Chapter 19
Accumulation of Okadaic Acid and Detoxifying Enzymes in the Digestive Gland of *Mytilus galloprovincialis* During Exposure to DSP

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Introduction

In the Galician Rias, *Dinophysis acuminata* and *Dinophysis acuta* are the main species of toxic microalgae, and have been shown to produce okadaic acid and dinophysistoxin-2 (Fernández et al. 1998). Okadaic acid, diniphysistoxin-1 (DTX-1) and dinophysistoxin-2 (DTX-2) have been identified in most Diarrhetic Shellfish Poisoning (DSP) episodes in Galician mussels (Gago-Martinez et al. 1996), as well as being found in Irish (Carmody et al. 1996) and Portuguese (Vale and Sampayo 2002) shellfish.

OA and Dinophysistoxins (DTX's) are the principal toxic compounds causing DSP in humans (Yasumoto et al. 1985). These compounds are lipophilic polyether molecules produced by dinoflagellates *Dinophysis* sp. and *Prorocentrum* sp. genera (Yasumoto et al. 1978; Murata et al. 1982).

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Mussels, like other bivalves, can accumulate DSP toxins during algal blooms of these microalgal species. Accumulation takes place mainly in the digestive gland (Blanco et al. 2007) and causes a negative economic impact to the shellfishing industry in the Galician rias on an annual basis. Following ingestion of contaminated mussels by humans the DSP toxins can cause a gastrointestinal disease and result in a serious public health problem.

The genetic selection of molluscs with a greater capacity for detoxification, in order to reduce depuration times, could help to reduce the resultant significant economic and health problems.

There are few studies concerning the metabolism of biotoxins in bivalves, although some authors have suggested involvement of detoxification enzymes (Kodama and Sato 2002; FRS Marine Laboratory 2004) and described the induction of some antioxidant enzymes in crustaceans and scallops in the presence of toxic dinoflagellates (Campa-Córdova et al. 2009).

This work is a preliminary study of the metabolism of okadaic acid in *Mytilus galloprovincialis*. We analysed a diverse range of enzymes potentially involved in xenobiotic metabolism and follow enzyme activity variation in relation to OA accumulation during three different toxic blooms of *D. acuminata* and *D. acuta* in the Ria de Vigo.

Material and Methods

Samples

Mussels (*Mytillus galloprovincialis*) were sampled fortnightly from floating rafts of the Vigo estuary (NW Spain) from June 2001 until August 2002. For each sampling, 80 adult individuals of 6–8 cm in length were randomly collected. The digestive glands of 30 mussels were dissected, immediately frozen in liquid nitrogen, pooled and stored at −80 °C until the enzymatic assays. A section of mantle tissue was also dissected, fixed in Bouin's solution and histologically processed using routine histological techniques (paraffin embedded, 5 μm sectioning, stained with Harris' haematoxylin-eosin solution and analysed microscopically) to determine the sex of each mussel.

The soft tissues of the other subsample (50 mussels) were pooled, homogenized, lyophilized and stored for drying until toxin analysis. In *Mytilus*, enzymatic activity varies with the reproductive status, sex and environmental parameters (Borkovic et al. 2005; Bochetti and Regoli 2006; Monserrat et al. 2007; Verlecar et al. 2008; Cravo et al. 2009). In this study, the histological analysis showed that most of the mussels collected on the same date were at the same gametogenic stage. Because of this and because we separated males and females following collection, we felt justified in pooling each sample from the same sampling location.

Enzymatic Analysis

Following sex determination the digestive glands were pooled for each sampling date and by sex and then homogenized in 20 mM Tris-HCl buffer pH7.6 (1:4, w:v) containing 0.5 M sucrose, 0.15 M potassium chloride (KCl), 1 mM ethylene-diaminetetraacetic acid (EDTA), 1 mM â-mercaptoethanol and 0.1 mM of the protease inhibitor phenylmethylsulfonyl fluoride (PMSF). The homogenate was centrifuged at 4 °C at 500 × g for 30 min and the resulting supernatant further centrifuged at 12,000 × g for 45 min. This last supernatant was considered to be the post-mitochondrial fraction, on which all enzymatic activities were assayed by spectrophotometric methods at 25 °C. Assays were performed in triplicate for each sample.

