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# Semiquantitative Immunoblot Detection of 70 kDa Stress Proteins in the Carp *Cyprinus carpio*

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Stress proteins, also known as heat shock proteins or chaperonins, are a group of intracellular proteins which are both constitutively expressed and induced by most forms of environmental stress (Sanders 1993). They are divided into families according to their molecular weight. This study deals with the detection of 70 kDa stress proteins, called hereafter HSP70. Stress proteins play a role in the maintenance and protection of important cellular functions like protein folding, translocation and protein repair (Morimoto et al. 1994). HSP70 are stress proteins which are induced after environmental insult (Dietz and Somero 1993; Ku et al. 1994; Lindquist 1986; Sanders 1993; Welch 1992). This stress protein response to environmental insult shows a high evolutionary conservation and could therefore be a potentially very useful biomarker in monitoring pollution and other forms of anthropogenic environmental stress (Sanders 1990, 1993).

A good non specific indicator of sublethal levels of environmental stress should meet the following criteria : 1) synthesis is induced by many different stressors, 2) it shows an increased response for long duration after exposure, and 3) a quantitative relationship exists between response and degree of stress (Sanders 1993; Huggett et al. 1992). Heat shock proteins may meet these criteria, supporting its potential as a biomarker, although more scientific information is urgently needed since data on quantitative dose-respons relations are very scarce (Huggett et al. 1992). This may be partly due to the lack of good quantitative methods to measure specific heat shock proteins levels. This paper focuses on the methodological optimalisation of semiquantitative detection of carp (*Cyprinus carpio*) HSP70 by immunoblot.

## MATERIALS AND METHODS

Adult carp *Cyprinus carpio* of approximately 100 g (± 18 cm long) were used as test organisms. Fish were kept in aquaria with aerated recirculated medium-hard filtered tap water at 25°C with a trickle filter and fed daily dry pellets (Tetramin) until acclimation and exposure in the test aquarium. Animals were acclimated to the test aquaria, with medium hard filtered tap water, for 2 wk prior to experiments. A strain of carp (Group 1) bought as juveniles (±2cm) from the

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Agricultural University of Wageningen (The Netherlands) were raised in the laboratory. These animals were used for the optimalisation of the immunoblot technique. Heat shock proteins were induced by exposing these fish to a heat shock of 33 °C for up to 96 hr. A second source of animals (Group 2) was a local fish farm. These animals were acclimated to laboratory conditions and handling for 6 weeks before exposure to a salt stress of 1% NaCl for 28 d. In this experiment, fish from both sources were used as control animals.

Animals were gently caught and rapidly anesthesised in a solution of 200 mg/L of 3-aminobenzoic acid (MS222). Animals were killed and dissected on ice. For the optimalisation of the immunoblot protocol only white muscle of heat shocked animals was used. In the salt exposure experiment muscle, liver, kidney, brains, and gill were dissected out. Tissue parts were quickly rinsed in icecold 10 m*M* EPPS buffer containing 0.25 *M* sucrose, pH 7.4, dried with paper tissues and either directely processed or frozen in liquid nitrogen and stored at -80 C untill processed.

Approximately 0.35 g tissue was homogenised in 3.5 mL of icecold Tris buffered saline (TBS : 10 m*M* Tris, 150 m*M* NaC1, pH 7.4) or in phosphate buffered saline (PBS : 0.9 m*M* $CaCl_2$, 2.7 m*M* KC1, 1.5 m*M* $KH_2PO_4$, 136 m*M* NaC1, 8.1 m*M* $Na_2HPO_4$, 0.24 m*M* $MgC1_2$, pH 7.4) with an ultraturax (Ika Labortechnik). After homogenisation, samples were centrifugated for 20 minutes at 10,000 x G (Sorval RC5 superspeed refrigerated centrifuge) and the supernatans recentrifuged for 60 minutes at 100,000 x G (Beckman L5 75 Ultracentrifuge) at 4°C. The supernatans was immediately used for electrophoresis or frozen and kept at -80 °C until used. To improve antigen retrieval, sample supernatans was used either untreated or cooked for 5 minutes in loading buffer (4% SDS, 10% mercaptoethanol, 0.5 M Tris HCL, 20% glycerol) or PBS.

