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Changes in mussel *Mytilus galloprovincialis* protein profile as a reaction of water pollution

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Abstract

In the present report the changes in mussels *Mytilus galloprovincialis* protein profile, as a reaction of water pollution, is presented. The antioxidant capacity was determined by the efficacy to scavenge the derived radical in mussel samples using the Trolox Equivalent Antioxidant Capacity (TEAC) assay. The highest percentage of inhibition was estimated in protein mussels from the dirty area (DM) in comparison with the clean samples (CM). The amounts of both zinc and copper determined by atomic spectroscopy were significantly higher in the protein mussels from the dirty than from the clean areas ($P<0.05$).

Fourier Transform Infrared (FT-IR) spectroscopy and fluorescence revealed specific qualitative changes in secondary and tertiary structures of mussel proteins from the dirty area by the shift in the amides I and II positions and fluorescence intensity. Estimated temperature and enthalpy of denaturation in the protein mussels well correlated with fluorescence and spectroscopic measurements and showed the changes influenced by water pollution.
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1. Introduction

Reaction of mussel proteins and antioxidant compounds to different types of marine pollution was well described by Porte et al. (2001) and Lopez et al. (2002).These authors have examined how some protein parameters are influenced by pollution. The results of the total oxyradical scavenging capacity (TOSC) showed significantly higher levels of DNA damage in mussels collected from the inner parts of the lagoon compared to specimens from more external sites (Regoli et al., 2002; Frenzilli et al., 2001). TOSC has a greater predictive value on the health condition of the organisms and allows to discriminate the different role of specific ROS in oxidative stress syndrome (Regoli et al., 2002). The efficiency of antioxidant defenses in *Mytilus galloprovincialis* were investigated in a number of research papers (Camus et al., 2004; Cavaletto et al., 2002; Mourgaud et al., 2002; Geret et al., 2002).

The metallothionein content showed an increase by 43% after 4 days of exposure (Cavaletto et al., 2002). TOSC was proved to be an interesting health index parameter to measure pollution impact in a transplantation study (Camus et al., 2004).

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High levels of metals (particularly Cr and Cu) were recorded in the digestive gland of Alboraya mussels, which also had elevated metallothionein content. The results support the usefulness of the biomarker approach to assess and diagnose environmental pollution (Porte et al., 2001). Irato et al. (2003) and Viarengo et al. (2000a,b) described metal accumulation and binding protein induction in *M. galloprovincialis*, *Scapharca inaequivalvis*, and *Tapes philippinarum* from the Venice lagoon and other areas. In our recent investigations on mussels we have studied the antioxidant activities of the whole tissue (Moncheva et al., 2004; Gorinstein et al., 2003). The use of mussels as an indication of water pollution, as it was mentioned, well described by many researchers including Viarengo et al. (2000a), Panfoli et al. (2000) and Dafre et al. (2004). There are no reports concerning property changes in mussel proteins. Some fish protein properties were described by Huang et al. (2004).

Additional biomarkers are needed to draw a conclusion on the health of the mussels.

Therefore the relationships between water pollution and the change in the protein profile, free radical scavengers, copper and zinc contents in common mussel *M. galloprovincialis* collected from dirty and clean areas of the Bulgarian Black Sea coast were studied. Denaturation-induced secondary and tertiary changes in protein by increased fluorescence intensity and shift in the wavelength maximum, including the thermodynamic and spectroscopic measurements, are presented in this report for first time.

2. Materials and methods

2.1. Reagents

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

2.2. Collection and preliminary characteristics of mussel samples

Animals (*M. galloprovincialis*) were collected as previously described (Gorinstein et al., 2003). Two regions of Bulgarian Black Sea coast were investigated: clean (Cape Galata) and dirty (Port Varna) areas. Total and individual polycyclic aromatic hydrocarbons (PAHs) and the concentration of metals in area of Port Varna were much higher than in other places such as Cape Galata, which is open to sea. PAHs, total PAHs (T-PAHs), polychlorinated biphenyls (PCBs), benzo(*a*)pyrene hydroxylase (BPH) and biochemical oxygen demand were reported previously (Gorinstein et al., 2003). The characterization of the area of investigation (Moncheva et al., 2004) is similar to Porte et al. (2001) and Orbea et al. (2002).