Cytochrome P450 reductase (CPR) was determined as described by Livingstone and Farrar (1984) using NADPH and NADH as electron donor and cytochrome c as substrate. 7-ethoxi-resorufine-O-deetilase (EROD) was assayed by the method of Burke and Mayer (1974). Xanthine oxidoreductase (XDH) was determinated following the method described by Lallier and Walsh (1991). DT-diaphorase activity (DTD) was determined as described by Ernster (1967) and Livingstone et al. (1992). Glutathione-S-transferase (GST) was determined as the conjugation enzyme by the method of Habig and Jakoby (1981) Among antioxidant enzymes, superoxide dismutase (SOD), total and selenium-dependent glutathione peroxidase (GPx-tot; GPX-Se); glyoxalases I and II (GLO I, GLO II) and Catalase (CAT) were assayed respectively, by the methods described by Ewing and Janero (1995), Lawrence and Burk (1976), Principato et al. (1983) and Aebi (1984). Glutathione reductase (GR) was assayed by the Ramos-Martínez et al. method (1983), as an enzyme involved in the redox cycle of glutathione, which yields an adequate concentration of reduced glutathione for GPX and GLO I activities. Activities were expressed in UI or mUI per gram of tissue.

Extraction and Analysis of Toxins

Toxin extraction for each sample date was carried out with 0.4 g of lyophilized mussel (equivalent to 2 g of wet weight). Three extractions were carried out adding 80 % MeOH at a ratio of 1:4 (weight: volume) according to Quilliam 1995. Extracts were clarified by centrifugation (10,000 × g for 10 min at 20 °C) and transferred into a volumetric flask through syringe filters of 0.22 μm. The extracts were evaporated in a rotavapor, resuspended freshly in MeOH 80 %, and then filtered through ultrafree centrifugal filters of 0.45 μm.

Aliquots of each extract were hydrolysed at 75 °C for 40 min. with 2.5 M NaOH, and the reaction stopped by adding 2.5 M HCl.

The presence of okadaic acid in the samples was determined by HPLC–MS/MS with a Surveyor MS HPLC system, coupled to a Deca XP plus ion trap mass

spectrometer (Thermo Fisher Scientific) with an electrospray interface, following the method of Gerssen et al. (2009). The chromatographic separation was carried out using a Gemini NX C18 column. The mobile phase consisted of 100 % water (A) and acetonitrile: water (95:5 %) (B), both containing 2 mM ammonium formate and 50 mM formic acid (pH 2.6). An isocratic elution consisting of a 10 % mobile phase B and 90 % mobile phase A, was run. The mobile phase flow was 0.4 mL/min and the injection volume was 20 μL.

OA was quantified by comparison with reference materials from NRC-CRM, Canada.

Results and Discussion

During our study three toxic episodes, caused by *D. acuminata* and *D. acuta*, occurred between June 18, 2001 and August 6, 2002 (INTECMAR: www.intecmar.org), during which our results showed that OA was accumulated by mussels (Fig. 19.1). DTXs were not detected in any samples.

Considering all enzymatic activities evaluated, only CPR, GST, GR, GLO I, GPXtot and CAT showed significant variations, which were related to the accumulation of OA in mussels.

