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Cellular and molecular responses of haemocytes from *Ostrea edulis* during in vitro infection by the parasite *Bonamia ostreae*

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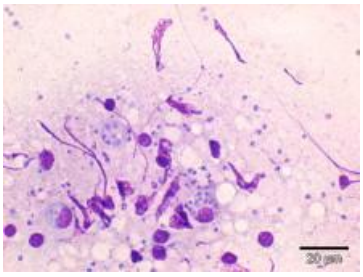
Abstract :

Bonamia ostreae is a protozoan, affiliated to the order Haplosporidia and to the phylum Cercozoa. This parasite is intracellular and infects haemocytes, cells notably involved in oyster defence mechanisms. Bonamiosis due to the parasite *B. ostreae* is a disease affecting the flat oyster, *Ostrea edulis*. The strategies used by protozoan parasites to circumvent host defence mechanisms remain largely unknown in marine bivalve molluscs. In the present work, in vitro experiments were carried out in order to study the interactions between haemocytes from *O. edulis* and purified parasite, *B. ostreae*. We monitored cellular and molecular responses of oyster haemocytes by light microscopy, flow cytometry and real-time PCR 1, 2, 4 and 8 h p.i. Light microscopy was used to measure parasite phagocytosis by oyster haemocytes. Parasites were observed inside haemocytes 1 h p.i. and the parasite number increased during the time course of the experiment. Moreover, some bi-nucleated and tri-nucleated parasites were found within haemocytes 2 and 4 h p.i., respectively, suggesting that the parasite can divide inside haemocytes. Host responses to *B. ostreae* were investigated at the cellular and molecular levels using flow cytometry and real-time PCR. Phagocytosis capacity of haemocytes, esterase activity and production of radical oxygen species appeared modulated during the infection with *B. ostreae*. Expression levels of expressed sequence tags selected in this study showed variations during the experiment as soon as 1 h p.i. An up-regulation of galectin (*OeGal*), cytochrome p450 (*CYP450*), lysozyme, omega GST (OGST), super oxide dismutase Cu/Zn (*Oe-SOD Cu/Zn*) and a down-regulation of the extracellular super oxide dismutase SOD (*Oe-EcSOD*) were observed in the presence of the parasite. Finally, the open reading frames of both SODs (*Oe-SOD Cu/Zn* and Oe-EcSOD) were completely sequenced. These findings provide new insights into the cellular and molecular bases of the host–parasite interactions between the flat oyster, *O. edulis*, and the parasite, *B. ostreae.*

Graphical abstract :



Highlights

► Multiplication of parasite Bonamia ostreae occurs inside haemocytes at 2 h p.i. ► Diminution of esterases and reactive oxygen species in the host at 1 h p.i. ► Over-expression of the galectin gene (OeGal) at 1 h p.i. ► Up-regulation of cytoplasmic super oxide dismutase (*OeSOD Cu/Zn*) at 2 and 4 h p.i.

Keywords : *Bonamia ostreae* ; Protozoan ; *Ostrea edulis* ; Haemocytes ; Real-time PCR ; Flow cytometry ; Super oxide dismutase

1. Introduction

Diseases due to infectious agents (virus, bacteria or protozoan) might curb oyster production and contribute to financial losses. A better understanding of interactions between the immune system of oysters and pathogens is necessary to control the development of these diseases. However, these interactions are poorly documented in oysters in contrast with other invertebrate species such as insects and nematodes (Gravato-Nobre and Hodgkin, 2005 ; D'Argenio et al., 2001). The immune defence of invertebrates, including oysters, is based on innate immune responses.

In Europe, since 1979 the flat oyster production has experienced high mortality outbreaks associated with protozoans including *Bonamia ostreae* (Pichot et al., 1979). This protozoan is an intracellular parasite and belongs to the order of Haplosporidae and the phylum of cercozoan (Cochennec et al., 2000 ; Carnegie et al., 2000 ; Cavalier-Smith and Chao 2003). *B. ostreae* infects the haemocytes, cells notably involved in oyster defence mechanisms (Cheng, 1981). Three different types of haemocytes have been described in the flat oyster, *Ostrea edulis*: granulocytes, large hyalinocytes and small hyalinocytes (Auffret, 1985 ; Bachère et al., 1991 ; Chagot et al., 1992 ; Mourton et al., 1992 ; Xue et al., 2001). These cells are responsible for wound repair, phagocytosis and encapsulation (Cheng, 1981 ; Fisher, 1986). The haemocytes are carried by the haemolymph in a semi-open system. Some soluble immune components such as lectins, lysosomal enzymes, and antimicrobial peptides have already been identified in the haemolymph of different bivalves species (Olafsen et al., 1992; Bachère et al., 2004 ; Xue et al., 2004).

