

70 kD stress protein (Hsp70) analysis in living shallow-water benthic foraminifera

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Abstract

Hsp70 is a phylogenetically highly conserved protein family present in all eukaryotic organisms tested so far. Its synthesis is induced by proteotoxic stress. The detection of Hsp70 in foraminifera is presented here for the first time. We introduce a standard immunoblotting protocol modified for the detection of Hsp70 in shallow water benthic foraminifera. Additionally, we showed a temperature-dependent expression pattern of Hsp70 in *Ammonia tepida*.

Key words: foraminifera, benthos, heat shock proteins, HSP 70, protein content

Introduction

In stressful situations (like elevated temperatures), many organisms strongly increase the production of stress proteins or heat shock proteins (=Hsps), that bind to partially denatured proteins preventing their aggregation, and directly mediate the correct folding (Gething and Sambrook 1992, Parsell and Lindquist 1993). Members of the class of Hsps have been found in all eukaryotic organisms tested thus far (Lindquist and Craig 1988). These proteins are phylogenetically highly conserved, especially the Hsp70-family (Gupta and Golding 1993). They can be used as biomarkers to monitor physiological stress caused by changing environmental conditions or pollution (e.g. Köhler et al. 1996). The aim of this study is to verify the existence of Hsp70 in foraminifera. Therefore, a standard protocol for immunological detection of Hsp70 (Köhler et al. 2005) was optimized for these organisms. Total protein contents of different foraminiferal species (*Ammonia beccarii* Linnaeus, 1758, *Ammonia tepida* Cushman, 1926, *Elphidium crispum* Linnaeus, 1758, and *Massilina secans* d'Orbigny, 1826) were determined, which can help to estimate the number of individuals needed for immunological studies.

Material and methods

Sediment Samples

Field sediments containing *Massilina secans*, *Ammonia beccarii* and *Elphidium crispum* were collected on the rocky shore near the Laboratory of Marine Bio-Indicators (LEBIM) on Yeu Island (France) in July 2007 and May 2008. *Ammonia tepida* was collected in the muddy shore in the Bay of Aiguillon (France) in June 2008. At both stations, the surface sediment was collected with a spoon and sieved; the >150 µm fraction was transferred to transport boxes (250 ml PE bottles, Kautex, Bonn, Germany) and carried to our lab in Tübingen, Germany.

Foraminifera Treatment, Protein Extraction and Concentration

In the laboratory the sediment was transferred to 2 L glass aquaria. Aquaria were kept under stable conditions (20°C, 34‰ salinity, 11:13 h light-dark cycle) up to 4 months in order to acclimate the foraminifera to laboratory condition. Sediment subsamples were shortly washed, and living foraminifera were picked and cleaned from small particles with a fine brush in an embryo dish filled with filtered seawater. The foraminifera were transferred into glass vessels filled with 100 ml seawater and sealed with parafilm. For a possible induction of stress proteins, vessels were placed in incubators adjusted to

higher (max. 45°C) or lower temperatures, or left at control conditions at 20°C. The experimental setups are summarized in Table 1.

For the extraction of the proteins, foraminifera were taken out of the vessels, and transferred with the smallest possible amount of water to a 1.5 ml Eppendorf cup. Extraction buffer (80 mM potassium acetate, 5 mM magnesium acetate, 20 mM Hepes pH 7.5) and protease inhibitor (Protease Inhibitor Cocktail, product no. P8340, Sigma, St. Louis, Missouri, USA) were added, and the foraminifera were mechanically homogenized with a plastic pestle. To remove the shell fragments the homogenate was centrifuged at 4°C at 153 g for 10 minutes and the supernatant was transferred to a new vial. A 4 µl aliquot of the supernatant was reserved for quantification of total protein content. The rest of the supernatant was mixed with sodium dodecyl sulfate (SDS) -containing buffer (20% glycerol, 3% SDS, 0.3% β-mercaptoethanol, 10mM Tris pH 7) in the ratio 2:1 (two parts supernatant : one part buffer), heated to 100°C for five minutes, and kept for further analysis at -20°C. For the protein concentration after extraction we used ultrafiltration units for centrifuges (Roti®-Spin Mini-30, Roth, Karlsruhe, Germany) with a nominal molecular weight cut off of 30 kDa and centrifuged the samples (experiment I: 5 min at 5,000 g; experiment II: 9 min at 4,000 g). During centrifugation, all proteins larger than 30 kDa pass through the filter, and Hsp70 can accumulate in the supernatant.

