Variability of heat shock proteins and glutathione S-transferase in gill and digestive gland of blue mussel, *Mytilus edulis*

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**Abstract**

Glutathione S-transferase (GST) and heat shock proteins (hsp) 40, 60, 70 and 90 were determined by immunoblotting using actin as an internal control in *Mytilus edulis* from one station outside (site1) and three stations within (sites 2–4) Cork Harbour, Ireland. Comparisons were made between gill and digestive gland and between sites. Gill shows generally higher hsp 60, 70 and 90 while digestive gland has higher hsp 40. Site 1 showed higher gill hsp 40 and 70 than sites 2–4 while gill GST was higher in sites 3 and 4 than 1 and 2. Comparison with sites in the North Sea (site 5: outside Tjärnö in The Koster archipelago in the Skagerack) and Baltic Sea (site 6: Askö island) also revealed lower hsp 40 and 70 in site 6 (low salinity) than site 5 (high salinity) although hsp 60, 70 and 90 were detectable in digestive gland unlike sites 1–4. Previously, only hsp 70 had been studied at these sites [Mar. Environ. Res. 39. (1995), 181]. At the mRNA level, gill hsp 70 is 80-fold higher at Tjärnö than Askö. These data suggest that, while salinity may slightly decrease hsp 40 and 70, both hsp 70 and GST are.

**Abbreviations:** CDNB, 1-chloro-2, 4-dinitrobenzene; GST, glutathione S-transferase; hsp, heat shock protein; SDS PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis

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selectively up-regulated by approx. 10- and 3-fold, respectively, at Tjärnö compared to the other sites which we attribute to exposure to more widely fluctuating pollution levels.

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## 1. Introduction

Heat shock proteins (hsps) are ubiquitous in eucaryotic species, being expressed in highly-conserved families denoted by their apparent $M_r$ on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE; for reviews see; Craig, Weissman, & Horwich, 1994; Getting & Sambrook, 1992; Hartl, 1996; Hofmann, 1999). Among the best-studied are hsps 40, 60, 70 and 90. Though originally discovered as proteins induced by heat treatment, these proteins have since been reported to be up-regulated by a range of other stressors including oxidative stress (Freeman, Borrelli, Meredith, & Lepock, 1999), radiation (Trautinger, Kindás-Mügge, Knobler, & Höngsman, 1996), xenobiotics (Wolfe, Olsen, & Tjeerdema, 1996) and exercise (McArdle, Vasilaki, & Jackson, 2002). Some hsps are expressed constitutively, presumably because they carry out important endogenous functions in cells including acting as chaperones in co- and post-translational protein folding, protein translocation into mitochondria and triggering and control of apoptosis (Craig et al., 1994; Creagh, Sheehan, & Cotter, 2000; Getting & Sambrook, 1992; Hartl, 1996).

Because of their sensitivity to environmental pollutants such as heavy metals, several researchers have quantified hsps 60 and 70 in bivalve sentinel species (Brown, Bradley & Tedengren, 1995; Brown & Lluoma, 1995; Sanders, Martin, Sanders, Phelps, & Welch, 1991; Nelson & Martin, 1993; Werner & Hinton, 1999, 2000). These studies have been carried out in the field (Brown et al., 1995; Brown & Lluoma, 1995; Werner & Hinton, 1999, 2000), in controlled exposure studies in holding tanks (Sanders et al., 1991; Werner & Hinton, 1999) or on archived samples (Sanders & Martin, 1993). In most cases, the methodology used has involved immunoblotting with antibodies raised originally to mammalian proteins, taking advantage of the extensive structural conservation found in hsp families. The usual approach is to create a serial dilution of a particular sample of known protein loading and then to quantify the lowest dilution giving a visible band by densitometry. This measurement depends on accurate and reproducible sample loadings and uniform transfer efficiency during blotting. A somewhat more sophisticated pulse-chase type experiment has been used to quantify radiolabelled hsp70 in *Mytilus californianus* gill slices in a seasonality and acclimatization study in which hsp70 levels were expressed relative to an unidentified 46 kDa protein (Roberts, Hofmann, & Somero, 1997).

