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Hsp70 instability and induction by a pesticide in *Folsomia candida*

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The heat shock protein Hsp70 has been shown to be a promising biomarker in aquatic and terrestrial organisms. However, its analysis in the soil insect *Folsomia candida* (Collembola) poses many problems as the protein is particularly unstable in this species. Western blotting has shown that the principal degradation fragment has a size of 48 kDa. We have developed a Western blot method that avoids the degradation of Hsp70 and was successful in detecting the protein in the springtail *F. candida* after a heat shock (12, 18 and 24 h at 32°C). In the second part of the study the organisms were exposed to artificial compressed soil contaminated with the dinitrophenol dinoseb (10, 15 and 20 μg g^{-1} dry weight [DW]). Hsp70 was analysed in pooled samples (40 to 150 collembola according to age) after 1, 2, 3, 4, 5, 6, 7, 11 and 14 days. The only significant induction was observed after 5 days at 20 μg g^{-1} DW of dinoseb. The induction patterns over time were dissimilar for the different concentrations and a relatively high variability between the replicates was observed. Our results show that we must be cautious when interpreting biomarker results, especially those for Hsp70.

Keywords: Collembola, organic xenobiotic, biomarker, unstable heat shock protein 70, dinoseb

Introduction

The toxic effects of a pollutant on soil fauna can be detected at biochemical, physiological and ecological levels. The biomarker approach was developed in order to detect and evaluate rapidly the impact of a pollution on living organisms. A biomarker is a parameter reflecting the interaction between a biological system and an environmental agent and is generally determined at a biochemical level. The measurement of the quantity and/or activities of molecules serving as biomarkers needs to be linked to the concentration of the pollutant.

Stress proteins are promising biomarkers of exposure and possibly of effects. These proteins are synthesized in a large number of organisms (from bacteria to humans) after exposure to a large variety of physical or chemical stressors. One family of stress proteins, the heat shock proteins, were discovered following the exposure of *Drosophila melanogaster* to an increase in temperature (Ritossa 1962). This exposure induces proteotoxicity, i.e. the denaturation or wrong folding of proteins leading to non-functionality. These abnormal proteins activate by phosphorylation a protein called heat shock factor, which then binds to a conserved

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sequence on a heat-inducible gene known as the heat shock element. The activation of this gene induces the synthesis of heat shock proteins (Sanders 1990, 1993), whose role is to refold correctly the damaged proteins. The induction of these proteins by a heat shock has been observed in many invertebrates, including the copepod *Eurytemora affinis* (Hakimzadeh and Bradley 1990), the chironome *Chironomus thummi* (Carretero *et al*. 1991), the isopod *Oniscus asellus* (Köhler *et al*. 1992) and the collembola *Orchesella bifasciata* and *Tomocerus flavescens* (Köhler *et al*. 1999). To our knowledge, the induction of heat shock protein 70 by heat shock has not been studied before in the springtail *Folsomia candida*.

As heat shock proteins are not only induced by heat but also by pollutants, they are increasingly used as biomarkers. The induction and accumulation of a heat shock protein with a molecular weight of 70 kDa (Hsp70) following exposure of soil invertebrates to various pollutants has been studied by many authors (Köhler and Alberti 1992, Köhler *et al*. 1996, 1998, Zanger *et al*. 1996, Köhler and Eckwert 1997), but not in the collembola species *Folsomia candida*. Springtails, and especially this species, are promising test organisms for biomarker studies. *F. candida* has many characteristics required for an ecotoxicity test species (Riepert and Kula 1996). A standardized test protocol permits the evaluation of the effect of xenobiotics on the reproduction and mortality of *F. candida* (ISO 1999). Léon and van Gestel (1994) (cited in van Gestel 1998) developed test selection criteria for the comparison and scoring of different test protocols. Among the tested species, *Eisenia fedita*, *F. fimetaria* and *F. candida* obtained the best scores of 72/100, 63/100 and 60/100, respectively (van Gestel 1998).

