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Partial Characterization of Proteins from Mussel *Mytilus galloprovincialis* as a Biomarker of Contamination

S. Gorinstein,[1] S.-T. Jung,[2] S. Moncheva,[3] P. Arancibia-Avila,[4] Y.-S. Park,[5] S.-G. Kang,[6] I. Goshev,[7] S. Trakhtenberg,[8] J. Namiesnik[9]

[1] Department of Medicinal Chemistry and Natural Products, School of Pharmacy, The Hebrew University-Hadassah Medical School, POB 12065, Jerusalem 91120, Israel
[2] Department of Food Engineering, Mokpo National University, Muan Jeonnam 534-729, Korea
[3] Institute of Oceanology, Bulgarian Academy of Sciences, 9000 Varna, Bulgaria
[4] Department of Basic Sciences, Universidad del Bio-Bio, Chillan, Chile
[5] Department of Horticultural Science, Mokpo National University, Muan Jeonnam 534-729, Korea
[6] Food Industrial Technology Research Center, Mokpo National University, Muan Jeonnam 534-729, Korea
[7] Institute of Organic Chemistry and Center of Phytochemistry, Bulgarian Academy of Sciences, 1040 Sofia, Bulgaria
[8] Kaplan University Medical Center, Rehovot, Israel
[9] Chemical Faculty, Gdansk University of Technology, Gdansk, 80952, Poland

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Abstract. Preservation of a healthy environment is a very important task, especially in the time of the total industrial revolution. Therefore, attempts to find new additional biomarkers of contamination are welcomed. For this aim, the functional and antioxidant properties of mussel *Mytilus galloprovincialis* proteins and the heavy metals Cd and Pb were explored. Mussels were collected in contaminated and noncontaminated sites in the Varna area of the Bulgarian Black Sea coast. Proteins were extracted from mussel entire soft tissue and analyzed using instrumental (Fourier transform infrared [FT-IR] spectroscopy, fluorescence, atomic absorption spectrophotometry), and biochemical (2,2′-azinobis 3-ethylbenzothiazoline-6-sulfonate) radical cation [$ABTS^{\bullet+}$]) methods. It was found that mussel proteins from the contaminated sites had specific qualitative changes: partial unfolding of the α-helix, slight shift in amide I bands, increased hydrophobicity, and fluorescent intensity in native and denatured samples. In the same mussel samples, an increased radical-scavenging capacity and increased contents of Cd and Pb in entire soft tissue were registered. Therefore, the above-mentioned indices could be used as additional biomarkers of sea water contamination.

Preservation of a healthy environment including prevention of sea contamination is one of the important goals of mankind, especially in times with negative results of industrial development. In order to control and fight for a healthy environment, monitoring of sea contamination is very important. The influence of different pollutants on mussels and as result some biomarkers of sea pollution were proposed (El Ghazi *et al*. 2003; Soazig and Marc 2003; Gorinstein *et al*. 2003; Moncheva *et al*. 2004; Domouhtsidou *et al.* 2004; Ciocan and Rotchell 2004). Regoli *et al.* (2004) and Olsson *et al.* (2004) have described the reaction of the mussel proteins to different pollutants. The response of mussel proteins and their antioxidants to marine contamination was demonstrated (Wilhelm *et al*. 2001; Lopez *et al.* 2002). Also, protein synthesis regulation as a component of cellular stress was used for this purpose (Kalpaxis *et al.* 2004).

The connection between occurrence and distribution of heavy metals in benthic organisms and sea contamination was described by Andreev *et al*. (1994). Some researchers demonstrated that the concentrations of metallothioneins (MTs) can be used as indicator of the degree of sea pollution (El Ghazi *et al*. 2003; Domouhtsidou *et al.* 2004; Santamaria-Fernandez *et al*. 2004; Rainbow *et al*. 2004). In our most recent investigations (Gorinstein *et al*. 2003; Moncheva *et al*. 2004) we have determined the antioxidant activity of different mussel samples and have concluded that total and free polyphenols can be used as a biomarker of contamination. As was mentioned, Monirith *et al*. (2003), Olsson *et al*. (2004), Kalpaxis *et al*. (2004), and Rainbow *et al.* (2004) and many others have widely used changes in mussels as biomarkers of sea contamination. Some authors showed that heavy metals that have been used to incubate healthy specimens of the freshwater mussels contributed to a decrease of the metabolic activity, and thus to mineralization in the exposed animals. The most disturbing pollutants were Pb, Zn, Cr, and Cd, which caused greatly decreased overall components, namely, with respect to protein and glucosamine (Moura *et al*. 2000; Raspor *et al*. 2004). The main purpose of our study was to control the changes in the protein functionality by the influence of pol-