Similar length and size (4.37±0.5 cm), which was 75–85% of the maximum size reached within each population, presented the main parameters of analyzed organisms (Moncheva et al., 2004). Whole soft tissue from 30 specimens of each population: CM=clean mussels area and DM=dirty mussels were rapidly frozen in liquid nitrogen to prevent oxidation and stored at −80 °C.

2.3. Extraction of proteins

Samples of 1 g from CM and DM mussel 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 obtained dry extracts were used for further analyses.

2.4. Determination of trace metals

Trace metals (Cu and Zn) were determined as follows. The samples were lyophilized and then mineralized in microwave oven for 15 min with concentrated HNO_3 according to Jurkiewicz et al. (2004) with our modifications. The concentrations of all above mentioned elements were estimated by a Perkin-Elmer 5100 ZL atomic absorption spectrometer (Perkin-Elmer Ltd., Beaconsfield, Buckinghamshire, England), using the flame method.

2.5. Structural properties of proteins

The whole soft tissue of glands and gills was investigated for proteins. Portions of 10 ml 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 with preliminary calibration of the column with 0.2 M NaCl and Blue Dextran. The gel chromatographic fractions were pooled and freeze-dried. Each sample (5 mg) was used for determination of protein content by Bradford (1976).

Fluorescence emission spectra were measured at excitation wavelengths (nm) of 274 and 295 and recorded from the excitation wavelength to wavelength of 450 nm.

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. Treatment of mussel proteins involved the addition of denaturants to the protein solutions in concentration of 8 M urea and denaturation was determined after incubation of protein with denaturants for 1 h (Gorinstein et al., 2001).

To record IR spectra a Bruker Optic GMBH Vector FT-IR spectrometer (Bruker Optic GMBH, Attingen, Germany) was used. Lyophilized material of 2 mg was mixed with 100 mg of KBr, and the pellet was pressed at 10000 kg/cm^2 for 15 s (Calero and Gasset, 2005).

2.6. Differential Scanning Calorimetry (DSC)

Denatured samples were prepared by homogeneous mixture of native and denaturated proteins in dry state. Urea as a denaturant was used. The mixed sample of 1 mg (sample:urea=1:1) was sealed in aluminum pan in the same way as the native one. As reference an empty pan was used. The conditions were in the range of 30–120 °C at a heating rate of 10 °C/min with a Perkin Elmer DSC System 4. Indium standards were used for temperature and

energy calibrations. *Td* and ΔH were calculated from the thermograms (Wright et al., 1977).

2.7. Determination of antioxidant capacity

The second peak of gel filtration chromatography was used for the determination of the antioxidant capacity by $ABTS^{\cdot+}$ radical scavenging method.

2,2′-azino-bis (3-ethyl-benzothiazoline-6-sulfonic acid) diamonium salt ($ABTS^{\cdot+}$) radical cation was generated by the interaction of ABTS (250 μM) and $K_2S_2O_8$ (40 μM). After addition of 990 μl of $ABTS^{\cdot+}$ solution to 10 μl of different extracts (0.2 mg/ml) or Trolox standards (final concentration 0–20 μM) in phosphate buffered saline (PBS), the absorbance was monitored exactly 1, 3 and 6 min after the initial mixing. The percentage decrease of the absorbance at 734 nm was calculated and plotted as a function of the concentration of the extracts and of Trolox for the standard reference data (Pellegrini et al., 1999).

2.8. Statistical treatment of results

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 and discussion

3.1. Trace metals

Mussel proteins from the dirty area had significantly higher concentration of copper than the clean ones. Similar picture was fixed in the concentration of zinc in the relevant areas ($P<0.05$). According to previous investigations (Gorinstein et al., 2003; Moncheva et al., 2004) it was supposed that the concentration of such metals will be higher in the dirty mussels than in clean (Fig. 1). Our results show that the change in the concentration of the important trace metals is in the range of the data presented by Viarengo et al. (2000a). Cu^{2+} and Zn^{2+} show different protective effect and the intensity of these effects was correlated with the metal affinity for sulfhydryls. Zn^{2+} protects lysosomes against the