Most studies on the induction of Hsp70 concern heavy metals, but some investigations have been conducted with organic pollutants. The induction of Hsp70 in invertebrates has been shown for the following organic xenobiotics: dimethoate in the centipede *Lithobius mutabilis* (Pyza *et al*. 1997), PCB 118 in the marine sponge *Geodia cydonium* (Wiens *et al*. 1998) and benzo[*a*]pyrene, PCB 152, lindane and pentachlorophenol in the isopod *Oniscus asellus* (Köhler 1999). In the study presented here we used dinoseb, a dinitrophenol selective contact herbicide used against the pre- and post-emergence of weed (grasses and mustards) in different cultures (soy bean, vegetables, fruits, etc.). Dinoseb is also used as a defoliant for tubers and leguminous plants (potatoes, peas) and has fungicide and insecticide properties (Etoxnet 1999). In 1986, United States Environmental Protection Agency (USEPA) proscribed the use of all products containing dinoseb; it was shown to have adverse effects on fertility as well as teratogenic effects in mammals, and it provokes cataracts in the users. At the present time it is still used in many countries such as Switzerland for the defoliation of potatoes.

The objective of this study was to show the presence and induction of Hsp70 in *F. candida*. With this aim in mind, we adapted a Western blot method in order to quantify the protein in the organisms after a heat shock of 32°C. The aim of the second part of the study was to determine whether dinoseb induces the synthesis and accumulation of Hsp70 and to establish the relevance of this biomarker for the evaluation of a stress produced by an organic pollutant in *F. candida*. The organisms were exposed to substrate (artificial soil) contaminated with dinoseb, as work done in our laboratory has shown that *F. candida* is more sensitive to substrate rather than food contamination (unpublished data).

Methods

Breeding of F. candida

F. candida were generously provided in 1996 by Dr Riepert (Biologische Bundesanstalt für Land- und Forstwirschaft, Berlin, Germany). The culture was maintained in plastic boxes (180 × 135 × 60 mm with transparent covers) containing a substrate of plaster of Paris and activated charcoal (120 and 15 g, respectively) (ISO 1999). Laboratory conditions were constant (20 ± 2°C and 600 lux, with a light:dark cycle of 16:8 h). Humidity inside the boxes was kept between 70 and 80% by enclosing the covers in Parafilm®. Twice a week the containers were aerated, some drops of bidistilled water were added to maintain humidity, and food was added (granulated dry yeast, courtesy of Dr Oetkerbrand, Germany). Unconsumed yeast was removed each week to avoid the development of bacteria and fungi, which might be harmful to collembola. Once a month the collembola were transferred into new boxes.

Obtaining organisms of homogeneous age

Adults were placed on a new breeding substrate in order to stimulate oviposition, which usually occurred 2 days after transfer. Seven days later the clusters of eggs were transferred with a humid paintbrush to a fresh substrate. The eggs hatched 2–3 days after transfer. As soon as the first juveniles appeared, the remaining eggs were removed from the containers. The juveniles were used for the experiments at the age of 10 days.

Preparation of substrate and pesticide application

Artificial ISO soil (ISO 1993) was used for the contamination experiments. The soil was composed of 70% quartz sand (50% 40–100 mesh, Fluka 84878 and 50% ⩾ 230 mesh, Fluka 83340), 20% kaolinite clay (Fluka 60609) and 10% sphagnum peat. The peat (Weißmoortorf, Sphagnum extra, Rastede, Germany) was air dried, ground and sieved to 1 mm. Sufficient $CaCO_3$ was added to reach a pH of 6 ± 0.5 (ISO 1994). A quantity of bidistilled water corresponding to 50% of the water-holding capacity (ISO 1999) was added to the different soil constituents, which were then mixed thoroughly.

As the water solubility of dinoseb is low (0.05 mg ml^{-1}), a stock solution of 0.5 mg ml^{-1} of dinoseb (99.7%, Promochem, code IPO 147) was prepared in acetone and kept at 4°C. For the preparation of the contaminated artificial soil, the required quantity of the stock solution was added to the quartz sand. The acetone was evaporated in a rotary evaporator and the container was then placed under a fume hood for 30 min to allow all acetone residues to evaporate. Finally, the contaminated quartz sand was mixed thoroughly with the other soil constituents.

Experiments

The tests were conducted in mini petri dishes (25 mm in diameter × 25 mm in height).

