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RESEARCH ARTICLE

P. R. Costa · R. Rosa · M. A. M. Sampayo

Tissue distribution of the amnesic shellfish toxin, domoic acid, in *Octopus vulgaris* from the Portuguese coast

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Abstract Domoic acid (DA), the amnesic shellfish toxin, is a food-web-transferred algal toxin that has been detected in many marine organisms from copepods to whales. However, cephalopods, which are important members of the food chain, have never been implicated in DA transfer or accumulation. Here, we present data showing relevant values of DA detected in the common octopus (*Octopus vulgaris*) from the Portuguese continental coast. Even though DA is hydrophilic and is not expected to be accumulated in the tissues, DA was always detected in our octopus tissue samples. Tissue distribution of DA revealed that the digestive gland and the branchial hearts are the main organs of DA accumulation. Highly variable DA concentrations, ranging from 1.1 to 166.2 μg DA g^{-1}, were observed in the digestive glands. Low levels of DA were detected in the digestive tract (stomach and intestine) and could be a consequence of high digestion rates or a result of non-exposure to toxic vectors during the sampling period. In fact, octopus prey, such as bivalves, crustaceans and fishes, are known to occasionally work as DA vectors. Consequently, DA uptake into octopus tissues is likely sporadic. Similar low levels were detected in the kidney, gills, systemic heart, posterior salivary glands and mantle, and no DA was found in either the gonads or the ink sac. These data are the necessary first step towards achieving an understanding of the accumulation of phycotoxins in *O. vulgaris*.

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P. R. Costa (✉) · M. A. M. Sampayo
Departamento de Ambiente Aquático,
IPIMAR, Avenida de Brasília,
1449-006 Lisbon, Portugal
E-mail: prcosta@ipimar.pt
Tel.: +351-21-3027072
Fax: +351-21-3015948

R. Rosa
Departamento de Inovação Tecnológica e Valorização dos Produtos da Pesca, IPIMAR,
Avenida de Brasília,
1449-006 Lisbon, Portugal

Introduction

Domoic acid (DA), a naturally produced phycotoxin with neurotoxic properties, is responsible for the illness amnesic shellfish poisoning (ASP). In 1987 on Prince Edward Island, Canada, at least 3 people died and >100 became ill, suffering neurological problems after consuming blue mussels (*Mytilus edulis*) contaminated with DA (Quilliam and Wright 1989; Todd 1993). Several species of the diatom genus *Pseudo-nitzschia* have been shown to produce this neurotoxin (Subba Rao et al. 1988; Bates et al. 1989; Garrison et al. 1992), which may accumulate in filter-feeding bivalves such as the mussels. Although bivalves were the vectors in the first ASP event, subsequent DA-poisoning events have revealed that many other marine organisms could also be vectors. Small and simple herbivorous organisms such as copepods and krill have been shown to accumulate DA (Lincoln et al. 2001; Tester et al. 2001; Bargu et al. 2002, 2003). Planktivorous fishes have been identified as DA vectors (Buck et al. 1992; Fritz et al. 1992; Lefebvre et al. 2001, 2002a; Vale and Sampayo 2001), with devastating effects on piscivorous predators like sea birds and sea lions (Work et al. 1993; Sierra Beltrán et al. 1997; Lefebvre et al. 1999; Scholin et al. 2000). The toxin permeates both benthic and pelagic members of the food web and has been detected in crustaceans (Wekell et al. 1994; Altwein et al. 1995; Costa et al. 2003) as well as whales (Lefebreve et al. 2002b). Despite all of these studies, DA has never been reported in cephalopods. It seems that there has been less attention focused on this molluscan group and studies characterising the presence and movement of the toxin through this member of the marine food web are needed.

In order to evaluate the presence of DA in cephalopods, we examined the tissue distribution of the amnesic shellfish toxin in the common octopus (*Octopus*

vulgaris). This species has a world-wide distribution in temperate, subtropical and tropical waters of the Atlantic, Indian and Pacific Oceans; it is also present in the Mediterranean Sea (Mangold 1998). It is a common and opportunistic predator on a wide variety of prey such as crustaceans, molluscs and fish, in many marine intertidal and subtidal communities (Altman 1967; Nigmatullin and Ostapenko 1976; Guerra 1978; Smale and Buchan 1981; Ambrose and Nelson 1983; Nixon 1987; Sánchez and Obarti 1993). On the other hand, octopuses are important in the diet of fish and marine mammals, playing an important role in the food chain and in the ocean's ecology (Boyle and Boletzky 1996; Caddy and Rodhouse 1998; Piatkowski et al. 2001).