Correspondence to: S. Gorinstein; *email:* gorin@cc.huji.ac.il

lutants; therefore, some of them, such as Cd and Pb, have to be studied. However, until now there has been no information in the literature about the influence of sea pollutants on the mussel *Mytilus galloprovincialis* protein structure. Therefore, it was decided to explore the use of possible functional changes in these mussel proteins, their antioxidant activity, and contents of heavy metals Cd and Pb as new biomarkers of sea water contamination.

In order to achieve this goal, mussel proteins were extracted from the entire soft tissue without distinguishing between glands and gills from samples of contaminated and noncontaminated areas. For detection of the above-mentioned changes, the following methods were used:

1. Fourier transform infrared (FTIR) spectroscopy for search of mussel proteins structural changes
2. Fluorescence for search of changes in hydrophobicity and intrinsic fluorescence intensity in proteins
3. Atomic absorption spectrophotometry for search of changes in heavy metals concentration
4. 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonate) radical cation ($ABTS^{\bullet+}$) have been for determination of changes in antioxidant capacity.

As far as we know, there have been no such investigations.

Materials and Methods

Animals and Sites of Collection

Mussels (*Mytilus galloprovincialis*) were collected in two regions of the Bulgarian Black Sea coast: from an ecologically noncontaminated (Cape Galata) and contaminated (the area of Port Varna) sites in 2003, during summer. Port Varna is close to the open sea and is characterized by high industrial, harbor, and urban contamination: it has the highest loading of biochemical oxygen demand, oil total suspended sediment, and metals (Balashov *et al.* 1998; Monirith *et al*. 2003) and also a chronic exposure to petrogenic and pyrolitic hydrocarbons (Gorinstein *et al*. 2003; Moncheva *et al*. 2004).

The collected mussels (*M. galloprovincialis*) from both contaminated and noncontaminated sites were characterized by a similar maximum length and size of analyzed organisms (4.37 ± 0.5 cm): it was 75–85% of the maximum size reached within each population. This approach guaranteed that the compared mussels had similar metabolic conditions, and the influence of physiological differences between two populations was less pronounced (Moncheva *et al*. 2004). The samples were designated as follows: NCont for noncontaminated and Cont for contaminated sites. The entire soft tissue from 30 specimens of each population was rapidly frozen in liquid nitrogen and stored at –80°C. Then the samples were dried in glass flasks on Lyovac GT-2 equipment (Finn-Aqua, Hürth, Germany) for 36 h.

Extraction of Proteins from Mussel Samples

The entire soft tissue from mussels of NCont and Cont areas was separately subjected to extraction with 0.05 M Na_2HPO_4 buffer (two portions of 20 ml each) at 4–6°C for 48 h under constant stirring. Centrifugation (K-24 D centrifuge, Janetzki, Germany) at 15,000 rpm for 45 min at 4–6°C was applied at the end of each extraction step and the supernatants were combined and freeze-dried. The obtained dry extracts were used for further analyses (Gorinstein *et al*. 2003).

Gel Filtration Chromatography

Ten-milliliter portions of phosphate buffer extracts were applied on a Sephadex G-25 column (bed volume 130 ml) at a flow rate of 40 ml h^{-1} at 20°C. The eluant profile was monitored at 280 nm on LKB 2510 Uvicord SD (LKB-Produkter, Bromma, Sweden). The column was preliminarily calibrated with 0.2 M NaCl and Blue Dextran.