Heat shock. The petri dishes were half filled with the plaster–charcoal breeding substrate. Forty adult collembola were introduced into the petri dish and placed in an incubator at 32°C. After 6, 12, 18 and 24 h the organisms were frozen in liquid nitrogen until protein extraction was performed (within 1 h). Five replicate dishes were used for each time period and for the control.

Dinoseb exposure. The petri dishes were filled with 2 g of contaminated artificial soil. The soil was compressed in order to prevent the collembola from burying into the soil. Ten-day-old collembola were exposed via the substrate to sublethal dinoseb concentrations of 10, 15 and 20 μg g^{-1} dry weight (DW) (analysed concentrations). For each concentration, for the control and for each exposure time, five replicate dishes were used. The test containers were maintained in the same conditions as for breeding. Each week the petri dishes were aerated, one drop of bidistilled water was added with a Pasteur pipette and the collembola were fed with dry yeast. After different exposure times (1, 2, 3, 4, 5, 6, 7, 11 and 12 days for the control and 10 μg g^{-1}; 1, 2, 5 and 6 days for 15 and 20 μg g^{-1}), collembola were frozen in liquid nitrogen until protein extraction was performed (within 1 h).

The collembola being exposed for more than 6 days were transferred into new petri dishes (contaminated the same day as the other dishes and kept under the same conditions) in order to avoid the appearance of juveniles.

Dinoseb analysis

Twenty petri dishes were contaminated on the first day of the dinoseb exposure experiments in order to analyse the exact dinoseb concentration in the artificial soil using the method of Spack (1997). Ten petri dishes with 10 μg g^{-1} DW of dinoseb and 10 dishes with 20 μg g^{-1} DW were maintained in the same conditions as the exposure experiment. After 0, 7, 14 and 21 days they were stored at −20°C until extraction (three replicates for 0 and 7 days, and two replicates for 14 and 21 days). The dinoseb was extracted by agitation (20 min, 420 shakes per min) with acetone and 5% trifluoroacetic acid. The extract was passed over a regenerated cellulose filter (0.2 μm) and analysed by high pressure liquid chromatography (Vydac C18 column, 25 cm × 4.6 mm × 3 μm, porosity 9 nm) equipped with a diode array

detector (HPLC-DAD, Hewlett Packard). The samples were analysed under acidic conditions (40% acetonitrile, 40% water, 20% trifluoroacetic acid) and detected by ultraviolet light with a wavelength of 270 nm (recovery = 90 ± 4.6%, two concentrations, $n = 6$).

Hsp70 analysis

The method used was adapted from Köhler *et al.* (1992). Between 40 and 150 collembola (according to age, to give a sufficient biomass to provide a detectable quantity of proteins) were introduced into Eppendorf tubes and homogenized in liquid nitrogen using a Teflon pestle tissue homogenizer. The tubes were placed into an isotherm rack in order to maintain them at 0°C. Then 20 µl of extraction buffer (80 mM potassium acetate, 4 mM magnesium acetate, 20 mM HEPES, 1/2 protease inhibitor cocktail tablet in 12.5 ml, pH 7.5; Boehringer, Germany) and 10 µl of denaturation buffer (glycerol, sodium dodecyl sulphate, 2-mercaptoethanol, blue bromophenol, pH 7) were added. The anti-proteolytic cocktail used is a mixture of several inhibitors with a broad inhibitory specificity (serine, cysteine and metalloproteases). The samples were heated at 95°C for 5 min in order to denature the proteins and deactivate the remaining proteases. After centrifugation (10 min, 20,000 *g* at 4°C) the supernatant was recovered for the determination of the total protein content using the method of Bradford (1976) and for Hsp70 analysis.