Materials and methods

Collection and preparation of octopus samples

Ten samples of *Octopus vulgaris* comprising a total of 90 individuals, were collected by commercial vessels (with traps and clay pots) during the period between February and May 2003. Eight of them were collected in Peniche (NW coast) and two in Olhão (south coast) (Table 1).

All octopuses were frozen (−20°C) and defrosted just before being prepared for analysis. A total of 40 specimens were dissected for: (1) the digestive gland, (2) the branchial hearts, (3) the kidney, (4) the stomach, caecum and intestine, (5) the gills, (6) the systemic heart, (7) the posterior salivary glands, (8) the mantle, (9) the gonads and (10) the ink sac. These tissues were homogenised, and a 5 g aliquot of each (or the amount available) was weighed separately.

Toxin extraction and HPLC analysis

Extractions were carried out according to the method of Quilliam et al. (1995) with some modifications (Vale and Sampayo 2001). The extraction was performed with aqueous 50% methanol (ratio 1:4) at 20,000 rpm for 1 min with a homogeniser probe. After 10 min of centrifugation at 4,000 rpm, the supernatant was filtered into a screw-cap autosampler vial with a nylon (0.22 μm, 13 mm diameter), disposable syringe filter. The equivalent of 1.0 mg extract (5 μl) was injected on the column without any further clean-up.

Liquid chromatography (LC) was performed on a Hewlett-Packard (HP) model 1100, equipped with in-line degasser, quaternary pump, autosampler, oven and diode-array detector (DAD); data collection and treatment of the results were performed by the HP "Chemstation" software. The column used was a Nucleosil 100-5C-18 (125×3 mm, 5 μm), with a guard-column Lichrospher 100 RP-18 (4×4 mm, 5 μm), both heated to 40°C. The flow rate was set at 0.45 ml min^{-1} of acetonitrile:0.1% formic acid (10:90, v/v) throughout the run. The injection volume was 5 μl, and the analysis time was set at 10 min. Detection wavelength was set at 242 nm with a 10 nm bandwidth, and reference wavelength was set at 450 nm with a 100 nm bandwidth. A confirmatory wavelength at 262 nm was used.

Calibration was performed with a full set of calibration standards of DA (0.5, 2, 4 and 10 μg ml^{-1}). Samples over the calibration curve were diluted. Calibration curves were always linear, with correlation coefficients >0.99. A single-point calibration, with a working solution of 4 μg DA ml^{-1} in 10% acetonitrile was performed after six consecutive samples. Under these conditions the detection limit was 0.04 μg ml^{-1}, which corresponded to 0.2 μg g^{-1} in tissue.

Solvents used for the HPLC analysis were methanol, acetonitrile and formic acid of LC grade supplied by Merck and Millipore-Q cleaned water. DACS-1D-certified DA standard was purchased from the National Research Council of Canada (NRC).

Mass spectrometry analysis

Analysis was performed as described in Vale and Sampayo (2001). The same chromatograph system as above was used, coupled with a HP model 1100 series single quadrupole mass spectrometer, through an ionspray LC-MS interface, operated in the positive ion mode. High-purity nitrogen was used as the nebulising gas, and a potential of 5,000 V was applied to the interface needle. Selected ion monitoring was used to record the signals from the ($[M+H]^+$) ions at m/z 312 and 266. The trace in figures shows only the m/z 312 signal.

Toxins were separated at 40°C on a Lichrospher 100 RP-18 (125×2 mm, 5 μm) column, protected by the same guard column as above. The mobile phase consisted of acetonitrile:0.1% formic acid (10:90, v/v).

Results

LC-UV analysis of octopus digestive gland extract showed that the compound identified as DA had the same retention time as DA in the calibration standard. Other peaks eluting close to DA were observed in the octopus digestive gland extract, and their retention times also matched the peaks observed in the chromatogram of the calibration standard, corresponding to isodomoic acid D (iso-D), isodomoic acid A (iso-A) and the C5′-diasteromer of DA (epi-DA). In the UV spectrum, the

Table 1 Sampling locations of *Octopus vulgaris* (*dash* no depth measurement taken)

Sample	Sampling location	Date (2003)	Depth (m)	Octopus weight (g, mean ± SD)	No. of individuals dissected for:	
					Digestive gland	Other tissues
P1	Peniche	12 Feb	46	1,651 ± 255	13	6[a]
O2	Olhão	12 Feb	–	2,894 ± 884	3	0
P3	Peniche	28 Feb	41	1,560 ± 265	8	4
O4	Olhão	7 Mar	–	1,076 ± 285	4	0
P5	Peniche	12 Mar	55	839 ± 73	14	7
P6	Peniche	26 Mar	39	3,123 ± 839	9	4
P7	Peniche	9 Apr	46	1,526 ± 361	17	5
P8	Peniche	23 Apr	41	2,566 ± 282	9	5
P9	Peniche	9 May	48	1,315 ± 346	9	5
P10	Peniche	23 May	41	2,995 ± 764	4	4

