Pergamon

Chemosphere, Vol. 37, Nos 14-15, pp. 2773-2783, 1998

0045-6535/98/ $ - see front matter

PII: S0045-6535(98)00320-8

TOTAL OXYRADICAL SCAVENGING CAPACITY IN MUSSEL *Mytilus* sp. AS A NEW INDEX OF BIOLOGICAL RESISTANCE TO OXIDATIVE STRESS

F. Regoli [1]*, G.W. Winston [2], V. Mastrangelo [1], G. Principato[1], S. Bompadre[3]

[1] Istituto di Biologia e Genetica, Università di Ancona, Via Ranieri Monte D'Ago, I-60100 Ancona, Italy;

[2] Department of Toxicology, North Carolina State University, Box 7633, Raleigh, NC 27695-7633, USA;

[3] Istituto di Scienze Biomediche, Università di Ancona, I-60100 Ancona, Italy

* Author for correspondence

ABSTRACT

The Total Oxyradical Scavenging Capacity (TOSC) has been characterized in various subcellular fractions from digestive gland of the mussel *Mytilus* sp.. A simple and sensitive gas-chromatographic assay is used for quantitating this new biochemical parameter.

The method is based on the reaction between peroxyl radicals and α-keto-γ-methiolbutyric acid (KMBA) which is oxidized to ethylene : in the presence of cellular antioxidant competing with KMBA for peroxyl radicals, ethylene formation is quantitatively reduced.

Whole cytosol from *M. edulis* exhibited slightly higher TOSC values than the Mediterranean species. Soluble antioxidants provided greater protection than proteins accounting for 70 % of oxyradical scavenging capacity of whole cytosols. TOSC assay was suitable also for lipid-soluble antioxidants, and the analysis of the microsomal fraction confirmed the importance of membrane-associated antioxidants in protection against toxicity of reactive oxygen species (ROS).

The analysis of TOSC in sentinel organisms is proposed as a new index of contaminant exposure, one which permits quantifiable assessment of the biological resistance to oxidative stress.

INTRODUCTION

Mussels, *Mytilus* sp., are commonly used as bioindicator species for monitoring environmental quality. Their use is based on the ability to accumulate pollutants and the several biological responses of this organism to xenobiotic chemicals that can be exploited as biomarkers of exposure or toxicity induced by these substances [1]. Several classes of pollutants are known to enhance the formation of reactive oxygen species (ROS), i.e. trace metals through the Fenton reaction or organic compounds which undergo redox cycling. Toxicity of ROS is counteracted by the cellular antioxidant system which is based on both specially adapted enzymes and low molecular weight scavengers. Oxidative stress occurs when the balance between prooxidant forces and antioxidant defenses is overcome [2].

The main components of the antioxidant system of *Mytilus* sp. have been in large measure characterized and the influence of several natural and biological variables has been assessed [3-7]. Variations in the levels or activities of the antioxidant defenses have been demonstrated in several field and laboratory studies and proposed as biomarkers of pollutant mediated oxidative stress [8-10]. Although data obtained on single antioxidant mechanisms may be useful in understanding specific responses to chemical insult, it is difficult to evaluate quantitatively the biological significance of such data in terms of resistance to toxicity induced by ROS.

We have recently described a new method for measuring the total absorbance capacity of oxyradicals by a tissue or biological fluids [11]. This method is based on the reaction between peroxyl radicals and α-keto-γ-methiolbutyric acid (KMBA) which is oxidized to ethylene gas. In the presence of antioxidants that compete with KMBA for peroxyl radicals ethylene production is quantitatively reduced. The application of a similar approach on marine invertebrates would allow better understanding of the effects of environmental conditions on the redox status of the organisms and on their susceptibility to oxidative stress.

In this paper we report the first results showing the Total Oxyradical Scavenging Capacity (TOSC) in the digestive gland of mussels *Mytilus* sp.. TOSC values were obtained for the microsomal and cytosolic fractions and, in the latter, the contributions of soluble and proteic fractions were further characterized. The results demonstrate the suitability of the Total Oxyradical Scavenging Capacity Assay for marine invertebrates and provide the first baseline data for an important bioindicator species, such as *Mytilus* sp..

