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Research Report

Biochemical and histological alterations of *Mytilus galloprovincialis* digestive gland after exposure to okadaic acid and derivatives

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Abstract

Electrophoretical and histological analysis were performed on *Mytilus galloprovincialis* digestive gland samples, in order to detect the presence of a previously identified protein ca 30 kDa MW, synthesized during *Dinophysis spp*. blooms, and assess a possible correlation between the occurrence of this protein and okadaic acid (OA) exposure by ingestion. Mussels were sampled monthly from July 2000 to November 2001 in the Gulf of Trieste (upper Adriatic Sea) and immediately processed. Parallel samples were maintained in sea water plastic tanks and fed with marine invertebrate feed mixed with OA and derivatives at different concentration of toxins for each experimental group (25 μg, 50 μg, 100 μg). In tank reared mussels fed with OA, degeneration of digestive cells and appearance of 24.6 kDa protein were observed, while in wild mussels, neither histological alterations nor presence of a 24.6 kDa protein, were detected. A correlation between the toxins concentration and time of appearance was highlighted, to demonstrate this protein is synthesized in response to OA and derivatives exposure. About the identity of 24.6 kDa protein, it could be an enzyme involved in detoxification reactions, probably Glyoxalase I.

Key words: mussels; digestive gland; okadaic acid; DSP; *Mytilus galloprovincialis*

Introduction

The ingestion by mussels of *Dinophysis spp.* (marine dinoflagellates) is the main consequence for mussel toxicity and it is responsible for Diarrhetic Shellfish Poisoning (DSP), a human toxic syndrome, caused by the ingestion of contaminated shellfish. Also in Italy, the DSP causes public health concern and economical problems with sale blockage. The more common DSP toxins belong to the okadaic acid group: okadaic acid (OA), dinophysistoxin-1 (DTX-1), dinophysistoxin-2 (DTX-2), dinophysistoxin-3 (DTX-3), dinophysistoxin-4 (DTX-4) (Yasumoto and Murata, 1993).

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In order to detect the presence of DSP toxins in mussels, Italian Government (Health Ministry Decree 1/9/1990, modified on 31/7/1995) enforced the Yasumoto mouse bioassay (Yasumoto *et al*., 1984). This test provides only qualitative information on toxin presence, has a low sensitivity (limit detection 20 μg/100 gr of mussel digestive gland) and can give false positive results. In relation with this problem, others tests aimed to detect the DSP toxins were proposed in the last twenty years. Monoclonal antibody ELISA test detects OA and DXT-1 in toxic mussels, but it underestimates total toxin presence and its sensitivity is 10 μg/100 gr of shellfish digestive gland (Uda *et al*., 1989; Morton and Tindall, 1996). HPLC-fluorescence detection assay is the most universally used analytical technique for the determination of OA and DXT-1, it is more sensitive than ELISA (detection limit 1 μg/100 gr) but it is an expensive test and not easy to apply (Lee *et al*., 1987; Nunez and Scoging, 1997). Liquid chromatography-electrospray ionization mass spectrometry (LC-ESI-MS test) gives quantitative/qualitative information about DSP toxins

and it is more precise than HPLC method but has the same applicative problems (Lee *et al*., 1989; Suzuki and Yasumoto, 2000; Goto *et al*., 2001). Tissue culture assay is based on direct microscopic observation of toxin-induced morphological changes in Buffalo green monkey cell cultures (Croci *et al*., 1997).

Finally, the fluorescence protein phosphatase (PP-2A) inhibition assay is based on knowledge that serine/threonine protein phosphatases are inhibited by DSP toxins. It detects OA and DTX-1 in mussels down to 1 μg/100 gr of digestive gland tissue (Tubaro *et al*., 1996; Mountfort *et al*., 2000).

In the Gulf of Trieste (upper Adriatic Sea), Avian *et al*. (1993) identified, by histological analysis, alterations in the digestive gland of the mussel *Mytilus galloprovincialis*, during *Dinophysis spp.* algal blooms. This histological study was later supported by a qualitative electrophoretical analysis of water–soluble proteins of digestive gland, in order to investigate a possible modification in protein pattern as a consequence of toxin presence (Amirante *et al*., 1994; Bonivento *et al*., 1993, 1995, 1997). In this analysis the presence of a low-molecular weight protein was detected, only in mussels during *Dinophysis spp.* algal blooms, synthesized *ex novo, which* could counteract to metabolic alterations. In order to confirm this observation and to verify the relation between DSP toxins and the appearance of this protein and finally to identify its role, in controlled conditions, OA and derivatives mixed with marine invertebrate feed were given to mussels.

