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Relationship between seawater pollution and qualitative changes in the extracted proteins from mussels *Mytilus galloprovincialis*

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Abstract

The aim of this study was to find a reliable biomarker of seawater pollution. For this purpose the contents of Zn and Cu, proteins and antioxidant activity in mussels *Mytilus galloprovincialis* collected from polluted and non-polluted sites of the Bulgarian Black Sea coast were compared. To determine the above-mentioned indices atomic spectroscopy, Fourier Transform Infrared (FT-IR) spectroscopy, fluorescence, differential scanning calorimetry (DSC), and two antioxidant tests were used. It was found that the amounts of Zn and Cu were significantly higher in the mussel proteins from the polluted than from the non-polluted sites ($P < 0.05$). FT-IR spectroscopy and fluorescence revealed specific qualitative changes in secondary and tertiary structures in mussel proteins in the samples from polluted sites. The thermodynamic properties of proteins and the changes upon denaturation were correlated with the secondary structure of proteins and disappearance of α-helix. Purified protein scavenging activity against 2, 2′-azinobis (3-ethylbenzothiazoline-6-sulfonate) radical cation ($ABTS^{\bullet+}$) was significantly higher in mussel samples from polluted than from non-polluted sites. Therefore, the changes in Zn and Cu concentration, in protein's secondary

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and tertiary structures and antioxidant activity in mussels *M. galloprovincialis* from polluted sites can be a reliable biomarker of the level of the seawater pollution.

Keywords: *Mytilus galloprovincialis*; Pollution; Proteins; Structural changes; Metals; Antioxidants activity

1. Introduction

The use of the inducible isoform of the Cu/Zn superoxide dismutase in the blue mussel *Mytilus edulis* as a tool for the marine environment monitoring was studied by Manduzio et al. (2003). Protein parameters influenced by contamination as a response of mussel proteins and antioxidant compounds to different types of marine pollution were shown in several reports (Lopez et al., 2001; Porte et al., 2001). Metal accumulation and binding protein induction and the rate of metal uptake in *Mytilus galloprovincialis* is presented in many reports (Romeo et al., 2003, 2005; Dragun et al., 2004; Rivera-Duarte et al., 2005).

The successful use of mussels by these and other authors (Goldberg, 1986; Goldberg et al., 1978; Viarengo et al., 1991; Cajaraville et al., 2000) influenced our decision to evaluate the relationships between seawater pollution and the changes in heavy metals Cu and Zn, antioxidant compounds and free radical scavengers in common mussel *M. galloprovincialis* collected in polluted and non-polluted sites of the Bulgarian Black Sea coast. Our recent studies on mussels have been focused only on the antioxidant activities of the whole tissue (Moncheva et al., 2004; Gorinstein et al., 2003). Togashi et al. (2002) described some changes in fish proteins. Lopez et al. (2001) and Mosquera et al. (2003) showed genetic variability and differences in protein expression between intertidal and cultured mussels *M. galloprovincialis* by two-dimensional gel electrophoresis. Hamer et al. (2004) discussed the usefulness of stress-70 proteins as biomarkers of environmental pollution. From the recent research in literature it can be concluded that there are no data concerning the direct changes in the properties of mussel proteins. Therefore in this report for the first time the changes in denaturation-induced secondary and tertiary structural changes of proteins as revealed by measurements of fluorescence intensity, wavelength of peak output response, Fourier Transform Infrared (FT-IR) spectroscopy and differential scanning calorimetry (DSC) were described.

2. Materials and methods

2.1. Reagents

All reagents used in this investigation were of analytical grade. Trolox (6-hydroxy-2,5,7,8,-tetramethylchroman-2-carboxylic acid) was purchased from Aldrich Chemical Co. (Milwaukee, WI) and Sigma Chemical Co. (St. Louis, MO, USA) and 2,2′-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) diamonium salt (ABTS)—from Fluka Chemie, Buchs, Switzerland.