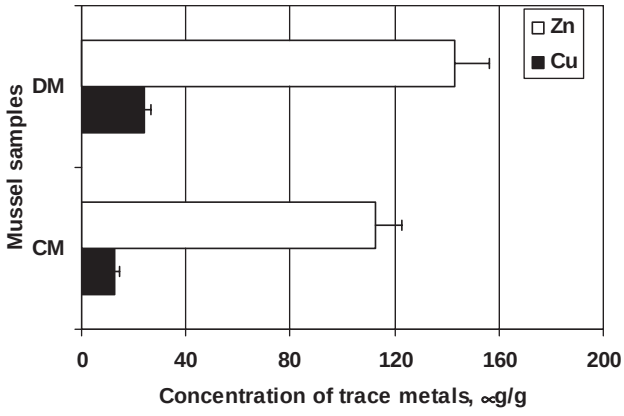

Fig. 1. Concentration of trace metals in clean and dirty mussel samples.

effects of Cd^{2+} and Cu^{2+}. The metals in this report were studied in connection with the changes in the protein profile of mussels as a reaction of water pollution. Metallothioneins (MT) are sulfhydryl-rich proteins binding essential and non-essential heavy metals and their antioxidant properties mainly derive from sulfhydryl nucleophilicity, but also from metal complexation. The effect of the metal may be derived from lipid peroxidation due to Cu^{2+}-induced oxyradical production (Panfoli et al., 2000). Cu^{2+} has much higher complex protein ability than Zn^{2+}. Binding of transition metals displaying Fenton reactivity (Fe, Cu) can reduce oxidative stress. Cu^{-+}-MT exhibits antioxidant activity, possibly due to differences in metal binding domains (Viarengo et al., 2000b). Our results are in accordance with Viarengo et al. (2000a,b) and Panfoli et al. (2000) and based on the ability of Cu^{2+} to form the complex-metal-protein and its high antioxidant activity as a free or bound metal.

3.2. Proteins

The fluorescence spectra exhibited a tryptophan-characteristic intrinsic fluorescence (Viarengo et al., 1997) with maximum (nm) excitation at 295 nm (Fig. 2A) and 274 nm (Fig. 2B). At excitation of 295 nm it was a small shift between the samples CM (maximum peak of 335 nm, fluorescence intensity 0.1282) and DM (maximum peak of 351 nm, fluorescence intensity 0.1532) and an increase in the fluorescence intensity (Fig. 2A and B). Urea denaturation showed bigger shift in the wavelengths maximum for the clean samples than for the dirty ones and as well as bigger increase in the fluorescence intensity in comparison with the dirty samples. Nearly the same results were obtained at excitation of 274 nm.

As can be seen the proteins of the DM samples showed lower increase in the intensity in comparison with the proteins of the CM samples. The same tendency was observed after denaturation with 8M urea, showing probably more stable position which can be explained by the involvement of metals in the protein complexes.

The two samples of mussels showed broad amide I (AI) bands at 1648 cm^{-1}, typical of proteins with high α-helical content (Fig. 3). High ratio of AII to AI bands in the CM sample can be attributed to the high content of α-helix. 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 (Calero and Gasset, 2005; Suci and Geesey, 2001).

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 dirty sample. The amide II band is shown at 1556 and 1540 cm^{-1}. The differences in the FT-IR data between the CM sample and DM were shown only in a small shift of the bands of amides I and II, characterizing the decrease in α-helical content.

The DSC thermograms (Fig. 4) have shown that mussel protein structure was stable up to a critical temperature and then became disrupted with intense heat absorption. Urea destabilized the protein conformation of mussel proteins as reflected by the marked decrease ΔH (native dirty mussel protein of 227.4 kcal/mol and 111.2 after denaturation with urea; native clean mussel protein of 103.4 kcal/mol and 66.6 after denaturation with urea) and *Td* values (native dirty mussel protein of 59.3 °C and 53.1 after denaturation with urea; native clean mussel protein of 55.1 °C and 48.9 after denaturation with urea) for the investigated samples.