Bradford Assay

Due to the detection range of the analysis, the Bradford analysis had to be conducted prior to sample ultrafiltration. The total protein content was measured two times per sample. For each measurement 2 µl of the protein solution together with 23 µl of extraction buffer 1:10 were transferred to a 96-well microplate. For the standard curve a serial dilution of bovine serum albumin (BSA) (0.4, 0.2, 0.1, 0.05, 0.025 mg/ml) in extraction buffer 1:10 was used. Subsequently 250 µl of Bradford reagent was added to each well. Measurements were made with an automated microplate reader (Elx 8006, BioTek, Winooski, Vermont, USA).

Immunological Analysis

Immunological analysis followed the protocol of Köhler et al. (2005). Extracted proteins were analyzed by minigel SDS-PAGE (12% acrylamide (w=v), 15 min at 80 V and 90 min at 120 V). Protein was transferred to nitrocellulose by semi-dry blotting (Western blotting after Burnette 1981), and the filter

was blocked for 2 h in 50% horse serum in TBS (50 mM Tris pH 7.5, 150 mM NaCl). After washing in TBS, either (a) the monoclonal antibody “mouse anti-human Hsp70 (MA3-006)”[†]; Dianova, Hamburg, Germany, dilution 1:5000, or, alternatively, (b) the monoclonal antibody “mouse anti-chicken Hsp70/Hsc70 (SPA-822)”[†]; Dianova, dilution 1:1000 (experiment II), or (c) a combination of both antibodies in the given concentrations (experiment I) was added. The filter was then incubated on a lab shaker at room temperature overnight.

After repeated washing in TBS for 5 min, the nitrocellulose filter was incubated with the secondary antibody solution (goat anti-mouse IgG coupled to peroxidase, Dianova, dilution 1:1000 in 10% horse serum in TBS) for 2 h at room temperature. After subsequent TBS washing, the antibody complex was detected by the staining solution (1 mM 4-chloro(1)naphthol and 0.015% H₂O₂ in 30 mM Tris pH 8.5 containing 6% methanol).

To quantify the amount of protein in the bands, the optical volumes (area (number of pixels) x average grey scale value) of the protein bands in experiment I were measured after background subtraction with a densitometric image analysis system (Herolab E.A.S.Y.). For comparison, the optical volumes were normalized by dividing them through the amount of total protein transferred to each lane.

Results

Protein Contents

Protein concentrations were quantified by the Bradford test. For each species, the mean total protein content per individual was calculated (Table 2). Highest protein content per individual (0.231 µg) was extracted from *M. secans*, followed by *A. beccarii* (0.085 µg), *A. tepida* (0.066 µg) and *E. crispum* (0.040 µg).

Hsp70-Immunoassay

One first weak band of foraminiferal Hsp70 was detected when 386 individuals of *M. secans*, 357 individuals of *A. beccarii*, and 300 individuals of *E. crispum* were pooled together and incubated for 3 h at 39°C. We transferred 47µg total protein to the gel, limited by the size of the gel pockets. After including an additional step of protein concentration by ultrafiltration, we were able to increase the

amount of total protein (>30 kD) on the gel. This modification significantly enhanced detection of Hsp70.

Temperature Expression

In experiment I, four pools of foraminifera were prepared and exposed to different stress temperatures: 10°C, 20°C, 35°C, and 45°C (Table 1). Proteins were extracted and concentrated. Expressed Hsp70 was shown in Fig. 1. At 45°C, no Hsp70-band was visible (Fig. 1, lane 4) and no intact proteins were detected by the Bradford test (Table 1). The amount of total extracted proteins (Table 1) and therefore of concentrated proteins (>30 kD) loaded in the different lanes was not equal. In order to normalize the results, the optical volumes of lanes 1-4 were divided through the corresponding transferred protein mass (Fig. 2). The amount of detected Hsp70 was lowest at 20°C (adequate to normal “unstressed” culture temperature), followed by 10°C, and a maximum was found at 35°C.

