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The 70 kD Heat Shock Protein (hsp 70) in Soil Invertebrates: A Possible Tool for Monitoring Environmental Toxicants

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Abstract. The expression of hsp 70 after heat shock or exposure to heavy metals/molluscicides was investigated by fluorography or immunoblot in three diplopods *(Glomeris marginata, Cylindroiulus punctatus, Tachypodoiulus niger)*, two slugs *(Deroceras reticulatum, Arion ater)*, and one isopod *(Oniscus asellus)*. In *O. asellus*, hsp 70 expression occurred after heat shock and also after lead treatment, whereby a solution of 100 mg/kg Pb^{2+} was sufficient. Animals of the same species taken from a heavy metal polluted site in the vicinity of a lead/zinc smelter also showed the presence of hsp 70. The comparison of laboratory and field experiments demonstrated the suitability of *O. asellus* for monitoring tests. In contrast, the blot pattern after contamination with 1,000 mg/kg Pb^{2+} (in the mentioned diplopods) or different concentrations of the molluscicide *Cloethocarb* (BASF, FRG) (slugs) showed no differences compared to the respective control group.

The indication of hazardous substances in the environment by faunistical, cytological, and spectroscopic methods is widely distributed. On the contrary, only little attention has been given to biochemical and immunological methods in this respect. This is all the more astonishing since proteins expressed under stress conditions had been discovered more than fifteen years ago (Tissières *et al.* 1974). Originally, sudden temperature increase was used as stressor, hence the term *heat shock proteins* (hsp). However, these proteins are not only inducible by thermal shock, but also by viruses or several chemicals (oxidants, chelators, amino acid analoga, steroids, antibiotics, and heavy metals) (Ashburner and Bonner 1979; Garry *et al.* 1983; Hightower and White 1982; Ireland and Berger 1982; Levinson *et al.* 1980). Therefore, these proteins might be used in monitoring the influence of different environmental factors on animals.

Until now, considerable basic research has been done concerning hsp 70, the most prominent eucaryotic protein of this group with a relative mass of 68–74 kD, which is phylogenetically highly conserved (Kelley and Schlesinger 1982). It is the aim of this study to elucidate the suitability of this protein as an indicator of the toxic effect of hazardous substances in soil. The inducability of hsp 70 in six phytophagous or saprophagous soil animal species (three diplopods, two slugs, one isopod) were compared.

Materials and Methods

Conditions and Contamination

Mature specimens of the following species were collected from a nearly uncontaminated beech forest site (near Heidelberg, FRG; leaf litter contamination: 1.6 mg/kg Cd, 55.5 mg/kg Pb, 293 mg/kg Zn (Köhler, unpublished data)): *Glomeris marginata* (Villers), *Cylindroiulus punctatus* (Leach), *Tachypodoiulus niger* (Leach) (Diplopoda), and *Oniscus asellus* Latreille (Isopoda). Additionally, the slugs *Deroceras reticulatum* (Müller) and *Arion ater* (L.) (Pulmonata) from an uncontaminated laboratory hatchery were tested.

In laboratory tests, diplopods and isopods were kept on leaf litter particles soaked with a solution of 1,000 mg/kg Pb^{2+} as $Pb(NO_3)_2$ at 15°C for 41 days. Specimens were fixed after different exposure times. The slugs were fixed either without contamination or after treatment with the carbamate molluscicide *Cloethocarb* [2-(2-chloro-1-methoxy-ethoxy-) phenyl-*N*-methylcarbamate, BASF, FRG] (*A. ater:* 20,000 mg/kg at 15°C for 4 h, *D. reticulatum*: 10 mg/kg at 15°C for 3 d).

Specimens of *O. asellus* from a heavy metal contaminated spoil heap (Braubach near Koblenz, FRG, leaf litter contamination: 41.9 mg/kg Cd, 628.4 mg/kg Cu, 1,658 mg/kg Pb (Dallinger and Prosi 1988) were kept under the same conditions as the isopods mentioned above. Finally, we tested a series of 1; 10; 100; 1,000 and 10,000 mg/kg Pb^{2+} (as $Pb(NO_3)_2$) respectively in *O. asellus* to find out the minimal concentration of lead inducing hsp 70.