Determination of Protein Content

The chromatographic fractions were pooled, freeze-dried, and 5 mg of each sample was used for determination of protein content according to Bradford's method (1976).

Urea Treatment

Treatment of proteins involved the addition of denaturants to the protein solutions in concentrations (M) such as urea 8. Denaturation was determined after incubation of protein with denaturants for 1 h (Chmelik 1989).

Fluorometry

The protein concentration corresponded to an absorbance less than 0.1 in a 1-cm pathlength. This range of concentration guarantees a linear increase in the relative fluorescence intensity. Absorbance values were measured using a Uvikon 930 UV spectrophotometer (Kontron AG Instruments, Zürich, Switzerland). Intrinsic fluorescence measurements of proteins were done using a Model FP-770 Jasco-Spectrofluorometer (Japan Spectroscopic Co., Ltd., Hachioji City, Japan). Proteins were dissolved in 0.01 M phosphate buffer, pH 7.2. For fluorescence measurements, protein solutions were 0.15 mg/ml. Fluorescence emission spectra were determined at excitation wavelengths (nm) of 274 and 295 and recorded from the excitation wavelength to wavelength of 450 nm. A thermostatically controlled cell holder kept the temperature of the samples at 30°C (Chmelik 1989; Gorinstein *et al*. 2001).

Fourier Transform Infrared Spectroscopy (FTIR)

A Bruker OpticGMBH Vector FT-IR spectrometer (Bruker Optic GMBH, Attingen, Germany) was used to record the IR spectra. Lyophilized material was mixed with KBr, and the pellet was pressed at 10,000 kg/cm^2 for 15 s. A potassium bromide microdisk was prepared from finely ground powder of 2 mg of lyophilized sample with 100 mg of KBr (Kaiden *et al*. 1987; Kato *et al*. 1987).

Hydrophobicity (S_0)

S_0 was determined by 1-anilino-8-naphthalenesulfonate (ANS)-fluorescent probe measurements with 0.01 M phosphate buffer, pH 7.0, from 0.001 to 0.02% protein concentrations at λex 357 nm. The fluorescence intensity was measured at 513 nm. The index of protein hydrophobicity was calculated as the initial slope of fluorescence intensity versus protein concentration (%) plot (Gorinstein *et al*. 2001).

Determination of Antioxidant Capacities

The antioxidant capacities of the proteins from the entire soft mussel tissue were determined by two methods:

1. 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt ($ABTS^{\bullet+}$) radical cation was generated by the interaction of ABTS (250 μM) and $K_2S_2O_8$ (40 μM). After addition of 990 μl of $ABTS^{\bullet+}$ solution to 10 μl of different extracts (0.2 mg/ml) or Trolox standards (final concentration 0–20 μM) in ethanol or phosphate buffered saline, the absorbance was monitored exactly 1 and 6 min after the initial mixing.
2. $ABTS^{\bullet+}$ was prepared as well by passing a 5 mM aqueous stock solution of ABTS through manganese dioxide on a Whatman no. 5 filter paper. Excess manganese dioxide was removed from the filtrate by passing it through a 0.2 μM Whatman PVDF syringe filter. This solution was then diluted in a 5 mM phosphate buffered saline, pH 7.4 to an absorbance of 0.70. The percentage decrease of the absorbance at 734 nm in A) and B) was calculated and plotted as a function of the concentration of the extracts and of Trolox for the standard reference data (Miller *et al.* 1996; Pellegrini *et al.* 1999).Trolox was used for the standard curve in the calculation of the antioxidant capacity; on the other hand, glutathione was used for comparison of the antioxidant activity of mussel proteins.

Determination of Heavy Metals

Metals (Cd and Pb) were determined by atomic absorption spectrophotometry with flameless atomization (Varian Spectra AA 300 Zeeman) in the extracted proteins from the entire soft tissue. Prior to the measurements, samples were digested with nitric acid (Andreev *et al.* 1994; Ciocan and Rotchell 2004; Santamaria-Fernandez *et al.* 2004).

