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Western blotting versus ELISA detection of stress proteins induced in the blue mussel *Mytilus edulis* exposed to cadmium and tributyltin

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Stress proteins of the Hsp70 family induced in the blue mussel *Mytilus edulis* exposed in the laboratory to increased concentrations of cadmium (Cd) or tributhyltin (TBT) were analysed using Western blotting and/or ELISA tests. Statistical evaluation of results indicated that increased concentrations of Hsp70 were detected by means of the ELISA tests as compared with control organisms in extracts from the gills of mussels exposed to both Cd or TBT ($p = 0.022$). Results of analysis by means of Western blotting showed no differences in the levels of Hsp70 in the extracts ($p = 0.151$). It was concluded that the ELISA test allowed a more sensitive detection of Hsp70 than did Western blotting.

Keywords: immunodetection, hsp, methods comparison, quantitative analyes

Introduction

Exposure biomarkers, defined as the measurable effects of toxic substances and their influence on the living organisms at subcellular or physiological level, are thought to be sensitive indicators of environmental contamination (Depledge and Fossi 1994). Among these toxicants, heavy metals attract interest because low concentrations are harmful to biota. Moreover, metal ions discharged in the environment persist there indefinitely since they are not subject to degradation (Salmons and Förstner 1984). There are several biomarkers of excessive exposure to metals (Regoli 1998) including metallothioneins (Viarengo *et al*. 1995) and stress proteins (Sanders *et al*. 1994, Depledge *et al*. 1995, Tendegren *et al*. 1999). The latter were reported to be sensitive indicators of cadmium and copper accumulation by marine mussels (Sanders *et al*. 1994), vertebrates (Pedersen *et al*. 1997) and soil worms (Köhler *et al*. 1996), although other reports indicate that the insufficient sensitivity of stress protein induction and analysis limits the application of stress proteins as biomarkers of environmental metal exposure in the case of mussels (Lundebye *et al*. 1995, Bradley *et al*. 1998, Radłowska and Pempkowiak 1998).

The usual approach to the stress protein analysis is based on one-dimensional SDS-PAGE electrophoresis, followed by Western blotting and immunodetection with mono- or polyclonal antibodies. Most often heat shock proteins (Hsp) of the 70-kDa family have been analysed (Sanders *et al*. 1994, Dunlop and Matsumura 1997, Radłowska and Pempkowiak 1998). Recent reports have indicated that the

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ELISA test has potential for detecting stress proteins, including Hsp70, at concentrations substantially smaller than those detected by the common SDS-PAGE/Western blotting approach (Yu *et al.* 1994, Vanickova *et al.* 1995, Pyza *et al.* 1997). However, no comparison of the sensitivity of both analytical techniques applied to the Hsp detection is available as yet, based on the parallel analysis of the same materials.

In the present paper, the results of applying both ELISA test and SDS-PAGE/Western blotting as methods of stress protein detection are presented. The blue mussel *Mytilus edulis*, known for its ability to accumulate cadmium (Pempkowiak and Kożuch 1996), was used as a test organism. Stress proteins were induced in *M. edulis* by means of incubating mussels in seawater enriched with cadmium or tributhyltin (TBT). It is concluded that the ELISA test is more suitable for analysis of stress proteins in extracts from the gills of the mussels due to its increased sensitivity.

Materials and methods

Mussels collecting and exposure to toxicants

Sea mussels, *Mytilus edulis*, were collected from the Bay of Puck, Southern Baltic, in May 1997. The average shell length of mussels selected for experiments was between 25 and 35 mm. Twelve animals were kept in an aquarium filled with 15 litres of continuously aerated seawater (salinity 7.0), at 10°C, without being fed, for 1 week's acclimatization prior to the experiment. After acclimatization the animals were incubated for 7 days in seawater enriched with cadmium chloride or TBT at concentrations of respectively 50 µg Cd(II) litre^{-1} or 50 µg TBT litre^{-1}. Details of the procedure are described by Pempkowiak and Kożuch (1996).

Composite samples of gills dissected from five mussels were prepared for analysis. The tissues were homogenized in Tris-HCl buffer and aliquots of homogenate were centrifuged. The extracted proteins were solubilized in SDS-sample buffer and heated at 80°C for 5 min. The total protein concentration was determined by Bradford's method (1976) using bovine serum albumin as standard.

SDS-PAGE and Western blotting analysis

One-dimensional sodium dodecasulphate-polyacrylamide gel electrophoresis was performed according to the Laemmli (1970) procedure. Proteins were separated on 10% resolving gel with 5% stacking gel. A total of 22 µg proteins was applied to each well. A mixture of proteins with known molecular weights was used as molecular weight markers. After separation, the proteins were transferred to immobilon using a semi-dry unit, the membranes stained for 5 min with Ponceau S to visualize molecular weight standards and all was rinsed with water to remove Ponceau S. The blots were incubated in Tris-buffered saline (TBS) containing 3% non-fat dry milk to block non-specific binding sites. Immunological detection of Hsp70 proteins was performed by using a commercial antiserum against Hsp70: clone 3a3 (Affinity BioReagents, Golden, CO, USA) overnight in a 1:5000 dilution in TBS-milk solution. The blots were then reacted with commercial goat anti-mouse antibody conjugated to alkaline phosphatase. The immunocomplex was developed using the *p*-nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate visualization system (Smerdon *et al.* 1995). The bands were quantitated by scanning with a Laser Densitometer (Epson GT, Amsterdam, The Netherlands), and readings were expressed as percentages of controls.