MATERIAL AND METHODS

Organisms and sample preparation

Mussels, *Mytilus edulis* and *Mytilus galloprovincialis* were obtained respectively from the Gulf of Mexico (USA) and from Tyrrhenian Sea (Northern Mediterranean). Digestive glands were rapidly excised, frozen in liquid nitrogen and maintained at –80°C till processed for analyses.

Tissues were homogenized in 4 vol. of 50 mM K-phosphate buffer pH 7.5, containing 2.5% NaCl, aprotinin (1μg/ml), leupeptin (1μg/ml) and pepstatin (0.5μg/ml). This homogenization buffer was without effect on the TOSC assay.

After a preliminary centrifugation at 13,500 xg (4°C) for 25 min, the obtained supernatants were centrifuged at 100,000 xg at (4°C) for 1h. Supernatants (cytosolic fractions) were aliquoted and stored at -80°C. Cytosols were further separated into soluble and proteic fractions by using microconcentrators with membrane cut-offs of 3, 10 and 30 Kda (Microcon, Amicon Inc., Beverly, MA, USA). Devices were spin-rinsed prior to use with deionized water to remove traces of glycerol from the membranes, which was found to interfere with the assay.

For *M. galloprovincialis*, the 100,000 xg pellets were thrice resuspended in the homogenization buffer and recentrifuged. The pellets were finally resuspended in 1/5 of the original volume of homogenization buffer (without protease inhibitors) and stored at –80°C.

TOSC-Assay

Peroxyl radicals were generated from thermal decomposition of 2,2'-azobis-amidinopropane (ABAP). A final volume of 1 ml contained 20 mM ABAP and 0.2 mM KMBA in 100 mM potassium phosphate buffer, pH 7.4. Cytosolic and microsomal fractions were diluted to obtain a concentration of 63 ± 12.9 μg of protein in the assay. All reactions were carried out in 10 ml rubber septa-sealed vials maintained at 35°C in a shaking water bath to allow a constant generation of peroxyl radicals from ABAP. Ethylene formation was measured by gas chromatographic analysis of 1 ml of head space of reaction vessels and 8-10 serial samples were sequentially monitored every 12 minutes for a total assay duration of 96-108 min from the addition of ABAP. Detailed instrumental conditions have been described elsewhere [11]. The difference

of ethylene formation between control (absence of peroxyl radical scavengers) and samples containing biological tissues, was quantified in TOSC units calculated according to the equation:

TOSC= 100-(∫SA / ∫CA * 100) where ∫SA and ∫CA are the mathematically calculated areas under the kinetic curves, respectively of sample and control reactions. In the absence of oxyradical scavenging capacity, ethylene formation is not reduced as compared to control (∫SA / ∫CA = 1) resulting in a TOSC value = 0. On the other hand, at the hypothetical ∫SA = 0 (i.e. total inhibition of ethylene formation) the TOSC value approaches 100.

RESULTS

The time-course of ethylene formation from different amounts of a typical cytosolic fraction is reported in Fig. 1. The presence of antioxidants in the biological sample that can compete with KMBA for peroxyl radicals caused a quantitative reduction of ethylene formation compared to the controls. Increasing the amount of biological sample in the assay resulted in the appearance of a lag-phase in which ethylene formation was completely inhibited. The presence of a lag-phase does not interfere with the quantification of the oxyradical scavenging capacity and the obtained TOSC values were linear over a wide range of dilutions for all the subcellular fractions investigated (Fig. 2).