Material and Methods

Samples

In this study 470 samples of digestive gland of *Mytilus galloprovincialis* (Lmk., 1819) were analyzed. From July 2000 to November 2001, every month, 10 mussels (range: 45-65 mm length) were collected in the Gulf of Trieste. Their digestive glands were analyzed, for both histological and biochemical studies. Parallel mussels groups, were kept in sea water 25 liters plastic tanks, of which 25 individuals were fed with marine invertebrate feed as control, and 25 fed with marine invertebrate feed mixed with OA and derivatives (Sigma-Aldrich Italy). A further assay was performed, treating 25 mussels with heavy metals. To prevent additional environmental stress in the mussels, the seasonal photoperiod and water temperature were maintained. Sea water was changed weekly (unless otherwise stated) and water salinity controlled daily.

The temporal relationship between the toxin ingestion and the presence of the low molecular weight protein was assessed every three days from the beginning of every administration. The digestive gland of treated and control mussels (n=5), was extracted and processed. One third of every mussel digestive glands treated with OA and derivatives was used for histological analysis and the rest for biochemical analysis.

Treatments

To investigate possible histological and biochemical alterations due to maintenance in tanks, in the first assay mussels fed with marine invertebrate feed, were compared with wild mussels.

In the second assay, mussels were fed with invertebrate feed mixed with OA, for a total amount of 25 μg administered in the time span of 30 days. To avoid the water insolubility of OA and derivatives, the chemicals were dissolved in ethanol, mixed with invertebrate feed and given to mussels after ethanol evaporation, on alternate days. Feed mixed with ethanol only was administered to control mussels.

In the two following assays, mussels were fed both with OA and DTX-1 (1:1). In third assay 25 μg of OA and 25 μg of DTX-1 (for a total toxin amount of 50 μg) were administered within a 30 day period. In the last one 50 μg of OA and 50 μg of DTX-1 (for a total toxin amount of 100 μg) were administered in the same time span.

To investigate if the protein under study appeared also as a consequence of different toxicants, the mussels were exposed for 30 days to Cu^{2+} (625 nM) and Hg^{2+} (200 nM). Metals were additioned as $CuCl_2$ and $HgCl_2$ in sea water every two days changes. Controls were kept in artificial uncontaminated sea water for the same period.

Protein analysis

Each sample of digestive gland (about 0.5 gr) was homogenized in the same volume of extraction buffer (Tris-HCl 50 mM pH 7.5, β-mercaptoethanol 10 mM, EDTA 1 mM, 3 % SDS, 8 % glycerol) and centrifuged at 4 °C for 20 min at 12.000 rpm. 10 μl of supernatant was added (1:1) to denaturant sample buffer (Tris-HCl 1 M pH 6.8, β-mercaptoethanol 10 mM, 10 % SDS, 8 % glycerol, 0.1 % bromophenol-blue) and denaturated at 100 °C for 3 min. The determination of the relative molecular mass of extracted proteins was performed in 16 % SDS-polyacrylamide gel according to Schagger and Von Jagow (1987) using the low MW kit for molecular weights 14.400-94.000 and the high MW-SDS calibration kit for MW 53,000-212,000 (Amersham-Pharmacia, Germany). Water soluble proteins were stained with Coomassie brilliant Blue G250 (Sigma) staining solution.

Histological analysis

For histological analysis, about 2 mm^3 of digestive gland of each specimen, were fixed in 2 % glutaraldehyde in 0.1 M cacodylate buffer pH 7.25. The tissue samples were washed in a 0.1 M cacodylate pH 7.25 buffer three times and post-fixed for one hour in 1% osmium tetroxide in the same buffer. After another wash cycle, the samples were dehydrated in ascending ethanol and embedded, via propylene oxide, in Embed812/Araldite (Electron Microscopy Sciences, Fort Washington, PA). For light microscopy, sections 1 μm thick were collected on slides, baked for 5 min at 75 °C, stained with 0.5 % toluidine blue in 0.1 % sodium carbonate solution at pH 11.1 at the same temperature.