The flat oyster *O. edulis* and its parasite *B. ostreae* represent a suitable model to study host-pathogen interactions in molluscs because of the possibility to reproduce the associated disease in experimental conditions (Mourton et al., 1992).

82 Intracellular parasites have developed sophisticated strategies to escape host defence mechanisms,
83 thereby finding unique niches where they can survive, and from which they can establish successful
84 infection. As previously described, the internalisation of *Bonamia ostreae* by flat oyster haemocytes
85 induces a diminution of esterase activities and reactive oxygen species (ROS) production after 2
86 hours of *in vitro* infection (Morga et al., 2009). Esterases are enzymes belonging to the group of
87 hydrolases catalysing the hydrolysis of ester bonds. The respiratory burst is a series of biochemical
88 reactions that produce highly microbicidal ROS including superoxide (O^{2-}), hydrogen peroxide
89 (H_2O_2), and hydroxyl radical (OH^+). Although radicals produced during the respiratory burst are
90 known to be involved in the destruction of parasites in different host species including molluscs
91 (Toreilles et al., 1996 ; Hahn and Bayne 2001 ; Humphries and Yoshino 2008), some intracellular
92 parasites like *Leishmania sp*, *Toxoplasma gondii* and *Perkinsus marinus* develop strategies to evade
93 this process which allows them to invade and multiply within host cells (Dermine and Desjardins
94 1999 ; Schott et al., 2003 ; Shrestha et al., 2006). Similar strategies seem to be developed by the
95 parasite *B. ostreae*. Recently, new data obtained on flat oyster genome have allowed identifying
96 some ESTs potentially involved in the host-parasite interactions, including the genes selected in the
97 present study (Morga et al., 2010a).
98 Previous *in vitro* studies have examined the specificity of the host responses to infection with the
99 parasite at the cellular or at the molecular level after 2 hours, but never by considering both
100 simultaneously and throughout time (Morga et al., 2009, Morga et al., 2010a). The specificity of the
101 haemocyte response has been tested using dead and live parasite.
102 In the present study, we have studied the kinetics of the response of haemocytes of *O edulis* to an *in*
103 *vitro* infection with the parasite *B. ostreae*. For that purpose, we combined flow cytometry to
104 measure some haemocyte activities and real-time PCR analysis to measure expression levels of
105 some genes of interest including galectin (OeGal), super oxide dismutase (Oe-SOD Cu/Zn),
106 extracellular super oxide dismutase (Oe-EcSOD), cytochrome p450 (CYP450), heat shock protein

90 (HSP90), omega gluthationne S transferase (OGST) and lysozyme. In addition, the complete open reading frames of two *Ostrea edulis* genes related to detoxification (Oe-SOD and Ec Oe-SOD) were determined and characterized.

This integrated study of the kinetics of the immune response of the *Ostrea edulis* to *Bonamia ostreae* may contribute to better understand the strategies developed by the parasite to escape host responses and by the oyster to eliminate the parasite and finally survive.

2. Material and methods

2.1 Biological material

2.1.1 Haemolymph collection

Two years old flat oysters *Ostrea edulis* were collected from the Bay of Quiberon (Southern Brittany, France), an endemic zone regarding bonamiosis.

Haemolymph was withdrawn from the adductor muscle using a 1mL syringe equipped with a needle (0.40x90 mm) and then filtered on a 75 µm mesh to eliminate debris. 10-15 samples of haemolymph were pooled together and maintained on ice to prevent aggregation (Auffret and Oubella. 1997). Haemocyte counts were performed using a Malassez cell.