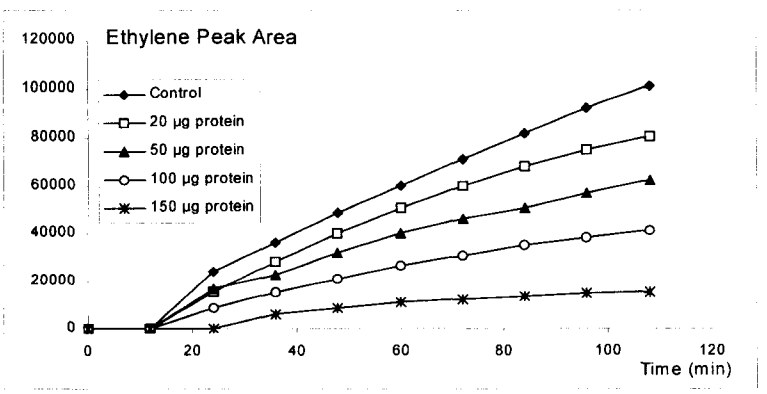

Figure 1. *Time-courses of KMBA oxidation by peroxyl radicals in the presence of different amounts of a typical cytosolic fraction from mussel digestive gland*

The oxyradical scavenging capacity of whole cytosols gave specific TOSC values of 0.47 ± 0.048 and 0.63 ± 0.073 per μg of protein, respectively for *M. galloprovincialis* and *M. edulis*.

Microconcentrators of different molecular weight cut-offs were used to separate cytosols into soluble and protein fractions. At the 3,000 Dalton exclusion limit, the percentage contribution of soluble antioxidants accounted for 67.5 ± 5.2 of the oxyradical scavenging capacity of the whole cytosol. A similar result (68.1 ± 4.7 %) was obtained using a microconcentrator with a molecular weight cut-off of 10 Kda. The contribution of soluble antioxidants significantly increased (81.3 ± 5.4 %) when the cytosolic fractions were separated with a 30 Kda cut-off microconcentrator.

The sum of the TOSC values obtained from the soluble and protein fractions was higher (121 ± 9.7 %) than those obtained for the corresponding cytosols, and a paired t-test confirmed the significance of these differences ($p<0.05$). On the other hand, when soluble and protein fractions were mixed together, the reconstituted samples gave a TOSC value comparable to those of cytosols ($p>0.05$).

Table 1 shows the results obtained with various combinations of pure antioxidants and biological fluids to detect possible synergistic interactions. The addition of a mixed solution of antioxidants (GSH 6.67μM, ascorbic acid 3.33 μM and uric acid 1.67 μM as final assay concentrations) resulted in an additive effect with whole cytosols and soluble antioxidants, while TOSC values lower than expected were obtained when the pure antioxidants were present combination with protein fraction.

Table 1. *TOSC values of biological fluids and combinations with a solution of pure antioxidants (GSH 6.67 μM, ascorbic acid 3.33 μM and uric acid 1.67 μM as final assay concentrations).*

	Obtained TOSC	Obtained/Expected TOSC
Cytosol (C)	34.5	
Soluble fraction (S)	22.0	
Proteic fraction (P)	18.4	
Antioxidant solution (A)	20.3	
C + A	53.8	98 %
S + A	42.5	100%
P + A	33.1	85 %

The TOSC-Assay was suitable also for measuring the antioxidant capacity of lipid-soluble antioxidants. Microsomal membranes from digestive gland of *M. galloprovincialis* exhibited TOSC values of 0.49 ± 0.041 per µg of protein. The effectiveness of this method towards lipid-soluble antioxidants was confirmed by the linearity of the TOSC-Assay over a wide range of biological dilutions (Fig. 2B).