Antibody Test

The cross-reactivity of the different primary antibodies used in the previous samples and the quality of the concentration step after extraction was tested in experiment II. Concentrated foraminiferal protein (>30 kD) was tested with each of the both primary antibodies used. The antibody “mouse anti-human Hsp70 (MA3-006)” detected a band of Hsp70, the antibody “mouse anti-chicken Hsp70/Hsc70 (SPA-822)” did not detect any band, also no standard was visible here. To check if there was a major loss of Hsp70 during ultrafiltration, the filtrate (containing proteins <30 kD) was also analyzed. No Hsp70 could be detected in the filtrate with neither of the two primary antibodies.

Discussion

Hsp70-Immunoassay

About 50 µg total protein is needed to detect Hsp70 in foraminifera by immunoblotting, which is remarkably more than used in the majority of studies dealing with other organisms (e.g. Knigge and Köhler 2000, Köhler et al. 2000). This could be explained by a considerably increased level of other essential proteins in foraminiferal cells, like actin and tubulin, which then lead to a proportionally low concentration of Hsp70 in the cytoplasm. Actin and tubulin are basic components of the cytoskeleton. Pseudopodial movement and the high dynamic cytoplasm motility of foraminifera need the presence of

many microfilaments composed of these two proteins (Travis and Bowser 1986). Another reason might be a low cross-reactivity of the used primary antibodies to bind to foraminiferal Hsp70, although our tested antibodies were developed to bind a large variety of Hsp70 proteins, as the target epitope of the amino acid sequence for the antibody to bind is located in the highly conservative ATP-binding region of the protein.

Temperature Expression

Experiment I investigated the relationship between stress temperature and expression of the Hsp70 protein (Fig. 1). Four pools of *A. tepida* (in total more than 5000 living foraminifera) were collected and exposed to different temperatures. The number of available foraminifera and the time-consuming process of isolating these out of the sediment limited the number of temperature steps. Because we decided to transfer the maximum amount of total extracted protein (>30 kD) of each pool to enhance Hsp70 detection, the amount of protein transferred to the different lanes differs and had to be normalized for comparison (Fig. 2).

The expression of Hsp70 was highest at 35°C. At a culture temperature of 20°C almost no Hsp70 was found, but at 10°C the Hsp70 had increased again. These results fit very well to the established statement that denaturated proteins caused by physiological stress, which can be created by higher temperatures as well as lower temperatures (Creighton 1990), induce Hsp70. The results also match to the Hsp70 reaction curve of Eckwert et al. (1997), showing the relationship of stress intensity and Hsp70 level.

No Hsp70 was detected at 45°C. Bradshaw (1961) determined the lethal temperature for *A. tepida* at 45°C, in which 50% of exposed specimens have been killed within 5 minutes. Our organisms have been exposed to this temperature for 4 hours, therefore we suppose that they did not survive and the proteins have likely been disintegrated.

Antibody Test

In the advanced experiment II either the MA3-006 was used as the first antibody, or SPA-822. Therefore, it was possible to judge on their respective potential to cross-react with foraminiferan antigen hsp70 of *A. tepida*, which would not have been possible, if the antibodies were applied as a mixture of them (experiment I). Since SPA-822 did not show any cross-reactivity with foraminiferan Hsp70, we

assume that the signals obtained in experiment I derive from MA3-006 cross-reaction solely. It was not possible to detect any Hsp70 in the filtrate after microfiltration with both antibodies. Therefore, we can assume that no or only a negligible amount of Hsp70 proteins were lost during filtration.

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Tables

Table 1. Overview of experimental conditions: assemblages of foraminifera/standards, duration and temperature of heat stress, primary antibodies, and amount of extracted total protein (analysis before ultrafiltration). Primary antibodies: “mouse anti-human Hsp70 (= MA3-006)”, “mouse anti-chicken Hsp70/Hsc70 (= SPA-822)”.