The CPR activity showed a significant increase, mainly in males, inversely related to decrease of OA accumulated (r: 0.375, $p < 0.05$) (Fig. 19.1a; Table 19.1). This enzyme provides electrons to different oxygenases from the endoplasmic reticulum, between them to the oxygenase- dependant cytochrome P450, which is

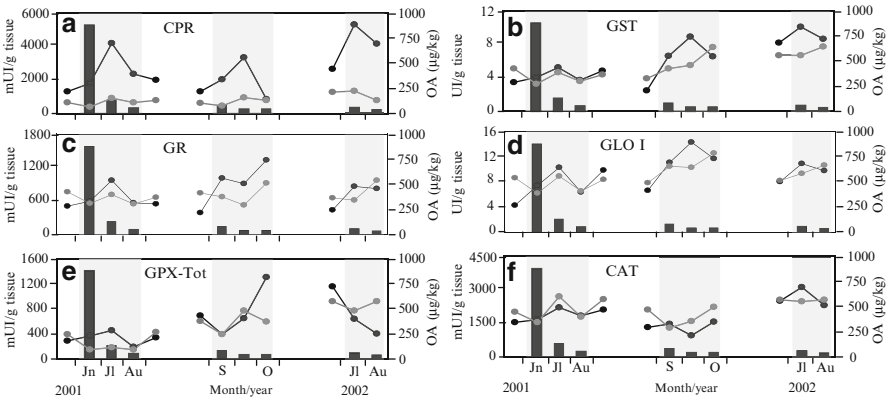

Fig. 19.1 OA accumulation and variation of CPR, GST, GR, GPX, CAT and GLO I activities from *M. galloprovincialis* digestive gland during three toxic episodes of *D. acuminata*. *Bars*: concentration of OA accumulated in mussel soft tissues. Enzymatic activities in females (○) and in males (●)

involved in xenobiotic metabolism, steroids and lipid signalling synthesis, sterol synthesis or the metabolism of desaturation or elongation of fatty acids, establishing a microsomal electron transport sequence known as "mixed function oxidase system". The, oxidation-reduction reactions of this system allow hydroxylation of multiple molecules and converts them into polar molecules in order to facilitate their elimination (Guengerich 1988). The relationship between CPR and OA in mussels is consistent with the involvement of some microsomal monooxygenases in the metabolism of OA. Other authors have described similar results in hepatocyte cultures from vertebrates (Tamaki et al. 2005; Guo et al. 2010).

The metabolites produced by the mixed function oxidase system are not easily eliminated and are usually more toxic than the initial compounds. Such metabolites can be conjugated with endogenous reduced glutathione to increase its polarity and hence make it easier to eliminate them. GST catalyzes the conjugation of a variety of endogenous and xenobiotic substrates with reduced glutathione (GSH) (Mannervik 1985; Listowsky et al. 1988). GST has an important role in preventing peroxidation and detoxification of toxic substances. In this work we observed an increase of this activity, parallel to decrease of OA and which was significantly correlated with CPR activity (r: 0.516, 0.544 in males and females, respectively; $p < 0.01$) (Fig. 19.1b; Table 19.1), suggesting its possible participation in OA metabolism.

The GST activity depends on the presence of reduced glutathione. The redox balance of cellular glutathione is maintained by glutathione reductase (GR). This enzyme catalyzes the reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH) (Ulusu and Tandogan 2007), which is also necessary for other enzymatic activities, such as glutathione peroxidase and glyoxalase I. In our study, GR activity showed a different behaviour in relation to OA accumulation, which was dependant on the sex of the mussel. In males we observed an increase of GR activity at the beginning of intoxication and then a decrease parallel to GST activity, when the OA began decreasing. Thus our results show a significant correlation between GR and GST activity (r: 0.498, $p < 0.01$) and between GR and OA accumulated (r: 0.483, $p < 0.01$). In female mussels there are no notable differences with males, although our results shows a GR activity inhibition at the beginning of the toxic episodes (Fig. 19.1c), which is negatively correlated with OA accumulation (r: −0.514, $p < 0.01$) (Table 19.1).

The oxidative reactions catalyzed by the mixed function oxidase system generate large amounts of reactive species of O_2 and other molecules, such as α oxaldeydes, which are highly toxic. Such molecules can be eliminated by the activity of other enzymes concerned with oxidative defense. Some authors have also described the induction by OA of these enzymes in vertebrate cell cultures (Fujii et al. 1994; Matias et al. 1999) and mussels (Auriemma and Battistella 2004).