Results and Discussion

In the first assay, mussels, fed with marine invertebrate feed only, did not show qualitative differences in the low MW proteins compared with

wild mussels in no algal bloom conditions, neither histological analysis showed significant alterations of the histology. In the second run of experimental rearing, mussels fed with 25 μg of OA, expressed a 24.6 kDa protein, never present in control mussels. This protein appeared in treated *M. galloprovincialis*, 28 days after the beginning of the exposure and disappeared 8 days after the end of OA administration.

In the third assay, the mussels, fed with 50 μg of toxins (25 μg OA and 25 μg DTX-1), expressed the 24.6 kDa protein (never present in control mussels) (Fig. 1), 16 days after the beginning of the administration and the protein disappeared within the same time of the previous test. During the last exposure experiment, performed with 100 μg of DSP toxins (50 μg OA and 50 μg DTX-1), the protein was detected 12 days after the beginning of the treatment, and disappeared within 8 days post exposure. In graph (Fig. 2) is represented the percentage of mussels that presented the 24.6 kDa protein during the three essays. It is evident the temporal relationship between toxin administration dose and the appearance of the protein.

In the heavy metal assay, the 24,6 kDa protein appeared neither in treated mussels nor in control ones.

The parallel histological analysis on the same samples, confirmed a digestive gland alteration consequent to toxic treatment. In fact an increase of lipid vesicles both in digestive and basophilic cells was visible a week after the beginning of treatment. In some cases, the vesicles filled the cells cytoplasm with the progression of the toxin treatment (Figs 3, 4). In addition partial or total degeneration of digestive cells was observed in treated mussels starting from the second week (Fig. 5). However, lipid vesicles accumulation and digestive cells degeneration do not represent an anomalous condition in the physiological cycle of *M. galloprovincialis* digestive gland, but remarkable is its degree occurrence in treated mussels only.

From the results obtained, in *M. galloprovincialis* it is clear the correlation between the exposure to OA and derivatives and 24.6 kDa protein synthesis. In fact its presence was observed only in treated mussels. Moreover the observation of a temporal relationship between the dose dependency of toxins and the advanced appearance of the protein, further confirm their relationship. These results are in agreement with previous studies on *M. galloprovincialis* in the Gulf of Trieste during *Dynophisis spp.* algal bloom (Amirante *et al*. 1994; Bonivento *et al*., 1993, 1995, 1997). Histological analysis also showed that the treated mussels present an initial increase of lipid vesicles, especially originated in those digestive cells that during the toxin administration degenerate, up to their lysis.

The role of the 24.6 kDa protein, was preliminarily supposed that it might be involved in detoxification reactions or that it could be an enzyme, accumulated for indirect causes consequent OA poisoning. The principal toxic effect of OA is due to its inhibiting effects on protein phosphatase 1 (PP1) and 2A (PP-2A) in eukaryotic cells, causing changes on protein phosphorylation state of enzymes (Bialojan and Takai, 1988).

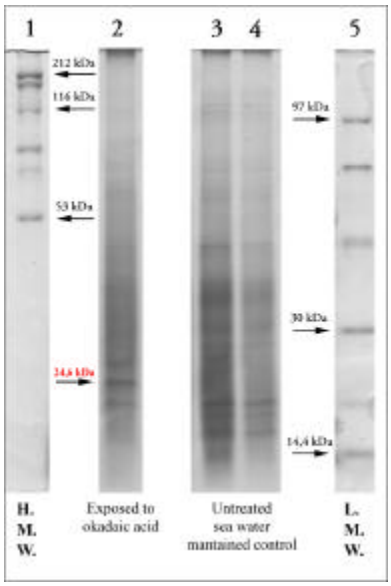

Fig. 1 SDS-PAGE electrophoretic pattern of exposed mussels (25 μg of OA and 25 μg of DTX-1) that present 24,6 kDa protein (line2), of untreated sea water maintained control (lane 3 and 4), HMW and LMW markers (lane 1 and 5).