Reagents

All the reagents used in this investigation were of analytical grade. Trolox (6-hydroxy-2,5,7,8,-tetramethyl-chroman-2-carboxylic acid) was purchased from Aldrich Chemical Co. (Milwaukee, WI), glutathione from Sigma Chemical Co. (St. Louis, MO), and 1-anilino-8-naphthalenesulfonate and 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) from Fluka Chemie, Buchs, Switzerland.

Statistical Analysis

The reported values are mean ± SD of five measurements. The analysis of variance and least significant difference tests were conducted to identify differences among means, while a Pearson correlation test was conducted to determine the correlations among means. Statistical significance was declared at $p < 0.05$.

Results

Protein Concentration

Protein concentrations in the main peak (μg/mg dry weight) of NCont were 210.256 and in Cont 87.515. The protein content of peaks 1 (protein-rich) in samples NCont and Cont was about 7–10 times higher than that of peaks 2. Peak 1 included MT-like proteins that were used in this report.

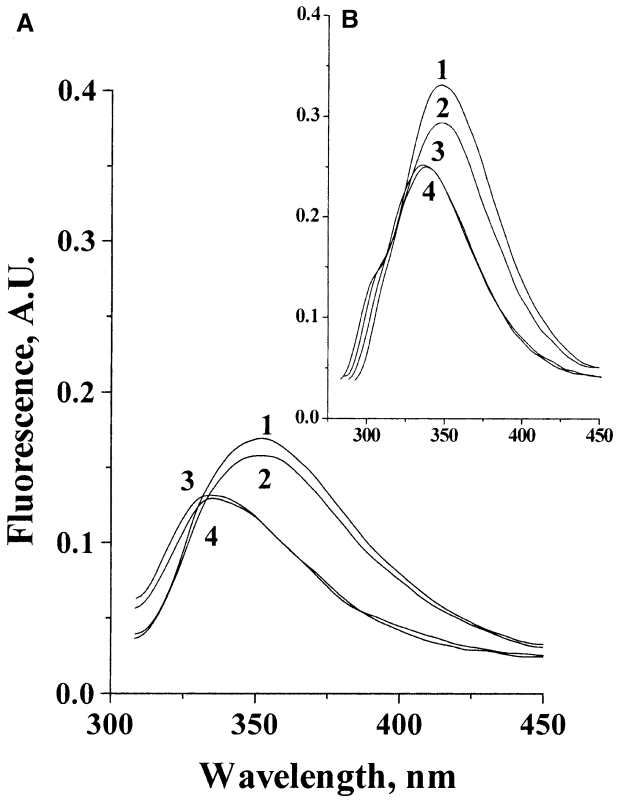

Fig. 1. Fluorescence spectra from noncontaminated (NCont) and contaminated (Cont) mussels as a function of fluorescence intensity for nondenatured and denatured proteins: (a) λ_{exc} 295 nm: 1, NCont+Urea (U); 2, Cont+U; 3, NCont; 4, Cont; (b) λ_{exc} 274 nm: 1, NCont+U; 2, Cont+U; 3, Cont; 4, NCont. All samples were dissolved in 0.01 M phosphate buffer at pH 7.2. The protein concentration was 0.15 mg/ml at 30°C.

Fluorescence Spectra

About 30 samples were analyzed and Figure 1 shows an average of these data. Fluorescence spectra of NCont and Cont samples demonstrated peaks (nm) at 334 and 335, respectively (Figure 1A, lines 3 and 4) and decreased intensity (0.1312 and 0.1282). It means that tryptophan residues are situated closer to the surface of the molecule in the case of NCont and consistent with the less compact and more hydrophobic structure in comparison with Cont. It showed a slight decreasing of intensity (0.1697 and 0.1581) and no shift in the maximum of emission reflecting the unfolding of these proteins with urea (U) (Figure 1A, lines 1 and 2). At λ_{exc} = 295 nm, the fluorescence intensities showed the following order: NCont + U > Cont + U > NCont > Cont. At λ_{exc} = 295 nm, tyrosine was not shown.