ELISA of Mytilus edulis *lysate samples*

For determination of *Mytilus edulis* Hsp70 levels, the design of the double-antibody sandwich enzyme-immunoassay was applied. For extraction of solubilized Hsp70 antigen from the samples, the solid-phase antibody was used (rabbit anti-Hsp70 serum diluted 1:400 in 0.05 mol litre^{-1} carbonate/biocarbonate buffer, pH 9.6, and incubated at 4°C overnight in wells of the NUNC (Rochester, NY, USA) Immunoplates Maxirosp; the serum was raised in a rabbit using recombinant human Hsp70 (Sigma, St Louis, MI, USA) and preliminary tested for recognition of the mussels Hsp70). After blocking with 2.5% casein (Sigma) for 2 h at room temperature, the microtitre plates were washed with wash buffer containing phosphate-buffered saline (PBS), pH 7.4, supplemented with Tween 20 (0.05% v/v). To each well, 100 µl extracts from *M. edulis* gills was added (before testing, samples were diluted to

the total protein concentration of 4 mg ml^{-1}) After an overnight incubation at 4°C the microtitre plates were washed with wash buffer and incubated for 1 h at room temperature with mouse monoclonal antibody detecting the extracted mussels Hsp70 (BRM-22 clone; Sigma) diluted 1:5000 with PBS-Tween. The second experiment used the mouse monoclonal antibody; the same commercial antibody as in the Western blotting analysis (clone no 3a3). After a further wash with PBS-Tween, the microtitre plates were incubated for 1 h with peroxidase-conjugated goat anti-mouse immunoglobulins (DAKO, Copenhagen, Denmark) in a 1:2000 dilution. The wells were washed four times and then 200 µl orthophenylene diamine (Sigmafast, Sigma) per well were added. After 10 min of incubation, the reaction was blocked with 50 µl 3 mol $litre^{-1}$ sulphuric acid. The absorbance was measured at 492 nm with a Microelisa Reader (Organon-Teknika, Copenhagen, Denmark) and means of triplicate wells were calculated. Results are means ± SD (Karawajczyk *et al.* 1998).

Results and discussion

Results of the Western blotting analysis accompanied by the results of densitometric analysis of blots are presented in figure 1. The results show that in the untreated individuals (controls), freshly collected (lane 2), after acclimatization (lane 3) and after incubation (lane 4), Hsp of apparent molecular weight 70 kDa was recognized. In the animals exposed to cadmium, there is evidence of the increased Hsp70 induction in gills, although the difference is not statistically significant. No difference in Hsp70 levels in the gills of control and test organisms exposed to TBT was detected.

Results of the ELISA tests with two monoclonal antibodies are presented in figure 2. Clear-cut differences are evident between control and test organisms in the exposure experiments, especially in the case of exposure to TBT. The result is noteworthy since TBT induced increases in Hsp70 levels reported in the literature were obtained only after exposing mussels to much higher concentrations of TBT (Lundebye *et al.* 1997).

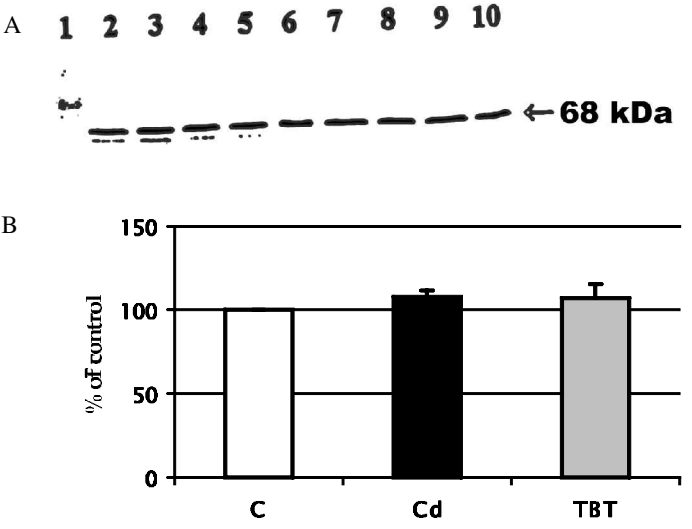

Figure 1. (a) Western blotting analysis of stress proteins in extracts from the gills of *Mytilus edulis*, control and exposed to Cd (50 µg/l, 7 days, $t = 10°C$, $s = 7.0\%$), or TBT (50 µg/l, 7 days, $t = 10°C$, $s = 7.0\%$); 1, molecular weight standards; 2–4, control; 5–7, Cd; 8–10, TBT. The arrow indicates the position of the 68-kDa molecular weight standard. (b) Results of the densitometric analysis of marks on blots expressed as a percentage of control (C); Cd, exposure to cadmium; TBT, exposure to TBT. Error bars indicate 1 SD of the mean calculated from three replicates.

