Antioxidant enzyme activities and lipid peroxidation in *Mytilus galloprovincialis* from the French Mediterranean coast

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Abstract

In the present work we have studied some of the indicators of oxidative damage of the digestive gland tissue of two populations of mussels *Mytilus galloprovincialis*: native mussels cultured in an aquaculture farm and contaminated mussels collected from the polluted marine area of the French Mediterranean coast located nearby Tamaris and La Seyne-sur-mer – small towns in close proximity to Toulon.

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Activities of antioxidant enzymes catalase (CAT) and glutathione peroxidase (GPx) were analyzed, as well as the amounts of glutathione (GSH) and malonyldialdehyde (MDA). Glutathione is the most abundant low molecular cellular antioxidant, while malonyldialdehyde is the main product of lipid peroxidation. Additionally, the activity of lactate dehydrogenase-LDH, a marker of cell membrane damage, was determined. We have found an elevated activity of CAT and GPx in mussels from the contaminated areas - 161.76 and 45.36 U mg\(^{-1}\) protein, respectively vs. 98.68 and 29.84 U mg\(^{-1}\) protein in the control mussels. The contaminated mussels also showed an elevated content of MDA – 9.32 nmol mg\(^{-1}\) protein) vs. 7.57 nmol mg\(^{-1}\) protein in the control glands. The concentration of heavy metals (Fe, Mn, Zn, Cu, Ni, Pb, Cd) in the digestive gland, except lead, was significantly higher in mussels from the polluted area. Contrarily, the level of GSH in contaminated mussels was about two-fold lower than that in the control mussels – 2.85 µmol GSH mg\(^{-1}\) protein vs. 5.81 µmol GSH mg\(^{-1}\) protein. At the same time, no significant differences in LDH activity were observed between the two mussel populations.

INTRODUCTION

Marine organisms, such as mussels and fish, have been successfully used as biomarkers in evaluating the biological effects of chemical pollutants. The mussels *Mytilus galloprovincialis* are widely distributed in meso- and eutrophic waters of the Mediterranean Sea. They are common in harbors and also in open waters, where they may characterize stable “facials” on the rocky substratum from 0 to 5 m deep (Ardizzone et al. 1996). Due to their widespread distribution and ability to accumulate organic and toxic compounds, mussels have long been used as sentinel organisms for indicating the levels of pollutants in the coastal environment (Charmasson et al. 1999). The measurement of cellular and subcellular responses to chemical contaminants in marine organisms allows the detection of early biological effects of their exposure to toxic anthropogenic chemicals. Many pollutants are known to enhance the formation of reactive oxygen species (ROS) (Gomez-Mendikute, Cajaraville 2003). Both antioxidant enzymes, such as superoxide dismutase, catalase and glutathione peroxidase, and low molecular weight antioxidants, such as glutathione, ascorbate and \(\alpha\)-tocopherol, belong to the cellular antioxidant system that counteracts the toxicity of reactive oxygen species (Santovito et al. 2005). Variations in antioxidant enzyme activity, the GSH level and lipid peroxidation have been demonstrated in several studies as biomarkers of pollutant-mediated oxidative stress (Porte et al. 1991, Sara et al. 1997).

The aim of the present work was to compare the level of some commonly accepted biomarkers of oxidative stress and cell damage (Sole et al. 2008) in the tissue of native and contaminated mussels. We investigated the activities of catalase, glutathione peroxidase and lactate dehydrogenase, the level of glutathione and lipid peroxidation, and the concentration of heavy metals in the digestive glands of native mussels, cultured in an aquaculture farm and in contaminated mussels collected from the polluted area of the French
Mediterranean coast – an old shipyard, continuously exposed to anthropogenic impact coming from the coastal marine area nearby the towns of Tamaris and La Seyne-sur-mer, located in the south of France, nearby Toulon.

**MATERIALS AND METHODS**

**Sampling**

Ten native (control) mussels of 10-15 mm shell length were obtained from the aquaculture farm close to Baie du Lazaret. Contaminated mussels (10 samples) were collected by snorkeling at two different sites of the harbor of La Seyne sur mer, situated on the French Mediterranean coast (Fig. 1). This is an area of industrial and urban activity (an old shipyard was located there), where the water is confined and considered highly polluted, mainly by heavy metals.

The subcellular fraction of the digestive gland was obtained by homogenizing the tissue in 1:9 (tissue weight: solution volume) ratio in 1.15% KCl in the ice bath. The homogenate was then centrifuged at 16000 × g at 4°C for 10 min in order to obtain the cytosolic fraction.