OA toxic effect allows to suggest that 24.6 kDa protein could be an enzyme correlated with glycogen synthase (GS) activity, because the inhibition of GS activity by the action of OA on PP1 and PP-2A, is demonstrated. (Haystead ***et al***., 1989; Pugazhenthi ***et al***., 1993). This hypothesis was rejected because following studies on *Mytilus edulis* (Svensson and Fõrlin, 1998) demonstrated that GS activity is not affected by OA levels that naturally occur in blue mussels. On the basis of these results, the Authors suggested that there may be a protective mechanism against harmful effects of OA in the blue mussels. On this point it is to note that digestive cells of mollusc digestive gland are particularly rich in lysosomes (Owen, 1974). These can accumulate and sequester foreign compounds such as lipophilic xenobiotics by increased autophagic activity. Such date prompted the hypothesis that 24.6 kDa protein could be a detoxificant enzyme.

Most common detoxificant enzymes in eukaryotic cells are the family group of cytochrome P-450, that mainly transform liposoluble toxins in hydrosoluble compounds easily eliminated. Studies performed on *M. galloprovincialis* by Peters ***et al.*** (1998) demonstrated the presence of five different types of cytochrome P-450 (CYP1A, 2B, 2E, 3A, 4A), about 50 kDa each, all involved in detoxificant reactions both of organic pollutants and heavy metals contamination. The identification of 24.6 kDa protein with one of cytochrome P-450 was excluded, since it is half the weight of cytochrome monomers, detected in *M. galloprovincialis.*

Another system of detoxificant enzymes is glyoxalase group (Glyoxalase I, GI, and Glyoxalase II, GII), glutathione-dependent. Glutathione (GSH) is

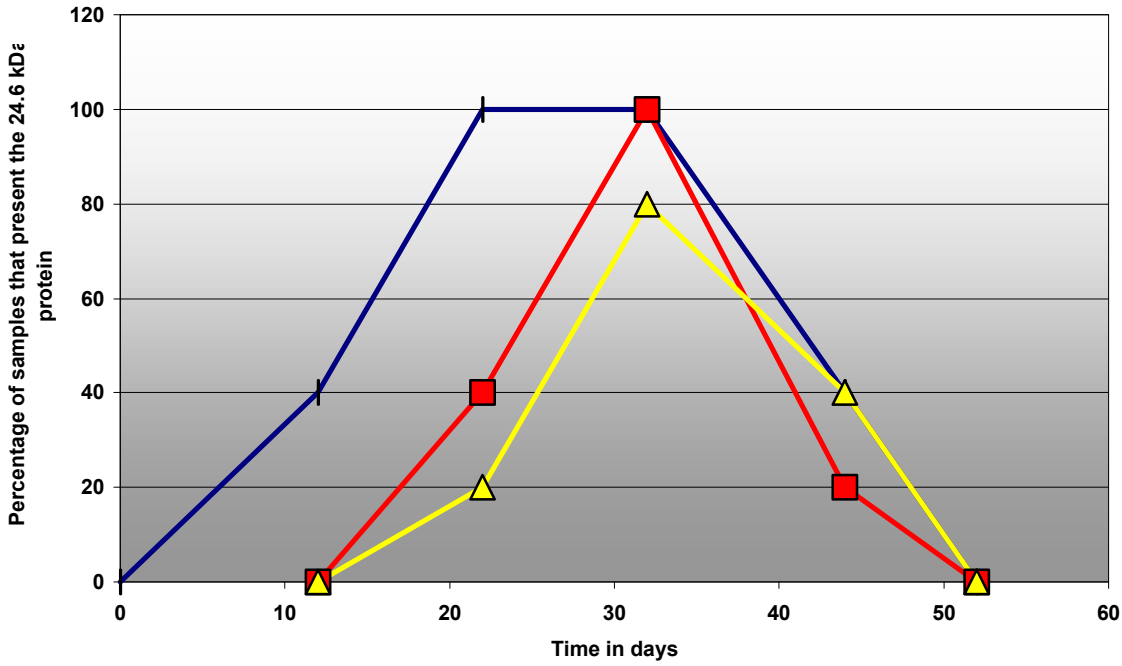

Fig. 2 Linear graph of percentage of mussels that present the 24.6 kDa protein in each assay. Yellow line: assay at 25 µg toxins, red line: assay at 50 µg toxins, blue line: assay at 100 µg toxins.