The Hsp70 level in the samples was determined by Western blotting. Samples with a constant protein content (50 µg per lane) were separated by a sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) minigel (12% acrylamide, 0.12% bisacrylamide [w/v], 10 min at 100 V, 1 h at 200 V) and transferred onto a nitrocellulose membrane (1 h 30 min at 150 mA). The membrane was agitated in a saturation solution (50% horse serum in Tris-Buffered Saline (TBS), 50 mM Tris, pH 5.7, 150 mM NaCl) for 2 h at 20°C to avoid non-specific binding of the antibodies. The membrane was washed in TBS and then incubated under agitation overnight at 4°C in the primary antibody solution (monoclonal mouse anti-human Hsp70, Affinity Bioreagents, USA; diluted 1:5000 in TBS containing 10% horse serum). After washing the membrane several times in TBS it was incubated for 2 h at 20°C under agitation in the secondary antibody solution (peroxidase-conjugated goat anti-mouse IgG (H+L), Jackson Immunoresearch Laboratories, USA; diluted 1:1000 in TBS containing 10% horse serum). After washing the blot several times in TBS the antigen–antibody complex was revealed by the reaction of the peroxidase with a chloronaphtol solution (9 mg of 4-chloro-1-naphtol in 3 ml of methanol, plus 50 ml Tris, pH 8.5, and 20 µl H_2O_2 30%). Human Hsp70 was used as a standard stress protein (Stress Gen SPP-755, distributed by Biomol, Germany).

The membranes were dried in the dark overnight at 4°C and then scanned using constant scanning conditions (resolution, light, contrast, etc.). The densitometric quantification of the protein band intensities was realized with a densitometric image analysis system (Pharmacia LKB Biotechnology, Sweden; Image master 3D software). The mean optic density of the background pixels was subtracted from the gel. The height and width of the bands was fixed and kept constant for all the gels, and the mean optic density per centimetre was calculated for each band.

The values obtained for the exposed samples were compared with those of the controls. At least five replicates were used for each control and for each exposure concentration and time.

Statistical analysis

Zanger and Köhler (1996) showed that the induction level of stress proteins might not follow a normal distribution. The values obtained for the exposed samples were therefore compared with those of the controls using the non-parametric Wilcoxon-Mann-Whitney U-test. The following statistical significance levels defined by Köhler (1999) were used: $p \leq 0.001$, highly significant; $0.001 < p \leq 0.01$, significant; $0.01 < p \leq 0.05$, slightly significant.

Results

Instability of Hsp70 in F. candida

The Western blot presented in figure 1 shows a strong reaction between the standard stress protein (human Hsp70) and the monoclonal anti-Hsp70 antibody (lane 3). The bands with a molecular weight of 48 kDa seen in lanes 1, 2, 4 and 5 correspond to the *F. candida* extracts. The observed cross-reaction between these polypeptides and the anti-Hsp70 antibody shows that the fragments are degraded Hsp70. The use of different extraction buffers (urea, Tris-HCl, and buffers with a high glycerol level or high salt content) and taking great care during the experiment (working in ice, use of a cocktail of protease inhibitors, centrifugation at 4°C)

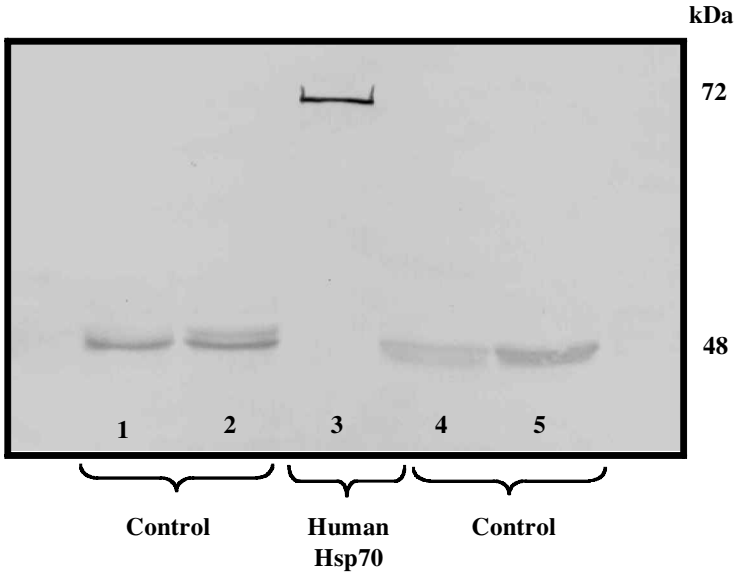

Figure 1. Western blot representing the instability of Hsp70 in *F. candida*. Lane 3 shows the cross-reaction between human Hsp70 and the antibody anti-Hsp70. Lanes 1, 2, 4 and 5 show the fragments of Hsp70 obtained after its extraction from non-exposed *F. candida*.