Samples were separated on a 12.5% or 7% SDS polyacrylamide gel with a 4% stacking gel. pH and buffer capacity of the initial gel buffer (25 m*M* Tris, 192 m*M* glycine, 0.1% SDS pH 7.5) were increased (50 m*M* Tris, 192 m*M* glycine, 0.1% SDS, pH 8.3) to achieve a better separation. Slots were loaded with 5µL loading buffer (4% SDS, 10% mercaptoethanol, 0.5 M Tris HCL, 20% glycerol) and 5µL protein standard solutions or supernatans. The first lane was loaded with molecular markers (SDS PAGE standards Low Range, BioRad). The other lanes were loaded with either a standard of commercial stress proteins (recombinant *E. coli* Human HSP70 , SPP755 StressGen) in TBS in the presence or absence of added protein (gelatine or skimmed milk), or samples in TBS. Empty slots were filled with water and loading buffer. Electrophoresis was performed at 4°C at 100V in the stacking gel (for approximately 15 min) and at 200V in the separating gel (for approximately 60 min) in Mighty Small II SE250/260 electrophoresis units (Hoefer).

The blotting membranes and SDS-PAGE gels were submerged for 10 min in transferbuffer (CAPS-buffer 10m*M*) prior to blotting. Transfer was performed at

50V for 3 hours at 4°C (Biorad TransBlot). Three different membranes were tested: Immun-Lite (BioRad), nitrocellulose (BioRad) and polyvinilidene difluoride membranes (PVDF) (PolyScreen, DuPont Nen). PVDF membranes were pretreated with 95% ethanol and rinsed with ultapure water (MilliQ 185 MilliPore) prior to submergence in CAPS buffer (Sigma). Originally the different washing and coating steps were performed in polyethylene bags, but were optimised by replacing the bags by plastic recipients. All steps were performed under continuous shaking with a rocker. Membranes were washed twice after transfer for 10 minutes with TBS, and incubated in a blocking solution. Four different blocking solutions were tested: (A) 6% skimmed milk powder in TBS; (B) 5% milk in TTBS (TBS with 0.5mL/L Tween 20); (C) 5% gelatine in TTBS; (D) 1% BSA in TTBS. The effect of blocking time was assessed by blocking at room temperature for 1, 2 or 16 hours. Membranes were washed again three times for ten minutes with TTBS.

Membranes were coated with diluted anti-HSP70 (Mouse anti bovine brain HSP70 ascites fluid BRM-22, Sigma H5147) in antibody buffer (ABB : 1% milk powder, thimerosal 0.02% in TBS) overnight at 4°C. Optimal concentration of the first antibody was determined with concentrations of 1/500, 1/1000, 1/1500 or 1/2000 (= 5.4, 2.7, 1.8 and 1.35 µg anti-HSP70/mL). The membranes were washed again three times for ten minutes with TTBS and coated with the second antibody. Dilutions of 1/1500 or 1/2000 or 1/5000 (Rabbit anti mouse IgG - Horseradish peroxidase conjugate, Sigma) were tested out for one hour at room temperature. Blotting membranes were again washed three times for ten minutes with TTBS and then washed twice with TBS for 30 minutes. Finally the membranes were put into the chemoluminiscence reagent for respectively 5 minutes (ImmunLite kit, BioRad) or 1 minute (Renaissance kit, DuPont Nen) at room temperature. The proteins were detected after exposure on a blue light sensitive film (DuPont Reflection NEF autoradiography film). Bands were scanned with a densitometer (LKB Bromma Ultrascan XL) for their intensities.