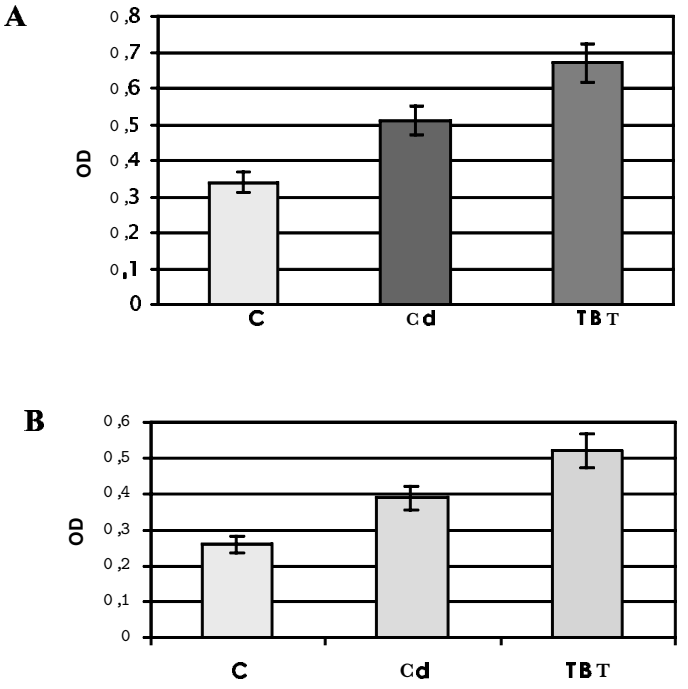

Figure 2. Results of the ELISA analysis of stress proteins in extracts from the gills of *Mytilus edulis* exposed to cadmium (Cd) or tributhyhyltin (TBT) expressed as optical density (OD). Experimental conditions as described in figure 1. C, control; Cd, exposure to cadmium; TBT, exposure to tributhyltin. Error bars indicate 1 SD of the mean calculated from three replicates. (a) Monoclonal antibody: clone no BRM22 (Sigma); (b) monoclonal antibody: clone no 3a3, Affinity BioReagents (Golden, CO, USA).

Statistically significant differences in the densitometric measurements of the Hsp70 obtained by means of Western blotting and ELISA tests in the lysate samples were evaluated by means of a Student's *t*-test. Assuming the acceptance level of the hypothesis that results between various experiments are different at $p \leq 0.05$, the following conclusions can be drawn:

- Applying Western blotting, no differences in levels of Hsp70 in test and control organisms can be detected ($p = 0.151$).
- Applying ELISA (BRM22 antibody) measured levels of Hsp70 in the TBT-exposed organisms are higher than in control ($p = 0.022$); p of the difference between TBT and Cd exposure exceeds the set-up limit of 0.05 ($p = 0.128$), while no statistically significant differences between Hsp70 in Cd exposed organisms and control can be documented ($p = 0.931$).
- The ELISA (3a3 antibody) is also capable of documenting increased levels of Hsp70 in TBT-exposed mussels as compared with control organisms ($p = 0.051$), while no statistically significant differences were found between the control and the cadmium exposed organisms ($p = 0.168$).

High amino acid homologies between the inducible and constitutively expressed Hsp70 isoforms give rise to possible immunochemical cross-reactivities. The background observed in both ELISA and immunoblot experiments might be

due to the cross-recognition of non-inducible Hsp species by the used antibodies. Applying antibodies specific only for the inductive Hsp70 isoform may improve the signal-to-noise ratio. But to our knowledge, at present no such antibodies to mussel Hsp70 are available. In preliminary tests we have found that commercially obtained antibody to human-inducible Hsp72 (SPA-812, StressGen, Canada) is not designed for mussel Hsp70 (data not shown). In this context, two-dimensional immunoblotting seems to be a separation and detection method by which discrimination between particular mussel Hsp species might be possible, even with antibodies cross-reacting with the constitutively expressed protein. Such an approach might circumvent problems arising from the relatively high background.

The present results confirm earlier reports suggesting high sensitivity of Hsp70 induction in *Mytilus edulis* exposed to cadmium (Radłowska and Pempkowiak 1996, 1998). Lundebye *et al.* (1997) reported positive results from SDS/PAGE-Western blotting tests on TBT induction of Hsp70 in *M. edulis*, but only at much higher exposure concentrations of TBT. In the present study, the ELISA test proved more useful in Hsp70 analysis than in SDS/PAGE-Western blotting due to increased sensitivity. This is documented by the increased difference in Hsp70 contents between control and test organisms in experiments with cadmium, and statistically significant differences between control and TBT-stressed organisms.

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