2.2. Collection and preliminary characteristics of mussel samples

The mussel samples were collected as previously described (Gorinstein et al., 2003). Animals (*M. galloprovincialis*) were collected in two regions of the Bulgarian Black Sea coast: from non-polluted (Cape Galata) and polluted (the area of Port Varna) sites. Port Varna is close to the sea and is characterized by high industrial, harbor and urban pollution. Therefore, the concentrations of total and individual polycyclic aromatic hydrocarbons (PAHs) and metals are much higher than in other places such as Cape Galata, which is open to sea (Balashov et al., 1998). PAHs, total PAHs (T-PAHs), polychlorinated biphenyls (PCBs), pesticides as well as biochemical markers, benzo (a) pyrene hydroxylase (BPH) in Port Varna and Cape Galata have been analyzed (Moore et al., 1998; Albalat et al., 2002). Cape Galata showed increased retention times in conjunction with relatively low inputs of biochemical oxygen demand (BOD) and oil (Shtereva et al., 1998). Other characteristics (Moncheva et al., 2004) of the Varna region correspond to similar and other regions (Porte et al., 2001; Albalat et al., 2002;

Andral et al., 2004). The collected mussels (*M. galloprovincialis*) from both polluted and non-polluted sites were characterized by a similar maximum length and the size of analyzed organisms (4.37 ± 0.5 cm), which 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 (Regoli, 2000; Moncheva et al., 2004). The samples were designated as follows: NP=non-polluted site and P=polluted site. Preliminary treatment of mussel samples was done as follows: whole soft tissue from 30 specimens of each population were rapidly frozen in liquid nitrogen and stored at -80 °C.

2.3. Mussel protein extraction

The extraction of mussel protein was done as follows: samples (1 g) of NP and P groups were extracted with 0.05 M Na_2HPO_4 buffer (two portions of 20 ml each) at 4–6 °C for 48 h (2×24 h) under constant stirring. Centrifugation (K-24 D centrifuge, Janetzki, Germany) at 17000 *g* 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 dry extracts obtained were used for further analyses.

2.4. Determination of Cu and Zn

Cu and Zn were determined by atomic absorption spectrophotometry with flame (IL model S11 with deuterium background corrector) atomization (Varian Spectra AA 300 Zeeman) in the extracted proteins from the whole soft tissue. Before the measurements, samples were digested with nitric acid.

2.5. Determination of the structural characteristics of proteins

The proteins were determined in the whole soft tissue without distinguishing between glands and gills. The gel filtration chromatography was exercised as follows: 10 ml portion of phosphate buffer extracts were applied on Sephadex G-25 column (bed volume 130 ml) at flow rate 40 ml h^{-1} at 20 °C. The effluent profile was monitored at 280 nm on LKB 2510 Uvicord SD. The column was preliminarily calibrated with 0.2 M NaCl and Blue Dextran. The chromatographic fractions were pooled, freeze-dried and 5 mg of each sample were used for determination of protein content according to the method of Bradford (1976). The changes in the protein structure were studied by Fourier Transform Infrared (FT-IR) spectroscopy and fluorescence. 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. Treatment of mussel proteins involved the addition of denaturants to the protein solutions in concentration of 8 M urea. Denaturation was determined after incubation of protein with denaturants for 1 h (Zemser et al., 1994).

A Bruker Optic GMBH Vector FT-IR spectrometer (Bruker Optic GMBH, Attingen, Germany) was used to record IR spectra. Lyophilized material was mixed with KBr, and the pellet was pressed at 10000 kg/cm^2 for 15 s. A potassium bromide microdisc was prepared from finely ground powder of 2 mg of lyophilized sample with 100 mg of KBr (Zemser et al., 1994).

2.6. Differential Scanning Calorimetry (DSC)

The denaturation of proteins was assessed with a Perkin Elmer DSC System 4. Lyophilized samples of about 1 mg were sealed in aluminum pans. Denatured samples were prepared by homogeneous mixture of native protein and denaturant in the dry state. Then the mixed sample of 1 mg was sealed in aluminum pan in the same way as the native one. As reference an empty pan was used. The scanning temperature was 30–120 °C at a heating rate of 10 °C/min. Indium standards were used for temperature and energy calibrations. T_d and ΔH were calculated from the thermograms (Wagner and Añon, 1985).

