Metallothionein Levels in the Bivalves *Callista chione and Venus verrucosa* from Two Mediterranean Sites

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Metallothioneins levels (MTs) in the clams *Callista chione* and *Venus verrucosa*, collected from two coastal sites in Greece, were determined and quantified by SDS polyacrylamide gel electrophoresis (SDS-PAGE) and a spectrophotometric assay (Ellman's reaction). SDS-PAGE separation in the digestive gland, which represents the hepato-pancreas in clams, demonstrated the presence of MTs similar to mammalian MT (rabbit liver Cd, Zn-thionein). No other SH-containing proteins apart from the MTs were detected. MT levels quantified by the Ellman's reaction indicated seasonal variation for both species. The highest values were recorded in the spring and the lowest in the autumn. The seasonal variation and the differences in the MT levels of the two areas seem to be related to the reproductive cycle of the organisms as well as to abiotic factors of each area. Our results show that both *C. chione* and *V. verrucosa* have the potential to be used as biomarkers of metal pollution, provided that the influence of the external factors is safely quantified.

Introduction

In the last decades there has been considerable scientific effort to elaborate biological mechanisms or biomarkers in order to assess and monitor various contaminants in the marine environment (Kramer Kees, 1994). Among those, metallothionein induction has been suggested as a biomarker of metal pollution in ecotoxicological and biomonitoring studies (George and Olsson, 1994). Metallothioneins (MTs) are low-molecular weight metal-binding proteins (6–7 kD), first discovered in the horse kidney cortex (Margoshes and Vallee, 1957). Later, they were described in a large number of animal species including mammals, reptiles, amphibians, invertebrates, plants and microorganisms (Hamer, 1986; Maroni, 1990; Riordan and Vallee, 1991; Carpene, 1993). In aquatic invertebrates MTs have been identified in approximately 50 different species, the majority of which are mollusks and crustaceans. MTs possess a high proportion of cysteine residues (30% of total amino acids) placed in specific sequences and classified into three classes according to their homology with mammalian. MTs in bivalves generally belong to class I (Roesijadi, 1992). Their role, though still controversial has been connected with the homeostasis of essential metals like Cu and Zn, detoxification of toxic metals like Cd, Ag and Hg and protection against free radicals (Roesijadi, 1992).

The use of MTs as biomarkers of metal pollution comes up against the problem of the complexity of mechanisms regulating their biosynthesis which is influenced by a wide range of other factors like hormones, second messengers, cytotoxic agents and physical stress (Hamer, 1986; Kägi, 1991; Gerpe *et al.,* 2000). The necessary criteria for species selection as models for metal pollution have truly been met only for a few fish and mussels, for other species either too much interference and competing sequestration systems are present or MTs studies have not been accomplished yet (George and Olsson, 1994).

In the present study we investigated the presence of MTs in the clams *Callista chione* and *Venus verrucosa*. These species are quite common in most of the Mediterranean coastal areas. They are found in sand, gravel and mud sea bottoms, down to depths of 30 m (Poppe and Goto, 1993). Both species sustain commercial fisheries in some areas of the Mediterranean basin such as the southern

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Adriatic and the Aegean Sea. Detailed studies on their biology are scarce. There is some information on the reproductive cycle of *V. verrucosa* from the south Adriatic and Thermaikos gulf (Marano *et al.,* 1982; Galinou-Mitsoudi *et al.,* 1997) and *C. chione* from the Gulf of Trieste (Valli *et al.,* 1984), but data on metal-binding proteins of these species is lacking. The objectives of this study were to identify and quantify MT levels in these species and compare the MT levels from the two areas. Two complementary approaches were adopted: identification of MTs using SDS polyacrylamide gel electrophoresis (SDS-PAGE) and quantification using a spectrophotometric assay (Ellman's reaction).