The data for comparison are very limited: rabbit muscle comprised at least 3 endothermic transitions with *T* max (temper-

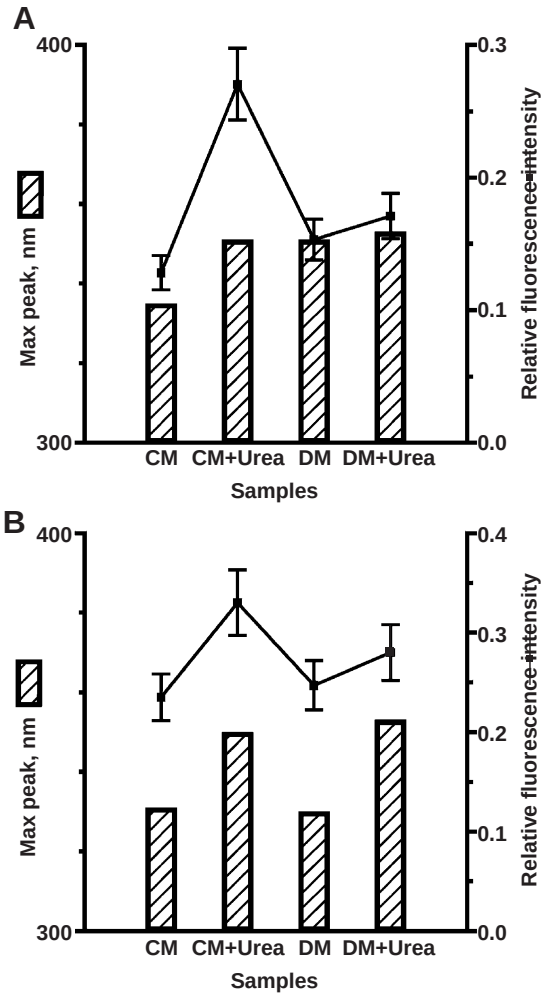

Fig. 2. Fluorescence intensity and maximum wavelength in clean (CM) and dirty (DM) mussel proteins before and after denaturation with 8 M urea: A, at λexc 295 nm; B, at λexc 274 nm.

ature of maximum rate of heat input) values of 60, 67, and 80 °C and which corresponded to denaturation of myosin. Our data are similar to these ones and correspond to myosin as well (Wright et al., 1977).

Hydrogen bonding is the main stabilizing force in protein stability. Hydrophobic interactions play an important role in the thermal stability of proteins and probably in mussel proteins. Addition of protein denaturants such as urea led to a decrease in enthalpy and *Td*, indicating protein denaturation and loss of cooperatively. Our data show that DSC can be used to study the effect of medium composition and heating on the tertiary and quaternary structures. The complexes of proteins with metals in dirty areas are more stable than in the clean ones. These results correspond with Irato et al. (2003) and Viarengo et al. (2000a,b), who have studied the correlations between metallothionein levels for some metals (copper, zinc and others) that are known to bind to this protein. Even in moderately dirty areas, measurement of the protein level in the soft tissue of studied mussels 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. Broadening of the peak was also demonstrated (Fig. 4). The decrease in ΔH indicates denaturation and less stable structure. It means that conformation of the protein molecule has shifted toward the unfolded state. Thermal protein denaturation involves the rupture of disulfide and hydrogen bonds (Makhatadze et al., 1994).

Our calculations show that during denaturation the rupture of 51 hydrogen bonds was involved in dirty in comparison with 20 in clean protein samples. It was assumed that during thermal denaturation only the rupture of hydrogen bonds is involved. With previous urea-induced denaturation the number of hydrogen bonds was reduced to about 30–50%. This trend is associated with the disruption of hydrogen bonds during heat denaturation and reflects a decrease in α-helix content of denatured protein, which was shown in our FTIR and fluorescence measurements (Huang et al., 2004; Wright et al., 1977). The heat denaturation is well approximated by a two-state transition and is accompanied by a significant increase of heat capacity. The contributions of hydrogen bonding and hydrophobic interactions to the stability of protein–urea–metal complex can be compared to those for other globular proteins. It was shown that the Gibbs energy of a hydrogen bond in a beta-sheet structure is greater than in alpha-helices (Makhatadze et al., 1994). In this report the main changes were in alpha-helices and this is confirmed by the shifts of amide I and amide II bands.

3.3. Total radical scavenging capacity

The efficacy of the samples from dirty and clean areas in buffer and water extracts to scavenge ABTS was lower in the water extracts by about 10% than that of the buffer ones (Fig. 5A, B).