[a]Systemic heart was not dissected for analysis

iso-D and epi-DA peaks and the iso-A peak had maxima at 244 and 242 nm, respectively, as reported by Quilliam and Wright (1989). The UV/diode-array spectra of the DA peak—maximum at 242 nm—from the octopus digestive gland extract matched (>99%) the spectra acquired for the DA standard. Further evidence for the identification of DA was provided by LC-MS analysis. Retention times of the peaks in the m/z 312 ($[M+H]^+$) ion chromatogram of the digestive gland extract matched those of the DA standard (Fig. 1).

DA was found in all 90 octopus digestive glands analysed and was highly variable, with values ranging from 1.1 to 166.2 μg g^{-1} (Fig. 2). Furthermore, DA was detected in the digestive gland of specimens collected on both the west and the south coast.

Highly variable DA levels were also found in the branchial hearts, ranging from 3.0 to 67.1 μg g^{-1}. It is worth noting that in 60% of the analysed cases ($n=40$), DA levels in the branchial hearts were higher than DA levels in the digestive glands of the same octopus (Fig. 3).

In the remaining tissues, DA levels were lower than those detected in digestive glands and branchial hearts. Nevertheless, DA was always detected in the 40 kidneys analysed. Kidney DA levels ranged from 0.2 to 3.5 μg DA g^{-1} and had lower variability than in other tissues (Fig. 4a). DA was found in the stomach, spiral caecum and intestine, which were analysed together, of 26 octopuses. Highly variable levels were again observed, and ranged from 0.4 to 7.0 μg DA g^{-1} (Fig. 4b). DA was detected in the gills of 33 specimens, and the values were always <2.0 μg DA g^{-1} (Fig. 4c). Systemic heart presented even lower concentrations of DA, which ranged, when detected, between 0.2 and 1.1 μg g^{-1} (Fig. 4d). For this organ only 34 specimens were analysed, and DA

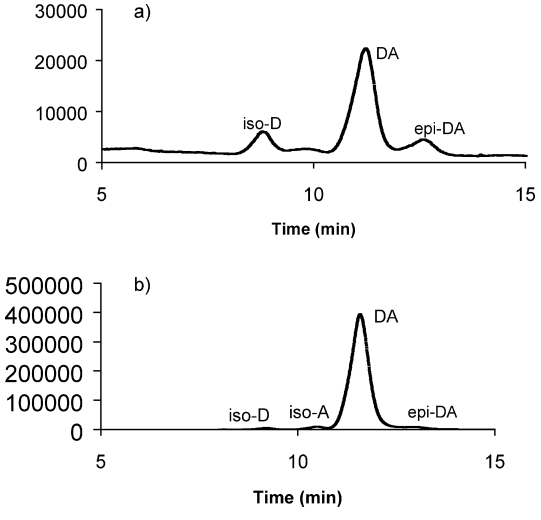

Fig. 1a, b *Octopus vulgaris*. Comparison of $[M+H]^+$ (m/z 312) chromatograms obtained from selected ion-monitoring LC-MS analyses of: **a** octopus digestive gland and **b** certified DA standard (*DA* domoic acid; *iso-D* isodomoic acid D; *iso-A* isodomoic acid A; *epi-DA* C5′-diasteromer of DA)

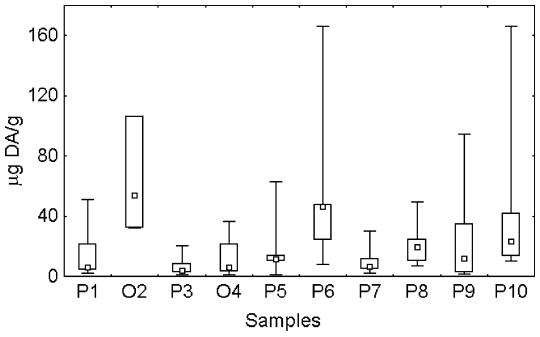

Fig. 2 *Octopus vulgaris*. Domoic acid (*DA*, μg g^{-1}) detected in octopus digestive gland (median, 25 and 75 quartiles, minimum and maximum, total $n=90$). Details on samples, see Table 1

was detected in 24. In the posterior salivary glands only half of the cases showed DA. However, values detected in this organ were higher than those observed in the gills, the systemic heart and even the kidney. The maximum value detected was 5 μg DA g^{-1} (Fig. 4e). In the mantle, which is muscle tissue, the maximum DA detected was 0.4 μg g^{-1} in specimens from samples P6 and P8. DA was not detected in the ink sac or in the gonads.

