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NOTE

**MICROENCAPSULATION OF OKADAIC ACID AS A
TOOL FOR STUDYING THE ACCUMULATION OF DSP
TOXINS IN MUSSELS**

Araceli E. Rossignoli¹, David Fernández¹, Carmen P. Acosta¹, Juan Blanco^{1*}.

¹ Centro de Investigacións Mariñas (CIMA). Pedras de Corón, s/n. Apdo. 13, 36620
Vilanova de Arousa (Pontevedra). España

Running title: Microencapsulation of okadaic acid.

* Corresponding author: Juan Blanco, Centro de Investigacións Mariñas (CIMA).

Pedras de Corón, s/n. Apdo. 13, 36620 Vilanova de Arousa (Pontevedra). Tel. +34

886206364; Fax: +34 886206372 E-mail: juan.blanco@cimacoron.org

1 **ABSTRACT**

2 The possibility and effectiveness of microencapsulation of okadaic acid (OA) in
3 gelatin acacia microcapsules has been studied. The encapsulation efficiency was *ca.*
4 33%. The microcapsules were shown to be very stable, not leaching more than 9% of
5 the encapsulated OA in a 20-hour period. OA from the microcapsules was absorbed by
6 the mussels very efficiently, accumulating -after 3 days of feeding and one of
7 depuration- 65% of the OA in microcapsules and 22% of the total OA used at the
8 beginning of the microencapsulation process. These efficiencies and the possibility of
9 encapsulating single DSP toxins and derivatives constitute a valuable tool for the study
10 of the accumulation and biotransformation of DSP toxins in bivalves.

11
12 **Keywords:** Microcapsules, okadaic acid, absorption efficiency, biotransformation,
13 mussels.

Diarrhetic shellfish poisoning (DSP) is a syndrome caused by some polyether compounds -okadaic acid (OA), analogs and derivatives- that produce diarrhoea, nausea, vomiting, headache, chills and moderate to severe abdominal pain in humans (Yasumoto et al., 1978). The responsible toxins are produced by several species of dinoflagellates belonging to the genus *Dinophysis* and *Prorocentrum* (Yasumoto et al. 1980, Murakami et al., 1982; reviewed in Blanco et al., 2005) and are accumulated by shellfish that feed on these species. DSP toxicity probably has the highest impact on bivalve aquaculture, as it is frequently found in areas in which that activity is intense, such as Galicia (Spain) or southern Chile.

Mitigating the consequences of these blooms requires understanding the mechanisms involved in the absorption, elimination and transformation of this group of toxins by the bivalves. Experimental approaches are hampered by the difficulty of obtaining cultures of DSP-producing organisms and even when those are obtained, the cells usually contain a complex mixture of toxins and toxin derivatives that makes interpretation of the results difficult.

In order to supply the bivalves with food containing individual toxins or derivatives, we have studied the possibility and the effectiveness of microencapsulation of okadaic acid in gelatin-acacia microcapsules.

To obtain okadaic acid, naturally contaminated mussels were dissected, weighed and their digestive glands homogenized in methanol in a proportion of 1 g of tissue (4 mL of extraction solvent), with an Ultraturrax T 25 Basic (IKA). The obtained homogenate was centrifuged at 48000 g for 20 min to clarify the solution (Beckman Avanti J-25). The extract was diluted to 50% methanol and subjected to liquid-liquid partitions with hexane and dichlorometane. The absence of conjugated forms (acyl derivatives or diol-esters) in the extract was checked by means of alkaline hydrolysis,

that transforms them to free OA. The dichloromethane fraction was evaporated to dryness and redissolved in 0.5 mL of olive oil. Aliquots of 0.25 mL were used to quantify OA in the extract and to be microencapsulated respectively

Gelatin-acacia microencapsulation was based on the complex coacervation method used by Planas et al. (1990) but with slight modifications (Figure 1). Once the microcapsules had been produced they were washed by allowing them to decant, removing supernatant water, adding new water and shaking the suspension gently. This operation was repeated each day during the experiment in order to avoid lump formation.

The encapsulation efficiency was quantified by the proportion of OA that was successfully encapsulated. OA contained in the microcapsules was quantified by extraction of *approx.* 1 g of microcapsules, with methanol 100% (1:4, v/v) and clarification by centrifugation for 20 min. at 48000 g.