Materials and Methods

Specimens of *C. chione* and *V. verrucosa* were hand collected by scuba divers and were brought alive to the laboratory. Collection areas were the Gulf of Elefsis (Neraki) and the Gulf of Chalkis (Chalia). Care was taken to use individuals of the same size, since size roughly represents the age of the organisms. The specimens from each area were found no more than 100 meters apart from each other. The MT levels were measured in the digestive gland of the species based on an adapted protocol for estimation of metallothioneins in marine invertebrates (Viarengo *et al.,* 1997). All analytical grade reagents were acquired from Merk, except those used for the electrophoresis which were from BioRad. The bromobimane (B-4380), the rabbit liver Cd, Zn-thionein (M-7641) and the RNA (R-7125) were purchased from Sigma.

Sample preparation

All specimens were rapidly dissected. Their digestive gland was removed and samples of 1 g (pool of 6–7 glands) were homogenized in a Potter-Teflon homogenizer in three volumes of 0.5 M sucrose, 20 mM Tris-HCl (pH 8.6) containing 0.006 mM leupeptine, 0.5 mM phenylmethylsulphonylfluoride (PMSF) and 0.01% β-mercaptoethanol. The homogenates were centrifuged at 30,000×*g* for 20 min to obtain a supernatant containing the MTs. The supernatants were treated with ethanol/chloroform solution (Kimura *et al.,* 1979). To aliquots of 1 ml supernatant 1.05 ml of cold (−20 °C) absolute ethanol and 80 (µl of chloroform were added. The samples were then centrifuged at 6,000×*g* for 10 min at 4 °C. Three volumes of cold absolute ethanol were added to the collected supernatant. Exceptionally, 1 mg of RNA with 40 µl 37% HCl was added to the supernatants used for the SDS-PAGE electrophoresis (Viarengo *et al.,* 1997). All samples were maintained at −20 °C for 1 h, then they were centrifuged at 6,000×*g* for 10 min at 4 °C. The supernatants were discharged and the metallothionein-containing pellets were washed with a cold (−20 °C) solution containing ethanol, chloroform and homogenization buffer (87:1:12, v/v). A centrifugation at 6,000×*g* for 10 min followed and the pellets were dried under nitrogen gas.

SDS-PAGE separation

The pellets were re-suspended with 50 µl of a solution containing 5 mM Tris, 1 mM EDTA, pH 7. To these, 50 µl of 12 mM fluorescent compound thiolyte were added. Thiolyte was freshly prepared from a 92 mM bromobimane stock solution in acetonitrile. The samples were then incubated in the dark at room temperature for 30 min. A volume of 100 µl 4% SDS was added to the samples. After incubation in a water bath at 37 °C for 30 min, 200 µl of glycerol were added and the samples were stored at −20 °C for one week before the electrophoresis. The Thiolyte-labelled metallothioneins were separated by 10% SDS polyacrylamide gel electrophoresis (Laemmli, 1970) utilizing 0.025 M Tris pH 8, 0.2 M glycine and 0.1% SDS as an elecrophoresis buffer. For the electrophoresis we used the PROTEAN II xi Cell apparatus of BIO-RAD set at 80 Volts for the first 15 minutes and at 150 Volts for the following 3:45 hours. After electrophoresis, the gel was maintained in a solution of methanol:acetic acid:water (45:10:45 v/v). The fluorescence of the protein bands in the gel was evidenced with an UV transilluminator and photographed on a positive/negative Polaroid 667 film using a Polaroid DS-34 camera.

Spectrophotometric assay (Ellman's reaction)

The pellets were re-suspended in 300 µl of 5 mM Tris-HCl buffer, 1 mM EDTA, pH 7. A volume of 4.2 ml DTNB (5,5-dithiobis-2-nitrobenzoic acid) in 0.2 M Na-phosphate buffer, pH 8 was added (Ellman, 1958). The MT content was evaluated spec-

trophotometrically at 412 nm and the metallothionein concentration was estimated utilizing reduced glutathione (GSH) as the reference standard. The amount of metallothionein was calculated assuming an arbitrary SH content of 21 SH/mole with a molecular weight of 7 kDa (Mackay *et al.,* 1993).

