Haloperoxidase from the marine sponge *Erylus discophorus* (Schmidt, 1862)

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Abstract: The presence of halogenated compounds is well documented in the phylum Porifera, although little is known about the biosynthetic pathways leading to their production. Studies with sponge enzymes are scarce, in particular those involving halogenating enzymes. Preliminary results with sponge crude extracts revealed haloperoxidase activity in some sponges. From *Erylus discophorus* (Schmidt, 1862) we extracted and partially purified a haloperoxidase with iodo and bromoperoxidase activities. This very unstable enzyme is a glycoprotein with a molecular mass higher than 200 kDa, as determined by gel filtration. A UV-visible spectrum with a shoulder in the Soret region, and the observed inhibition by azide ions, makes the hypothesis of a heme protein more likely.

Keywords: Bromoperoxidase, *Erylus discophorus*, halogenation reactions, Northeastern Atlantic, Porifera

Introduction

The oceans are the single largest source of biogenic organohalogens, which are biosynthesized by a myriad of seaweeds, sponges, corals, tunicates, bacteria, and other marine life (Gribble 2003).

Due to their habitat, sponges rely heavily on chemicals for survival, and many of these compounds contain halogen(s). Examples of sponge halogenated compounds include fatty acid derivatives (Pham *et al.* 1999), pyrroles (Cafieri *et al.* 1998), indoles (Qureshi *et al.* 1999), phenol derivatives (Utkina *et al.* 2005), tyrosine derivatives (Nicholas *et al.* 2001, Saeki *et al.* 2002, Kijjoa *et al.* 2002), terpenes (Miyaoka *et al.* 2006), diphenyl ethers (Vetter and Jun 2003, Hanif *et al.* 2007), and even dioxins (Utkina *et al.* 2001). The formation of these compounds probably involves an enzymatic halogenation. Haloperoxidases (HPO) catalyse, in the presence of hydrogen peroxide, the oxidation of halides (X⁻: iodide, bromide or chloride) to their corresponding hypohalous acids or to a related electron-oxidised halogenating intermediates such as OX, X³⁻ and X⁺. Most peroxidases are able to catalyze the formation of a carbon-halogen bond with a suitable halide, in the presence of a halide acceptor, such as tyrosine (Henderson and Heinecke 2003). Concerning the cofactor nature, three major classes of HPO are known: HPO containing no prosthetic group, the heme-containing HPO and the vanadium-dependent HPO (VHPO) that binds a vanadate ion (HVO,²⁻). Enzymes representing these two latter classes differ, at least, in two aspects - catalytic mechanism and stability. Heme-containing HPO catalyse the formation of hypohalous acid by a redox mechanism, whereas in VHPO the vanadate group does not change its redox state but may function as a Lewis acid. Unlike heme-containing HPO, VHPO exhibit a high level of thermostability, and good tolerance to organic solvents, as well as to high concentrations of their substrates and products (Almeida *et al.* 2001).

Haloperoxidases from a variety of plant, animal and microbial sources have been identified and studied. These include haloperoxidases from horseradish, milk, saliva, tears, red and white blood cells as well as fungi, marine algae, and invertebrates (Alexander 1959, Shaw and Hager 1959, Archer *et al.* 1965, Ilgner and Woods 1985).

As far as sponges are concerned, just one report was found: a labile chloroperoxidase-like activity in a subtropical marine sponge (Baden and Corbett 1979).

In this paper we present a survey of haloperoxidase activities detected in sponges from the Northeast Atlantic and the extraction and partial purification of a haloperoxidase from the sponge *Erylus discophorus* (Schmidt, 1862).

Material and methods

Chemicals: Analytical grade inorganic salts were purchased from Merck. Diethylaminoethyl (DEAE)-Sephacel, Sephacryl S-300, hydrogen peroxide, high molecular weight gel filtration and Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis, (SDS-PAGE) standards, orcinol, bovine serum albumin (BSA), glucose, 2-(N-morpholino)ethanesulfonic acid (MES) and Coomassie brilliant blue G-250 were purchased from Sigma.

Biological specimens: Sponge specimens were collected by SCUBA diving in several areas of two Portuguese marine natural reserves (Arrábida and Berlengas) in the period May-
July 2003, at Ferrol (Spain) in April 2004, and at Gorringe (a large seamount located off the south west coast of Portugal) in June 2006 (Fig. 1).

After collection, the specimens were transported to the laboratory in refrigerated containers and frozen at -20ºC until required. From all the samples, a voucher was preserved in absolute ethanol for identification purposes. The samples were named according to the respective collection site: B for Berlengas, A for Arrábida, F for Ferrol and G for Gorringe, followed by a number that corresponds to the order of collection.