## RESULTS AND DISCUSSION

Electrophoresis with 7% polyacrylamide gels resulted in a better separation of the proteins of interest than with 12% polyacrylamide gels. Two protein bands were detected in muscle of heat shocked animals and a low but consistent binding of the anti-HSP70 antibody to bovine serum albumin was noted. The bands detected most probably correspond to HSP70 and HSP72 detected in a *Cyprinus carpio* fibroblast cell line (Ku et al. 1994). The use of ultra pure water (18.2 µSiemens, < 5ppb organic carbon) resulted in much lower background signals compared to the use of demineralised water.

Immun Lite and nitrocellulose membranes proved to be ineffective for the detection of carp HSP70 while the use of PVDF membranes resulted in a sensitive detection. One hour blocking proved to be sufficient. No increase in signal intensity or decrease in background signal was found with blocking for 2 hours or overnight in comparison with 1 hour. The membranes were best blocked with 6 % skimmed

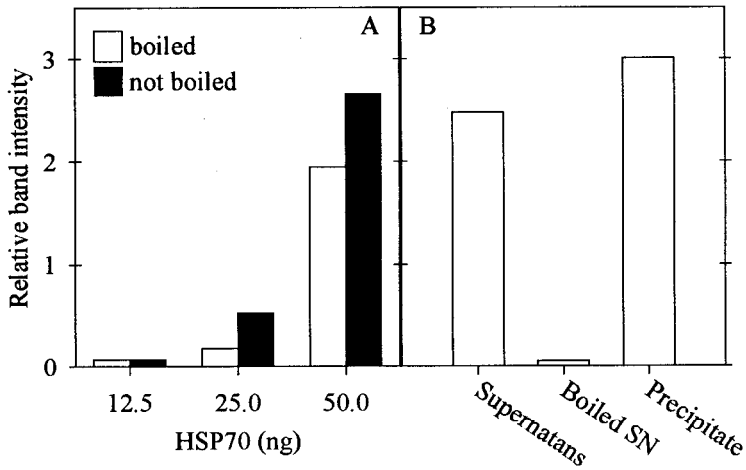


**Figure 1.** Relative band intensity of commercial recombinant HSP70 (A) : effect of boiling in loading buffer (B) Relative band intensities of HSP70 in white muscle of heat shocked carp (*Cyprinus carpio*) in sample supernatans not boiled, sample supernatans boiled in PBS buffer or the precipitate of boiled sample supernatans.

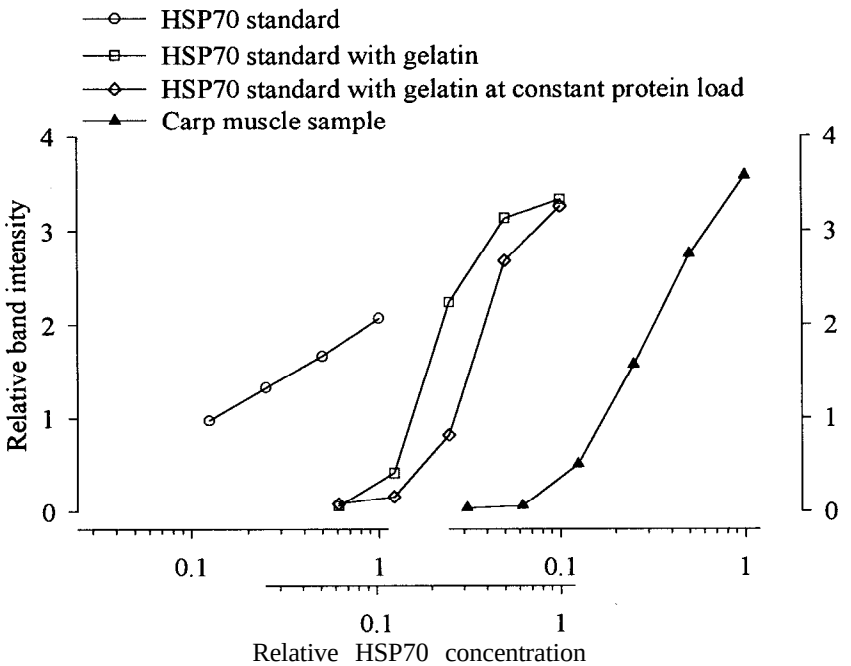


**Figure 2.** Relative band intensity of commercial recombinant HSP70 in different matrices compared with HSP70 band intensity in the white muscle of heat shocked carp (*Cyprinus carpio*).

milk powder in TBS.