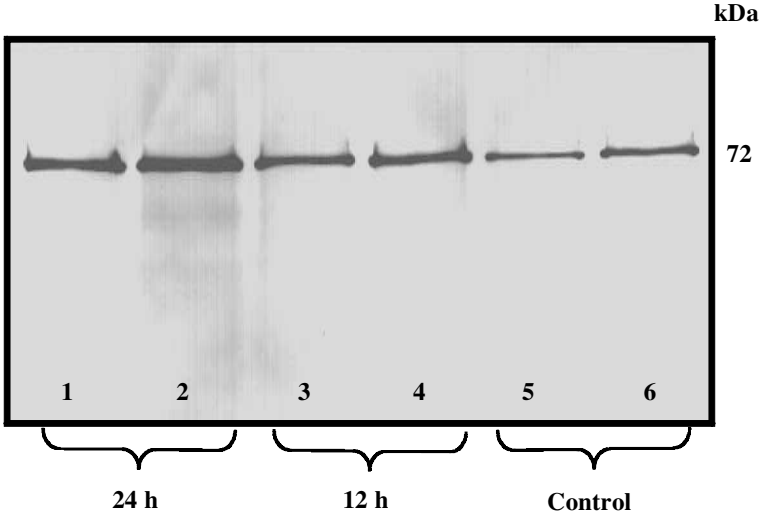

Figure 2. Western blot representing intact Hsp70 in *F. candida* after a heat shock at 32°C.

did not improve the stability of Hsp70. Only the following extreme conditions enabled us to detect non-degraded Hsp70 (figure 2): homogenization in liquid nitrogen, conservation of the tubes at 0°C in an isotherm rack, separation and electrotransfer realized on ice, and denaturation of the proteins before centrifugation with 2-mercaptoethanol.

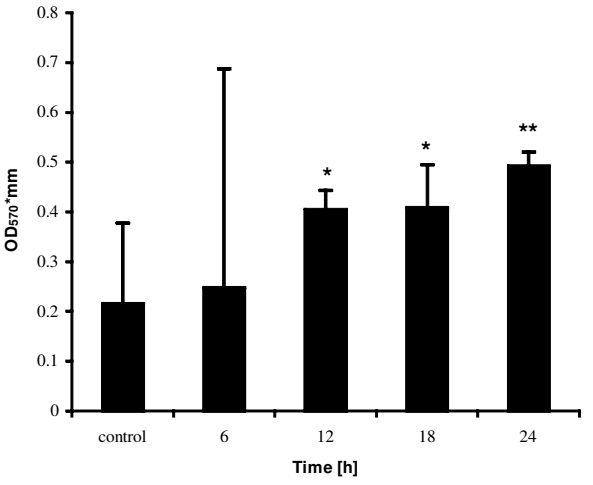

Figure 3. Induction of Hsp70 in *F. candida* after a heat shock of 32°C at 6, 8, 12, 18 and 24 h. **Significant and *slightly significant compared with control.

Heat shock

The exposure of the adult collembola to 32°C induces the synthesis of Hsp70. The induction depends on the exposure duration: as seen in figure 2, the intensity of the bands increases with time. After 12 h of incubation at 32°C a slightly significant effect and after 24 h a significant effect can be observed (figure 3). The mean optical density is 2.3 times higher than in the controls after 24 h. A consequence of the high variability obtained for the controls is that no results are highly significant. Moreover, the high variability of the results obtained after 6 h of incubation might mask a positive effect, as in other organisms induction is already observed after a few hours (Hakimzadeh and Bradley 1990, Sanders *et al.* 1992).

Effect of dinoseb

The exposure of *F. candida* having the same age to different dinoseb concentrations in the substrate does not induce a highly significant effect on the synthesis of Hsp70 (figure 4). Only the peak obtained after 5 days at 20 µg g^{-1} is slightly significant. The peak observed after 2 days at 15 µg g^{-1} is not significant as the values obtained for the controls at that time have a very high variability. However, interesting tendencies can be observed when adjusting a polynomial third order function showing a peak after 2–3 days for a concentration of 15 µg g^{-1} DW and after 4–5 days for a concentration of 20 µg g^{-1} DW (figure 5). In both cases we obtain an induction factor of about 1.4 compared with the control. For a concentration of 10 µg g^{-1} no significant effect is obtained, even after 14 days of exposure. An increase in concentration seems to have an effect on the intensity of the response. The peak observed for each concentration is followed in time by a decrease in heat shock proteins compared with the control.