Among the other enzymes of oxidative defense, the glioxilases I and II catalyze the coordinated detoxification of reactive α oxaldeydes with mutagenic and cytotoxic activity, converting them into their corresponding α -hydroxy acids (thiol ester intermediaries) (Regoli et al. 1996).

The glioxalase I is also a GSH-dependent enzyme. α oxaldeydes and glutathione form spontaneously an intermediary hemithioaceatal, which is transformed

Table 19.1 Correlation factors between diverse enzyme activities related to xenobiotic metabolism from mussel digestive gland and accumulation of OA

	Males						Females					
	CPR	GR	GST	CAT	GPX-tot	GLO I	CPR	GR	GST	CAT	GPX-tot	GLO I
GR	–											
GST	0.516**	0.498**					0.544**	–				
CAT	0.338*	–	0.416**				–	–				
GPX-tot	–	–	0.430**	–			0.440**	–	0.608**	0.317*		
GLO I	–	0.771**	0.737**	–	0.412**		0.334*	0.557**			0.312**	
OA	0.375*	0.483**	–	–	–	0.370*	–	−0.514**	–	–	−0.366**	–

Spearman coefficient: *$p < 0.05$; **$p < 0.01$

to S-D-lactoilglutathione by the GLO I activity. This S-D-lactoilglutathione is subsequently hydrolyzed to D-lactate and glutathione (GSH) by GLO II enzyme. Our results show an increase of GLO I activity with OA intoxication in both sexes (Fig. 19.1d) with a statistically significant correlation in males (r: 0.370, $p < 0.05$) (Table 19.1). This enzyme also shows a significant correlation with GR (r: 0.771, $p < 0.01$) and GST (r: 0.737, $p < 0.01$) activities in males, and with CPR (r: 0.334, $p < 0.05$) and GR (r: 0.557, $p < 0.01$) activities in females (Table 19.1). These results are consistent with induction by OA of GLO I in *Mytilus* as also obtained by Auriemma and Battistella (2004).

Other oxidative defence enzymes that seem to be related to the episodes of intoxication by OA in mussels are glutathione peroxidase (GPXtot) and catalase (CAT). GPXtot activity increases in both sexes during intoxication, showing in females a negative correlation with accumulated OA (r: −0.366, $p < 0.01$) (Fig. 19.1e; Table 19.1) and positive one with CPR (r: 0.440, $p < 0.01$), GST (r: 0.608, $p < 0.01$) and with GLO I (r: 0.312, $p < 0.0$) activities. In males its activity is only correlated with GST (r: 0.430, $p < 0.01$) and GLO I (r: 0.412, $p < 0.01$) (Table 19.1). On the other hand, CAT activity has no correlation with accumulated OA (Fig. 19.1f), but shows significant correlation with CPR (r: 0.338, $p < 0.0\ 5$) and GST (r: 0.416, $p < 0.01$) in males and with GPXtot (r: 0.317, $p < 0.05$) in females.

Despite the preliminary nature of this work, the results obtained suggest the involvement of the microsomal monooxygenase enzymatic system dependent on cytochrome P450 in the okadaic acid biotransformation in *Mytilus galloprovincialis*. Moreover, the different enzymatic correlations in males and females seem to indicate sexual differences in the metabolic pathways followed. However, to confirm this and to define other possible enzymes and pathways involved in OA degradation and elimination in mussels further studies will be required.