At λ_{exc} = 274 nm for Cont (fluorescence intensity) = 0.2527 and NCont = 0.2509 samples (Figure 1B, lines 3 and 4), very slight shoulders were seen in the samples at 308 nm, which was evidence of tyrosine as well as a shift of about 2 nm to the side of 335 nm appeared. The fluorescence intensities showed the following order: NCont + U > Cont + U > NCont > Cont. On the basis of measured and calculated data of all samples, the percentage of denaturation for NCont and

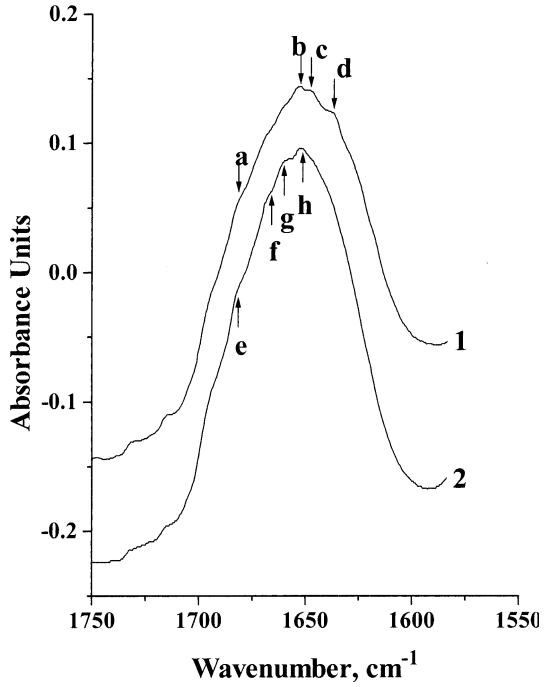

Fig. 2. Fourier transform infrared spectra from 1750 to 1550 cm^{-1}. (1) Noncontaminated (NCont) protein. (2) Contaminated (Cont) protein. The following peaks were shown: 1a–1681, 406; 1b–1652, 224; 1c–1647, 256; 1d–1637, 011. 2e–1681, 140; 2f–1667, 145; 2g–1660, 327; 2h–1650, 997.

Cont samples of proteins with 8 M urea was 22.7% and 18.9 %, respectively (Chmelik 1989; Gorinstein *et al.* 2001).

FTIR Spectra

Amide I (Al) bands (in the range of 1650–1681 cm^{-1}) differ for spectra of NCont (Figure 2, line 1) and Cont (Figure 2, line 2) (Kaiden *et al.* 1987; Kato *et al.*1987). NCont (Figure 2, line 1) demonstrated three bands in the AI (1b-1652, 224; 1c-1647, 256; 1d-1637, 011) and a slight one (1a-1681, 406). The same bands were shifted for the Cont sample (2e-1681, 140; 2f-1667, 145; 2g-1660, 327; 2h-1650, 997). The FTIR spectrum of Cont (Figure 2, line 2) demonstrated a shift in the AI band from 1647 cm^{-1} to 1651 cm^{-1}. The band of high-frequency components in the amide I band at 1669 cm^{-1} can be assigned to turns and elements of β-sheet. About 30 samples were investigated and an average spectrum is presented. These data indicate that the mussel proteins show typical protein peaks and the changes in the peaks were influenced by the pollutants.

Surface Hydrophobicity

Surface hydrophobicity (S_0) was the highest for Cont protein sample (S_0 = 9.94554 ± 1.3; R^2 = 0.98907) in comparison with NCont (S_0 = 6.28218 ± 0.7; R^2 = 0.99664). After denaturation with urea (S_0 = 2.36634 ± 0.5; R^2 = 0.95173), NCont had a slightly lower value than Cont (S_0 = 2.65842 ± 0.3; R^2 = 0.97016). NCont has more compact tertiary structure and less hydrophobic surface than Cont. The increase in surface hydrophobicity was not correlated with the increase in the extent of protein denaturation. It can be explained by the altered, partially unfolded proteins as well as by the increased amount of heavy metals (Yusof *et al.* 2004).

