1} prot.) in anterior and posterior gills of the Chinese mitten crab, *Eriocheir sinensis*, exposed to Cd in freshwater. C0, controls (n = 4, anterior gills; n = 5, posterior gills); CA0, 500 µg Cd l^{-1} for 3 days (n = 5); C50, 50 µg Cd l^{-1} for 30 days (n = 7); CA50, 50 µg Cd l^{-1} for 30 days and then 500 µg Cd l^{-1} for 3 days (n = 6). The asterisks denote significant difference from controls (* $p < 0.05$; ** $p < 0.01$)

clearly distinguished by their ultrastructure and physiology (Barra et al., 1983). While posterior gills actively pump ions from the surrounding water, anterior gills are considered as largely respiratory with passive movements of Na^+ only (Péqueux and Gilles, 1981; Gilles et al., 1988). Consequently, even if anterior and posterior gills are functionally different organs, they have similar levels of HSP70, suggesting a similar basal defense level against protein denaturation.

After three days of Cd exposure at 500 µg l^{-1}, the HSP70 level in anterior gills significantly increased to 1.4 ± 0.1 µg equiv. mg^{-1} prot. ($p < 0.05$) whereas no significant modification of this protein expression was observed in posterior gills. Similar levels were reported for crabs exposed to 50 µg Cd l^{-1} for 30 days (C50) and for crabs challenged with the same exposure followed by an acute exposure (CA50). Under these latter conditions, HSP70 levels in anterior gills reached 1.5 ± 0.1 µg equiv. mg^{-1} prot. for both C50 and CA50, and were significantly higher than controls (C0) ($p < 0.01$ and $p < 0.05$, respectively). On the other hand, the HSP70 level in posterior gills was not modified.

The observed differences in HSP70 induction between both kind of gills can be related to a higher sensitivity to Cd (Silvestre et al., 2005b) and to a higher permeability (Silvestre et al., 2004) and accumulation of Cd (Silvestre et al., 2005a) in anterior gills compared to posterior ones. For example, the maximum Cd concentrations in gills were measured for the CA50 group and reached 188 µg g^{-1} w.w. in anterior gills but only 36 µg g^{-1} w.w. in posterior ones. The reasons for the observed differences between the gills are not clear but a possible role of the cuticle in preventing the entry of cadmium inside posterior gill has been advanced as a possible explanation.

In response to Cd exposure, induction of the chaperone HSP70 was initiated in anterior gills, indicating Cd-induced protein denaturation inside this kind of gills. This induction seems rather rapid since it was observed after three days of exposure and participates in the short-term response against Cd accumulation, as was reported for the induction of MTs (Silvestre et al., 2005a). Moreover, HSP70 was at the same level after 30 days of Cd exposure at 50 µg l^{-1} and after a chronic followed by an acute exposure, which suggests that HSP70 level has reached saturation after three days of exposure and that long-term exposure fails to induce more HSP70. Studies on *Drosophila* (Feder et al., 1992) and on algae (Lewis et al., 2001) suggested that a high production of HSP70 may be energetically costly and could have a negative impact if increased HSP70 was not necessary to counteract protein denaturation. Consequently, it is possible that the HSP70 level in anterior gills of the Chinese crab exposed to Cd is at a maximum related to energy demand, and that induction of more HSP70 would be too costly. The persistent high level observed in anterior gills from chronically exposed organisms indicates that these proteins also act as a long-term defense mechanism. However, the lack of difference between acclimated crabs and organisms exposed to acute Cd indicates that HSP70 is not sufficient to explain increased resistance.

In *E. sinensis*, we previously showed that exposure to 50 µg Cd l^{-1} for 30 days increased its resistance to this metal (Silvestre et al., 2005b). A previous proteomic analysis showed that some protein expressions were modified during cadmium acclimation (Silvestre et al., 2006). However, HSP70 was not identified as being overexpressed. In fact, the overexpression reported in the present study was between 26 and 34% only. This confirms that the proteomic analysis had sensitivity limitations, partly because of a high variability, that made it difficult to detect expression modifications below 100%. According to the present results, HSP70 is both a short- and long-term response to Cd exposure but other mechanisms which necessitate a chronic exposure to be initiated explain the acclimation process. This strengthens the hypothesis that Cd toxicity is partly due to proteotoxic effects.