The primary purpose of the experiments reported here was to build on previous approaches by using the “housekeeping” protein actin as an internal control to which intensities of hsp bands may be compared. Using this methodology we...
wanted to determine relative levels and normal variation of hsps 40, 60, 70 and 90 in gill and digestive gland of *Mytilus edulis* sampled from Cork Harbour, Ireland, to determine whether other hsps apart from hsp 60 and 70 might vary with pollution status. As well as revealing important differences between the two tissues, our results show modest down-regulation of hsp 40 and 70 within Cork Harbour possibly as a result of low salinity in this estuary. We explored this possibility by comparing samples from sites at Tjärnö (North Sea) and Askö (Baltic Sea) in which hsp 70 alone had been previously studied (Brown et al., 1995). Our experiments suggest that, of the proteins studied, hsp 70 is most variable being approximately 10-fold higher in the Tjärnö (North Sea) site than in sites at either Cork Harbour or Askö (Baltic Sea).

2. Materials and methods

2.1. Materials

Antibodies were either available in our laboratory (GST; Fitzpatrick, Krag, Horup, & Sheehan, 1995) or were purchased (hsps 40, 60, 70, 90 and actin) from Stressgene Inc. (Victoria, BC, Canada). Enhanced chemiluminescence kits were obtained from Amersham-Pharmacia Biotech (Little Chalfont, Bucks, UK). Chemicals for electrophoresis, blotting and other analyses were of analytical grade and obtained from Sigma chemical Co. (Dublin, Ireland).

2.2. Animals and sampling areas

Triplicate groups of five individual animals were sampled in and around Cork Harbour, Ireland (sites 1–4) and at single sites at Tjärnö (North Sea; site 5) and Askö (Baltic Sea; site 6) in Winter of 2001/2 (Fig. 1). Locations of sites were as follows: 51°38′N 8°34′W (site 1), 51°49′N 8°18′W (site 2), 51°52′N 8°23′W (site 3), 51°51′N 8°11′W (site 4) 58°53′N, 11°09′W (Tjärnö; site 5) and 58°49′N, 19°39′W (Askö; site 6). Samples from sites 5 and 6 were frozen on sampling and shipped in dry ice to our laboratory for analysis. After holding at 4°C overnight, gill and digestive glands were dissected and pooled. Tissues were homogenised by polytron in 10 mM Tris/HCl, pH 7.2, buffer containing 500 mM sucrose and 1 mM dithiothreitol. A cell-free extract was collected by centrifugation at 20,000g for 1 h. Supernatants of these extracts were immediately used for electrophoretic analysis by diluting samples directly into sample buffer (1:3; Laemmli, 1970) to achieve a loading of approx. 20 µg protein. The precise loading was later calculated from the Bradford Assay (see below).

2.3. Immunoblotting

Electrophoretic separation was carried out with aliquots of approximately 20 µg protein in 10% (hsps) or 15% (GST) polyacrylamide gels containing SDS (Laemmli,
Proteins were then transferred onto nitrocellulose membranes, non-specific binding sites were blocked with 5% skim milk and washed with appropriately diluted antibodies to the protein of interest (Burnette, 1981). Dilutions used were: 1:1,000 (GST); 1:1500 (hsp 40 and 60); 1:2000 (actin and hsp 70 and 90). Specific bands were revealed by washing membranes with a 1:2000 dilution of anti-rabbit IgG labelled with horseradish peroxidase followed by enhanced chemiluminescence. Blots were then stripped by incubation in 62.5 mM Tris–HCl containing 100 mM 2-mercaptoethanol, 2% SDS, pH 6.7 at 50 °C for 30 min followed by washing with Tris-buffered saline-tween. Membranes were then re-probed with antibodies to actin.

Fig. 1. Sampling area and sampling sites. The North Sea site is located at Tjärnö (site 5) while the Baltic site is located at Askö (site 6).
2.4. Biochemical determinations

GST activity was determined with 1-chloro-2,4-dinitrobenzene (CDNB) by the spectroscopic method of Habig, Pabst, and Jakoby (1974). Protein was estimated by the method of Bradford (1976).

2.5. Quantification of proteins

X-ray films showing positive bands were scanned electronically and bands were quantified using the Scion Image analysis programme (Release beta 4.0.2; Scion Corp., Frederick, MD, USA). This allows representation of each band as a densitometry trace with calculation of peak area corresponding to the band of interest. Three traces were taken across each band and these generally agreed well with each other. The average of the three peak areas was determined for each sample and for each protein. Finally, results for each sampling site were expressed as a ratio to the densitometry area for the actin blot from that sample. Densitometry data expressed as a ratio of each protein studied to total protein actually loaded on electrophoresis gels agreed closely with the ratio to actin suggesting that this protein is appropriate as an internal standard in our samples (not shown).