Immunological Analysis

After fixation in liquid nitrogen, the specimens were homogenized and centrifuged. Total protein from the extracts was analyzed by SDS-PAGE (12% acrylamide, 0.12% bisacrylamide (w/v), 5h at 250V). The proteins were transferred to nitrocellulose and blocked for 60 min at 25°C with 50% horse serum in Tris-buffered saline (TBS) (50 mM Tris pH 7.5, 150 mM NaCl). After removal of the blocking solution primary antiserum (rabbit anti-human hsp 70, 1 : 1,000 dilution) in 10% horse serum/TBS with 0.04% NaN_3 (w/v) was added and left

overnight at 25°C. Subsequently, the blot was washed with TBS. Secondary antibody (goat anti-rabbit IgG (H + L) coupled to peroxidase (Dianova, FRG), 1 : 2,000 dilution) in 10% horse serum/TBS with 1% Tween 20 was added and after 3 h incubation the blot was washed with TBS. The antibody complex was detected by chloronaphthole. Using the polyclonal antibody rabbit anti-*Astacus* (crayfish) hemocyanin (1 : 1,000 dilution) as primary antiserum, determination of the background proteins with a molecular weight of about 75 kD was performed in the same way (Stöcker et al. 1988).

Fluorography

Adults of *O. asellus* were individually kept in six-chamber tissue culture plates on wet filter paper. All animals were fed a piece of leaf litter soaked in a ^{35}S-Met solution (2μCi per specimen). After heat shock (30°C for 26 h) or lead contamination (1,000 mg/kg Pb^{2+} for 13 d), fixation of the animals and SDS-PAGE analysis was performed as mentioned above. Subsequently, the gel was stained with Serva Blue, destained in 5% methanol/7.5% acetic acid, and soaked with Amersham Amplifier. The dried gel was exposed to Kodak Diagnostic Film for 7 d at −80°C.

All specimens were examined individually; every lane represents one single animal. For all data at least 5 (fluorography: 3) up to 10 replicates were examined.

Results

Under heat shock conditions *O. asellus* exhibits two major protein bands in the 65-70 kD region in the fluorograph. Since the smaller of these proteins (about 65 kD) also occurs in the control, it seems very likely that only the larger protein (about 68 kD) can be assigned as a heat shock induced member of the hsp 70 family. The other protein seems to be a constitutively expressed gene product of one of the so-called hsc *(heat shock cognate)* genes (Craig *et al.* 1983), especially since it also crossreacts with the anti-hsp 70 antibody in the immunoblot. In the specimens treated with lead, the same protein pattern shows the induction of hsp 70 as well (Figure 1).

The hsc band (but not hsp 70) is demonstrable as a rule (but not in every case) in untreated specimens by immunoblot. In lead-treated isopods, additionally, hsp 70 is present in all examined samples—directly after induction and also after 41 d lead treatment. The rapid appearance and the long persistence of this protein band is conspicuous (Figure 2). Hsp 70 is also present in isopods taken directly from heavy metal contaminated mining waste. After taking the isopods from the contaminated area to the laboratory and placing them on lead enriched leaf litter this pattern does not change. Hsp 70 is still visible after 41 d (Figure 3).

In isopods which were not pretreated a contamination of the food with a solution of 100 mg/kg Pb^{2+} seems to be sufficient for the expression of hsp 70 (Figure 4).

In both slugs, a double band similar to *O. asellus* is visible after molluscicide treatment in the immunoblot. Since the same proteins, however, are present under laboratory control conditions as well, it is unclear whether a inducible hsp occurs in the examined species. Furthermore, the three diplopod species show only one prominent protein band in the 68 kD region-under lead conditions but also in the control group.

Unspecific crossreactions of the polyclonal anti-hsp 70 antibody occur predominantly in *O. asellus* proteins with a molecular weight of about 75 kD. This effect is most probably due to high hemocyanin concentrations in the homogenized material. The mentioned bands are shown to be subunits of hemocyanin (Stöcker *et al.* 1988) (Figure 1).

Discussion

Hsp 70 is a widely distributed protein in tissues of numerous animals as well as in cultured cells (Anderson *et al.* 1982; Currie and White 1981; Voellmy and Bromley 1982). The induction of a protein with a relative mass of 70 kD, caused by heavy metal ions, is also a well known phenomenon described previously (Levinson *et al.* 1979, 1980; Heikkila *et al.* 1982; Winter *et al.* 1988). Three stress proteins (but not hsp 70) have been described in cell cultures of rat fibroblasts and kidney