Detection of halogenating activity: A small volume of sponge (circa 2.5 mL) was homogenized in 30 mL of 0.2 M Tris-SO₄ buffer (pH 8.3). After centrifugation, to remove cell debris, this crude extract was used to test the triiodide formation from I⁻ and H₂O₂, catalysed by a iodoperoxidase (IPO) and followed at 350 nm, (Björkstén 1968). If the sample showed positive activity (+), then the consumption of monochlorodimedone (the assay to test bromoperoxidase (BrPO) activity) will follow (Hager et al. 1966). If this test was positive (++), bromide was replaced by chloride and the crude extract tested again, now for the presence of chloroperoxidase (ClPO) activity and assign (+++), if positive.

For quantitative purposes, one enzyme unit was defined as the amount of enzyme which catalyses the formation of 1 µmol of product per minute.

Enzyme extraction and purification: Fresh sponge of *E. discophorus*, specimen FE05 (10g) was triturated and homogenized with 40 mL of 0.2 M Tris-SO₄ buffer (pH 8.3), for 30 minutes, at 4°C, followed by centrifugation (35 minutes, 5500 g, 4°C), to remove cell debris and clarification.

This crude extract was then applied to a DEAE-Sephacel column equilibrated with 0.2 M Tris-SO₄ buffer (pH 8.3). The column was eluted with 200 mL of 0.2 M Tris-SO₄ (pH 8.3) buffer, followed by 400 mL of a linear gradient 0→2 M NaCl in 0.2 M Tris-SO₄ buffer (pH 8.3), and finally eluted with 100 mL of 2 M NaCl in 0.2 M Tris-SO₄ buffer (pH 8.3), at 1 mL.min⁻¹ flow. The fractions presenting the highest iodoperoxidase activity were pooled and concentrated by ultrafiltration using a 10 kDa membrane cut-off.

This concentrated fraction was then introduced into a gel filtration column (Sephacryl S300) equilibrated and eluted with 165 mL of 0.2 M NaCl in 0.2 M Tris-SO₄ buffer (pH 8.3), using a 0.5 mL.min⁻¹ flow. The fraction showing the highest iodoperoxidase activity was pooled and stored at 4°C.

In the purification procedures, all the activity determination were done using the IPO assay, since this determination gives the highest absolute values and allow us to monitor the purification with more precision.

Protein determination: Protein quantification was based on the Bradford microassay method, using bovine serum albumin (BSA) as a standard (Bradford 1976).

Total glycid determination: Glycidic content was determined by the orcinol method using glucose as a standard (White and Kennedy 1986).

The pH dependence of iodoperoxidase activity: Iodoperoxidase activity tests were performed using the following buffer solutions: 0.1 M sodium acetate (pH 4.0, 4.5, 5.0 and 5.5), MES (pH 5.0 and 5.5) and 0.2 M citrate-phosphate (pH 6.2), at 25°C.

Temperature dependence of iodoperoxidase activity: The iodoperoxidase activity tests were performed in 0.1 M sodium acetate buffer (pH 5.0) after incubation of the samples, for 30 minutes, at 4, 15, 25, 30, 35, 40 and 45°C.

Results

Haloperoxidase survey

The survey conducted on the presence of haloperoxidase activity in the sponges of the Portuguese coast (Berlengas and Arrábida) revealed that this activity could be found in many sponge species (Table 1).

In line with these results, we choose the sponge *E. discophorus* to extract and purify the haloperoxidase, since it was the sponge that presented the highest activity values and also is quite abundant in some of the collection sites.

The results of the activity tests are presented in Table 2 for the nine examined samples of *E. discophorus*, expressed as specific activity, i.e., the number of activity units per mg of protein in the sample.

Purification of *Erylus discophorus* haloperoxidase

For enzyme extraction, several conditions were tested. The most efficient conditions were those described in the materials and methods section. Once the crude extract was obtained, it
Table 1: Presence of haloperoxidase activity in marine sponges.