The capability of the microcapsules to retain the toxins when they are suspended in seawater was also studied. Microcapsules were placed in seawater and samples of this water were taken at different times (0, 1, 2, 3, 4 and 20 h). An initial blank of the seawater was also analysed. OA was extracted from the water by liquid-liquid partition with dichloromethane, evaporation of the organic fraction, and resuspension in 100% methanol

Finally, we have shown that the toxins from microcapsules can be absorbed by mussels and they can be traced after being fed. OA-containing microcapsules were fed to two groups of six mussels obtained from the same batch which contained low levels of toxins (Table I). The mussels were placed in a beaker and were fed with a diet constituted by 10 mL of the microcapsules suspension (approximately 1 g of microcapsules) plus 25 mL of a culture of the microalgae *Tetraselmis* sp. (3×10^6

cells·mL⁻¹) plus 0.05 g of clay. The food was supplied twice a day during 3 days. After the third day of feeding, mussels were maintained without food for an additional day, in order to remove most undigested food from the digestive system before analysis. This step was needed because very likely not all microcapsules are digested and, if they are not removed from the gut, the toxin they contain would be measured jointly with the absorbed one thus making it impossible to discriminate the absorbed from the ingested toxin. Furthermore, feces were collected from the bottom of the beaker every day and pooled the last day of the experiment. At the end, the digestive glands were homogenized in methanol (100%) in a proportion of 1:4, w:v, with an Ultraturrax T 25 Basic (IKA) and the homogenate obtained was centrifuged at 48000 g for 20 min to clarify the solution. This extract was analyzed to quantify free OA. In order to quantify the total amount of toxin (OA and its conjugated forms (CF)) the methanolic extracts were subjected to an alkaline hydrolysis (after Vale and Sampayo, 1999, but using a temperature of 80°C). The extracts were desalted and partially cleaned with polymeric reverse-phase SPE cartridges Strata X (60 mg/3 mL) (Phenomenex). Feces were subjected to the same procedure, with the exception that the proportion of methanol added to extract the toxins was 4 mL of methanol per mL of feces, instead of per gram as in digestive glands.

OA concentration in each extract was determined by HPLC-ESI-MS with a Thermo LCQ-Deca XPplus ion trap mass spectrometer with electrospray ionization coupled to a Thermo Surveyor HPLC. The chromatographic separation was carried out using the method of Quilliam (2003) slightly modified to adapt it to a fused core column (HALO C8 50x2.1mm 2.7 µm). The two mobile phases were: A= H₂O: F (95:5) and B= CH₃CN: F (95:5), where F was 40 mM ammonium formate and 1M formic acid in water. The gradient program went from 5% to 100% B over 5 min, hold for 5 min and

then re-equilibrate at the starting conditions for 5 min prior to the next injection. Flow rate was $400\mu\text{L min}^{-1}$ and injection volume $5\mu\text{L}$. The mass spectrometer was operated in negative ionization mode and SIM (selected ion monitoring) for $803.5\ m/z$. Quantification of the OA was carried out by comparing the resulting response with that of the reference materials (OA-1b) obtained from IMB - NRC, Canadá. The presence of matrix effect was checked by serial dilutions of the samples and corrected when needed by using the results obtained with the largest dilution.

Some modifications to the microencapsulation technique of Planas et al. (1990) were made in order to adapt the microcapsule size to the requirements of mussels. In this sense we have used a pH of 7 for the step of mixture of colloids and of 4.2 for the emulsification, obtaining microcapsules with a diameter about $50\ \mu\text{m}$. Also a homogenization with Ultraturrax at 24000 rpm (instead of the sonication of the original technique) was introduced to favor a correct mixture between the OA-olive oil and the acacia solution.

The efficiencies of encapsulation of the oils (olive and cod liver oil) were always high and similar to the ones obtained by Planas et al. (1990) encapsulating cod liver oil (50 to 85%) or by Knauer and Southgate (1997) encapsulating lipids (ca. 80%). However the encapsulating efficiency of OA was lower, ranging from 33 to 53%. This difference is probably due the partially hydrophilic nature of OA, (its partition coefficient between water and olive oil which is roughly 0.3, as we have estimated in a preliminary experiment in which ca. 30% of okadaic acid remained in water after a 1:1 water:olive oil liquid-liquid partition).

The stability of the microcapsules in seawater was good, as the OA they contain did not leach substantially. Only about 8.5% of the toxin contained in the microcapsules was lost as soon as they were suspended in seawater (0 hours), probably due to an

insufficient washing, and no significant loss (c.a. 0.5%) could be detected after that time. Planas et al. (1990) found a similar leaching percentage for cod liver oil but during an 8-hour period notwithstanding, with no noticeable loss at 0 hours.

