

Heat shock proteins in *Daphnia* detected using commercial antibodies: description and responsiveness to thermal stress

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Received: 4 September 2008 / Accepted: 4 February 2009 / Published online: 26 February 2009
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Abstract It has previously been shown that the level of *Daphnia* proteins detected by commonly used anti-HSP antibodies, with molecular weights corresponding to those of heat shock proteins, can be modified by various kinds of stress, including the threat of predation. In this study, we demonstrate for the first time that these proteins change their expression in response to thermal stress. Thus, it is concluded that *Daphnia* heat shock proteins, as components of the cellular stress defense, are involved in both, abiotic and biotic stress.

Keywords *Daphnia* · Heat shock proteins · Thermal stress

Introduction

Daphnia is one of the key model animals in evolutionary ecology (Lampert 2006). Due to its parthenogenetic reproduction, it is possible to test thousands of genetically identical individuals and study all aspects of their phenotypic plasticity. Numerous investigations have shown that *Daphnia* can adaptively modify morphology, life history and behavior, adjusting its phenotype in response to the quantity and quality of available food (Lampert and Brendelberger 1996), predation risk (Riessen 1999),

parasitic infection (Chadwick and Little 2005), abiotic factors such as temperature, oxygen and pH conditions (see e.g., Fox and Phear 1953), or the presence of dissolved harmful chemicals (see e.g., Coors et al. 2004). Growing interest in *Daphnia* has resulted in an increasing number of molecular studies on this genus (Fox 1947; David et al. 2003; Little et al. 2004; MacFadyen et al. 2004; Nunes et al. 2005; Kato et al. 2007). The culmination of these studies is the *Daphnia* Genome Project (Colbourne et al. 2005; see also <http://wfleabase.org/database/>).

Induction of the expression of heat shock proteins (HSP) expression is one of the most universal stress answers in organisms. These proteins were found in almost all taxonomic groups, with their expression often upregulated due to the exposition of individuals to various kinds of abiotic and biotic stress factors (see the comprehensive review of Sorensen et al. 2003). Despite many studies describing the reaction of *Daphnia* to environmental stress (including thermal stress: see e.g., Paul et al. 2004), information on the expression of heat shock proteins (also called stress proteins) is sparse. Following the initial report of Chen et al. (1999), there have only been a few publications describing changes in presumed HSP expression caused by biotic factors, such as predation risk (Pijanowska and Kloc 2004; Pauwels et al. 2005) or parasitic infection (Pauwels et al. 2007). Reliable data on the modification of HSP expression by thermal stress in cladocerans are scarce. In the two earlier studies on HSP expression in thermally stressed *Daphnia* (Bond et al. 1993; Bond and Bradley 1995), the proteins have been identified on the basis of molecular weight, solely. These results are not unambiguous because, as we demonstrate in our study, molecular weight of *Daphnia* HSP differs from that in other animals. Thus, proteins defined as HSP in Bond's studies may belong to other families of proteins.

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Earlier studies on *Daphnia* exposed to stress other than temperature were conducted using antibodies specific for proteins from other taxonomic groups. The cross-reactive *Daphnia* proteins, revealed by these antibodies, usually differ in molecular weight from the animal HSPs to which the antibodies were raised. It, therefore, remains uncertain whether the proteins induced by stresses other than temperature are indeed HSPs.

The aim of this study was to catalogue the *Daphnia* proteins detected with the most commonly used anti-HSP antibodies (anti-Hsp60, anti-Hsp70 and anti-Hsp90) and determine whether their expression is modified by thermal stress.

Methods

Experiments were carried out with genetically identical individuals of a *Daphnia magna* Straus clone originating from Binnensee (shallow coastal lake located in northwest Germany). *Daphnia* from this lake are well known for their strong phenotypic reaction to environmental stress. A single cohort of experimental animals was cultured at 20°C under constant dim light in three 0.8 L glass jars (20 animals per jar) from birth until first reproduction. They were fed green algae (*Scenedesmus obliquus*) at a concentration of 1 mg C L⁻¹, and the growth medium was changed daily. After having released the first clutch, animals from the jars were randomly transferred to six glasses (five females per glass), each containing 200 mL of medium. After 24 h, 100 mL of the medium was removed from each glass, and the same amount of fresh medium was returned to three of the glasses. The contents of the other three glasses were carefully completed with 100 mL water at 40°C and quickly transferred to a water bath set at 30°C. After 1 h, these glasses were removed from the water bath and slowly cooled to 20°C. *Daphnia* from each glass were collected 6 h after the start of heating, dried on the filter (sieve) and subsequently transferred to 1.5 mL microfuge tubes and homogenized in 75 µL of RIPA buffer (0.15 M NaCl, 1% Deoxycholate Na salt, 1% Triton X-100, 0.1% SDS, 0.01 M Tris-HCl, pH 7.2) plus complete protease inhibitor (Roche 11836153001) applied according to the producer's instruction. The homogenates were centrifuged for 2 min at 10,000 rpm, and the supernatants were transferred to fresh tubes. For measurement of the total protein concentration, 10 µL samples of each supernatant were removed to fresh tubes for later use in the DC protein assay (Bio-Rad). The remaining supernatants were mixed with an equal volume of SDS-PAGE sample buffer (Sigma), boiled for 5 min and then frozen. Samples containing an equal mass of protein (3 µg) were subjected to 25% SDS-polyacrylamide gel electrophoresis. Separated proteins were transferred to nitrocellulose membrane, and