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References

Aebi H (1984) Catalase in vitro. Methods Enzymol 105:121–126

Auriemma R, Battistella S (2004) Biochemical and histological alterations of *Mytilus galloprovincialis* digestive gland after exposure to okadaic acid and derivatives. Invertebr Survive J 1:66–71

Blanco J, Mariño C, Martín H, Acosta CP (2007) Anatomical distribution of diarrhetic shellfish poisoning (DSP) toxins in the mussel *Mytilus galloprovinciallis*. Toxicon 50:1011–1018

Bochetti R, Regoli F (2006) Seasonal variability of oxidative biomarkers, lysosomal parameters, metallothioneins and peroxisomal enzymes in the Mediterranean mussel *Mytilus galloprovincialis* from Adriatic. Chemosphere 65:913–921

Borkovic SS, Saponjic JS, Pavlovic SZ, Blagojevic DP, Milosevic SM, Kovacevic TB, Radojicic RM, Spasic MB, Zikic RV, Saicic ZS (2005) The activity of antioxidant defense enzymes in the mussel *Mytilus galloprovincialis* transplanted into the northwest Mediterranean Sea. Comp Biochem Physiol 138C:411–427

Burke MD, Mayer RT (1974) Ethoxyresorufin: direct fluorimetric assay of a microsomal O-dealkylation which is preferentially inducible by 3-methylcholanthrene. Drug Metab Dispos 6(2):583–588

Campa-Córdova AI, Núñez-Vázquez EJ, Luna-González A, Romero-Geraldo MJ, Ascencio F (2009) Superoxide dismutase activity in juvenile *Litopenaeus vannamei* and *Nodipecten subnodosus* exposed to the toxic dinoflagellate *Prorocentrum lima*. Comp Biochem Physiol C Toxicol Pharmacol 149(3):317–322

Carmody EP, James KJ, Kelly SS (1996) Dinophysistoxin-2: the predominant diarrhetic shellfish toxin in Ireland. Toxicon 34:351–359

Cravo A, Lopes B, Serafim A, Company R, Barreira L, Gomes T, Bebianno MJ (2009) A multi-biomarker approach in Mytilus galloprovincialis to assess environmental quality. J Environ Monit 11:1673–1686

Ernster L (1967) DT-diaphorase. Methods Enzymol 10:309–317

Ewing JF, Janero DR (1995) Microplate superoxide dismutase assay employing a nonenzymatic superoxide generator. Anal Biochem 232:243–248

Fernández ML, Míguez A, Moroño A, Cacho E, Martínez A, Blanco J (1998) Detoxification of low polarity toxins (DTX3) from mussels *Mytilus galloprovincialis* in Spain. In: Reguera B, Blanco J, Fernández ML, Wyatt T (eds) Harmful algae. Xunta de Galicia and Intergovernmental Oceanographic Commission of UNESCO, Spain

FRS Marine Laboratory (2004) Marine biotoxins. Fisheries Research Services. Report AE 14/08, Aberdeen

Fujii J, Nakata T, Miyoshi E, Ikeda Y, Taniguchi N (1994) Induction of manganese superoxide dismutase mRNA by okadaic acid and protein synthesis inhibitors. Biochem J 301:31–34

Gago-Martinez A, Rodriguez-Vazquez JA, Thibault P, Quilliam MA (1996) Simultaneous occurrence of diarrhetic and paralytic shellfish poisoning toxins in Spanish mussels in 1993. Nat Toxins 4:72–79

Gerssen A, Mulder PPJ, McElhinney MA, de Boer J (2009) Liquid chromatography-tandem mass spectrometry method for the detection of marine lipophilic toxins under alkaline conditions. J Chromatogr A 1216:1421–1430

Guengerich FP (1988) Cytochromes P-450. Comp Biochem Physiol 89C:1–4

Guo F, An T, Rein KS (2010) The algal hepatoxoxin okadaic acid is a substrate for human cytochromes CYP3A4 and CYP3A5. Toxicon 55:325–332

Habig WH, Jakoby WB (1981) Assays for differentiation of glutathione S-transferases. Methods Enzymol 77:398–405

Kodama M, Sato S (2002) Metabolism of the toxins responsible for paralytic shellfish poisoning. In: Proceedings of coastal management and sustainable development. UNU-Iwate-UNESCO joint international conference on conserving our coastal environment, Tokyo, Japan