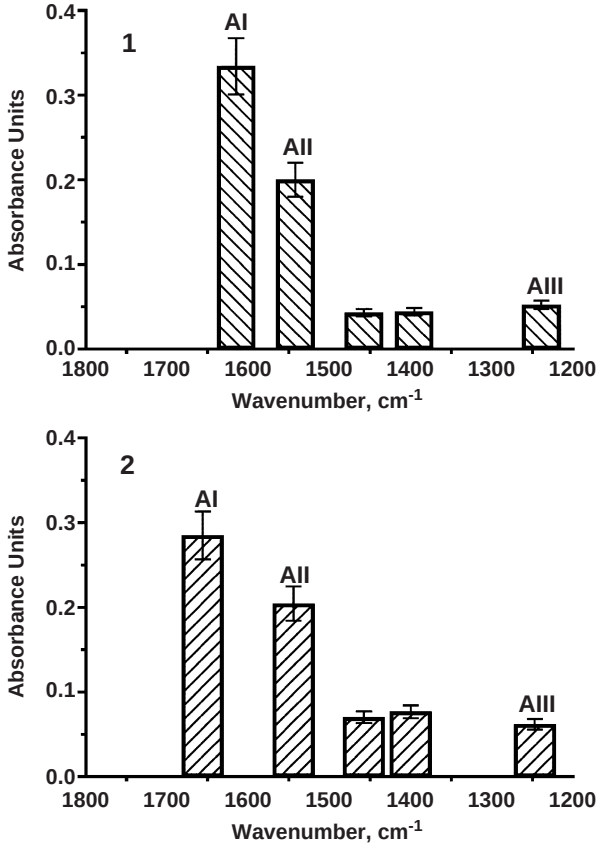

Fig. 3. FTIR-spectroscopic characterization of mussel proteins from clean (1) and dirty (2) areas. AI, amide I bond; AII, amide II bond.

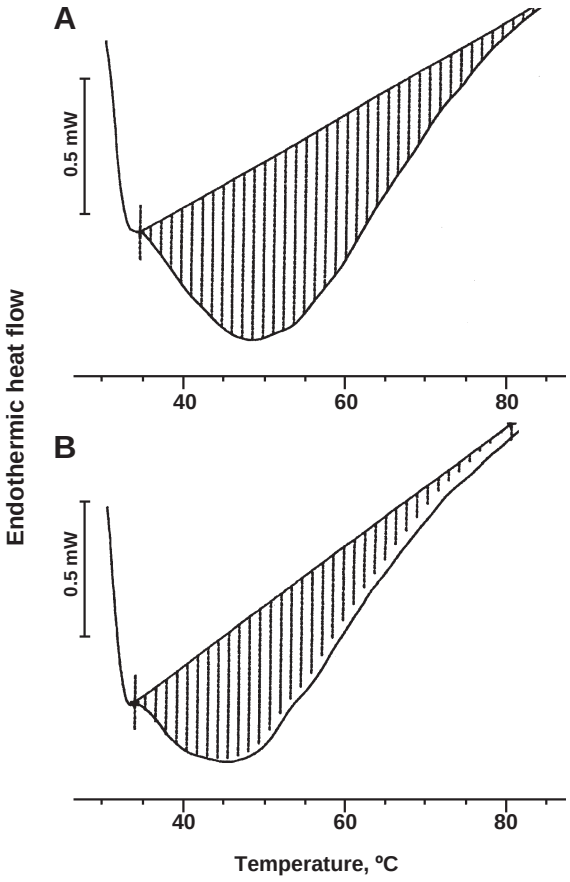

Fig. 4. Differential scanning calorimetry thermograms of mussel proteins denatured with urea: in dirty (A) and clean areas (B).

Each point on the curve was the average of five determinations of the assay procedure. The highest percentage of inhibition in dirty protein mussels was estimated in buffer fraction. Probably with the water extraction not all proteins were dissolved and the salt soluble globulin-like proteins were not reacting in the Trolox Equivalent Antioxidant Capacity (TEAC) assay. The antioxidant activity of Trolox was compared with the mussel samples. The contribution of total polyphenols to the antioxidant potential of dirty and clean samples was high.

The relative radical scavenging capacity of individual mussel extract against different testing radicals may be explained by the mechanisms involved in the radical-antioxidant reactions. In this study, the $ABTS^{\cdot+}$ was generated by incubating ABTS with potassium persulfate. Chemical compounds that inhibit the potassium persulfate activity may reduce the production of $ABTS^{\cdot+}$. This reduction results in a decrease of the total $ABTS^{\cdot+}$ in the system and contributes to the total $ABTS^{\cdot+}$ 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.