2.1.2 Parasite

B. ostreae was purified according to the protocol of Mialhe et al., (1988). Briefly, heavily infected oysters were selected by examination of heart tissue imprints using light microscopy. After

132 homogenization of all the organs except the adductor muscle, the parasites were concentrated by
133 differential centrifugation on sucrose gradients and then purified by isopycnic centrifugation on a
134 Percoll gradient. Finally, the purified parasites were suspended in filtered sea water (FSW).
135 *Bonamia ostreae* cells were then counted using a Malassez-cell. Suspensions of purified parasites
136 were stored at 4°C and used within the 24 hours following the purification for *in vitro* infection
137 experiment.
138
139 *2.2 In vitro infection protocol*
140
141 Haemocytes (5.10^5 cells mL^{-1}) were incubated with live parasites at the ratio 5:1 (parasites per
142 haemocyte) at 15 °C and analysed after 1h, 2h, 4h and 8h of *in vitro* infection. The control consisted
143 of haemocytes alone suspended in FSW (5.10^5 cells mL^{-1}). The whole experiment was carried out
144 three times and included each time duplicate (n=6).
145
146 *2.3 Flow cytometry analysis*
147 Protocols and methods used for the flow cytometry analyses have previously been described in
148 Morga et al., 2009. For each sample, 5000 events were counted using an EPICS XL 4 (Beckman
149 Coulter). Based on size discrimination, parasites or other small particles were not counted, only
150 haemocytes were taken into account for cell activity measures. Results were depicted as cell
151 cytograms and reported in log scale fluorescence levels for each marker used. Fluorescence
152 depended on the monitored parameters: non specific esterase activities, ROS production and
153 phagocytosis were measured using green fluorescence while cell mortality was measured using red
154 fluorescence.
155
156

157 *2.4 Light microscopy*

158

159 One hundred µl of cell suspensions were cytocentrifugated (100 × g, 4 °C, 1 min), stained with
160 Hemacolor® (Merck) and examined using light microscopy. Before the infection experiment, pools
161 of haemolymph were examined in order to check the absence of the parasite. Then, for each time of
162 contact, 150 haemocytes were observed, and number of infected haemocytes, number of parasites
163 per infected hameocytes and the total number of di and trinucleated parasites were reported.

164

165 *2.5 Gene selection*

166

167 Seven ESTs were selected from *O. edulis* cDNA databases because of their potential involvement in
168 response to infection with *Bonamia ostreae*. Among these genes, one encodes a cell recognition
169 protein, the galectin (OeGal) and four are involved in post-phagocytosis degradation and cellular
170 protection mechanisms: super oxide dismutase (Oe-SOD Cu/Zn), extracellular super oxide
171 dismutase (Oe-EcSOD), cytochrome p450 (CYP450), omega gluthationne s transferase (OGST).
172 The expression of two other genes implicated in defence mechanisms (lysozyme) and stress (heat
173 shock protein 90, HSP90) was also studied.

174

175 *2.6 RNA extraction and reverse transcription*

176

177 Total RNA was extracted from haemocytes using Trizol (Trizol® reagent, Invitrogen™) and treated
178 with RQ1 RNAse-free DNAse (Promega) to remove remaining genomic DNA. RNA concentrations
179 were measured before and after DNase treatment. Reverse transcription (RT) was carried out as
180 previously described Morga et al., (2010) using the oligo(dT) anchor primer (5'-GAC

CACGCGTATCGATGTCGACT(16)V-3'). Reverse transcriptase was performed using SuperScript III (Invitrogen) according to the manufacturer's recommendations.

2.7 Expression analysis of the selected ESTs by quantitative real-time PCR

For each of the selected ESTs, forward and reverse primers were designed using primer3 software (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) (Table 1). Real-time quantitative PCR reactions were duplicated and performed in a total volume of 25 µL using a Mx3000 Thermocycler sequence detector (Stratagene) in 96-microwell plates. Each well (25 µL) contained 5 µL of cDNA dilution (1/30), 12.5 µL of Brilliant® SYBR® Green II PCR Master Mix (Stratagene), 2.5 µL of each diluted primer (3µM) and 2.5 µL of distilled water. Thermal cycling conditions were: 1 cycle of activation of Hot start Taq polymerase at 95 °C for 10 min; 40 cycles of amplification at 95°C for 10 s, 60°C for 30 s; and melting temperature curve analysis at 95°C to 60°C by 0.5°C decrease of the temperature every 10 s. In all cases, negative controls (without cDNA) were included to rule out DNA contamination.