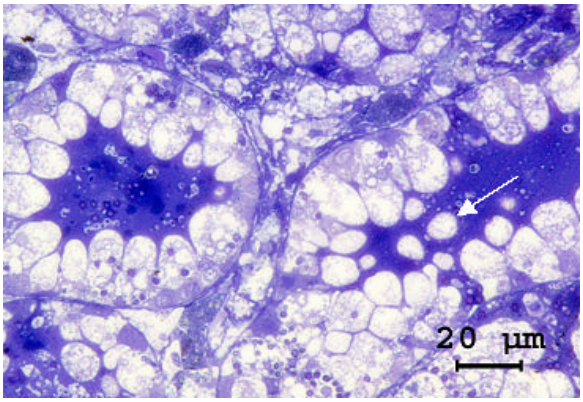

Fig. **3** Cross section of digestive tubules of an untreated sample. Some digestive cells, that release fragmentation spherules, are evident (arrow).

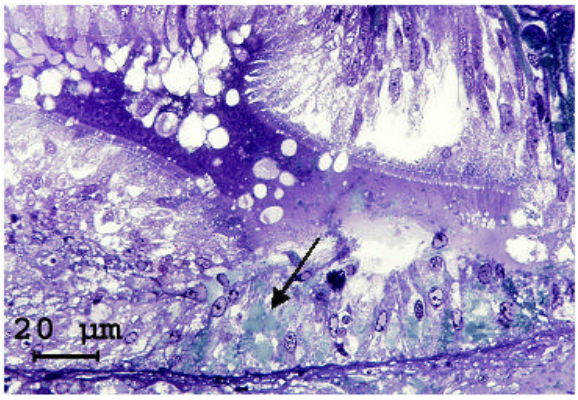

Fig. 4 Sample about one week after the beginning of treatment with 25 µg toxins. Oblique section of a digestive tube. Lipid vesicles (in greenish blue) inside digestive cells are evident (arrow).

a ubiquitarian tripeptide and it is a cofactor of many enzymes catalyzing the detoxification and excretion of several toxic compounds; in particular it is a coenzyme of GI. The GI activity is demonstrated also in *M. galloprovincialis* (Fitzpatrick *et al*., 1995). In addition Regoli *et al.* (1996) performed a first purification and characterization of Glyoxalase I from the digestive gland of the same species. From this study it results that the pure enzyme is a 48 kDa protein with an heterodimeric quaternary structure and in denaturant conditions (SDS-PAGE) it is composed of 24 and 25 kDa subunits.

Taking into account that: GI is an enzyme involved in several detoxification reactions, and 24.6 kDa protein appears after toxin (OA, DTX-1) treatment; GI consists of 2 subunits of MW very close and similar to the protein detected in the present study, and finally the staining used to evidence low MW proteins in the present work (Blue Comassie staining), does not allow to discriminate two subunits running so close in SDS-PAGE, we could suppose that 24.6 kDa protein is the GI.
Further analysis will be necessary to confirm whether it is actually the GI. If this hypothesis will be

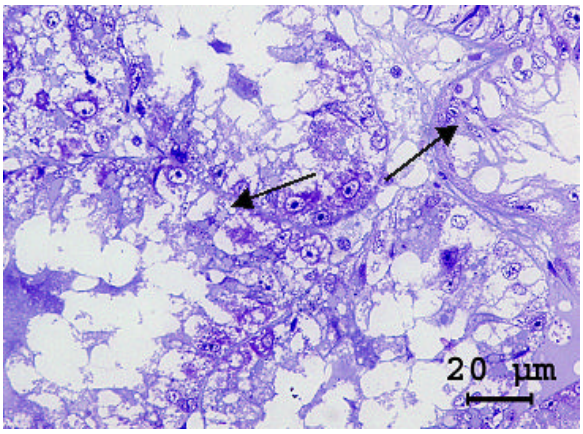

Fig. 5 Sample 20 days after the beginning of treatment with 50µg of toxins (25 µg of OA and 25 µg of DTX-1). Digestive cells are pyknotic and/or lytic (arrows).

tested and proved, it will be the first connection between this enzyme and the OA contamination. This could be a result of particular interest, because the detoxificant mechanism against OA and derivatives in *M. galloprovincialis* results still unresolved (Blanco *et al*., 2002). Besides, it will be interesting to evaluate if the 24.6 kDa protein synthesis is induced also by others DSP toxins, like yessotoxins and pectenotoxins. Indeed, were this induction demonstrated, this protein may represent a useful biomarker in biomonitoring studies, as an indirect index of DSP risk for all DSP toxins groups.

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