Boiling standards in loading buffer increased the signal (Fig. 1A), but boiling in PBS buffer resulted in precipitation of the proteins (Fig. 1B), which were detectable in the sediment. Detection of the proteins was optimal at a concentration of 5.4 µg/mL (dilution 1/500) for the anti-HSP70 and a 1/2000 dilution of the second antibody (anti IgG).

The effect of protein concentration in calibration curves on band intensity is shown in Figure 2. It was found that HSP70 calibration curves did not resemble dilution curves of carp muscle samples. Addition of gelatine, in a concentration equal to the protein concentration (71 µg protein / 100 µL) of the muscle samples resulted in response curves with a course similar to the dilution curve of muscle sample. The source of proteins in the calibration curves did not influence the band intensity or the shape of the response curve. No difference was found between addition of gelatine or skim milk powder. A significant decrease in band intensity was however noted when comparing 25 ng HSP70 in buffer with proteins added to 25 ng HSP70 standards without protein added.

In a study to test reproducability of band intensity after blotting and detection, it was found that variability was highest between days and lowest between different electrophoretic runs (Table 1). A 15 % coefficient of variation (standard deviation / average*100) was found between bands within one blotted electrophoresis gel.

Sixty nine hours heat shock at 33°C resulted in a more than 15 fold increase in undiluted samples in HSP70 band intensity (Fig. 3). Addition of 5 ng of HSP70 standards resulted in heat shocked samples in a decrease in band intensity while addition to non heat shocked animals resulted in an increase in band intensity. A 28 d exposure to 1% NaCl resulted in a significant increase in HSP70 band intensity in the liver of the salt exposed animals (Fig 4) (Mann Whitney U test, $p<0.05$). In the liver 2 or 3 bands were found. One of these bands was the constitutive HSP70 while the second was the salt induced band. If high HSP70 levels were detected a third band also appeared. These results support the findings in other organisms and fish of tissue specific induction of stress proteins (Dietz and Somero 1993; Dyer et al. 1991, 1993). These results also confirm the observation that ion and water balance is only disturbed in the liver of salt exposed animals (Peeters 1996, *De Boeck et al.* 1997 *MAGMA in Press*). Band intensities in different tissues of

**Table 1.** Percentage coefficient of variation (%CV) of HSP70 band intensity of a heat shocked carp white muscle tissue sample

| comparison | %CV (n) |
|---|---|
| between 5 bands, within one eletrophoresis gel | 15.4 ± 5.2 (5) |
| between averages of gels, within one electrophoresis | 13.4 ± 9.8 (2) |
| between averages of electrophoresis, within one day | 6.31 ± 4.0 (2) |
| between averages of different days | 23.0 ± 9.4 (4) |

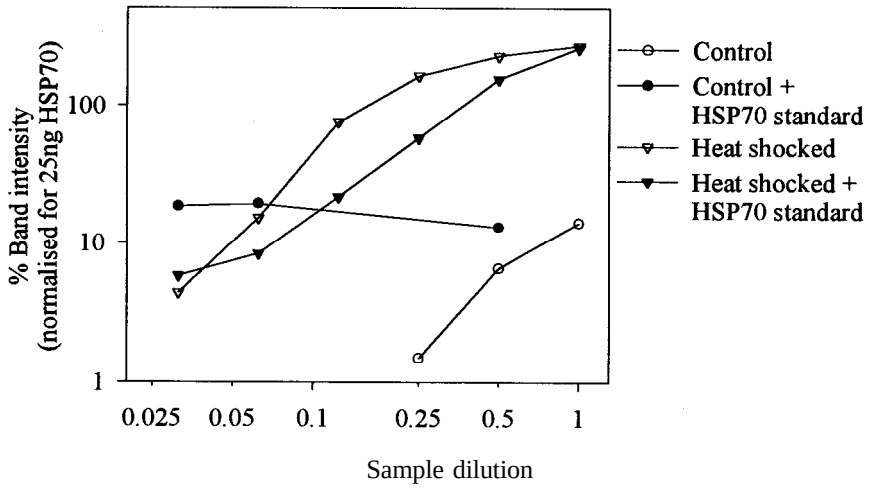


**Figure 3.** Relative band intensity of dilution series of carp (*Cyprinus carpio)* white muscle HSP70 of heat shocked animals and control animals with and without commercial recombinant HSP70 added