For each candidate gene, melting curve and gel picture were analysed in order to verify the specificity of the amplified products and that a single PCR product was amplified. PCR efficacy (E) was calculated for each primer pair by determining the slopes of standard curves. These curves were generated using a serial dilutions analysis of plasmid containing the insert of interest. Amplification efficacies were calculated according to the following equation $E=10^{(1\text{-slope})}$ (Pfaffl, 2001).

The expression of the candidate genes was normalized using the elongation factor 1 alpha (GenBank, accession n° EU651798) as housekeeping gene. The elongation factor alpha has previously been identified as the most stable housekeeping gene for similar study (Morga et al. 2010b). Haemocytes alone were used as calibrator. Primers are shown in Table 1. Fold units were calculated using the double delta Cts method described by Pfaffl (2001).

206

207 *2.8 Identification and characterization of immune-related genes Oe-SOD Cu/Zn and Oe-*
208 *EcSOD*

209

210 In order to obtain the complete open reading frame (ORF) of *Oe-SOD Cu/Zn* and *Oe-EcSOD*,
211 RACE reactions were carried out using SMART RACE cDNA Amplification Kit from Clontech
212 according to the manufacturer's instructions. 5' and 3' primers were designed using primer 3
213 software (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) (3'Oe-SOD Cu/Zn
214 TGATTAACCTGGCTGGTCCACAGTC, 5'Oe-SOD Cu/Zn
215 GACTGTGGACCAGCCAGGTTAATCA and 3'Oe-ECSOD
216 GTGCACATGGAGGTCATGTTGACTG, 5'Oe-ECSOD
217 TGACAGGATGGTCCTCTTCCTCCTC) and synthesized by Eurogentec. Race PCR products
218 were cloned using TOPO Vector System (Invitrogen). Several clones were selected and amplified
219 with TOPO R and F primers in order to control the size of the insert in the plasmid.

220

221 *2.8.1 Sequencing and sequence analysis*

222

223 Selected clones were first amplified using TempliPhi™ DNA Sequencing Template Amplification
224 Kit (Amersham's). PCR products isolated from individual clones were sequenced in both ends with
225 TOPO R and F primers (Table 1) with the sequencing kit ABI BigDye® terminator version 3.1
226 using an ABI PRISM® 3130 XL-Avant Genetic Analyzer, a 36 cm capillary array and POP 7
227 polymer. Chromatograms were analyzed with Chromas 231 software. Sequences were then
228 analyzed with BlastX algorithm available from the National Center for Biotechnology Information
229 (NCBI) and the EST sequences were then submitted to dbEST and GenBank databases

(http://www.ncbi.nlm.nih.gov/blast/). Complete sequences (Oe-SOD and Oe-EcSOD) were deposited in GenBank and assigned under the accession numbers GU320695 and GU320696.

2.8.2 Phylogenic analysis of Oe-SOD Cu/Zn and Oe-EcSOD

The sequence of the complete ORF of *Oe-SOD Cu/Zn* was aligned with homologous genes from GenBank database (*Homo sapiens sapiens* NP000445.1, *Rattus norvegicus* CAA79925.1, *Bos taurus* NM174615, *Salmo salar* NP00111780.1, *Pagrus major* AA015363.1, *Mytilus edulis* CAE46443.1, *Crassostrea ariakensis* ABF14366.1 and *Crassostrea gigas CAD427221).*

The sequence of the complete ORF of *Oe-EcSOD* was aligned with homologous genes from GenBank database (*Homo sapiens sapiens* NP0003093.2, *Rattus norvegicus* CAA64149.1, *Bos taurus* NP001076079.1, *Salmo salar* NP001134324.1, *Perna canaliculus* AF273766.1, *Crassostrea virginica* BAF30874.1 *Crassostrea gigas AAY60161.1 Saccostrea glomerata* ACQ73551.1 and *Barbula unguiculata* BAC53790.1 (outgroup)).

Sequences were aligned using ClustalW (Thompson et al., 1997) from MEGA 4 (Tamura et al., 2007). Phylogenic trees based on deduced amino acid sequences were constructed using the Neighbour-Joining (NJ) (Saitou et al., 1987) and the Maximum parsimony (MP) method (Eck and Dayhoff 1966) with the MEGA 4 software program. Statistical confidence on the inferred phylogenic relationships was assessed by bootstrap of 1000 replicates.