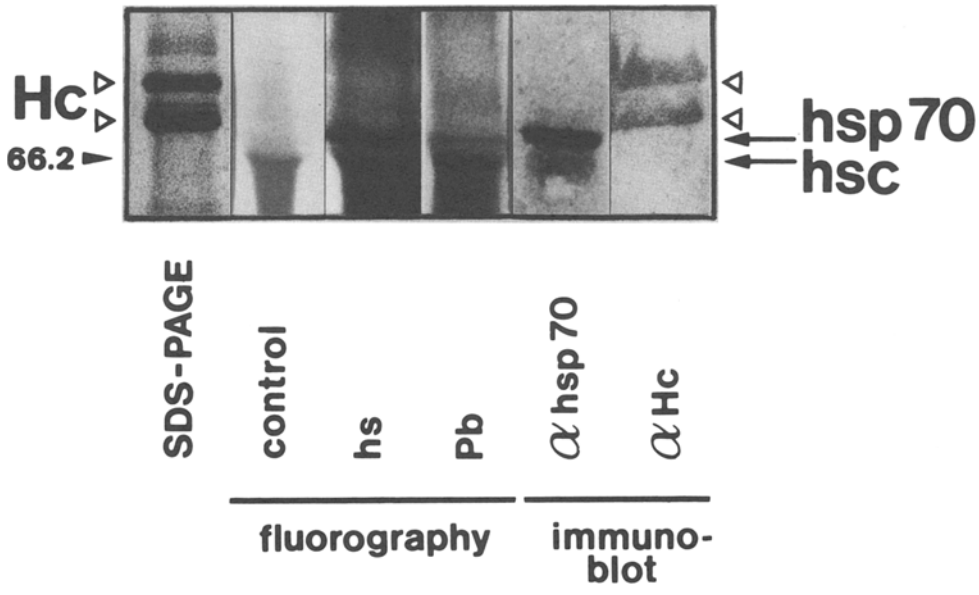

Fig. 1. Serva Blue-stained SDS-PAGE, fluorographs, and immunoblots of specimens of *O. asellus*. The prominent protein bands of the SDS-PAGE (triangles) are identified as components of hemocyanin (α Hc: immunoblot with rabbit anti-*Astacus* hemocyanin as primary antibody). Incubation with rabbit anti-human hsp 70 (α hsp 70) reveals the two protein bands of hsp 70 (upper) and hsc (lower). The same proteins are visible in the fluorographs under heat shock (hs) and lead contamination (Pb). In the control appears only hsc