In all performed experiments, the digestive gland of each of the collected mussels was studied separately.

**Mineralization of tissue**

The digestive glands were prepared from ten native mussels (the control group) and ten mussels collected from the contaminated area (the shipyard). The fresh tissue was dried to dry residue and stored in the perchloric acid/nitric acid mixture (v/v, 1:2) at room temperature for 24 hours and then mineralized by boiling for 12 hours until the appearance of white mist.

**Determination of the protein concentration**

The protein concentration was evaluated by the spectrophotometric method of Lowry et al. (1959) using Folin’s reagent. The protein concentration was calculated on the basis of the calibration curve performed for different concentrations of bovine albumin as a standard.

**Measurement of catalase activity**

The activity of catalase was measured spectrophotometrically according to Beers and Sizer (1952). The method is based on the decomposition of hydrogen peroxide, which is indicated by a decrease in absorbance. The assay mixture consisted of homogenate, Na/K phosphate buffer (50 mM, pH 7.0) and hydrogen peroxide (0.1%) in the final volume of 3 ml. The absorbance was read
at 240 nm. Catalase activity was calculated as µmol H₂O₂ consumed per minute per milligram of cellular protein.

**Measurement of glutathione peroxidase activity**

The activity of glutathione peroxidase was measured using the spectrophotometric method described by Sedlak, Lindsay (1968). Cell lysate was mixed with Tris-HCl buffer (50 mM, pH 7.6), glutathione and cumene hydroperoxide as a substrate. After 5 min incubation at 37°C, the enzymatic reaction was stopped by adding an ice-cold solution of 20% trichloroacetic acid. Then, the assay mixture was centrifuged at 3000 × g for 10 min and DTNB solution (5′,5′-dithio-bis (2-nitrobenzoic acid) was added. The absorbance was

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*Fig. 1. Location of sampling stations S1 (uncontaminated, control mussels) and S2 (contaminated mussel).*
read at 412 nm. The enzymatic activity was expressed as µmoles of glutathione converted per minute per milligram of cellular protein.

**Measurement of lactate dehydrogenase activity**

Lactate dehydrogenase activity was measured by the method of Wroblewski, La Due (1955). The relative activity of the enzyme was determined from the rate of oxidation of NADH to NAD⁺. The reaction was carried out in the sodium phosphate buffer, 0.1 M, pH 7.4, in the final volume of 3 ml containing 0.1 mM of NADH, 0.8 mM of sodium pyruvate and homogenate. Before starting the enzymatic reaction by the addition of the substrate (sodium pyruvate), the mixture was incubated at room temperature for 20 min.

The formation of NAD⁺ was estimated by the measurement of absorbance at λ=340 nm for 5 min. The tissue LDH activity was expressed in U mg⁻¹ protein.

**Determination of glutathione concentration**

The level of reduced glutathione was determined according to the method of Ellman et al. (1961). Homogenates were deproteinized by addition of 25% TCA to the final 3% concentration and centrifuged (600 × g for 10 min). Phosphate buffer (0.5 M, pH 8.0) and DTNB (5,5’-dithio-bis(2-nitro-benzoic acid)) (5 mM) were added to the translucent supernatant (1:0.1:1). The formation of 5-thio-2-nitrobenzoic acid, which is proportional to GSH concentration, was monitored at 412 nm. The concentration of glutathione was calculated using 5-thio-2-nitrobenzoic acid extinction coefficient 13.6 mmol⁻¹ cm⁻¹ and expressed as mmol mg⁻¹ protein.

**Determination of thiobarbituric reactive substances**

Thiobarbituric acid was used to estimate cellular lipid peroxidation. The amount of thiobarbituric acid reactive substances was measured spectrophotometrically at 532 nm (Stocks, Dormandy 1971) with modification of Rice-Evans et al., (1991). Homogenates were mixed with 20% TCA, 2% BHT in ethanol and 0.37% TBA in 0.25 mol l⁻¹ HCl (1:1:0.0075:1). Samples were boiled at 100°C for 10 min and then centrifuged (600 × g for 10 min). The absorbance of supernatant was measured at 532 nm. Lipid peroxidation was calculated in nmol MDA per mg protein, using MDA extinction coefficient 156 mmol⁻¹ cm⁻¹.

**Determination of heavy metals**

The analysis of metals (Fe, Mn, Zn, Cu Pb, Cd, Ni) was performed in an atomic absorption spectrophotometer Varian Spectra A300/400. Estimation of
heavy metal concentrations was performed in an electric atomizer (GTA 96) in a graphite cuvette. The concentration of metals was expressed in µg g⁻¹ of tissue dry weight.