The radical $ABTS^{\cdot+}$ has been widely used in model systems to investigate the scavenging capacity of several natural compounds such as phenolic compounds, anthocyanins, or crude mixtures such as methanol extracts of plants (Pellegrini et al., 1999). However, some studies have reported on proteins with regard to the subject of direct antiradical effects and TOSC in mussel (Regoli, 2000; Frenzilli et al., 2001; Camus et al., 2004; Viarengo et al., 2000a,b). Therefore, we used these proteins to test the scavenging capacities against the $ABTS^{\cdot+}$ radical. The scavenging capacity of proteins against the $ABTS^{\cdot+}$ radical is concentration-dependent. This is the first report that these proteins could capture the $ABTS^{\cdot+}$ radical.

Monitoring of the marine pollution is very important for the future of the mankind and therefore many scientists are searching for reliable biomarkers of the water pollution (Porte et al., 2001; Irato et al., 2003). Therefore the differences in both zinc and copper contents, the changes in proteins and their functional properties, and antioxidant capacity of mussel *M. galloprovincialis* from dirty (the area of Port Varna) and clean (Cape Galata) areas of the Bulgarian Black Sea were studied by atomic spectroscopy, Fourier Transform Infrared (FT-IR) spectroscopy, fruorescence, DSC and total scavenging radical activity.

The mussels from dirty areas are characterized by high copper concentration and high TBARS levels and catalase activity and are involved in metal homeostasis and detoxification. These partly results are corresponding with Irato et al. (2003) and Mourgaud et al. (2002).

So far nobody has studied mussel proteins in the dirty and clean areas using a combination of FT-IR spectroscopy, fluorescence, DSC and scavenging tests, therefore we could not compare the above-mentioned qualitative changes found by us with other research reports.

It is most probably that in total antioxidant capacity 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 Mourgaud et al. (2002) and our own studies (Gorinstein et al., 2003; Moncheva et al., 2004) that the total scavenging capacity

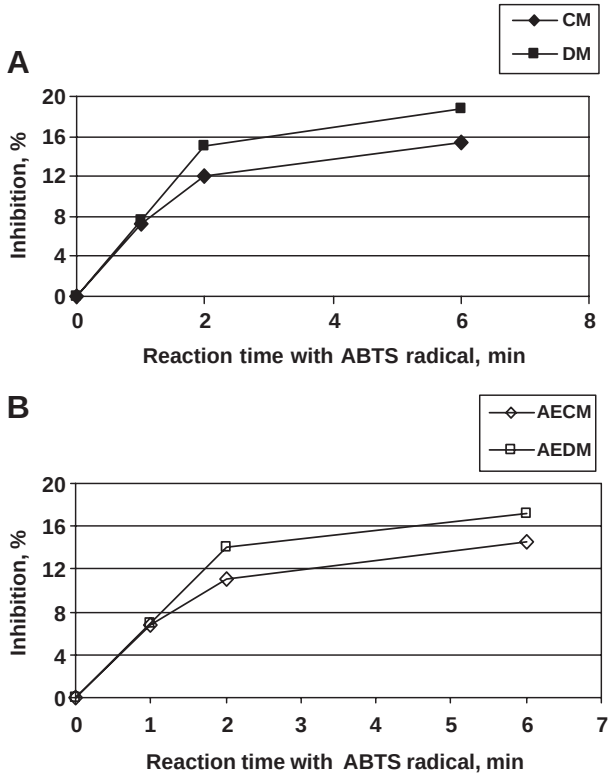

Fig. 5. Inhibition of (A) clean and dirty mussel, (B) aqueous extracts of clean (AECM) and dirty (AEDM) mussel against ABTS radical scavenger. Each point of measurements is the average of five determinations.

towards different forms of oxyradicals is confirmed as a useful biomarker with predictive validity at the organism level.

In conclusion, there are some quality structural changes in mussel proteins, their antioxidant capacity and the concentration of copper and zinc in mussels from dirty areas, which are significantly different than in samples from clean areas. These changes in the protein profile of mussels can be used as an indicator of water pollution.

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