2.7. Determination of antioxidant activity

Similar profiles (data not shown) were obtain for all samples in gel filtration chromatography. They were very similar and differ to a certain extent only in the shape of the second peak, which contained low molecular weight substances (salts, vitamins, phenolic compounds, short chain protein fragments, etc). Both fractions, obtained from the samples NP and P, were freeze-dried and then tested for antioxidant activity.

The antioxidant activity in the whole soft mussel tissue was determined by two methods:

a) 2,2′-azino-bis (3-ethyl-benzothiazoline-6-sulfonic acid) diamonium 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 (PBS), the absorbance was monitored exactly 1 and 6 min after the initial mixing.
b) $ABTS^{\bullet+}$ 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. To calculate the Trolox equivalent antioxidant coefficient (TEAC), the slope of the plot of the percentage inhibition of absorbance vs. concentration for the antioxidant was divided by the slope of the plot of Trolox. This gives the TEAC at the specific time point (Miller et al., 1996). The higher the TEAC value of the sample means the stronger the antioxidant ability.

2.8. Statistical analysis

The reported values are mean ± SD of 5 measurements. The analysis of variance ANOVA was conducted to identify differences among means, while a Pearson correlation test was done to determine the correlations among means. Statistical significance was declared at $P<0.05$.

3. Results

3.1. Metals

The concentrations of metals in proteins were (μg/g dry weight): 24.2 ± 2.2 and 12.9 ± 1.4 and 143 ± 13.3 and 112.4 ± 10.3, for Cu and Zn in P and NP, respectively. As was expected, the contents of both studied metals were significantly higher in mussels of the polluted than in mussels of the non-polluted areas ($P<0.05$).

3.2. Proteins

The protein concentrations in the main peak (μg/mg dry weight) of NP and P were 210.256 and 87.515, respectively. The protein content of peaks 1 (protein-rich) in samples of NP and P is about 7–10 times higher than that of peaks 2 (low molecular weight substances). The spectra exhibited a tryptophan-characteristic intrinsic fluorescence with maximum (nm) excitation at 295 nm (Fig. 1). The emission peak centered for protein from non-polluted mussels at 335 nm with intensity of 0.1282 and for polluted ones at 351 nm with intensity of 0.1532 and showed an increase in fluorescence intensity of 1.1952 (Fig. 1, curves 4 and 3). At 274 nm (Fig. 1, curves 4 and 3) the spectra showed peaks at 330 and 331 nm and an increase in intensity as reported above for 295 nm. In NP samples after urea denaturation the intensity has increased to about 1.38 and the emission peak has shifted from 335 to 351 nm at 295 nm, and for the polluted ones—the intensity has increased of about 1.12 and the emission peak has shifted from 351 to 353 nm. For 274 nm for the NP samples two peaks appeared at 331 and 330 nm and showed the same increase in FI (1.4) as was shown at 295 nm and for polluted ones the increase was about 1.13 and peaks of 350 and 353 nm.

NP samples (Fig. 2, curve 1) showed similar bands at amide I (AI), amide II (AII), and amide III (AIII) bands (in the range of 1650, 1530, and 1300–1250 cm^{-1}) but differ slightly for spectra for P one (Fig. 2, curve 2). The two samples of mussels showed broad amide I bands at 1648 cm^{-1}, typical of proteins with high α-helical content. The rather high ratio of AII to AI bands in the NP sample can be attributed to the high content of α-helix. Some other spectral bands were located at frequencies of 1681, 1669, 1659 and 1632 cm^{-1}. The band at 1651–1652 cm^{-1} originates from α-helical and/or random structures. 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 (Jackson et al., 1989; Lee et al., 1990). Amino acid side chains such as those from Arg, Asn, Gln, and