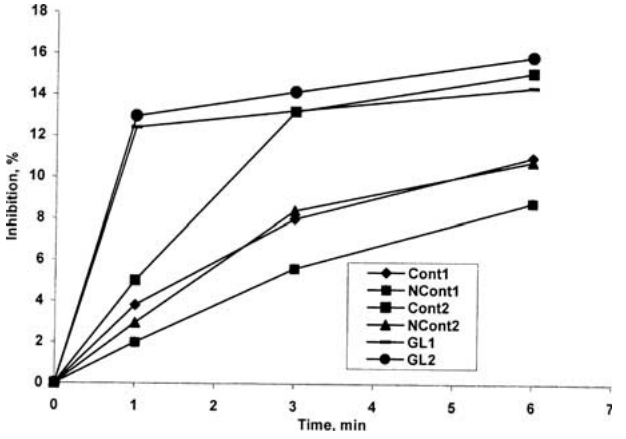

Fig. 3. Kinetics of ABTS scavenging effect of mussel protein extracts. The concentration of proteins is 0.025 mg/ml. NCont1, Cont1, GL1: samples of proteins extracted from noncontaminated and contaminated areas, glutathione, respectively, and persulfate ammonium was used for antioxidant determination; NCont2, Cont2, GL2: samples of proteins extracted from noncontaminated and contaminated areas, glutathione, respectively, and manganese dioxide was used for antioxidant determination.

Antioxidant Capacities

The obtained results with manganese dioxide (NCont2 and Cont2) were compared with the results of another variation of ABTS decolorization assay where ABTS radical cation was produced by reacting ABTS with potassium persulfate (NCont1 and Cont1). The antioxidant capacity of proteins from Cont and NCont had comparative results against ABTS at the end point of 6 min as determined by spectrophotometric measurement (Figure 3). According to our results, Cont2 had the highest percentage of inhibition (15.1%). The other extract such as NCont showed the lowest percentage of inhibition (8.8%). The proteins were examined at a concentration of 0.25 mg/ml and were comparable with glutathione of the same concentration.

Cd and Pb Concentration

The concentration of Cd and Pb in mussel proteins from the Cont was significantly higher than in mussel's proteins from the NCont areas (0.8 ± 0.04 and 59.1 ± 6.2 ng g^{-1} vs. 0.5 ± 0.01 and 45.2 ± 5.1 ng g^{-1}, respectively.

The correlation coefficients between the scavenging percentage of ABTS and the contents of Cd and Pb (Figure 4) were 0.9782 and 0.9820.

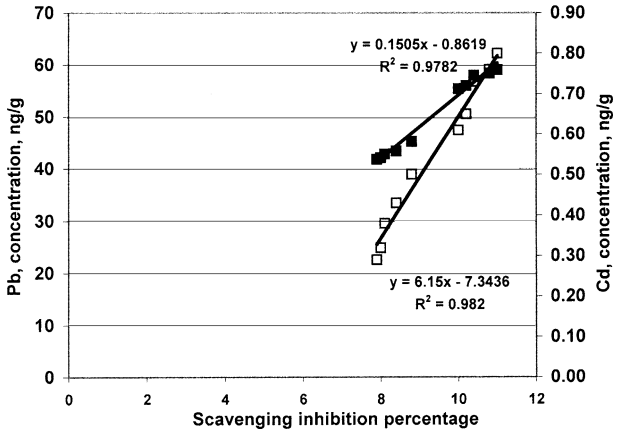

Fig. 4. Correlation between ABTS (Inhibition %, X) and (■) the content of Cd (ng/g, Y_1) and between ABTS (Inhibition %, X) and (□) the content of Pb (ng/g, Y_2).