Experiment	Specimen	Stress	Primary antibody	Total protein (µg)
I	1,300 <i>A. tepida</i>	4h / 10°C	MA3-006	77.9
	1,300 <i>A. tepida</i>	4h / 20°C	+ SPA-822	106.0
	1,300 <i>A. tepida</i>	4h / 35°C		79.8
	1,300 <i>A. tepida</i>	4h / 45°C		--
	<i>D. rerio</i> (standard)			40.0
II	<i>D. rerio</i> (standard)		MA3-006	40.0
	<i>D. rerio</i> (standard)		SPA-822	40.0
	1,057 <i>A. tepida</i>	4h / 35°C	MA3-006	57.9
	1,057 <i>A. tepida</i>	4h / 35°C	SPA-822	57.9
	filtrate	4h / 35°C	MA3-006	-
	filtrate	4h / 35°C	SPA-822	-

Table 2. Average total protein content per individual of the different species (SD = standard deviation).

Species	Number of measurements	Total number of specimen	Total protein per individual (SD) (μg)
<i>A. tepida</i>	4	7,070	0.066 (0.012)
<i>M. secans</i>	3	315	0.231 (0.034)
<i>A. beccarii</i>	1	370	0.085 (-)
<i>E. crispum</i>	1	600	0.040 (-)

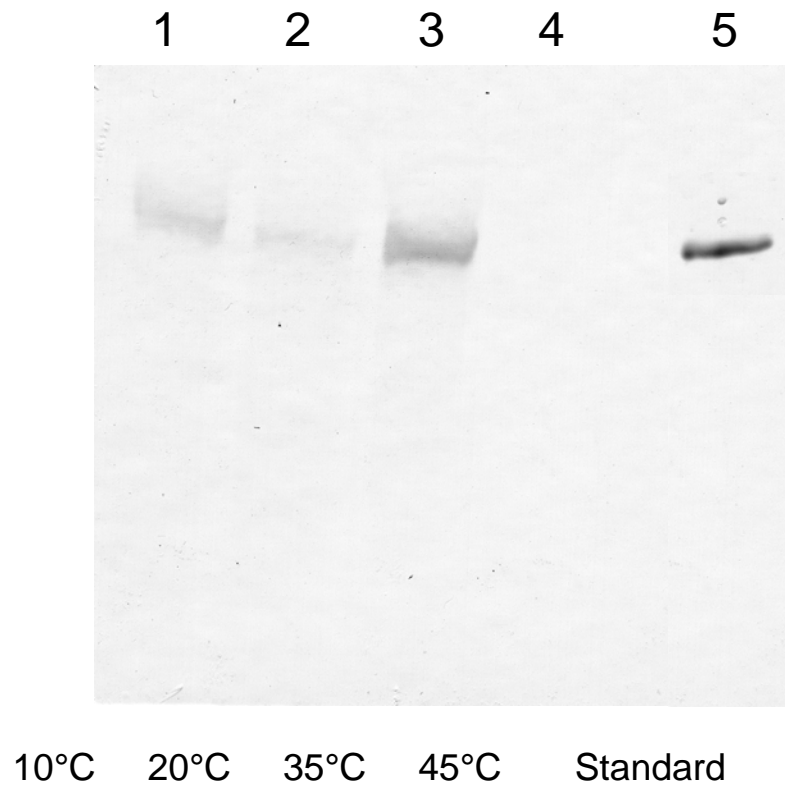
Figure Legends

Figure 1. Temperature depending expression pattern of *A. tepida* (experiment I). Immunodetection of Hsp70 by Western blotting. antibodies: MA3-006 + SPA-822. Lane **1-4**: *A. tepida*, lane **5**: Standard (*D. rerio*).

Figure 2. Temperature depending expression pattern of *A. tepida* (experiment I). Hsp70 level was quantified by optical volume of the bands on the resulting filter with a densitometric image analysis system (Herolab E.A.S.Y.) and normalized with the amounts of total protein transferred to each lane.

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Figure 1



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Figure 2