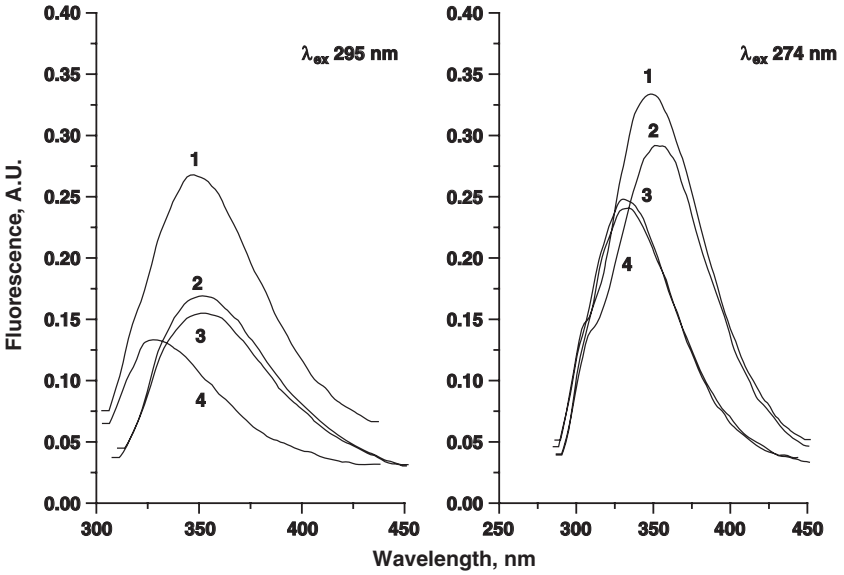

Fig. 1. Change in the fluorescence spectra of proteins from non-polluted (NP) and polluted (P) mussels as a function of urea denaturation at λ exc 295 nm: 4, NP; 1, NP+urea; 2, P+urea; 4, P; λ exc 274 nm: 1, NP+urea; 2, P+urea; 4, NP; 3, P. All samples were dissolved in 0.01 M phosphate buffer at pH 7.2. The protein concentration was 0.15 mg/ml at 30 °C.

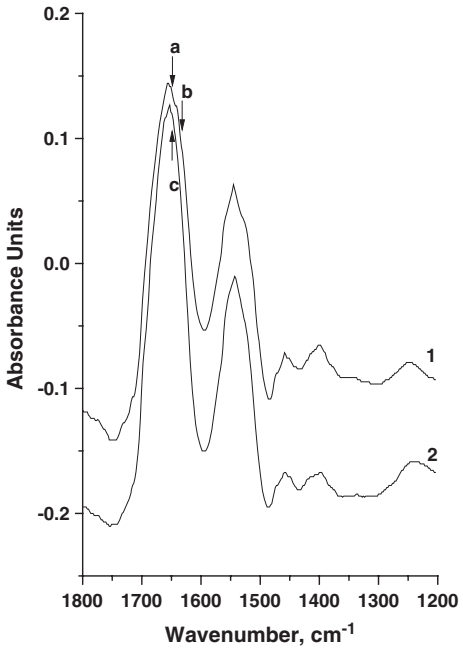

Fig. 2. Absorption spectra from 1800 to 1200 cm^{-1}. (1) Non-polluted protein. (2) Polluted protein. 1a=1648.531; 1b=1632.697; 2c=1648.104.

Lys absorb along the whole amide I band and may affect specially the intensity of the latter two bands and that at 1632 cm^{-1} (see a band with very low intensity on line 1, Fig. 2), and their whole contribution to the amide I and II bands is estimated to be around 20% of the total absorbance. The band at 1632 cm^{-1} was not shown in polluted sample. The amide II band is shown at 1556 and 1540 cm^{-1}. The differences in the FT-IR data between the NP sample and P one were shown only in a small shift of the bands of amides I and II, characterizing the decrease in α-helical content.