Discussion

The total industrial revolution led to negative changes in environment in general and in seawater status in particular. Therefore, the monitoring of these changes using *inter alia* biomarkers is one of the important necessities of our times (Gorinstein *et al.*, 2003; Kalpaxis *et al.* 2004; Moncheva *et al.* 2004; Domouhtsidou *et al.* 2004). The search for reliable biomarkers of sea pollution continues. Some investigators have studied the influence of different pollutants on mussels including *Mytilus edulis* L and *M. galloprovincialis* and have found that changes in mussel proteins and the antioxidant capacity could be suitable biomarkers of sea pollution (Gorinstein *et al.* 2003; Moncheva *et al.* 2004). Also, the changes in the concentration of heavy metals could be used for this aim (Bebianno and Serafim 2003; Soazig and Marc 2003). Therefore, we decided to compare the possible changes in functional and antioxidant properties of *M. galloprovincialis* proteins and concentration of the heavy metals Cd and Pb from contaminated areas with these indices in mussels from noncontaminated areas. The studies began with the extraction procedures. These procedures differ from one report to another. In this study, the procedures and extractant solution [10 mM Tris-HCl buffer at pH 7.4 with 5 mM 2-mercaptoethanol (2-MCE), 0.01 mM phenylmethyl-sulfonylfluoride (PMSF), and 25 mM sodium chloride] used were similar to those of Santamaria-Fernandez *et al.* (2004). In order not to change the mussel proteins' secondary structure by destroying the sulfur bridges, we did not use 2-MCE for the extraction. It was found that the amount of proteins in mussels from NCont is higher than that in mussels from Cont areas. These data are in agreement with data of Ciocan and Rotchell (2004).

The intrinsic fluorescence and FTIR spectra of the studied proteins were measured to compare their structure. It was found that there are some structural differences in the samples from the contaminated and noncontaminated areas. It was observed that only mussel proteins from contaminated sites had specific qualitative changes: partial unfolding of α-helix, slight shift in amide I bands, increased hydrophobicity, and fluorescent intensity in native and denatured samples. It is tempting to speculate that the changes in the protein structure of Cont and NCont samples observed by fluorescence measurements can result from the partial unfolding of α-helix. The fluorescence intensity increased gradually with the urea denaturation. Most probably these changes are due to the decrease in distance between the tryptophan and the tyrosine residues. The extent of denaturation was higher in NCont (22.7%) than in Cont (18.9%). The variation of the extent of denaturation between the protein samples may be explained by the differences in the amounts of amino acids, by the sulfur bridges existing in such proteins, and the protein–metal complexes that were formed in the Cont samples (Chmelik 1989; Gorinstein *et al.* 2001).

The radical $ABTS^{\bullet+}$ has been widely used in model systems for investigation of the scavenging activities of phenolic compounds, anthocyanins, or crude mixtures such as methanol extracts of plants (Pellegrini *et al.* 1999). However, a few studies have been reported on proteins, except antioxidative enzymes, with regard to the subject of direct antiradical effects (Lopez *et al.* 2002; Bebianno and Serafim 2003; Lesser and Kruse 2004). An increase in the radical-scavenging capacity and increased contents of Cd and Pb in whole soft tissue of mussels from contaminated sites were registered. Similar results were observed in our recent investigations using other methods (Gorinstein *et al.* 2003; Moncheva *et al.* 2004): the antioxidant activity of the whole soft mussel tissue from the contaminated site was significantly higher than from the noncontaminated site.

Antioxidant capacities determined by the $ABTS^{\bullet+}$ method and their correlations to the concentrations of the protein samples were about 0.74. The radical-scavenging capacity of individual mussel extracts against different testing radicals may be explained by the different mechanisms involved in the radical-antioxidant reactions (El Ghazi *et al.* 2003; Gorinstein *et al.* 2003; Moncheva *et al.* 2004). In this study, the $ABTS^{\bullet+}$ was generated by incubating ABTS with potassium persulfate (Miller *et al.* 1996; Pellegrini *et al.* 1999). Chemical compounds that inhibit the potassium persulfate activity may reduce the production of $ABTS^{\bullet+}$. This reduction results in a decrease of the total $ABTS^{\bullet+}$ in the system and contributes to the total $ABTS^{\bullet+}$ scavenging capacity. Other factors, such as stereoselectivity of the radicals or the solubility of mussel extracts in different testing systems, may also affect the capacity of mussel extract to react and quench different radicals (Regoli *et al.* 2004; Wilhelm *et al.* 2001; Lopez *et al.* 2002; Monirith *et al.* 2003; Lesser and Kruse 2004). Besides single antioxidants, the total scavenging capacity depends on the whole antioxidant system to neutralize specific forms of radicals: these data were further integrated by measurement of DNA integrity, oxidized bases, and the impairment of lysosomal membrane stability in hemocytes (Frenzilli *et al.* 2004; Regoli *et al.* 2004). The hypothesis of reactive oxygen species–mediated toxicity is supported by the appearance of cell damages.