**Statistical analysis**

The results were expressed as mean ±SD and statistical significance of differences was evaluated using the Student’s t-test.

**RESULTS**

We have found a substantial difference in the values of all estimated biomarkers (except the activity of lactate dehydrogenase) between native and contaminated mussels. The activity of catalase was significantly higher in contaminated mussels (161.76 U mg⁻¹ protein, p<0.01) than in the control mussels (98.68 U mg⁻¹ protein) (Fig. 2A). Similar changes were observed for glutathione peroxidase activity – 28.94 U mg⁻¹ protein in the control organisms and 45.36 U mg⁻¹ protein in contaminated organisms, p<0.05 (Fig. 2B). The activity of lactate dehydrogenase was similar in both control and contaminated mussels (Fig. 2C). The glutathione level, however, was lower in contaminated organisms (2.85 µmol GSH mg⁻¹ protein, p<0.05) than in the control ones (5.80 µmol GSH mg⁻¹ protein), (Fig. 2D) in contrast to lipid peroxidation. The MDA content, which is a useful indicator of lipid peroxidation and correlates well with the degree of oxidative stress was enhanced in contaminated mussels (9.32 nmol MDA mg⁻¹ protein, p<0.05), (Fig. 2E).

In Table 1, there are presented concentrations of heavy metals in digestive glands of mussels. Generally, the concentrations of heavy metals in digestive glands, except lead was significantly higher in mussels from the polluted area of La Seyne sur mer as compared to the unpolluted area of Lazaret Bay.

**DISCUSSION**

In this study, we examined the effect of environmental pollution, mainly by heavy metals present in the seawater in the old shipyard area, on oxidative stress and its defense in mussels *Mytilus galloprovincialis*. Activities of glutathione peroxidase, catalase, lactate dehydrogenase and the amount of reduced glutathione were estimated as the most important endogenous antioxidants involved in the cellular antioxidant defense. The MDA content, the main product of lipid peroxidation, was measured as an indicator of cellular oxidative stress. In physiological processes, molecular oxygen is reduced in tetravalent reaction to water in the production of ATP. In the respiratory chain, however,
one electron reduction of molecular oxygen produces the superoxide anion radical. Spontaneous dismutation of this radical both on enzymatic (superoxide dismutase) and non-enzymatic ways leads to formation of hydrogen peroxide ($H_2O_2$, bivalent reduced state). In normal conditions (unpolluted environment), there exist a delicate balance between the production of reactive oxygen species and their detoxication. In the presence of heavy metals (Fe, Cu, Co, Ni, Cr, Ti
etc.), hydrogen peroxide is reduced to highly reactive hydroxyl radicals (Fenton reaction) (Borg, Schaich 1984; Halliwell, Gutteridge 1986). In the presence of these metals hydrogen peroxide can be reduced by the superoxide anion radical to the hydroxyl radical (Haber-Weiss reaction). In normal conditions, this reaction is kinetically very slow, but in the presence of traces of heavy metals it is an important source of the hydroxyl radical. Oxygen free radicals and other ROS molecules, such as singlet oxygen, hydrogen peroxide, hypochlorite acid, peroxynitrite, nitrogen dioxide and other, have the potential to cause damage to biomolecules, such as lipids, proteins and DNA.

The utility of mussels as sentinel organisms for monitoring the trace metal pollution in coastal environments has been established in many studies (Viarengo et al. 1990; Regoli, Principato 1995; Lionetto et al. 2003). Several classes of pollutants, such as heavy metals and organic compounds enhance oxidative stress in marine organisms (Linvingstone et al. 1990; Gomez-Mendikute, Cajaraville 2003). A higher level of reactive oxygen species increases lipid peroxidation and activity of antioxidant enzymes and decreases the GSH level (Porte et al. 1991, Sara et al. 1997). The toxicity of ROS can be mitigated by free radical scavengers, such as glutathione, ascorbic acid, α-tocopherol and by antioxidant enzymes. Glutathione peroxidase is of central importance to mammals in detoxifying hydrogen peroxide and lipid hydroperoxides, as its activity is more efficient as compared to the other key antioxidant enzyme, catalase, which also removes H₂O₂. We have found considerable differences in CAT and GPx increase of their activities between mussels from polluted and unpolluted sites. The increase in the activity of both enzymes was found in contaminated mussels. The comparison of enzyme distribution between the tissues, revealed a marked difference for catalase. Its activity in the digestive gland was greater by more than an order of magnitude as compared to that in gills. Similarly to the data reported by Winston et al. (1990) for Mytilus edulis, we obtained comparable activities of glutathione peroxidase and higher values for the catalase activity. Wenning et al. (1988),