Mussels fed well on microcapsule suspensions. At the end of each 24h feeding cycle no suspension in the water was apparent and no pseudofeces detected, indicating that all the food supplied was ingested. At the end of the experiment the six mussels had increased their total toxin content (OA free + OA Conjugated forms) in the digestive gland (DG) by 1.857 μg (Table 1). This accumulation by the mussels represents 63% of the microencapsulated toxin and 22% of the initial toxin amount. A large proportion of the toxin is therefore lost but enough of it was retained by mussel as to consider the technique useful to study toxin accumulation and bio-transformation. In feces, only a small percentage of free OA was found and most was found as conjugated forms. From this proportion of free OA an absorption efficiency higher than 88% could be estimated (assuming that the free okadaic acid is not transformed in the gut, and that consequently the free OA found in feces comes from the non-absorbed toxin plus the first absorbed and then excreted free toxin).

It can be concluded that microencapsulation is a viable way of supplying toxic food for studies of accumulation and biotransformation of biotoxins in bivalves. Mussels feed well on gelatin-acacia microcapsules if they are mixed with microalgae. The microencapsulated toxins can be readily absorbed allowing a substantial increase in toxin concentrations of the mussels in a few days.

It seems clear that this technique is more feasible for studying the biotransformation and the mechanisms involved in accumulation than using *Dinophysis* or *Prorocentrum* cultures and also than the study of naturally intoxicated populations. In all cases because the complexity of the toxin mixture could be controlled and

simplified. In the case of *Dinophysis* cultures, because the AO producing species in this genus have complex nutritional requirements. *D. acuminata*, for example, requires (as far as we know presently) a partially photosynthetic ciliate (*Myrionecta rubra*) as prey. The ciliate must obtain the chloroplasts from a cryptophyte (usually *Teleaulax*) (Park et al. 2006). Culturing this species requires consequently three steps (one for each species) that should be carefully synchronized. That is an expensive and time consuming process, which is additionally prone to fail due to contamination of the final *Dinophysis* culture with the cryptophytes. In relation to *Prorocentrum*, using microcapsules has advantages because the species in this genus are inefficiently digested by mussels, and (and perhaps because of), at least some of them produce a number of bioactive compounds (Cruz et al., 2008; Napolitano et al., 2009; Napolitano et al., 2010). Microcapsules can be used to supply mussels with individual DSP toxins derivatives that have lower polarity than okadaic acid, such as 7-O-acyl derivatives or diol esters, but its use with more polar derivatives, such as “DTX4” and “DTX5”, should be additionally evaluated.

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1

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FIGURE AND TABLE CAPTIONS

Figure 1: Schematic diagram of the preparation of okadaic acid gelatin-acacia coacervate microcapsules (Partially from Planas et al., 1990).

Table 1: Content of okadaic acid (OA) and their conjugated forms (CF) in the digestive gland of mussels and feces at the beginning and the end of the experiment.

DG = digestive gland; Day 1 = the first day that the mussels were fed. Day 4 = the day after the last feeding.

0.5 g of Acacia
dissolved in 50 mL
of water 40°C

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0.5 g of Gelatin
dissolved in 50 mL
of water 40°C

Mix

0.25 mL
dichloromethane
(okadaic acid)
fraction
redissolved in
olive oil.

Homogeneization
with Ultraturrax
24000 rpm
20 sec.

Adjust pH to 7
with NaOH 0.1N

Adjust pH to 7
with NaOH 0.1N

Emulsify

(Magnetic stirrer)
Stirring rate: 250 rpm.
30 sec.

Adjust pH to 4.2 with
HCl 0.1N.
Stirring rate: 250 rpm

Gelation of coacervates
by cooling in an ice bath
for 20 minutes.
Stirring rate: 100 rpm

Adjust pH to 9.0 with
NaOH 0.1N
Agitate for 30 minutes
Stirring rate: 100 rpm

Let stand the mixture for
2 hours at 4°C

	Type of Toxin	Toxin in Day 1 (T0) (µg)	Toxin Day 4 (TF) (µg)	Toxin increase (µg)
Mussels	OA	0.4750	0.7802	0.3052
	CF+OA	1.0973	2.9545	1.8572
Feces	OA	Not sampled	0.1663	-
	CF+OA	Not sampled	1.3706	-