2.9 Statistical analysis

A Mann-Whitney test was performed to test the difference between the mean numbers of parasites in infected haemocyte during the kinetic experiment. Flow cytometry results were expressed as percentages of positive cells. In order to detect an effect of tested conditions, a two factors ANOVA was performed using XLSTAT-Pro® version 7.5.3 software. Values were converted into r angular arcsinus √ (% of positive cells) before analysis to ensure the respect of *a priori* assumptions of normality and homogeneity. In the case of rejection of H_0, an *a posteriori* Tukey test was used to compare differences between means. Quantitative PCR results were also analysed by a two factor ANOVAs using XLSTAT-Pro® version 7.5.3 software. In the case of rejection of H_0, an *a posteriori* Tukey test was used to compare differences between means.

3. Results

3.1. Detection of parasites in haemocytes

Bonamia ostreae appeared engulfed by haemocytes whatever the tested contact period was. Percentages of infected haemocytes are 18% to 27% and mean number of parasites per infected haemocyte from 1.3 to 2.4 between 1h and 8h of contact (Fig. 1). The number of parasites observed inside the haemocyte was significantly more important at 4h and 8h compared to 1h ($p< 0,036$ and $p<0,016$, respectively). Number of binucleated parasites inside the haemocytes increased after one

hour of contact (Fig. 1). Moreover, two intrahaemocytic trinucleated parasites could be observed after 4h.

3.2 Flow cytometry analysis

3.2.1 Haemocyte mortality

Prior to haemocyte activity measurement, cell survival was checked by deducing cell mortality based on propidium iodide labelling. Whatever the tested condition was, percentage of live cells was never below 89,1%. Cell mortality was considered equivalent between all the tested conditions.

3.2.2 Esterases, ROS and phagocytosis activity

In presence of parasites, the percentages of positive haemocytes showing esterase activities were significantly lower compared to the control after 1h ($p < 0.033$), 2h and 4h ($p < 0.004$) of *in vitro* infection (Fig. 2a). Percentages of ROS positive haemocytes were significantly lower in presence of parasites than in controls at 1h, 2h and 8h ($p < 0.013$) (Fig. 2b). Lastly, a significant decrease of the percentage of haemocytes which have engulfed three beads or more was observed in haemocytes in contact with *Bonamia ostreae* after 2h of contact ($p < 0.02$) (Fig. 2c).

3.3 Identification and characterization of immune-related genes

3.3.1 Ostrea edulis Super Oxide Dismutase (Oe-SOD Cu/Zn)

RACE-PCR reactions allowed obtaining the full cDNA sequence of *Ostrea edulis* Super Oxide Dismutase Cu/Zn (*Oe-SOD Cu/Zn*). This sequence of 898 nucleotides contains a short 5'-

303 untranslated region (1-48 nt) followed by an ORF of 471 nucleotides (49-519 nt) and finished by a
304 3'-untranslated region (520-898 nt) showing a polyadenylation signal (AATAAA) starting 79 bp
305 upstream from the polyA tail (Fig 3). The deduced amino acid sequence presented a molecular mass
306 of 15 863.70 Da and a predicted isoelectric point of 5.93 (http://www.expasy.ch/tools/pi_tool.html).
307 SignalP analysis further confirmed that Oe-SOD Cu/Zn was a cytoplasmic protein. The deduced
308 amino acid sequence was devoid of signal peptide.
309 Multiple alignments of intracellular SOD identified in different invertebrate and vertebrate species
310 revealed the presence of several conserved motifs and SOD signature sequences in Oe-SOD Cu/Zn.
311 Two cysteine residues (C^{60} and C^{149}), involved in the formation of internal disulfide bond were fully
312 conserved in Oe-SOD Cu/Zn. Four histidine residues (H^{50}, H^{52}, H^{66}, and H^{123}) predicated to be
313 critical for copper (Cu) binding, and highly consistent with other invertebrate or vertebrate SODs,
314 are present in Oe-SOD Cu/Zn (Fig. 3). Moreover, four residues involved in Zinc (Zn) binding,
315 notably three histidine (H^{66}, H^{74}, H^{83}) and one aspartate (D^{86}), were also identified in Oe-SOD
316 Cu/Zn. Finally, Oe-SOD Cu/Zn amino acid sequence shows two Cu/Zn-SOD family signatures :
317 GFHVHQFGDNT and GNAGGRLACGVI.
318
319 *3.3.2 Ostrea edulis Extacellular Super Oxide Dismutase Oe-EcSOD*
320
321 RACE-PCR reactions allowed obtaining the full cDNA of *Ostrea edulis* Extracellular Super Oxide
322 Dismutase (*Oe-EcSOD*). *Oe-EcSOD* showed a 985 nucleotide sequence containing a short 5'-
323 untranslated region (1-42 nt) followed by an ORF of 794 nt (43-837 nt) and finished by a 3'-
324 untranslated region (838-985 nt) (Fig 4). The deducted amino acid sequence of the ORF region
325 showed a predicted molecular mass of 29 478.38 Da and an isoelectric point of 4.93
326 (http://www.expasy.ch/tools/pi_tool.html). Analysis of the deduced amino acid sequence using the
327 SignalP3.0 software (www.cbs.dtu.dk/services/SignalP/) revealed the presence of a putative signal