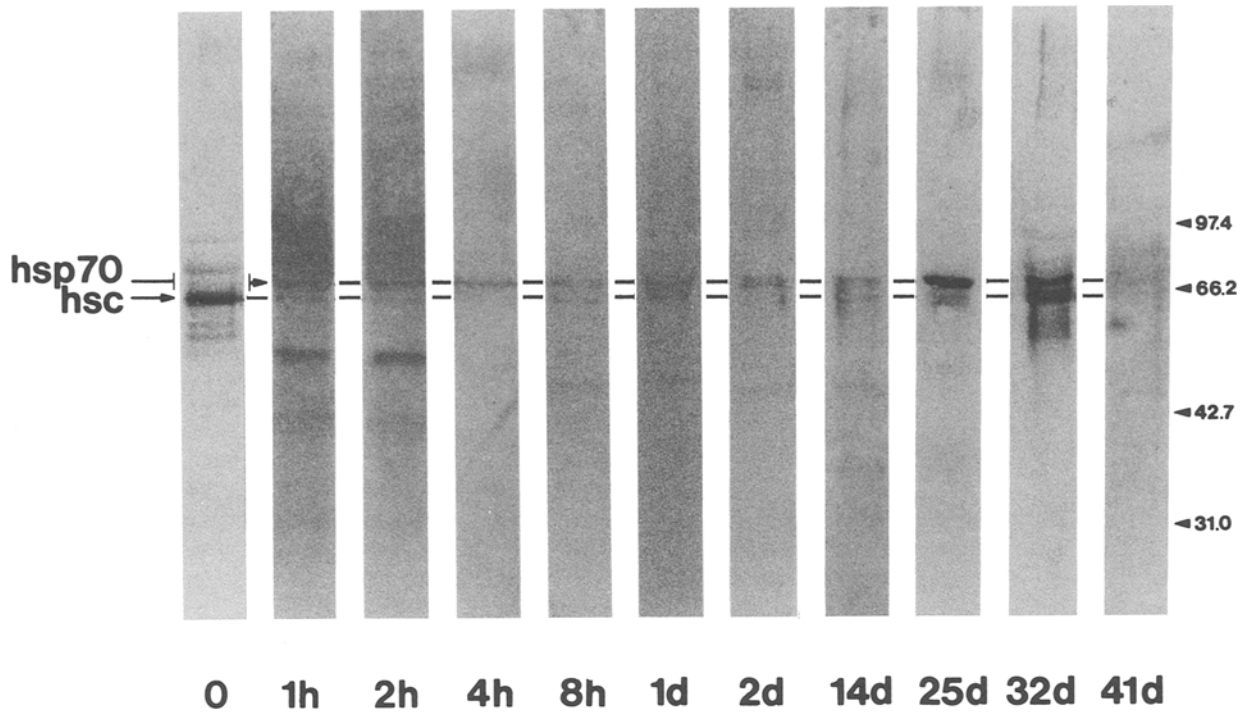

Fig. 2. Identification of hsp 70 and hsc by immunoblot in primary non-contaminated specimens of *O. asellus* after different times of artificial exposure to 1,000 mg/kg Pb^{2+}. Uncontaminated animals (line 0) only show the presence of hsc. Already 1 h after addition of lead, a weak band of hsp 70 can be detected. After several weeks of exposure hsp 70 is still present

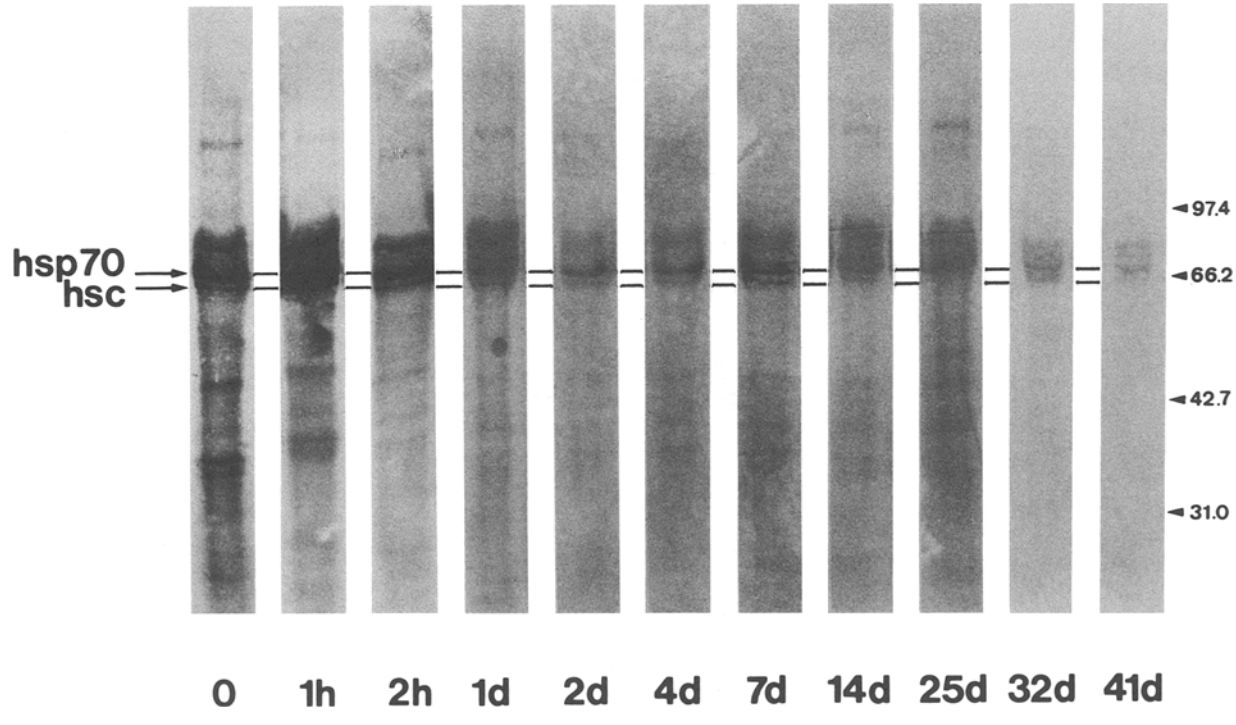

Fig. 3. Immunoblots of specimens of *O. asellus* taken directly from a heavy metal contaminated site (Braubach). Line 0: Fixation in the field. Hsp 70 as well as hsc can be detected without any additional stress. After laboratory lead treatment the protein pattern appears similar in all examined specimens