We speculated that maybe Cu and Fe can catalyze the production of reactive oxygen species, to which the organism reacts by increasing activities of the enzymes, thus preventing the accumulation of reactive oxygen species. Therefore, our explanation of the increase of the antioxidant activity of the proteins from two areas was based only on the increase of Cd and Pb. Others have shown (Giguere *et al.* 2003) that cadmium

and MT concentrations in the gill cytosol increased along the contamination gradient, but the distribution of Cd among the various cytosolic complexes remained quite constant: 80% in the MT-like pool, 7% in the low-molecular-weight pool (<1.8 kDa), and 13% in the high-molecular-weight pool (>18 kDa).

The heavy metals are bound to low-molecular-weight compounds in mussels, presumably MT-like proteins (Bebianno and Serafim 2003; El Ghazi *et al*. 2003; Soazig and Marc 2003; Ciocan and Rotchell 2004; Domouhtsidou *et al*. 2004). The concentration of Cd and Pb in the mussel proteins from the Cont area was higher than the mussel proteins from the NCont area. The determined amounts of Cd and Pb in this study were lower than those found by Andreev *et al.* (1994), Soazig and Marc (2003), and Santamaria-Fernandez *et al*. (2004). The changes in the amounts of metats can be explained by different extraction procedures and by different pollution levels. Our results corresponded with those of others (Ciocan and Rotchell 2004; Soazig and Marc 2003) who showed that Cd was observed in the digestive gland of exposed mussels, both adults and juveniles, up to 500 times higher than in the control. Another approach was shown by Bebianno and Serafim (2003) and Giguere *et al*. (2003), who reported that total metal concentrations decreased according to the sequence digestive gland > gills > remaining tissues for Cd. Cd was the only metal that significantly influenced MT synthesis in all the tissues. In some reports it was found that *M. galloprovincialis* is the best lead accumulator (Saavedra *et al*. 2004). In the present report, there is a linear relationship between Pb and the antioxidant capacity of the mussel proteins from the contaminated area. Our results of *M. galloprovincialis* are in agreement with the data of Saavedra *et al*. (2004), but in other cases the correlation was moderate or low, depending on mussel species. The induced and/or existent MT was sufficient to bind free Cd ions present in the cells, preventing any damage to cellular metabolism. The potential use of bivalves as suitable bioindicators was evaluated from correlation tests based on the concentrations of heavy and trace elements in the sediment-metals system (Rainbow *et al*. 2004; Yusof *et al*. 2004).

The aim of this study was to evaluate the influence of contamination of seawater on the functional and structural properties of mussel proteins. The mussels were screened for proteins from the contaminated and noncontaminated areas. The proteins were denatured with 8 M urea. Fluorescence was employed in order to elucidate urea-induced conformational changes and structural behavior of proteins. The measured fluorescence emission spectra were used to estimate the stability of native and denatured samples in contaminated and noncontaminated sites. It was found that the contaminated samples were more stable to urea denaturation than the noncontaminated ones. Some changes in native and denatured proteins were detected in contaminated samples: a tendency to higher stability and minor structural deviations. The obtained results may be used in clarifying protein structure–function properties as well as the additional biomarker of contamination. Even in moderately contaminated sites, measurement of the protein level in the soft tissues of mussels studied in this report was generally able to discriminate between different levels of contamination, allowing the use of a simplified procedure compared with dissection of the digestive gland.

In conclusion, specific qualitative protein changes: partial unfolding of α-helix, slight shift in amide I bands, increased hydrophobicity and fluorescent intensity, increased radical scavenging capacity, and increased contents of Cd and Pb in entire soft tissue of mussels could be used as additional biomarkers of seawater contamination.

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