328 peptide of 20 amino acids (MQSLIILVLAALFYVA), suggesting that *Oe-EcSOD* can be secreted.
329 The mature peptide contained a putative N-glycosylation site (NLS[41])
330 (www.cbs.dtu.dk/services/NetNGlyc/), suggesting that *Oe-EcSOD* is a glycoprotein (Fig. 4).
331
332 *3.3.3 Phylogenetic analysis of both Ostrea edulis SOD*
333
334 The phylogenic trees based on amino acid sequences of *Oe-SOD* and *Oe-EcSOD* from different
335 species have been generated using Neighbour-Joining (NJ) and the Maximum Parsimony (MP)
336 methods.
337 Both phylogenic trees (NJ) (Fig.5) and (MP) (data not shown) show two distinct groups including
338 the cytoplasmic SOD sequences and the extracellular SOD sequences. Extracellular SOD group and
339 cytoplasmic SOD present two sub group, the mammal group and the invertebrate group in both
340 trees (Fig. 5). In the extracellular SOD group, "mollusc species" formed a unique branch including
341 *Perna canaliculus*, *Crassostrea virginica, Ostrea edulis, Crassostrea gigas* and *Saccostrea*
342 *glomerata* (Fig. 5). In the cytoplasmic SOD group, "mollusc species" formed a unique branch
343 including *(Mytilus edulis, Crassostrea ariakensis, Ostrea edulis and Crassostrea gigas).*
344
345 *3.4 Expression patterns of selected genes*
346
347 At 1h of *in vitro* infection, Oe-Gal was significantly ($p<0.01$) over expressed (3.9) and its
348 expression slowly decreased afterwards. A significant over expression of OGST ($p<0.02$) was
349 observed at 1h (2.1) while it appears stable after that time. The lysosyme gene was significantly
350 ($p<0.01$) over expressed at 1h, 2h and 4h with a maximum expression at 2h (4.1). The CYP450
351 expression was significantly ($p<0.01$) up regulated at 1h (2.3) and 8h (2.5). Oe-SOD was
352 significantly ($p<0.006$ and 0.001) over expressed at 2h (1.9) and 4h (1.4). *Oe-EcSOD* was down

353 regulated at 1h and not significantly regulated at 2h, 4h and 8h. Finally, HSP 90 was not
354 significantly regulated during the course of the experiment (Fig. 6).
355

356 ## 4. Discussion

357

358 The present work aimed at better understanding the interactions between flat oyster *O. edulis*
359 haemocytes and the parasite *Bonamia ostreae*. Cellular and molecular responses were measured by
360 light microscopy, flow cytometry and real time PCR assays at different times post incubation
361 between hameocytes and parasites: 1h, 2h, 4h and 8h.
362 *In vitro* infection was performed at the ratio of five parasites per haemocyte at 15°C. The
363 internalization of the parasite by the haemocytes was monitored by examination of
364 cytocentrifugated cells.
365 Parasites were observed inside haemocytes as soon as 1h of infection. Previous studies
366 demonstrated that *B. ostreae* was internalized after 30 min of contact with haemocytes and was not
367 degraded after phagocytosis (Chagot et al., 1992 ; Mourton et al., 1992). Percentages of infected
368 haemocytes increased during the course of the experiment. Due to the difficulty to identify with
369 certainty haemocyte types for some cytocentrifugated cells, we did not compare percentages of
370 infected haemocytes considering cell types. However, all types of haemocytes (granulocytes, small
371 and large hyalinocytes) were found infected as it was previously observed in several studies
372 (Chagot, 1989 ; Cochennec et al., 2003). The mean number of parasites observed inside the infected
373 haemocytes was significantly higher after 4h and 8h compared to 1h. This result suggests that the
374 parasite multiplies inside the haemocytes, or that the parasites accumulate within a particular cell
375 during the time course of the experiment. Binucleated and trinucleated parasites were observed
376 inside hameocytes suggesting that the multiplication of the parasite rapidly occurs after the
377 internalization.