Lallier FH, Walsh PJ (1991) Activities of uricase, xanthine oxidase, and xanthine dehydrogenase in the hepatopancreas of aquatic and terrestrial crabs. J Crustac Biol 11:506–512

Lawrence RA, Burk RF (1976) Glutathione peroxidase activity in selenium-deficient rat liver. Biochem Biophys Res Commun 71:952–958

Listowsky I, Abramovitz M, Homma H, Niitu Y (1988) Intracellular binding and transport of hormones and xenobiotics by glutathione-S-transferases. Drug Metab Rev 19:305–318

Livingstone DR, Farrar SV (1984) Tissue and subcellular distribution of enzyme activities of mixed-function oxygenase and benzo(a) pyrene metabolism in the common mussel *Mytilus edulis L*. Sci Total Environ 39:209–235

Livingstone DR, Archibal S, Chipman K, Marsh JW (1992) Antioxidant enzymes in liver of dab, Limanda limanda, from the North Sea. Mar Biol 112:265–276

Mannervik B (1985) The isoenzymes of glutathione transferase. Adv Enzymol Relat Areas Mol Biol 57:357–417

Matias WG, Traore A, Bonini M, Sanni A, Creppy EE (1999) Oxygen reactive radicals production in cell culture by okadaic acid and their implication in protein synthesis inhibition. Hum Exp Toxicol 18:634–639

Monserrat JM, Martínez PE, Geracitano LA, Lund Amado L, Martínez Gaspar Martins C, Lopes Leaes Pinho G, Soares Chaves I, Ferreira-Cravo M, Ventura-Lima J, Bianchini A (2007) Pollution biomarkers in estuarine animals: critical review and new perspectives. Comp Biochem Physiol 146C:221–234

Murata M, Shimatani M, Sugitani H, Oshima Y, Yasumoto T (1982) Isolation and structural elucidation of the causative toxin of the diarrhetic shellfish poisoning. Bull Jpn Soc Fish 48:549–552

Principato GB, Locci P, Rosi G, Talesa V, Giovannini E (1983) Activity changes of glyoxalase I-II and glutathione reductase in regenerating rat liver. Biochem Int 6:249–255

Quilliam MA (1995) Analysis of diarrhetic shellfish poisoning toxins in shellfish tissue by liquid chromatography with fluorometric and mass spectrometric detection. J AOAC Int 78(2):555–569

Ramos-Martínez JI, Bartolomé TR, Pernas RV (1983) Purification and properties of glutathione reductase from hepatopancreas of *Mytilus edulis L.* Comp Biochem Physiol 75B:689–692

Regoli F, Saccucci F, Principato G (1996) Mussel glyoxilase I as a possible marker for eco-toxicological studies: purification and preliminary characterization. Comp Biochem Physiol 113C:313–317

Tamaki H, Samuka T, Uchida Y, Jaruchotikamol A, Nemoto N (2005) Activation of CYP1A1 gene expression during primary culture of mouse hepatocytes. Toxicology 216:224–231

Ulusu NN, Tandogan B (2007) Purification and kinetic properties of glutathione reductase from bovine liver. Mol Cell Biochem 303(1–2):45–51

Vale P, Sampayo MAM (2002) Esterification of DSP toxins by Portuguese bivalves from the Northwest coast determinated by LC_MS-a widespread phenomenon. Toxicon 40:33–42

Verlecar X, Jena K, Chainy G (2008) Seasonal variation of oxidative biomarkers in gills and digestive gland of green-lipped mussel *Perna viridis* from Arabian Sea. Estuar Coast Shelf Sci 76:745–752

Yasumoto T, Oshima Y, Yamaguchi M (1978) Occurrence of a new type shellfish poisoning in the Tohoku district. Bull Jpn Soc Sci Fish 44:1249–1255

Yasumoto T, Murata M, Oshima Y, Sano M, Matsumoto GK, Clardy J (1985) Diarrhetic shellfish toxins. Tetrahedron 41:1019–1025