Thermal properties of mussel proteins (Fig. 3) were stable up to a critical temperature. Urea destabilized the proteins which was shown in the decrease of enthalpy of denaturation (ΔH, kcal/mol): [native polluted mussel protein of 227.4 and 111.2 after denaturation with urea; native non-polluted mussel protein of 103.4 and 66.6 after denaturation with urea] and temperature of denaturation (T_d, °C) values [native polluted mussel protein of 59.3 and 55.1 after denaturation with urea; native non-polluted mussel protein of 53.1 and 48.9 after denaturation with urea] for the investigated samples. Comparison of the thermogrametric data (Fig. 3) of mussel proteins from P and NP sites showed the changes in T_d and ΔH for P and NP.

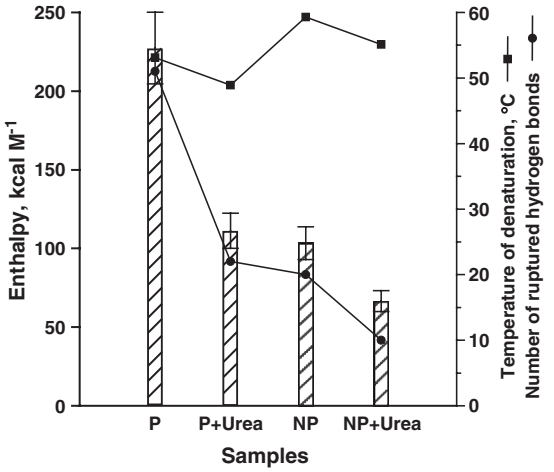

Fig. 3. Thermodynamic properties (enthalphy, temperature and number of rupture hydrogen bonds) of mussel proteins denatured with urea: in polluted (A) and non-polluted areas (B).

Thermal protein denaturation involves the rupture of disulfide and hydrogen bonds (Wagner and Añon, 1985). One disulfide bond contributes $\Delta H = 25$ kcal/mol and a negligible entropy (ΔS). The number n of hydrogen bonds corresponds to $\Delta H = 4$ kcal/mol and $\Delta S = 0.012$ kcal/mol/per protein molecule. Thus, the number n of broken hydrogen bonds can be calculated as $n = \Delta S / 0.012$ and $n = (\Delta H - 25)/4$, where ΔS is the entropy and ΔH is the enthalpy of denaturation. The entropy, which is associated with state transition and affirmed disordering of protein structure, was also calculated: ΔS (kcal/mol K) $= \Delta H / (273 + T_d\ ^\circ\mathrm{C})$. The calculations showed the changes in the ΔS for polluted site 0.34, for non-polluted of 0.21.

3.3. Antioxidant activity

The obtained results with manganese dioxide were compared with results of another variation of ABTS decolorization assay where ABTS radical cation was produced by reacting ABTS with potassium persulfate. The antioxidant activity of proteins from P and NP had comparative results against ABTS at the end point of 6 min as determined by spectrophotometric measurement (Fig. 4A and B). According to our results P with manganese had the highest percentage of inhibition (15.1%). The other extract such as NP potassium persulfate showed the lowest percentage of inhibition (8.8%). As it was shown on Fig. 4B, P manganese dioxide had the highest antioxidant activity as well [0.28 mM of trolox equivalent/ml (TE)]. The proteins were examined in three concentrations: 0.2, 0.4 and 0.8 mg/ml. The relationship between the