2.6. Quantification of gill hsp 70 mRNA

Sampled mussels were stored in RNAlater (Ambion, Huntingdon, Cambridgeshire, UK). RNA from parts of one gill (about 2 mg) of every mussel was eluted according to the procedure described in the RNAwiz™ manual (Ambion, Huntingdon, Cambridgeshire, UK). They were homogenised as five groups of three individual mussels so each replicate had mRNA from three individuals. Samples were loaded onto an agarose gel (1%) at 6 μg/lane. Size-fractionated RNA was transferred from the gel to a nylon membrane and mRNA was hybridised to a biotinylated hsp 70 or actin probe according to NorthernMax™-Gly Lit manual (Ambion, Huntingdon, Cambridgeshire, UK). The detection procedure was performed according to BrightStar™ BioDetect™ (Ambion, Huntingdon, Cambridgeshire, UK) and removal of the probe was achieved according to Strip-EZ™ RNA manual (Ambion, Huntingdon, Cambridgeshire, UK). Photographs of membranes were scanned into a computer and analysed using ImageMaster™ 1D Software version 3.0 (Amersham Pharmacia Biotech, Little Chalfont, Bucks, UK). The results are presented as a ratio of hsp 70 mRNA/actin mRNA.

2.7. Statistical analysis

Samples were compared using Kruskal–Wallace ANOVA. Site 1 at Cork Harbour was compared with the three in-harbour stations (sites 2–4) and with the Tjärnö (North Sea) and Askö (Baltic Sea) sites (i.e. sites 5 and 6). These latter sites were also compared to each other.
3. Results and discussion

Relatively little attention has been paid to quantitating hsp60s in the important bioindicator species *M. edulis* apart from hsp60 and 70. Here, with a novel method using levels of actin as a common point of reference, we explore for the first time three interconnected aspects of this group of proteins; (1) inter-tissue differences in hsp expression (2) relative abundance of the four hsp60s (3) inter-site differences in hsp levels.

Previous studies on *M. edulis* hsp60 and 70 have concentrated on immunoblots which have usually been quantitated by densitometric comparison of relative intensity. In the present investigation, we have used the housekeeping protein actin as an internal control allowing us for the first time to compare levels of different hsp60s across all sites by a common yardstick. This procedure avoids serial dilution of each sample (thus increasing the number which can be studied in each electrophoretic separation) and also minimises effects due to minor differences in sample loading and/or transfer efficiency. Notwithstanding this, comparison of our results expressed as a ratio to actin corresponded closely with those expressed as a ratio to protein loading (not shown). Typical immunoblots used for analysis are shown in Fig. 2. SDS PAGE analysis of samples used reveals that very similar protein loadings were used for each sample (Fig. 2).

There appear to be marked inter-tissue differences in expression of the panel of proteins studied in mussels sampled from Cork Harbour, Ireland (Fig. 3; sites 1–4) while inter-tissue differences are less clear in samples from Tjärnö (North Sea) and Askö (Baltic Sea) (sites 5 and 6; Figs. 2 and 3). In sites 1–4, three hsp60s (60, 70 and 90) and GST are expressed at significantly higher levels in gill than digestive gland (*P*< 0.05) while, in the latter tissue, hsp 40 is present at 2-fold higher levels than in gill (*P*< 0.05). Moreover, in gill, relative levels of individual hsp60s are mostly in the order hsp 40 > hsp 60 > hsp 70 > hsp 90 (range of ratios of hsp 40:hsp 60:hsp 70:hsp 90 found in this tissue for sites 1–4 were: 4–8.6:2.5–4.9:1.2–3.4:1). While previous studies in this field have concentrated mainly on hsp60s 60 and 70, our results suggest that hsp 40 is sometimes more abundant than these proteins. Indeed, in our study of sites 1–4, levels of hsps 60, 70 and 90 were not detectable in digestive gland while easily measurable in gill. Using a spectroscopic assay with CDNB, we have previously demonstrated that GST is usually expressed 2–3-fold more highly in gill than in digestive gland (Fitzpatrick, Sheehan, & Livingstone, 1995). Immunoblotting of GST (Figs. 2 and 3) agrees with these and other CDNB assay data (Fitzpatrick, O’Halloran, Sheehan, & Walsh, 1997; Fitzpatrick, Sheehan, & Livingstone, 1995) providing important confirmation of our overall methodology.