<table>
<thead>
<tr>
<th>Species</th>
<th>Specimens examined</th>
<th>Haloperoxidase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stelleta grubii Schmidt, 1862</td>
<td>3</td>
<td>+</td>
</tr>
<tr>
<td>Erylus discophorus (Schmidt, 1862)</td>
<td>7</td>
<td>++</td>
</tr>
<tr>
<td>Cliona celata Grant, 1826</td>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td>Cliona viridis (Schmidt, 1862)</td>
<td>3</td>
<td>+</td>
</tr>
<tr>
<td>Saberites carnosus (Johnston, 1842)</td>
<td>1</td>
<td>++</td>
</tr>
<tr>
<td>Ciocalepyta penicillus Bowerbank, 1862</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>Axinysa aurantiaca (Schmidt, 1864)</td>
<td>1</td>
<td>+</td>
</tr>
<tr>
<td>Halichondria (Halichondria) panicea (Pallas, 1766)</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>Myxilla (Myxilla) rosacea (Lieberkühn, 1859)</td>
<td>10</td>
<td>++</td>
</tr>
<tr>
<td>Crambe sp.</td>
<td>1</td>
<td>+</td>
</tr>
<tr>
<td>Hymedesmia sp.</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>Phorbas fictitius (Bowerbank, 1866)</td>
<td>3</td>
<td>++</td>
</tr>
<tr>
<td>Chalinula molitba (de Laubenfels, 1949)</td>
<td>2</td>
<td>++</td>
</tr>
<tr>
<td>Haliclona (Reniera) cinerea (Grant, 1826)</td>
<td>3</td>
<td>+</td>
</tr>
<tr>
<td>Spongia sp.</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td>Sarcotragus spinosulus Schmidt, 1862</td>
<td>3</td>
<td>+</td>
</tr>
<tr>
<td>Ircinia sp.</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>Dysidea fragilis (Montagu, 1818)</td>
<td>1</td>
<td>+</td>
</tr>
<tr>
<td>Aplysina sp.</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>Clathrina cerebrum (Haeckel, 1870)</td>
<td>1</td>
<td>+</td>
</tr>
<tr>
<td>Clathrina contorta Minchin, 1905</td>
<td>1</td>
<td>-</td>
</tr>
</tbody>
</table>

+ positive for iodoperoxidase (IPO) activity
++ positive for iodoperoxidase (IPO) and bromoperoxidase (BrPO) activity

Table 2: Haloperoxidase specific activities (enzymatic activity units per mg of protein) for the species E. discophoris.

<table>
<thead>
<tr>
<th>Sample reference</th>
<th>IPO (U/mg)</th>
<th>BrPO (U/mg)</th>
<th>CIPO (U/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B125</td>
<td>5.080</td>
<td>0.065</td>
<td>0.000</td>
</tr>
<tr>
<td>B172</td>
<td>3.275</td>
<td>0.217</td>
<td>0.000</td>
</tr>
<tr>
<td>B206</td>
<td>8.643</td>
<td>0.528</td>
<td>0.000</td>
</tr>
<tr>
<td>B329</td>
<td>1.969</td>
<td>0.032</td>
<td>0.000</td>
</tr>
<tr>
<td>B351</td>
<td>3.179</td>
<td>0.076</td>
<td>0.000</td>
</tr>
<tr>
<td>B358</td>
<td>10.535</td>
<td>0.249</td>
<td>0.000</td>
</tr>
<tr>
<td>B397</td>
<td>8.750</td>
<td>0.463</td>
<td>0.000</td>
</tr>
<tr>
<td>FE05</td>
<td>26.550</td>
<td>0.570</td>
<td>0.000</td>
</tr>
<tr>
<td>G06.01*</td>
<td>11.524</td>
<td>0.594</td>
<td>0.000</td>
</tr>
</tbody>
</table>

IPO - iodoperoxidase specific activity
BrPO - Bromoperoxidase specific activity
CIPO - Chloroperoxidase specific activity
* this specimen has been identified only to the genus level (Erylus sp.)

was subjected to several chromatographic steps. Fig. 2 shows a representative chromatogram, obtained from a weak anionic exchange chromatography, with DEAE-Septacel.

The chromatogram shows three different regions concerning the 280 nm absorption characteristics of the eluted fractions. For each region the total glycid and protein content was determined as well as it IPO specific activity (see insert in Fig. 2). These results clearly show that the eluted fractions corresponding to region 1 are composed essentially by sugars, fractions in region 2 contained, besides sugar and proteins, the iodoperoxidase fraction and the remaining region 3, is composed also by sugar and proteins.

This chromatographic step removed sugars and some contaminant proteins from the crude extract but the specific activity was just slightly increased. However, even keeping the fractions with IPO activity at 4°C, their activities were quickly lost (in a period of 24 hours activity decreases approximately 50%). We tried to freeze the fractions at -80°C, but the activity decreasing was even steeper, which may be due to thawing conditions.

The fractions with IPO activity were concentrated by ultrafiltration, using a 10 kDa membrane cut-off and further applied into a strong anionic exchange chromatography (Mono-Q), but, unfortunately none of the collected fractions presented IPO activity. So, the DEAE-Sephacel IPO containing fractions were, therefore, subject to a gel filtration chromatography with Sephacryl S-300. The results are shown in Fig. 3.