putative HSPs were detected by western blotting using anti-human primary antibodies: anti-Hsp60 (Stressgen, nos. SPA-805 and SPA-807), anti-Hsp70 (Stressgen, SPA-812) and anti-Hsp90 (Stressgen, SPA-846). Positive immunoreaction was identified by incubating the blots with alkaline phosphatase-conjugated secondary antibody and then using the chromogenic substrate NBT/BCIP. The intensity of specific bands on the blots was determined using a Gel Doc system with Quantity One analysis software (Bio-Rad). The amount of each putative HSP in the *Daphnia* samples was normalized relative to the intensity of HSP bands in a standard thermally stressed human HeLa cell extract on the same blots (Stressgen, LYC-HL101). The data were analyzed using the ANOVA model.

Results

All antibodies produced one or more clear immunoreactive bands on the *Daphnia* protein blots. All bands were considered, regardless of their conformity with the molecular weights of the human HSPs in the HeLa cell lysate detected using the same antibodies.

Anti-Hsp60 antibody SPA-807 revealed two distinct proteins at ~50 and ~56 kDa (Fig. 1a). They were slightly smaller than the corresponding human HSP (~58 kDa). Thermal stress was found to enhance the expression of both *Daphnia* proteins (Table 1).

Anti-Hsp60 antibody SPA-805 also revealed two distinct proteins. The protein at ~60 kDa was slightly smaller than the corresponding human HSP (~61 kDa), but the second protein (~103 kDa) was considerably larger (Fig. 1b). Thermal stress enhanced the expression of both *Daphnia* proteins (Table 1).

Anti-Hsp70 antibody SPA-812 revealed three immunoreactive protein bands (Fig. 1c). The first size of the 73 kDa was similar to that of the corresponding human HSP (~73 kDa), while the two others were larger (~74 and ~79 kDa, respectively). The levels of the 73 and 74 kDa proteins were significantly increased in heat-shocked *Daphnia* (Table 1), while the expression of the 79 kDa protein was apparently reduced, although this change was not statistically significant.

Anti-Hsp90 SPA-846 antibody revealed only one protein of molecular weight ~92 kDa (Fig. 1d), which was smaller than the corresponding human HSP (~96 kDa). Thermal stress enhanced its expression in *Daphnia* (Table 1).

Discussion

The expression of all proteins (except the 79 kDa form) detected in *Daphnia* using commercially available anti-HSP

Fig. 1 Proteins in *Daphnia* and heat-shocked human HeLa cell lysates revealed by western immunoblotting with anti-HSP antibodies: **a** anti-Hsp60 (SPA-807), **b** anti-Hsp60 (SPA-805), **c** anti-Hsp70 (SPA-812), **d** anti-Hsp90 (SPA-846)