Discussion

Portugal is located in the northern part of the climatic subtropical, high-pressure belt of the Northern Hemisphere. The upwelling events that migrate from the south of Morocco to the north of Portugal have a very well-defined maxima off the west coast in July, August and September (Fiúza et al. 1982). Such seasonal upwelling events are responsible for the occurrence of algal blooms, including *Pseudo-nitszchia* spp., which are the producers of the ASP toxin. Although the typical upwelling period lasts from July to September, it is not that unusual to detect DA in organisms at other times. In this study, DA was always found in some octopus tissues, but the persistence of such toxicity is not

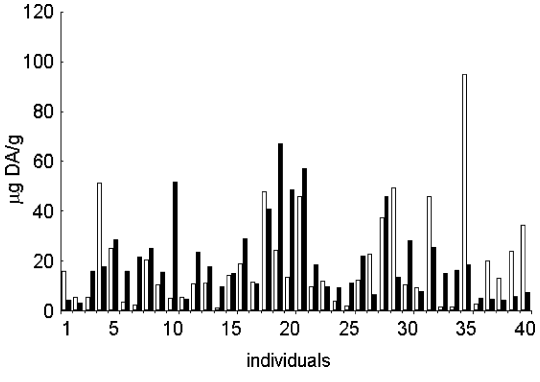

Fig. 3 *Octopus vulgaris*. Comparison of domoic acid concentrations (*DA*, μg g^{-1}) detected in octopus branchial hearts (*closed bars*) and in digestive gland (*open bars*) of 40 octopuses