In this chromatogram we observe 4 regions which included two peaks (named 2 and 3), with some degree of overlap. Only the first peak presented IPO activity, but solely around 70% of the initial IPO specific activity could be recovered. This fraction loses activity very fast. After 24 hours, IPO activity decreases to 50% and after 48 hours the IPO activity is completely lost. As for the weak anionic chromatography step, the glycid and protein content and IPO specific activity was determined for the several regions in the chromatogram (see insert in Fig. 3).

For molecular weight determination, this sample was subject to a gel filtration chromatography in a Sephacryl S-300 column, which had been previously calibrated using a wide range of molecular weight standards (6.5-669 kDa), and using dextran blue to determine the void volume of the column. However, the protein was eluted within this void volume meaning that its molecular mass should be between 700 and 2000 kDa (results not shown).

The SDS-PAGE of these fractions did not show any band staining with Coomassie brilliant blue or silver nitrate but, when the gel was stained for HPO activity with o-dianisidine, one well defined band was observed (Almeida et al., 2001). Also, a very faint band, just near the entrance well, was observed for the glycoprotein staining (results not shown). These results are in agreement with the lower 280 nm
absorption values (due to lower protein content), observed in all chromatographic steps and with a high specific IPO activity values also observed for the IPO containing fractions.

The UV-visible absorbance of the partially purified enzyme in 0.2 M Tris-SO$_4$ buffer (pH 8.3) was determined from 250-600 nm (Fig. 4). The spectrum showed a slight absorption at 410 nm (Soret region).

An optimum pH of 5.0, for IPO activity, was determined for this partially purified enzyme, as shown in Fig. 5. It is interesting to notice the absence of activity in MES buffer at pH higher than 6.0.

The enzyme presents the same activity from 4 to 25°C, but after 25°C quickly loses activity; at 45°C, 80% of activity is lost (Fig. 6). Addition of 0.3 mM of sodium azide completely inhibits the iodoperoxidase activity.

**Discussion**

Preliminary studies of haloperoxidase activity on sponges collected in Arrábida and Berlengas Archipelago, allowed us to identify some sponges from which haloperoxidase enzymes could be detected. It is interesting to notice a high percentage (ca. 50% of the samples examined) of sponge samples with haloperoxidase activity. Also, it was, the first time that sponges from the Calcarea class, have been screened for haloperoxidase activity. Both Calcarea samples belong to the Clathrina Gray, 1867 genus and, in one of them, haloperoxidase activity was detected. The remaining specimens belong to several orders of the class Demospongiae. Along with *E. discophorus*, other species also showed haloperoxidase activity, such as *Myxilla (Myxilla) rosacea* (Lieberkühn, 1859), *Cliona viridis*.
and Haliclona (Reniera) cinerea (Grant, 1826).

Considering that these results could be influenced by some form of consortia or association with other organisms, in particular microorganisms, we tried to collect the same species in several locations, geographically apart. Unfortunately, only two species (E. discophorus and Cliona celata Grant, 1826) could be collected in more than one site. All the examined samples of E. discophorus presented haloperoxidase activity, independently of the collection site. Samples of C. celata did not present any haloperoxidase activity at all.

The studies on the partial purification and preliminary characterization of the haloperoxidase from the sponge E. discophorus revealed a complex system to deal with. The rapid decrease in the activity during the purification steps was the major point that hampered this study. Several reasons may be ascertain for this fact, for example the possible involvement of an ion or molecule in the maintenance of the three dimensional structure of the enzyme that was removed during purification procedures. These same stability problems were also observed in an old work on the tropical marine sponge Iotrochota birotulata (Higgin, 1877) (Baden and Corbett 1979). Nevertheless, due to the high specific activity values showed by this enzyme a preliminary characterization could be performed. This sponge contains a haloperoxidase that shows iodo- and bromoperoxidase activities. The very faint shoulder in the Soret region of the UV-Vis spectrum, the inhibition by azide and the thermal instability may provide evidences that we are in the presence of a heme haloperoxidase. This fact would be also in close agreement with the known fact that in animal kingdom only heme haloperoxidases were found. However, other type of haloperoxidases may not be discharged.

This study, though still preliminary in its results, provides a background for the screening and study of the haloperoxidases in marine sponges and we hope that will stimulate more studies. Since marine sponges are a rich source of halometabolites, with diverse biological activities, the knowledge of the enzymes involved in their production in vivo might be of major importance not only from a biological point of view but also from a pharmaceutical approach.

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References

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