*M. edulis* is commonly used as a bioindicator organism and several previous studies have suggested that levels of biomarker proteins such as GST or hsp60s 60 and 70 vary in polluted sites compared to unpolluted ones (Brown et al., 1995; Brown & Lluoma, 1995; Fitzpatrick et al., 1997; Fitzpatrick, Sheehan & Livingstone, 1995; Sanders & Martin, 1993; Werner & Hinton, 1999). Our data for sites 1–4 indicate that hsps 40 and 70 are significantly higher in samples from the out-harbour station (site 1) compared to all three in-harbour stations (sites 2–4; *P*< 0.05). In a recent
Fig. 2. A. Immunoblots of hsps and GST in *M. edulis*. Representative results for (a) gill and (b) digestive gland pooled from groups of five individual animals are shown. In each case, samples from sites 1–6 were probed with antibodies to actin, hsps 40, 60, 70 and 90 and to GST Pi. Note that four groups were analysed for actin, hsp 40 and 60 in gill while determinations were performed in all other cases on three groups. ND, not detected. In practice, blots for each protein analysed were stripped and reprobed with antibodies to actin as described in Section 2. B. SDS Polyacrylamide gel electrophoresis analysis of *M. edulis* extracts stained with coomassie blue R-250 from (a) gill and (b) digestive gland. Approx 50 µg protein loadings were analysed on 15% gels. *M* denotes molecular weight markers and samples were loaded in the same order as in panel 1. *M* values (kDa) are shown for left-hand tracks. Note that four samples were analysed from sites 6 and 2.
Fig. 3. Levels of hsp50 and GST as a ratio to actin (arbitrary units) determined by immunoblotting. *
Significantly different to Site 1 ($P < 0.05$). †Significant difference between Tjärnö (site 5) and Askö (site 6).
study (Widdows et al., 2002), it was suggested that stress on mussels along the South Irish Coast revealed by scope for growth measurements may be due to untreated sewage from population centres such as Cork City which flows into Cork Harbour. Levels of chemical contaminants were found to be relatively low in these animals which might conceivably cause down-regulation of certain hsps such as hsps 40 and 70.

Alternatively, low salinity levels within Cork Harbour might selectively down-regulate the proteins. Previous investigations in other sites certainly point to salinity-mediated down-regulation of hsp 70 in bivalves. A comparison of \textit{M. edulis} hsp 70 between Tjärnö (North Sea) and Askö (Baltic Sea), suggested that the latter sea’s lower salinity might also be responsible for lower hsp 70 levels associated with greater sensitivity to cadmium toxicity at Askö (Brown et al., 1995). Recent work suggests that this difference is unlikely to be due to genetic differences between Baltic Sea and North Sea mussels (Riginos, Sukhdeo, & Cunningham, 2002) but most probably reflects adaptation to habitat (Tedengren, Olsson, Bradley, & Zhou, 1999; Tedengren, Olsson, Reimer, Brown, & Bradley, 1999). Both field and holding tank studies also found hsp 70 to be down-regulated in the pacific clam \textit{Potamocorbula amurensis} in a salinity gradient in San Francisco Bay (Werner & Hinton, 1999). Our findings with sites 1–4 raised the possibility that low salinity in Cork Harbour might also be responsible for down-regulation of hsps 40 and 70 with associated up-regulation of GST in \textit{M. edulis} gill.