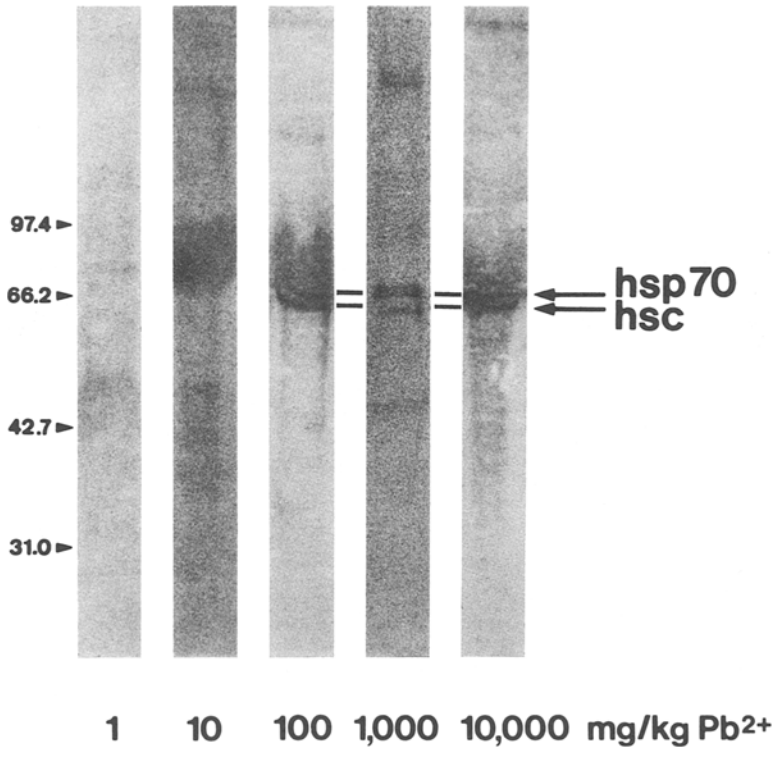

Fig. 4. Immunoblots of specimens of *O. asellus* treated with different concentrations of lead. Food contamination with a solution of 100 mg/kg Pb^{2+} at the minimum induces hsp 70 in all specimens. Minor contaminated animals lack hsp 70 (and also hsc, which did not necessarily occur in uncontaminated specimens)

epithelium cells treated with lead (Shelton *et al.* 1986). The mentioned induction of hsp 70 after lead treatment in the examined soil animals, however, is not necessarily primary. The expression of hsp 70 may also be due to a general physiological stress response to adversely affected life conditions. Nevertheless, the presence of this protein may indicate a reaction of the animal to a harmful stress situation, which cannot be evaluated by measurement of the stress-factor alone. However, investigations concerning the suitability of heat shock proteins in soil animals for the indication of environmental stressors have not been performed until now.

It is known from previous studies that ultrastructural alterations in the midgut of diplopods occur under heavy metal stress in laboratory as well as in the field (Köhler and Alberti, in press). The preferentially affected cell components (plasma membrane and microtubules) are assigned to be binding sites for heat shock proteins (summarized in Nover 1984). Therefore, a relation between the influence of hsp on these components and the observed ultrastructural alterations in certain cells seems to be possible.

Since control animals of *O. asellus* which were influenced by low lead concentrations (55.5 mg/kg dry weight) did not produce hsp 70, a threshold type of response seems likely. One, however, has to consider that a contamination of the food with a solution of 100 mg/kg Pb^{2+} (which was sufficient for hsp 70 induction) results in an approximate five-fold increase of the lead concentration in the food's dry weight (Köhler, unpublished data). Thus, the threshold value for induction of hsp 70 is suggested between 55.5 and 500 mg Pb/kg dry weight under the mentioned laboratory conditions.

Although it is likely that most polyclonal antisera against hsp 70 will also crossreact with the constitutively expressed cognate (hsc) gene product—e.g., the *Drosophila* genes hsc 4 and hsp 70 showed a homology of 82% (Craig *et al.* 1982, 1983)—the isopod *O. asellus* can still be used to evaluate the effect of heavy metal contamination. This is supported by recovery experiments showing the cessation of hsp 70 expression in lead-treated isopods after one or two weeks stress removal (Köhler, preliminary data). In contrast to some other phytophagous or saprophagous soil invertebrates, such as the examined diplopods and slugs which also produce hsp 70 under untreated laboratory conditions (possibly caused by a minor "captivity-stress"), the appearance of hsp 70 can be directly correlated with lead as a stress factor. The large amount of protein due to the size of isopods (compared with the mesofauna) allows examination at the individual level.

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