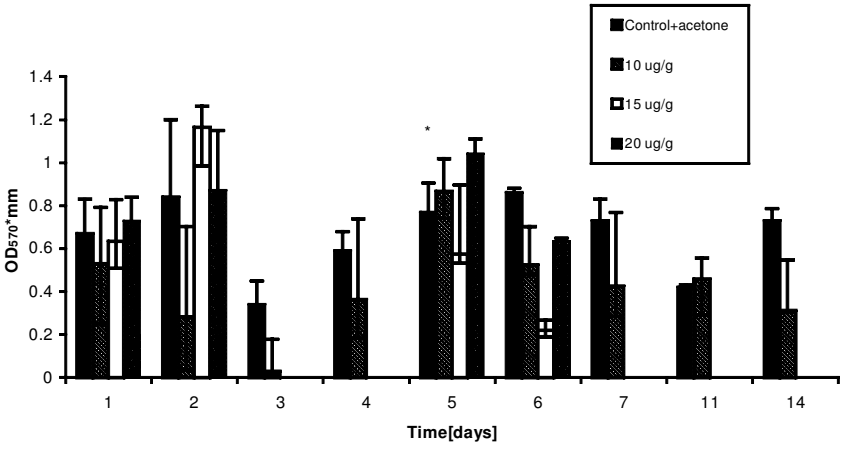

Figure 4. Median Hsp70 levels detected after *F. candida* exposure to substrate contaminated by dinoseb. *Slightly significant compared with control from the corresponding day.

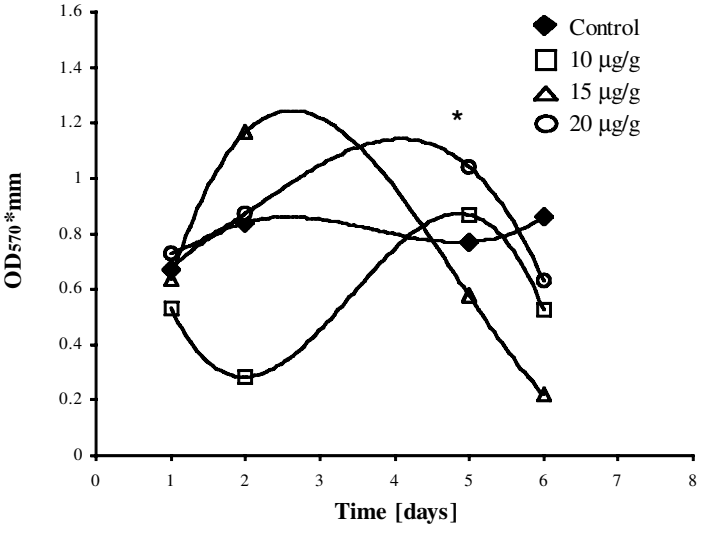

Figure 5. Adjustment of polynomial third order curves on the median levels of Hsp70 detected after 1, 2, 5 and 6 days of exposure to different dinoseb substrate concentrations. *Slightly significant compared with control from the corresponding day.

Degradation of dinoseb

Figure 6 shows the evolution of the dinoseb concentrations (10 and 20 µg g^{-1} DW), with a decrease in concentration over time. Two phases can be distinguished: a linear phase corresponding to a degradation of dinoseb, and a stationary phase. The quantity of dinoseb degraded over time (4 µg g^{-1} DW in 21 days) is not correlated with the concentration, which indicates that degradation is probably mainly due to microbial processes. After 14 days (the longest time period for the Hsp70 analysis), 42% of dinoseb is degraded at 10 µg g^{-1} and 26% at 20 µg g^{-1}.