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<th>Fe</th>
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<td></td>
<td>434.1 ±6.1*</td>
<td>12.1 ±1.8*</td>
<td>423.1 ±29.3*</td>
<td>32.8 ±1.7*</td>
<td>21.3 ±1.1*</td>
<td>3.7 ±1.1</td>
<td>6.6 ±2.1*</td>
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Table 1

Heavy metal concentrations (µg g⁻¹ of dry tissue weight) in the digestive gland of Mytilus galloprovincialis from both the control and contaminated area (bold), (p<0.05*).
described a significant increase in the catalase activity in the digestive gland of the ribbed mussel *Geukensia demissa* exposed to paraquat, in response to the induced oxidative stress by this pollutant in mollusks. Variations in the activity of these enzymes have been reported by Livingstone et al. (1990,) who found the increased catalase activity in the digestive gland of *Mytilus edulis* exposed to menadione, another oxidative agent. This finding suggests that some enhancement of antioxidant defense (increase in the antioxidant enzyme activity) may occur in polluted mussels. In contrast to activities of antioxidant enzymes, no changes in the activity of LDH were observed. This might be a result of some kind of “biochemical adaptation” of LDH response of mussels exposed to pollutants, which could explain the lack of the increase in LDH activity in contaminated mussels. Generally, during heavy metal intoxication, the activity of LDH increases (Santos et al. 2005, Elumalai et al. 2007, Vieira et al. 2009).

GSH is considered as one of the most important low molecular weight antioxidant in cells, involved in the protection of cell membranes from lipid peroxidation by scavenging oxygen radicals (Meister 1989). GSH is also a cofactor of many enzymes catalyzing the detoxification of numerous toxic compounds, including ROS (Gamble et al. 1995; Power, Sheehan 1996). From our results, it appears that one of the striking effects induced by a high level of toxic substances, mainly heavy metals, was the reduced GSH level, which could explain the enhancement of lipid peroxidation in organisms from contaminated sites. Depletion of GSH in senescent tissues may result from a cellular response to an increased rate of oxidation and degradation, or decreased synthesis of GSH (Canesi, Viarengo 1997). Heavy metal cations, such as Hg(II), Cu(II) Cd(II) Zn(II), have extremely high affinity to thiol groups of peptides and/or proteins, including glutathione (Rabestein et al. 1985; Viarengo, Nott 1993). On the other hand Cu(II), Fe(II) can catalyze oxidation of thiols to the thyil radical and hydroxyl radical (Halliwell, Gutteridge 1989). According to the review by Christie et al. (1984), metals promoting oxidation of glutathione include Cu(II), Co(II), Mn(II), Fe(II) and Cr(IV). These reactions could explain the observed decrease in glutathione content in digestive glands of contaminated mussels and the damage to cell membranes by lipid peroxidation.

In this work, the significant increase in MDA formation in mussels from polluted sites was also observed. Our results are similar to the results obtained by Viarengo et al. (1990), who observed the increased lipid peroxidation in the digestive gland of *Mytilus galloprovincialis* exposed to Cu (40 µg l\(^{-1}\) ind.\(^{-1}\)) for six days. Torres et al. (2002) observed a strong correlation between an increase in lipid peroxidation and higher concentration of Cu and Mn in the digestive gland of *Mytella guyanensis*. 
Determination of the amount of heavy metals in the mussels' tissue clearly shows their significant increase in the polluted area. Changes in the mussels' tissue observed in this work, were correlated with the presence of these metals in the polluted environment.

It was a surprise that the tissue of mussels collected from the unpolluted area displayed a higher level of lead than mussels from the polluted site. It seems that the high level of this metal can be accumulated by mussels cultured in the farm situated close to the water trail for ships, motorboat and other vehicles.

To sum up, our data obtained for the contaminated mussels, indicate both that the level of non-enzymatic antioxidants, such as glutathione, in concert with enzymatic antioxidant activities (catalase and glutathione peroxidase) and the indicators of damage to biomolecules, e.g. lipid peroxidation, are useful biomarkers of cellular oxidative stress, as well as that the mussels of *Mytilus* species can be used as an excellent bioindicator of industrial pollution.

**REFERENCES**


Lionetto M.G., Caricato R., Giordano M.E., Pascariello M.F., Marinosci L., Schettino T., 2003, *Integrated use of biomarkers (acetylcholinesterase and antioxidant enzymes activities) in*
Antioxidant enzyme activities and lipid peroxidation in *M. galloprovincialis* 43


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