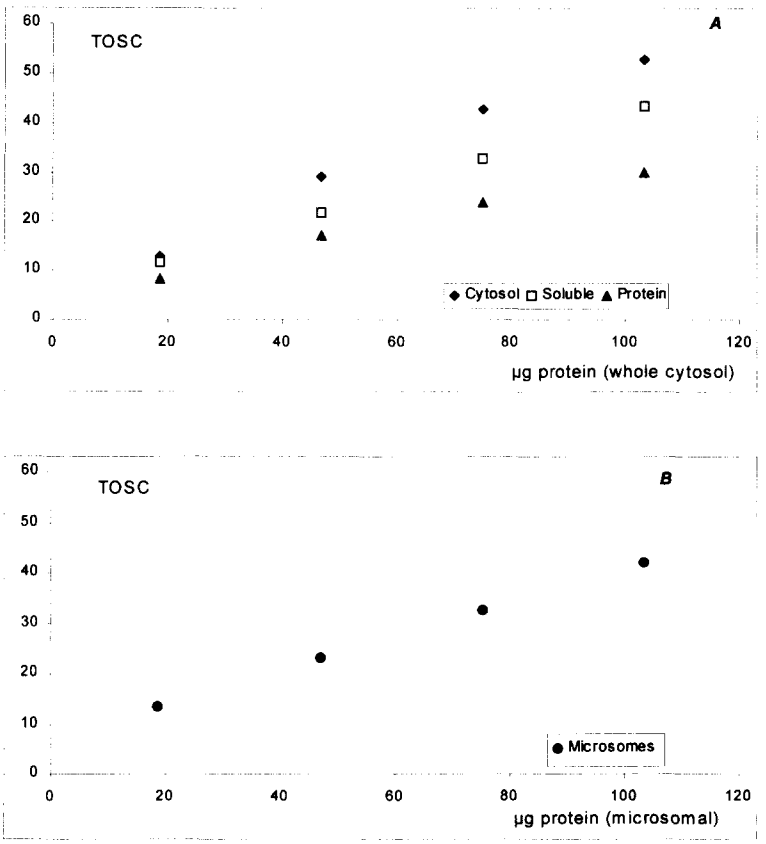

Figure 2. *Relationships of TOSC values with different amounts of biological samples: A: whole cytosol and corresponding soluble and protein fractions (same dilution factor); B: microsomal fraction*

DISCUSSION

Biochemical responses are now considered an important tool for ecotoxicologists dealing with the impact of pollutants on marine biota. In this respect, since oxidative stress is a general pathway of toxicity

induced by several chemical compounds, those biomarkers describing the redox status of the organisms may be used to reveal exposure and/or toxicity induced by xenobiotics.

Oxidative stress is usually investigated by the analysis of single antioxidant defenses and their responses to stress. These mechanisms, based on low molecular weight scavengers and specially adapted enzymes, have been extensively investigated in marine bivalves such as mussels [3-7].

Specific responses of antioxidants to pollutants are difficult to generalize since the same parameters have been reported to increase as an adaptive response or, in different circumstances, to be depleted as a toxic effect of contaminant exposure [9, 12-14]. Variations in the activity or components of the antioxidant system may be useful in understanding the mechanisms by which pollutants exert their effects and similar studies have been successfully proposed in several field and laboratory investigations for identifying or detecting biological impact of pollutants [9-10, 15-17].

However, considering the complexity of the whole antioxidant cellular system, from the analysis of specific, single defenses it is difficult to evaluate in a quantitative way the real susceptibility of a tissue to oxidative stress. In this respect, the integration with a method measuring the total absorbing capacity of oxyradicals would provide a better understanding of resistance to toxicity caused by these reactive species.

The TOSC-Assay is a simple gas chromatographic method based on the oxidation of KMBA to ethylene upon reaction with oxyradicals, and on the quantitative inhibition of this reaction in the presence of antioxidants. In respect to other methods which are similar in concept [18-22], this assay is suitable also for tissue and the obtained TOSC values are expressed per weight of protein or tissue which allows for easy comparison of variations in oxyradical scavenging capacity between different conditions (i.e. pollution), organisms and/or tissues.

With the TOSC assay good linearity was obtained over a range of assay values from 10 to 60 TOSC units. Our samples were diluted to obtain 20-40 TOSC units which roughly corresponded to 60 ± 20 μg of protein in the assay.

The Total Oxyradical Scavenging Capacity of whole cytosol was 0.63 ± 0.073 for digestive gland of *M. edulis* and 0.47 ± 0.048 per μg of protein for *M. galloprovincialis*. This difference could be "species"-dependent or, otherwise, related to seasonal variation of the redox status of the organisms. In this respect, *M.*

galloprovincialis was sampled during the winter season when the levels of the main antioxidants defenses are lower [7].