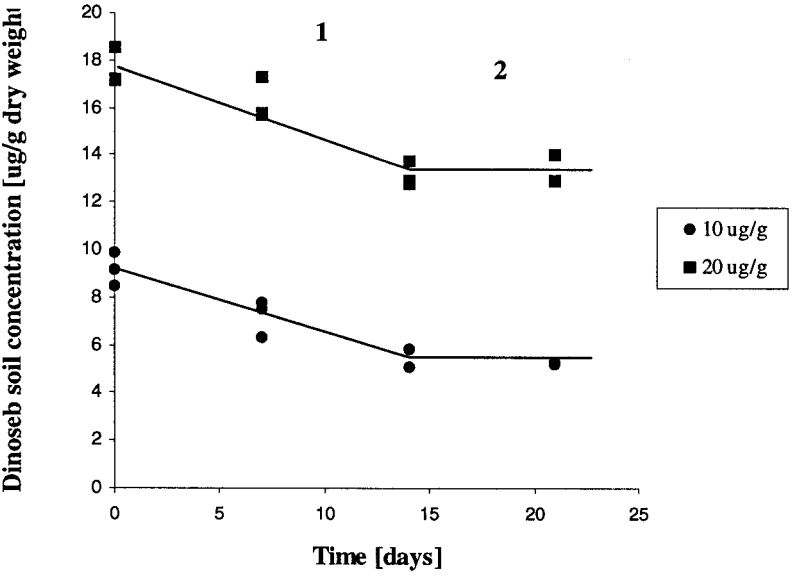

Figure 6. Evolution of soil dinoseb concentrations (μg g^{-1} DW) over time: 1, degradation phase; 2, stationary phase.

Discussion

Instability of Hsp70 and its induction by heat

The results show that a heat shock induces a protein with a molecular weight of around 70 kDa that cross-reacts highly with anti-Hsp70 antibodies. To our knowledge, this is the first successful detection of Hsp70 in the springtail *F. candida*, the most widely used collembola species in ecotoxicology.

However, the results of this work indicate that Hsp70 is an unstable protein in *F. candida*. This instability has already been observed in other species such as salmon hepatocytes of *Salmo salar* L. (Grøsvik and Goksøyr 1996) or *Drosophila melanogaster* (Mitchell *et al.* 1985). Even when working close to 0°C with a cocktail of protease inhibitors, decay of Hsp70 occurs in *F. candida*. We therefore conclude that this degradation is not due to a general protease, but to a specific proteolytic activity. Grøsvik and Goksøyr (1996) observed that the principal degradation product in salmon is a polypeptide of 45–46 kDa and Mitchell *et al.* (1985) described a peptide of 43 kDa in *D. melanogaster*. The band of 48 kDa observed in our experiments corresponds to their observations. According to Mitchell *et al.* (1985), many polypeptide chains have the capacity to fold after denaturation. In the case of Hsp70, part of the protein could refold to a product that has a self-proteolytic activity. The authors indicate that the amino acid sequence of Hsp70 in *D. melanogaster* contains the appropriate arrangements of serine, cysteine, histidine and aspartic acid residues in the carboxyl end that could become a 'serine' type protease if it were folded in a particular way. Hsp70 probably has a similar structure in *F. candida*, in which case it would have a self-degradation capacity that would lead to the observed instability. This would explain why the protein is difficult to stabilize in spite of all the precautions taken.

The increase in Hsp70 level caused by a fairly robust heat shock at 32°C is relatively moderate (two-fold induction). The synthesis of Hsp70 continues during

24 h after the shock. The increase between 18 and 24 h is low but the variability of the data diminishes, explaining the higher level of statistical significance. This low increase probably indicates that the protein level is close to stabilization. These results are in accordance with those obtained with the copepod *Eurytemora affinis*, where the heat shock proteins progressively disappear 24 h after a heat shock (Hakimzadeh and Bradley 1990). If the heat exposure ceases, the organisms can recover and survive due to the intervention of the heat shock proteins; otherwise the organisms die.

Effect of dinoseb

Sublethal concentrations of dinoseb do not substantially induce Hsp70. Only the peak obtained after 5 days at the highest concentration of 20 µg g^{-1} DW is slightly significant. Higher concentrations were not tested, as they have an effect on mortality (LC_{50} at 20 days = 25 µg g^{-1}, unpublished data). The observations of the peaks during the first days of exposure and the rapid decrease to a level below the control could indicate that dinoseb is quickly metabolized or excreted by *F. candida*. However, according to Kaufman (1975), in animals phenols have the tendency to be rapidly absorbed and distributed throughout the body and their excretion seems to be slow.