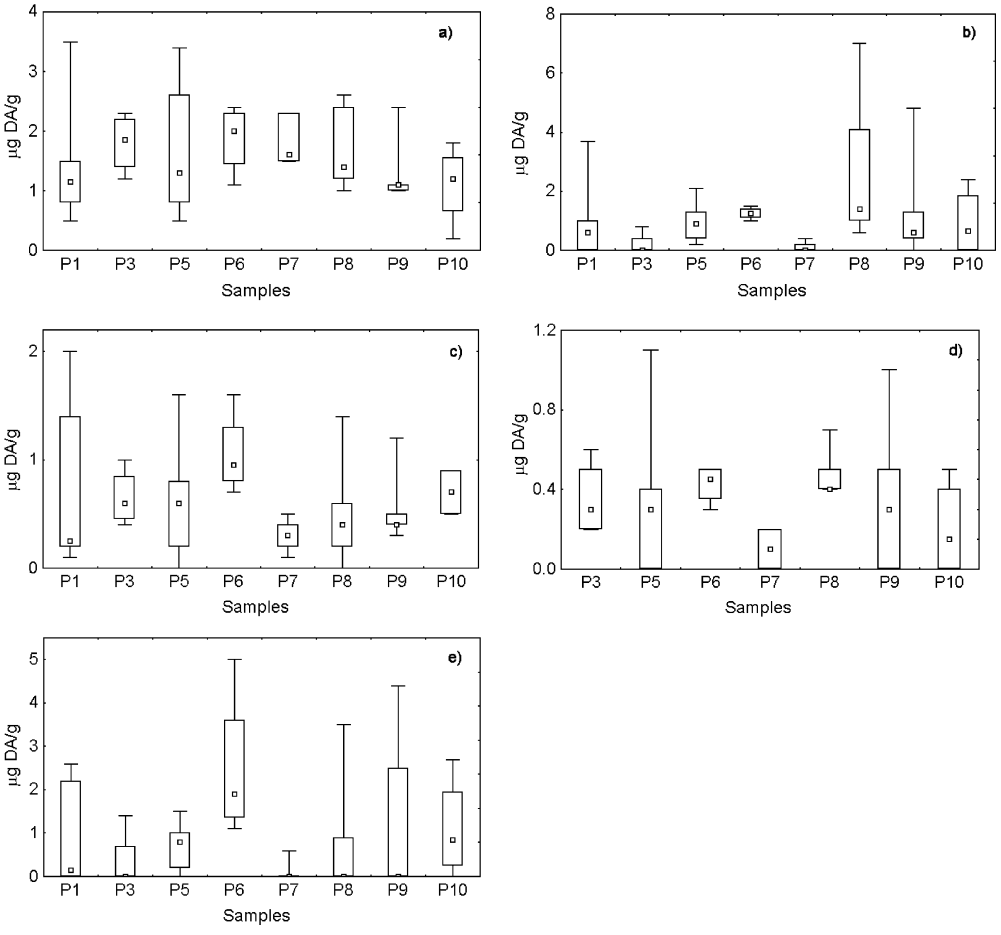

Fig. 4a–e *Octopus vulgaris*. Domoic acid (*DA*, µg g^{-1}) detected in octopus tissues (median, 25 and 75 quartiles, minimum and maximum): **a** kidney ($n=40$); **b** pooled stomach, caecum and intestine ($n=40$); **c** gills ($n=40$); **d** systemic heart ($n=34$); and **e** posterior salivary glands ($n=40$). Details on samples, see Table 1

common. Due to its hydrophilicity, DA is more likely to be released than to be accumulated (Wright et al. 1989; Novaczek et al. 1991). However, DA retention has also been reported in some molluscs, such as the king scallop (*Pecten maximus*) (Arévalo et al. 1998; Blanco et al. 2002), and the Pacific razor clam (*Siliqua patula*) (Drum et al. 1993; Horner et al. 1993). Our results are comparable to those obtained for king scallops, whereby the highest DA concentrations were found with high variability in the digestive gland. Octopuses probably do not represent new health hazards to humans, since DA was mainly detected in the digestive gland and not in the edible parts (muscle). However, when the whole body is consumed, *O. vulgaris* may act as a DA vector to predators such as marine mammals (Blanco et al. 2001; Piatkowski et al. 2001; Santos and Haimovici 2001).

DA was detected in several tissues in addition to the digestive gland. In many cases, DA levels detected in the branchial hearts were above DA levels detected in the digestive gland. However, the digestive gland is the largest octopus organ (just surpassed by the female gonads at the peak of maturity) and may act as a reservoir of the amnesic toxin, as it is for other substances (Grisley and Boyle 1988). The two branchial hearts are located at the base of the gills, receiving deoxygenated blood from the body tissues; from there blood then is sent to the gills where it is oxygenated. In addition to this pumping function, the branchial hearts have an excretory role (Cuénot and Bruntz 1908). Schipp and Hevert (1981) concluded that these organs might be involved in excrete-storage activities. Moreover, it has been demonstrated that these organs are able to accumulate high concentrations of some heavy metals (Nakahara et al. 1979; Miramand and Guary 1980; Nakahara and Shimizu 1985); they have been called the "kidneys of accumulation" (Martin and Aldrich 1970).

In spite of the low DA concentrations detected in kidney, this tissue showed DA whenever analysed, which might be due to its excretory function as well as to its connection to the branchial hearts by the pericardial ducts.

Results obtained in pooled stomach, caecum and intestine samples showed low DA concentrations. The lack of DA in these tissues suggests that the octopus may not have been exposed to DA when sampled, or may be a consequence of the high digestion rates that characterise cephalopods (Boucaud-Camou et al. 1976; Boucher-Rodoni and Mangold 1977). Along the Portuguese coast, the common octopus has a versatile diet, including osteichthyes (e.g. Clupeidae), crustaceans (e.g. *Liocarcinus* sp. and *Polybius henslowii*), bivalves (e.g. *Mytilus* sp.), cephalopods and gastropods (Rosa et al. 2004), which is consistent with the general view of cephalopods as opportunistic predators (Summers

1983). These prey can occasionally act as DA vectors (Vale and Sampayo 2001; Costa et al. 2003), resulting in sporadic uptake of toxin and bioaccumulation in octopus tissues.

Although the gills are connected with the branchial hearts and receive their venous blood, only low concentrations of DA have been detected in gill tissues. The systemic heart, which is essentially a purely muscular tissue, pumps oxygen-rich blood from the gills to the rest of the body and, when detected, presented low values of DA. This suggests that DA hardly reaches the blood system. However, small traces of DA were sometimes found in the mantle, but never were detected in the gonads.

Salivary glands did not always show DA, but, when the toxin was found, it was found at levels similar to those detected in the kidney. This organ, besides its digestive function, also appears to play a role in excretion as a path for the elimination of fluid wastes (Wells 1978). Evaluation of the toxin distribution showed that DA was mainly associated with tissues that take part in both digestion and excretion.

This is the first report of DA detected in a cephalopod species. Such data are the necessary first step towards achieving an understanding of the accumulation of DA in *O. vulgaris*, as well as its transfer to predators higher in the food web. Consequently, it is of great importance to intensify field and laboratory studies on trophic interrelationships in which the common octopus is involved.

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