Soluble antioxidants accounted for the major percentage of whole cytosol oxyradical scavenging capacity. In a previous paper from our laboratory [11, 23], the separation of soluble and protein fraction by the use of microconcentrators with a cut-off of 3 Kda gave similar results to ammonium sulphate precipitation technique. In this work, no significant differences were obtained using a molecular weight cut-off of 3 or 10 Kda indicating that the physiological pool of low molecular weight proteins have a negligible effect on the TOSC value of soluble antioxidants. The latter consideration may not apply for cadmium-exposed mussels, since in these organisms a protective antioxidant effect of Cd-induced metallothioneins has been proposed [24].

The sum of the separate TOSC values of soluble and protein fractions was consistently greater than those from the corresponding whole cytosols in agreement with previous results obtained on rat liver [11]. When the isolated protein fraction is analysed, the obtained TOSC value is a combination of specific and non-specific oxyradical scavenging, respectively reflecting -SH content and random protein damage [11]. On the other hand, in whole cytosols the presence of soluble antioxidants acting as fast-reacting molecules, minimize the aspecific reaction of protein with radicals.

This is confirmed also by data obtained adding a mixed solution of pure antioxidants to various biological samples. The combination with isolated protein fraction gave a TOSC value lower than that expected from a simple additive effect.

The TOSC-Assay has been shown to be effective in detecting the antioxidant capacity of lipid-soluble antioxidants since most of the primary radicals from ABAP are generated within or near the surface of the membrane micellar system [25]. The TOSC value of microsomes from mussel digestive gland was 0.487 ± 0.041 per μg of protein, confirming the importance of membrane-associated antioxidants in protection against ROS toxicity.

In conclusion, the data presented in this study demonstrates the suitability of the TOSC-Assay for quantification of the biological resistance to oxyradical toxicity, and also provides the first baseline data for an important bioindicator species such as *Mytilus* sp.

Due to the ease of the analytical procedure, high reproducibility of results and the possibility to analyze 8-10 samples in less than 2 hr, the TOSC assay may represent a valuable tool for ecotoxicologists working with biomarkers of contaminant-mediated oxidative stress in sentinel organisms.

REFERENCES

1. D.R. Livingstone, Biotechnology and pollution monitoring. Use of molecular biomarkers in the aquatic environment, *J. Chem. Technol. Biotechnol.* 57, 195-211 (1993)
2. G.W. Winston and R.T. Di Giulio, Prooxidant and antioxidant mechanisms in aquatic organisms, *Aquat. Toxicol.* 19, 137-161 (1991)
3. G.W. Winston, D.R. Livingstone and F. Lips, Oxygen reduction metabolism by the digestive gland of the common mussel, *Mytilus edulis* L., *J. Exp. Zool.* 255, 296-308 (1990)
4. A. Viarengo, L. Canesi, M. Pertica and D.R. Livingstone, Seasonal variations in the antioxidant defense system and lipid peroxidation of the digestive gland of mussels, *Comp. Biochem. Physiol.* 100C, 187-190 (1991)
5. A. Viarengo, L. Canesi, M. Pertica , D.R. Livingstone and M. Orunesu, Age-related lipid peroxidation in the digestive gland of mussels. The role of antioxidant defence system, *Experientia* 47, 454-457 (1991)
6. D.R. Livingstone, F. Lips, P. Garcia Martinez and R.K. Pipe, Antioxidant enzymes in the digestive gland of the common mussel *Mytilus edulis*, *Mar. Biol.* 112, 265-276 (1992)
7. F. Regoli, Trace metal and antioxidant mechanisms seasonal variations in gills and digestive gland of the Mediterranean mussel *Mytilus galloprovincialis*, *Arch. Envir. Contam. Toxicol.* in press (1997)
8. A. Viarengo, L. Canesi, M. Pertica , G. Poli, M.N. Moore and M. Orunesu, Heavy metal effects on lipid peroxidation in the tissues of *Mytilus galloprovincialis* Lam., *Comp. Biochem. Physiol.* 97C, 37-42 (1990)
9. C. Porte, M. Sole, J. Albaiges and D.R. Livingstone, Responses of mixed-function oxygenase and antioxidant enzyme system of *Mytilus* sp. to organic pollution, *Comp. Biochem. Physiol.* 100C, 183-186 (1991)