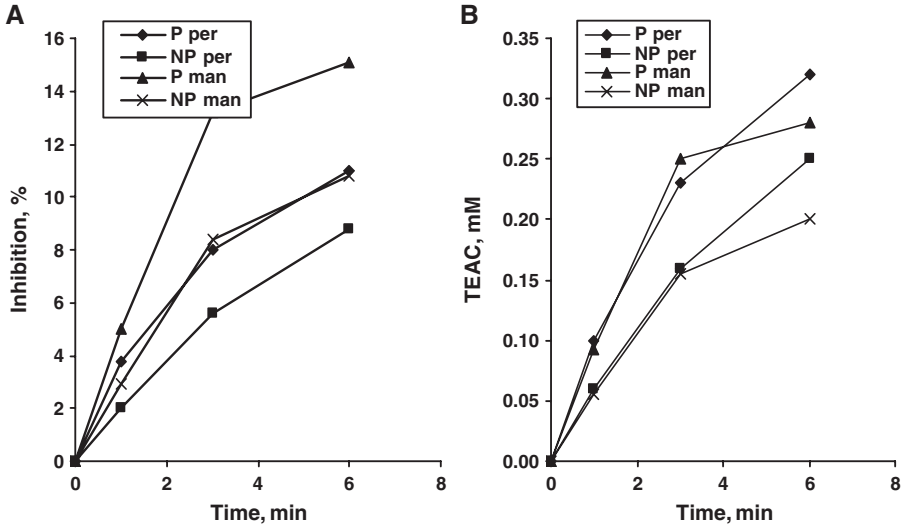

Fig. 4. Kinetics of ABTS scavenging effect of protein extracts (A). (B), relationship between time and antioxidant activity. The concentration of proteins is 0.02 mg/ml. P per, mussel protein from polluted area with potassium persulfate; NP per, mussel protein from non-polluted area with potassium persulfate; P man, mussel protein from polluted area with manganese dioxide; NP man, mussel protein from non-polluted area with manganese dioxide.

antioxidant activities and the protein concentration showed direct increase. In samples from P area the increase in the antioxidant activities and concentration was higher than in NP one.

Antioxidant activity determined by $ABTS^{\bullet+}$ method and their correlations to the concentrations were about 0.74. The manganese dioxide method gave slightly higher results than with potassium persulfate about 10% less than the persulfate.

As can be seen it was a relatively low correlation between the antioxidant activities determined in extracts with relatively low amounts of proteins (0.74). In this report only one concentration is shown in Fig. 4, 0.2 mg/ml, but the three concentrations were used.

4. Discussion

As was already mentioned in the Introduction, the monitoring of the marine pollution is very important and therefore many scientists are searching for reliable biomarkers of the seawater contamination (Lopez et al., 2001; Porte et al., 2001; Manduzio et al., 2003).

The differences in Zn and Cu content, the changes in proteins and their functional properties, and antioxidant activity of mussel *M. galloprovincialis* from P (the area of Port Varna) and NP (Cape Galata) sites of the Bulgarian Black Sea were studied for this aim using atomic spectroscopy, Fourier Transform Infrared (FT-IR) spectroscopy, fluorescence, DSC and two antioxidant tests.

It was found that the concentrations of Cu and Zn in mussel samples from P site are significantly higher than in samples from NP. As can be seen the proteins of the P samples showed a lower increase in the intensity than the proteins of the NP samples. The same tendency was observed after denaturation with 8 M urea, showing probably a more stable position which can be explained by the involvement of metals. The obtained results are similar to Manduzio et al. (2003), Romeo et al. (2003) and Dragun et al. (2004) who have shown that mussels from P sites are characterized by very high copper concentration and high catalase activity. Our results showed as well that the correlation between copper and proteins in P and NP samples from the soft tissue were in accordance with Viarengo and Nott (1993), Manduzio et al. (2003), Rivera-Duarte et al. (2005) and Romeo et al. (2005). These results were explained by binding to metallothioneins which is one of the important heavy metal cation homeostasis mechanisms identified in marine invertebrates, and the metallothionein detoxifying copper was found in *M. galloprovincialis*. Other mechanisms (compartmentalization within lysosomes and formation of mineral granules) exist in heavy metal homeostasis. Zinc, which is associated with membrane proteins and lipoproteins, is abundant in the contamination environment and its relatively high concentration is in the accepted ranges (Kaimoussi et al., 2001; Manduzio et al., 2003; Romeo et al., 2005).