Statistical analysis

The statistical analysis was carried out using SPSS (1996). All analyses were performed in four replicates from pooled tissue of 6–7 glands and the results reported as mean values ± SD. The statistical significance ($P<0.05$) of variation of MTs as function of season, species and area was assessed by one-way and two-way ANOVA (Dunnett test).

Results

Figure 1 shows the UV fluorescence bands of thiolyte-labelled MTs (separated by SDS 10% polyacrylamide gel electrophoresis) from purified rabbit liver Cd, Zn-thionein (A), from the digestive gland (ethanolic extracts) of *C. chione* and *V. verrucosa* (B, D), from the ethanolic extracts of *C. chione* and *V. verrucosa* after addition to rabbit liver Cd, Zn-thionein (C, E), and from the bovine serum albumin (H). The MTs extracted from the clam digestive glands show a relative mobility on the gel similar to that of the mammalian standard MT (rabbit liver MT). Known amounts of mammalian Cd, Zn-thionein (5 μl) added in clam samples increased the fluorescence of bands C and E. There are no fluorescent bands in the gel between the metallothionein bands and that of bovine serum albumin (H).

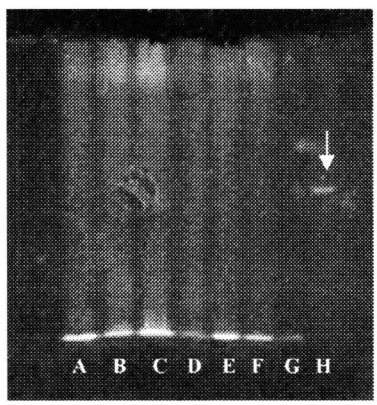

Fig. 1. UV fluorescence bands of thiolyte-labelled MTs separated by SDS-PAGE 10%.
(A) 10 μl of rabbit liver Cd, Zn-thionein, (B) 10 μl of ethanolic extracts of *C. chione*, (C) mixture of 5 μl of rabbit liver thionein added to 5 μl extracts of *C. chione*, (D) 10 μl of ethanolic extracts of *V. verrucosa*, (E) mixture of 5 μl of rabbit liver thionein added to 5 μl extracts of *V. verrucosa*, (F) 10 μl of rabbit liver Cd, Zn-thionein, (G) 5 μl of rabbit liver Cd, Zn-thionein, (H) 10 μl of bovine serum albumin. The arrow indicates the band for bovine albumin.

MT levels of *C. chione* and *V. verrucosa* quantified by the Ellman's reaction indicated peaks in spring (late April – early May) and in winter (middle February), while the lowest levels were found in autumn (middle October) (Fig. 2). The difference between the highest and lowest levels reached almost a factor of 2 for *C. chione* and 1.5 for *V. verrucosa.*

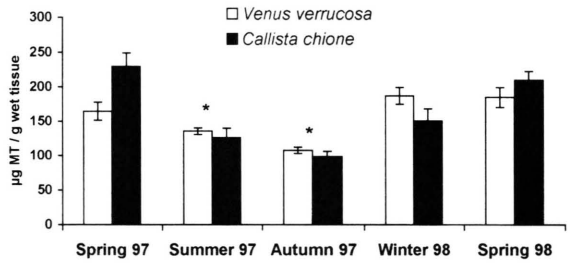

Fig. 2. Seasonal variation of the MT content in the digestive gland of V. veruccosa and C. chione. Metallothioneins were evaluated by spectrophotometric method utilizing GSH as reference standard. * Indicates significant difference at the $p<0.05$ level.

Similar seasonal variation pattern was found to exist in both investigated sites (Figs 3a and 3b). A significant difference, however, was observed between the two areas in spring of 1998 where MT levels in the Gulf of Elefsis were 100 μg/g wet tissue higher than those of Chalkis Gulf for both species.