10. F. Regoli and G. Principato, Glutathione, glutathione-dependent and antioxidant enzymes in mussel, *Mytilus galloprovincialis*, exposed to metals under field and laboratory conditions: implications for the use of biochemical biomarkers, *Aquat. Toxicol.* 31, 143-164 (1995)

11. G. W. Winston, F. Regoli, A.J. Dugas Jr, J.H. Fong and K.A. Blanchard, A rapid gas chromatographic assay for determining oxyradical scavenging capacity of antioxidants and biological fluids, *Free Radic. Biol. Med.* 24(3), 480-493 (1997)

12. R.J. Wenning, R.T. Di Giulio and E.P. Gallagher, Oxidant-mediated biochemical effects of paraquat in the ribbed mussels (*Geukensia demissa*), *Aquat. Toxicol.* 12, 157-170 (1988)

13. D.R. Livinstone, P. Garcia Martinez, X. Michel, J.F. Narbonne, S. O'Hara, D. Ribera and G.W. Winston, Oxyradical production as a pollutant-mediated mechanism of toxicity in the common mussel *Mytilus edulis* L., and other molluscs, *Funct Ecol* 4, 415-424(1990)

14. F. Regoli, M. Nigro and E. Orlando, Lysosomal and antioxidant responses to metals in the antarctic scallop *Adamussium colbecki*, *Aquat. Toxicol.* in press (1998)

15. D. Ribera, J.F. Narbonne, X. Michel, D.R. Livingstone and S. O'Hara, Responses of antioxidant and lipid peroxidation in mussels to oxidative damage exposure, *Comp Biochem Physiol* 100C, 177-181 (1991)

16. J.E Stein, T.K. Collier, W.L. Reichert, E. Casillas, T. Hom and U. Varanasi, Bioindicators of contaminant exposure and sublethal effects: studies with benthic fish in Puget Sound, Washington, *Environ. Toxicol. Chem.* 11, 701-714 (1992)

17. R.T. Di Giulio, C. Habig and E.P. Gallagher, Effects of Black Rock Harbour sediments on indices of biotransformation, oxidative stress and DNA integrity in channel catfish, *Aquat. Toxicol.* 26, 1-22 (1993)

18. D.D.M. Wayner, G.W. Burton, K.U. Ingold, K.U. and S.J Locke, Quantitative measurement of the total, peroxyl radical-trapping antioxidant capability of human blood plasma by controlled peroxidation, *FEBS Lett.* 187, 33-37 (1985)

19. R.J. De Lange and A.N. Glazer, Phycoerythrine fluorescence-based assay for peroxyl radicals: a screen for biologically relevant protective agents, *Anal. Biochem.* 177, 300-306 (1989)

20. G. Cao, H.M. Alessio and R.G. Cutler, Oxygen-radical absorbance capacity assay for antioxidants, *Free Radic. Biol. Med.* 14, 303-311 (1993)

21. A. Ghiselli, M. Serafini, G. Maiani, E. Azzini and A. Ferro-Luzzi, A fluorescence-based method for measuring total plasma antioxidant capability. *Free Radic. Biol. Med.* 18, 29-36 (1995)

22. S. Chevion, E.M. Berry, M. Kitrossky and R. Kohen, Evaluation of plasma low molecular weight antioxidant capacity by cyclic voltammetry, *Free Radic. Biol. Med.* 22, 411-421 (1997)

23. F. Regoli and G.W. Winston, Applications of a new method for measuring the total oxyradical scavenging capacity in marine invertebrates, *Mar. Environ. Res.* in press (1997)

24. A. Viarengo, J. Blasco, B. Burlando, E. Ponzano, B. Marchi, F. Trielli, Metallothioneins and oxidative stress in marine organisms, *PRIMO 9 (Pollutants Responses In Marine Organisms) Meeting*, Bergen, Norway, 27-30 April (1997)

25. W. Pryor, J. Cornicelli, L. Devall, B. Tait, B. Trivedi, D. Witiak and M. Wu, A rapid screen test to determine the antioxidant potencies of natural and synthetic antioxidants, *J. Org. Chem.* 58, 3521-3532 (1993)