The thermodynamic results suggest changes in molecular conformation of proteins and are consistent with those of other authors (Kato et al., 1990; Murphy and Gill, 1991; Togashi et al., 2002). A considerable number of protein molecules shift to a state that contributes much less to the unfolding transition, thus causing a significant decrease in the calorimetric enthalpy. The enthalpy changes of the initial and remaining DSC endotherm were measured and used for calculation of percentage of denatured proteins. The decrease in ΔH indicates denaturation and less stable structure. It means that conformation of the protein molecule has shifted towards the unfolded state. During thermal denaturation the breakage of hydrogen bonds was calculated and this number was as much as twice in polluted protein complexes than in non-polluted. These data are in accordance with the changes in fluorescence intensity and the shift of the wavelength maximum as well as in the shift of Amide II band and the decrease in α-helix (Kato et al., 1987). Protein thermal stability depends on hydrogen bonds and hydrophobic interactions. Mussel proteins reflect the same or similar properties as all animal proteins. Addition of protein denaturants such as urea led to a decrease in enthalpy and temperature of denaturation, indicating the loss of cooperatively. Therefore denaturation results in the decrease of enthalpy and such decrease effects the functional properties of proteins (tertiary and quaternary structures).

The study of the antioxidant activity has revealed a completely new phenomenon: the purified proteins have scavenging activities against $ABTS^{\bullet+}$ radical cation and the antioxidant activity in the mussel samples from P site are significantly higher than in mussel samples from NP. The radical $ABTS^{\bullet+}$ has been wide-

ly used in model systems to investigate the scavenging activities of several natural compounds such as phenolic compounds, anthocyanins, or crude mixtures such as methanol extracts of plants (Hou et al., 2001).

There are papers about metallothionein against oxidative stress in the mussel *M. galloprovincialis,* proteins and its antioxidant activity (Cajaraville et al., 2000; Mosquera et al., 2003; Dragun et al., 2004; Hamer et al., 2004), but the changes in protein structure were not studied. Therefore, we used these proteins to test the scavenging activities against the $ABTS^{\bullet+}$ radical. The scavenging activity of proteins against the $ABTS^{\bullet+}$ radical is concentration-dependent. This is the first report that these proteins could capture the $ABTS^{\bullet+}$ radical.

It is most probably that in total antioxidant activity of mussel extracts, polyphenols are playing a major role in comparison with other compounds such as proteins. This conclusion is in accordance with Regoli (2000) and our previous studies (Gorinstein et al., 2003; Moncheva et al., 2004) that the total scavenging capacity towards different forms of oxyradicals is confirmed as a useful biomarker with predictive validity at the organism level. In this study, the $ABTS^{\bullet+}$ was generated by incubating ABTS with potassium persulfate or manganese oxide. The production of $ABTS^{\bullet+}$ depends on the inhibitors during the reaction which contributes to the total $ABTS^{\bullet+}$ scavenging capacity. 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. Separation of soluble antioxidants from the protein fraction, suggested a greater depletion of low molecular weight molecules during the first phase of exposure to pollutants. Conversely, stress-70 proteins were significantly induced in the most polluted locations (Cope et al., 1997; Porte et al., 2001).

Similar results were registered in our recent investigations (Gorinstein et al., 2003; Moncheva et al., 2004) for the antioxidant activity in the whole soft mussel tissue from the polluted and non-polluted sites using other methods: the antioxidant activity of the whole soft mussel tissue from the polluted site was significantly higher than in non-polluted site. The measurement of the protein level in the soft tissues of mussels was studied in this report as generally able to discriminate between different levels of contamination, allowing the use of a simplified procedure compared with dissection of the digestive gland.

We did not find literature data to compare our findings of the qualitative changes in mussel proteins of the P site with the data of others: nobody has studied proteins in the P areas using a combination of FT-IR spectroscopy, fluorescence, DSC and two antioxidant tests.

In conclusion, there are some quality structural changes in mussel proteins, their antioxidant activity and the concentration of Cu and Zn in samples from polluted areas, which are significantly different than in samples from non-polluted areas.

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