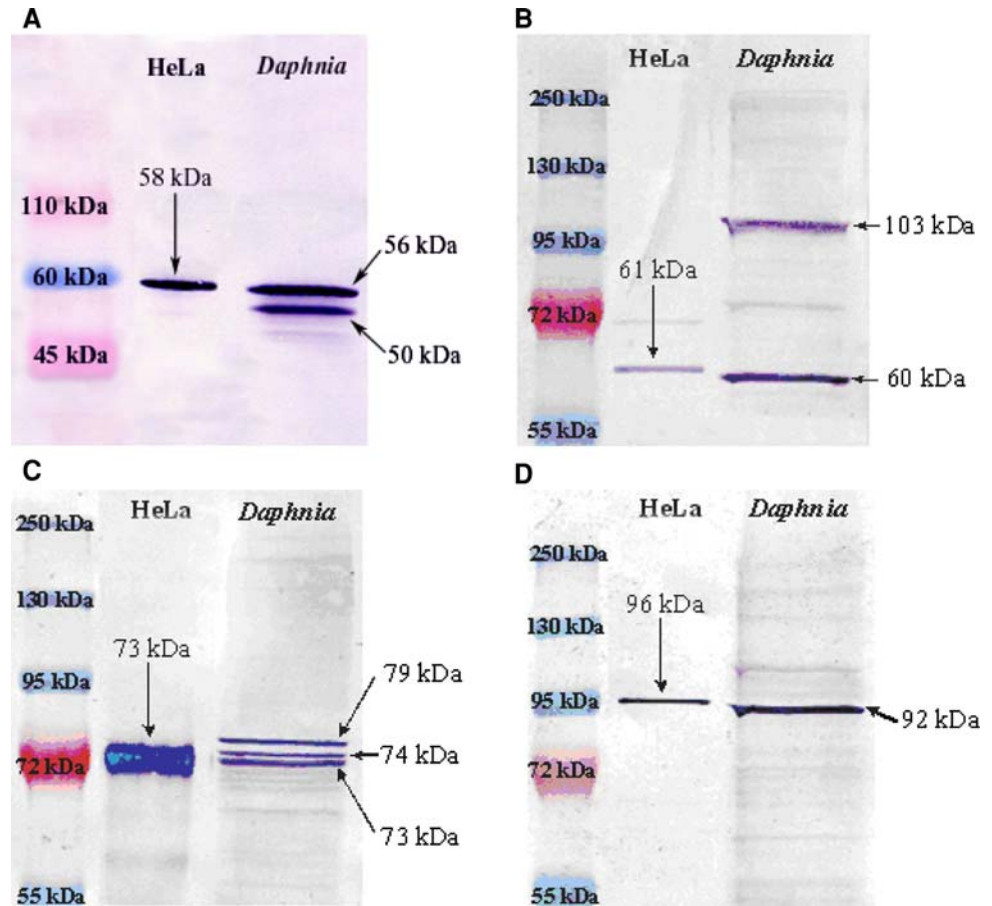


Table 1 The relative amount of proteins detected using anti-HSP antibodies in *Daphnia* cultured at 20°C (control) or 6 h after rapid transfer to 30°C (heat shock) (average \pm 1SD) and differences

between the expression of proteins in control and heat-shocked animals (ANOVA)

Protein MW	Antibody	The amount of proteins in <i>Daphnia</i> ^a		<i>F</i>	<i>df</i> effect	<i>df</i> error	<i>p</i>
		Control	Heat shocked				
50 kDa	Anti-Hsp60 (SPA-807)	28.55 \pm 3.44	63.06 \pm 17.08	11.77	1	5	0.0265
56 kDa	Anti-Hsp60 (SPA-807)	79.00 \pm 11.57	111.9 \pm 1.90	23.62	1	5	0.0083
60 kDa	Anti-Hsp60 (SPA-805)	64.02 \pm 5.40	144.59 \pm 24.99	29.78	1	5	0.0055
73–74 kDa	Anti-Hsp70 (SPA-812)	10.47 \pm 12.19	57.6 \pm 13.03	20.93	1	5	0.0102
79 kDa	Anti-Hsp70 (SPA-812)	27.71 \pm 9.17	17.93 \pm 6.43	2.28	1	5	0.2053
92 kDa	Anti-Hsp90 (SPA-846)	25.46 \pm 5.34	63.81 \pm 16.5	14.68	1	5	0.0186
103 kDa	Anti-Hsp60 (SPA-805)	15.90 \pm 8.20	41.65 \pm 12.5	8.90	1	5	0.0406

The intensities of immunoreactive *Daphnia* bands on western blots were normalized against those of bands detected in a standard thermally stressed human HeLa cell extract

^a Relative units

antibodies was enhanced by thermal stress. These proteins may now be classified as heat shock proteins.

Though the expression of the 73 and 74 kDa proteins was enhanced by thermal stress, the 79 kDa protein, also detected using the anti-Hsp70 antibody, was inhibited. It seems plausible that all three polypeptides arise from posttranslational modification of a single protein, and stress

stimulates the transformation of one form to another (e.g., by uncoupling of some functional groups).

The detection of a 103 kDa protein detected with the anti-Hsp60 antibody was rather unexpected, considering that its mass is drastically different from that anticipated. It may be the product of modification of a protein from the Hsp60 family (e.g., a fusion with another polypeptide).

The demonstration that *Daphnia* proteins, revealed by commercial anti-HSP antibodies, are indeed heat shock proteins allows further interpretation of previous data and will be of importance in future studies on adaptive responses to biotic stress. The modification of HSP expression caused by the presence of predators identified by Pijanowska and Kloc (2004) and confirmed by Pauwels et al. (2005) can now be considered as a typical cellular response to stress. Previously, HSPs were recognized as molecules involved in the protection of other proteins against chemical and physical stress factors (Ritossa 1962), in the recovery or degradation of damaged proteins (e.g., Jolly and Morimoto 2000; Pockley 2003) and in rearrangements of proteins leading to major structural changes in tissues, e.g., molting (Spees et al. 2003). Our findings demonstrate that the same HSPs that are apparently involved in the reaction to the presence of predator kairomones signaling a potential risk, but do not directly cause damage to cells are, in addition, involved in mediating responses to abiotic stresses. This role of HSPs in adaptive modifications of the *Daphnia* life history, and behavior in response to stress caused by predators provides important information about heat shock protein activity that will be of interest to molecular biologists studying the functions and evolution of these molecules.

The involvement of HSP in the response to thermal stress in cladocerans may be of particular importance considering the necessity to overcome the sharp thermal gradient of a stratified lake, by *Daphnia* performing diel vertical migrations (DVM) to avoid visually hunting predators at the surface during the day (for review see Gliwicz 2003).

In addition, *Daphnia* represents an excellent model for interdisciplinary studies on individual reactions to stress. The obvious role of HSPs in the expression of phenotypic plasticity is of importance to the evolution of responses to environmental stress.

Acknowledgment This study was supported by Grant KBN 2 PO4 036 26.

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