Discussion and Conclusions

The electrophoretic method allowed the identification of MTs in the ethanolic extracts of *C. chione* and *V. verrucosa* digestive glands (Fig. 1). No other SH-containing proteins different from MTs were present in the ethanolic extracts. This was confirmed by the fluorescent bands of proteins present in the control (A) and the ethanolic extracts. In the ethanolic extracts there were no

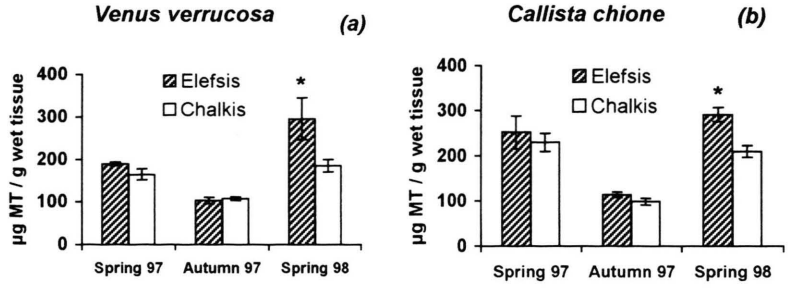

Fig. 3. MT content in the digestive gland of V. verrucosa (a) and C. chione (b) collected from the Gulfs of Elefsis and Chalkis (Eastern Mediterranean, Greece). * Indicates significant difference at the $p<0.05$ level.

other protein bands with higher molecular weight than that of the control. Therefore, the isolation of MTs in our samples was successful as under oxidizing conditions the large number of SH groups present in MT is possible to form disulfide bridges resulting in attachment of MT molecules to other MT molecules or to other proteins (Minkel *et al.*, 1980). Besides, since we analyzed MTs on two new species for which no information was available, it was important to reconfirm our results with the Ellman's reaction (Viarengo *et al.*, 1997). The SDS polyacrylamide gel demonstrated also that the fluorescence of the bands increased proportionally with the metallothionein content (Fig. 1).

The quantification of MT levels by the Ellman's reaction showed seasonal variation in both species (Fig. 2). Even though similar results were found with respect to seasonal variation for the two areas, there was a significant difference in MT levels in the spring 98 (Figs 3a and 3b). That difference could be related to the different state of metal pollution in the two areas. However, sedimentology and geochemistry studies based on the "Index of Geoaccumulation" (I_{geo}) have characterized the two areas of similar state of metal pollution (Karageorgis *et al.*, 1997; Anagnostou and Sioulas, 1997). Hence, we consider that factors other than the presence of metal pollution influenced the MTs differences between the two areas in the spring of 1998.

Besides metals, hormones are frequently cited as other factors able to induce MTs biosynthesis, especially in relation to the reproductive stages. In other species seasonal variation in MT concentrations has been correlated with the reproductive cycle of the organisms and maximum values have been coincided with the gonadal maturation period (Viarengo *et al.*, 1997; Baudrimont *et al.*, 1997). Digestive gland represents a significant site for biosynthesis of MT proteins according to its volume and is directly or indirectly linked to the gonad, via hormonal secretion. Additional factors like temperature, salinity, oxygen depletion and degree of transparency have been reported to interfere with the reproductive cycle of the species (Galinou-Mitsudi *et al.*, 1997). Therefore, we believe that the observed seasonal variations and the differences between the two areas are related to the reproductive activity of the species in each area. The variation of the MTs is in agreement with the reported reproductive cycles of *V. verrucosa* and *C. chione* (Marano *et al.*, 1982; Arneni *et al.*, 1998). These results suggest that the seasonal metabolic changes in *C. chione* and *V. verrucosa*, at certain periods of the year, have to be taken into consideration if these organisms are to be used as biomarkers of metal pollution. Also, is essential that the abiotic factors are carefully monitored in order to distinguish the influence of natural fluctuations from metal pollution.

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