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References

Barra JA, Péqueux A, Humbert W (1983) A morphological study on gills of a crab acclimated to fresh water. Tissue Cell 15: 583–596

Duffy LK, Scofield E, Rodgers T, Patton M, Bowyer RT (1999). Comparative baseline levels of mercury, Hsp70 and Hsp60 in subsistence fish from the Yukon-Kuskokwim delta region of Alaska. Comp Biochem Physiol C Toxicol Pharmacol. 124:181–186

Feder ME, Hofmann GE (1999) Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. Annu Rev Physiol 61:243–282

Gilles R, Péqueux A, Bianchini A (1988). Physiological aspects of NaCl movements in the gills of the euryhaline crab, *Eriocheir sinensis*, acclimated to fresh water. Comp Biochem Physiol 90A: 201–207

Klaassen CD, Liu J, Choudhuri S (1999) Metallothionein: an intracellular protein to protect against cadmium toxicity. Annu Rev Pharmacol Toxicol 39:267–294

McDonald DG, Wood CM (1993) Branchial mechanisms of acclimation to metals in freshwater fish. In: Rankin JC, Jensen FB (eds), Fish ecophysiology. Chapman & Hall, London, pp 297–321

Lewis S, Donkin ME, Depledge MH (2001) Hsp70 expression in *Enteromorpha intestinalis* (*Chlorophyta*) exposed to environmental stressors. Aquat Toxicol 51:277–291

Pedersen SN, Lundebye AK, Depledge MH (1997) Field application of metallothionein and stress protein biomarkers in the shore crab (*Carcinus maenas*) exposed to trace metals. Aquat Toxicol 37:183–200

Péqueux A, Gilles R (1981) Na^+ fluxes across isolated perfused gills of the Chinese crab *Eriocheir sinensis*. J Exp Biol 92: 173–186

Radlowska M, Pempkowiak J (2002) Stress–70 as indicator of heavy metals accumulation in blue mussel *Mytilus edulis*. Environ Int 27:605–608

Sanders BM (1993) Stress proteins in aquatic organisms : an environmental perspective. Crit Rev Toxicol 23:49–75

Scofield E, Bowyer RT, Duffy LK (1999) Baseline levels of Hsp70, a stress protein and biomarker, in halibut from the Cook Inlet region of Alaska. Sci Tot Environ 226:85–88

Silvestre F, Dierick JF, Dumont V, Dieu M, Raes M, Devos P (2006) Differential protein expression profiles in anterior gills of *Eriocheir sinensis* during acclimation to cadmium. Aquat Toxicol.76:46–58

Silvestre F, Duchêne C, Trausch G, Devos P (2005a) Tissue-specific Cd accumulation and metallothionein-like protein levels during acclimation in the Chinese crab *Eriocheir sinensis*. Comp Biochem Physiol C Toxicol Pharmacol. 140:39–45

Silvestre F, Trausch G, Devos P (2005b) Hyper-osmoregulatory capacity of the Chinese mitten crab (*Eriocheir sinensis*) exposed to cadmium. Acclimation during chronic exposure. Comp Biochem Physiol C Toxicol Pharmacol. 140:29–37

Silvestre F, Trausch G, Péqueux A, Devos P (2004) Uptake of cadmium through isolated perfused gills of the Chinese mitten crab, *Eriocheir sinensis*. Comp Biochem Physiol A Mol Integr Physiol 137:189–196

Snyder MJ, Mulder EP (2001) Environmental endocrine disruption in decapod crustacean larvae: hormone titers, cytochrome P450, and stress protein responses to heptachlor exposure. Aquat Toxicol 55:177–190

Vallee BL, Ulmer D (1972) Biochemical effects of mercury, cadmium, and lead. Annu Rev Biochem 41:91–128

Vedel GR, Depledge MH (1995) Stress–70 levels in the gills of *Carcinus maenas* exposed to copper. Mar Pollut Bull 31:84–86