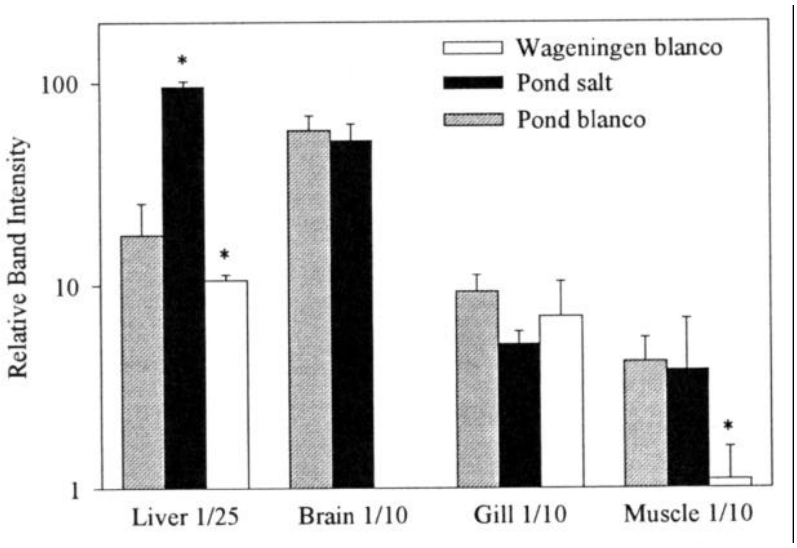


**Figure 4.** Relative band intensities of HSP70 in different tissues of the carp (*Cyprinus carpio*) in control animals from two different sources and animals exposed for 28 days to 1% salt. Significant differences (Mann Whitney U test, $p<0.05$) are indicated by an asterix

control animals from different sources were also significantly different ($p<0.05$, Mann Whitney U test). Higher levels were found in the liver and muscle of control fish retrieved from a local retailer, of which the history was not known, compared to levels of control fish obtained as juveniles from the agricultural university of Wageningen and which were grown in the lab to adult size. Within the context of the possible use of stress proteins as biomarkers it is imperative that the stress protein levels in control fish should be carefully checked before experiments are done, because stress protein levels may stay elevated for long periods of time after stress exposure (Sanders 1993).

The detection of increased HSP70 in heat shocked or salt exposed carp was expected in view of the large amount of evidence of the stress protein response in a wide variety of organisms (Sanders 1990) and the report of induction of HSP68, HSP70 and HSP72 in carp cell lines after heat shock (Ku et al. 1994). More important however is that our results have shown that the supernatans matrix interferes with the detection of HSP70 proteins in immunoblotting. Addition of gelatin or milk proteins decrease the signal intensity and modify the slope of the calibration curve, but make its course more similar to that of a dilution curve of fish tissue. Sample treatment by boiling showed to be beneficial for antigen retrieval. The interaction of added HSP70 standards with tissue samples is momentarely not clear and needs further investigation. The detection limit of HSP70 proteins with our method was around 6 ng HSP70 proteins in a solution of 710 µg total protein per ml. The method showed not only to be very sensitive but also showed a good level of reproducability. Although the method was developed for carp muscle tissue, is was also readily applicable for other carp tissues, including a metabolic active tissue as the liver. Therefore it can be concluded that immunoblot is a suitable and sensitive method for the detection and semiquatitative determination of stress protein in diverse tissues after proper sample treatment and construction of calibration curves containign the same total protein concentrations as the studied samples.

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