Köhler (1999) observed a difference in Hsp70 induction according to the lipophilicity of the substances. Extremely lipophilic chemicals could only induce a transient response, while less strongly lipophilic ones managed to elevate the Hsp70 level for at least 1–2 weeks. These observations could not be confirmed in our study. Even though dinoseb is moderately lipophilic (log Kow = 3.3–3.6 according to the pH), it only induces an increase in the Hsp70 levels for 4–5 days. The induction therefore more closely resembles extremely lipophilic substances such as benzo[*a*]pyrene or PCB52 (Köhler 1999).

Dinoseb has three mode of actions depending on the concentration at which it is present in the cells: uncoupler, inhibitor and narcotic agent (Escher 1995). Substances having similar characteristics have an impact on the induction of Hsp70 in *D. melanogaster* (Ashburner and Bonner 1979, Behnel and Seydewitz 1980). However, the mechanism of induction of the proteins is not yet elucidated. Moreover, dinoseb has a denaturating effect on proteins, but the mode of action is unclear (Kaufman 1975). For all these reasons we expected to observe a more intense and a more lasting Hsp70 induction.

The measurement of soil dinoseb concentrations over time is important and has to be included in the interpretation of the results. When contaminating the artificial soil with dinoseb, one fraction of the pollutant will adsorb onto the soil particles. This phenomenon is very rapid (between 24 and 48 h) (Martins and Mermoud 1998) and is therefore not visible in figure 6. The subsequent phase corresponds to a microbial and photolytic degradation of the available fraction of dinoseb. Since the quantity of dinoseb degraded over time is not correlated to the concentration, degradation is probably mainly due to microbial processes. This first phase is followed by a stationary phase, which can be explained by the fact that the remaining dinoseb is no longer available. This may be true for the photodegraded fraction, but, considering the relatively low lipophilicity of dinoseb (and therefore relatively low adsorption), this explanation is not satisfactory for the biological degradation. Another hypothesis is a limiting resource for the degrading

bacteria, such as a lack of organic carbon. In fact, most soil micro-organisms cannot degrade dinoseb without another carbon source (Martins and Mermoud 1998).

The results show that the collembola are not exposed to a constant concentration over time, which might enable them to recover after a long time period. As the dinoseb concentrations decrease, prolongation of the exposure time will not increase the induction of Hsp70.

Utility of Hsp70 as a biomarker

The utility of Hsp70 as an early warning system in *F. candida* is questionable. On the one hand, even by heat shock, the induction of Hsp70 is relatively moderate, which might indicate that other stress proteins could be involved such as small heat shock proteins, Hsp60 or Hsp90. On the other hand, concerning dinoseb toxicity, reproduction seems to be a more sensitive endpoint than Hsp70 induction (EC50 for reproduction at 28 days = 15.6 μg g^{-1} DW, unpublished data). Moreover, even though we worked with individuals from the same species that were the same age and were treated in the same way, the differences in Hsp70 response between pooled samples were very high. A high variability in Hsp70 response has also been observed between individuals of *D. melanogaster* by Feder *et al.* (1996). This natural variability obtained in the controls can in some cases mask a possible effect in the exposed samples and makes interpretation difficult.

The variability in the quantification of this protein is partly due to the Western blot method, which is only semi-quantitative. The Bradford method used for the determination of the total protein content is probably also responsible for some of the variability: the use of microplates enables work with very low sample volumes (2 μl), but has the consequence of diminishing the precision of the measurement. Moreover, the use of 2-mercaptoethanol decreases the sensitivity of the method and should be replaced in further work by another denaturating agent that will not interfere with the total protein determination.

Depending on the type of organism as well as the type and concentration of the tested pollutant, induction of Hsp70 does not occur after the same time period. If, as in the case of dinoseb, the response corresponds to a peak, the induction can easily be missed by measuring over the wrong time periods. It is therefore necessary to determine the protein levels regularly, with short time intervals (1–2 days). Considering all the points discussed above and knowing that, especially with *F. candida*, Western blotting is a relatively long, expensive and constraining method, the use of Hsp70 as an early warning system is compromised in this species.

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