To test this hypothesis we measured hsps and GST in mussels from Tjärnö (North Sea; site 5) and Askö (Baltic Sea; site 6) in which hsp 70 only had previously been studied (Brown et al., 1995). We reasoned that we would expect samples from the Baltic to display elevated GSTs if low salinity indeed caused up-regulation of this protein. Salinity data for five of our six sites summarised in Table 1 show that Askö is significantly less saline than Tjärnö and that these values are generally similar, respectively, to those inside and outside Cork Harbour where salinity can vary from as low as 5.9 parts per thousand at the top of the estuary to 33 parts per thousand at the harbour mouth (Environmental Research Unit Report, 1989). Immunoblotting determined that both hsp 70 and GST are higher ($P < 0.005$) in digestive glands sampled from the Tjärnö site (North Sea) than those from the Askö site (Baltic Sea) while gill hsp 70 is also higher in Tjärnö samples than those from Askö ($P < 0.005$; Figs. 2 and 3). The latter conclusion is supported by the finding that the hsp70 mRNA/actin mRNA ratio is approximately 80-fold higher in gills of animals from Tjärnö compared to those from Askö. Though differences in gill GST immunoblots were not statistically significant between these sites, enzyme activity with CDNB as substrate showed significantly higher levels in samples from the Tjärnö site (North Sea; mean specific activity: 45.82 ± 7.4 U/mg) than in those from Askö (Baltic Sea; mean specific activity: 16.3 ± 1.16 U/mg; $P < 0.005$). These findings suggest that GST and hsp 70 are controlled by distinct processes and that, while salinity appears to play some role in regulation of hsps 40 and 70, GST levels are not simply up-regulated as a result of low salinity.

Comparison of data from sites 1–4 on the one hand with sites 5 (Tjärnö) and 6 (Askö) on the other shows that gill hsps 70 and 40 are higher in the Tjärnö site (site
than either sites 1 or 6 (Fig. 3; \( P < 0.05 \)). Moreover, in contrast to sites 1–4, all four hsps are detectable in digestive gland from the Tjärnö and Askö sites (Figs. 2 and 3). Possible reasons for these differences include differing nutritional and reproductive statuses, lower water temperatures in sites 5 and 6 and variable exposure to pollutants from mountain run-off associated with the Tjärnö/Askö sites resulting in generally elevated hsps at sites 5 and 6 compared to sites 1–4.

Modest down-regulation of both hsp 40 and 70 in gill at sites 2–4 compared to site 1 is particularly interesting since these two proteins are known to work in concert as co-chaperones while also possessing individual chaperone activities (Craig, Weissman, & Horwich, 1994; Getting & Sambrook, 1992; Hartl, 1996; Quian, Hou, Zhengang, & Sha, 2002). However, the 2-fold higher levels of hsp 40 in digestive gland under conditions where hsp 70 is undetectable suggests that hsp 40 may be functionally independent of hsp 70 in this tissue. It should also be noted that there is not quite an inverse correlation between gill hsp levels and amounts of GST because site 2 has similar GST levels to site 1 but significantly lower hsp 40 and 70 in digestive gland (and hsp 40 in gill). This analysis provides additional evidence that GSTs on the one hand and hsps on the other are most likely varying due to distinct control processes.

In bivalves, the gill is the main feeding organ and we have suggested previously that this may explain high GST activities in this tissue (Fitzpatrick, Sheehan, & Livingstone, 1995). By contrast phase I detoxification enzyme activities are preferentially expressed in the digestive gland of mussels (Livingstone & Farrar, 1984). It is possible that the generally higher levels and complexity of expression pattern for hsps 60, 70 and 90 in gill may be connected with the fact that many xenobiotics are first encountered by Mytilus in this tissue. It has been reported that hsp 70 in gill is far more inducible in response to heat treatment than in either adductor muscle or mantle tissue which has been attributed to the physiological function of this tissue (Chapple, Smerdon, & Hawkins, 1997).

We have previously argued that extraneous factors such as seasonality, feeding and reproductive status can complicate use of proteins as biomarkers of pollution (Sheehan & Power, 1999). Recent work with mussels has suggested that hsp70 is subject to complex variation in response to spatial and temporal factors such as
existence of microhabitats, thermal history and seasonal temperature variations (Chapple, Smerdon, Barry, & Hawkins, 1998; Hofmann, 1999; Minier, Borghi, Moore, & Porte, 2000; Helmuth & Hofmann, 2001; Buckley, Owen, & Hofmann, 2001). Our present study suggests that, while salinity effects may be a factor in interpreting field studies of hsps 40 and 70 from mussels sampled in marine estuaries, the magnitude of variation (approximately 0.5-fold decrease in low salinity) is lower than that found for hsp 70 in a site experiencing a wider range of pollution (e.g. approximately 10-fold short-term increase in heavy metals at the North Sea site at Tjärnö). Levels of hsp 70 would therefore seem to be especially sensitive compared to other hsps.

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References