Haemocyte activities were monitored by flow cytometry according to protocols previously described (Morga et al., 2009). Haemocyte mortality never exceeded 89.1% during the experiment. The internalization of the parasite did not induce haemocyte death even after 8h of contact. Previous *in vitro* studies have examined the specificity of the host responses to infection with the parasite at the cellular level after 2 hours of contact, the specificity of the haemocyte response has been tested using live and dead parasite (Morga et al., 2009).

Non specific esterase activities were recognized as reflecting the global activity of haemocytes. Esterases may also be involved in the degradation of internalised particles after phagocytosis. In the present study, parasites induced a decrease of the percentage of esterase positive cells after one hour and more significantly after two and four hours of contact. These results, in addition to the lack of haemocyte mortality, suggest a direct impact of parasites on host cell esterase activities. Catalytic enzymes present in *B. ostreae* (Hervio et al., 1991) might be able to inhibit hydrolases (ie esterases) produced by haemocytes.

Similarly, the percentage of ROS positive haemocytes was lower after 1h, 2 h and 8h of incubation with parasites compared to the control. As no cell mortality was detected, parasites may have a direct impact on haemocyte ROS production. The inhibition of oxygen radical production has already been described and should facilitate intracellular survival of protozoan parasites including *Trypanosoma sp* (Penketh et al., 1987), *Toxoplasma sp* (Murray et al., 1980, Shrestha et al., 2006), *Leishmania sp* (Murray, 1981), *B. ostreae* (Morga et al., 2009) and *P. marinus* (Volety and Chu, 1997).

P. marinus and rickettsia-like organisms possess acid phosphatase, partly responsible for the suppression or inhibition of ROS production (Le Gall et al., 1991 ; Volety and Chu 1997). Hervio et al., (1991) demonstrated the presence of acid phosphatase activity in haplosporosomes of *Bonamia ostreae*. Acid phosphatase can inactivate the production of NAD(P)H oxidase and thus prevents

403 ROS production. The level of acid phosphatase activity in *B. ostreae* is equivalent (Hervio et al.,
404 1991) to levels found in several *Leishmania* species (Lovelace and Gottlieb, 1987) in which the
405 inhibitor role of the NAD(P)H oxidase has been shown.
406
407 Bead phagocytosis capacity of haemocytes was significantly lower after 2 hours in presence of
408 *Bonamia ostreae* compared to haemocytes alone. Cytocentrifugation observation revealed an
409 increase of the parasite number inside infected haemocytes between 1 and 2 hours post infection.
410 The decrease of bead phagocytosis capacity by infected haemocytes observed after 2 hours of
411 contact with parasites could be due to the presence of parasites inside haemocytes. Goedken et al.,
412 (2005) have shown that the internalisation of the parasite *P. marinus* by haemocytes from the oyster
413 *Crassostrea virginica* did not induce a difference of bead internalisation by haemocytes. The
414 decrease of bead phagocytosis capacity and the increase of infected haemocytes during the course
415 of the experiment support the hypothesis that the parasite *B. ostreae* mediates its own phagocytosis.
416
417 In addition to this cellular analysis of haemocytes response to an infection with the parasite *B.*
418 *ostreae,* some genes have been selected for their potential interest to better understand host parasite
419 interactions. Previous *in vitro* studies have examined the specificity of the host responses to
420 infection with the parasite at the molecular level after 2 hours of contact, the specificity of the
421 haemocyte response has been tested using live and dead parasite (Morga et al., 2010a).
422 Galectins are able to bind glycans present at the surface of micro organisms and favour their
423 entrance of the pathogens inside host cells (Tasumi and Vasta, 2007). In the present study the
424 expression level of Oe-Gal was highly up regulated at 1h and slightly up regulated at 2h. This result
425 suggests the participation of oyster galectin to the parasite internalization process facilitating its
426 binding to the host cell surface.

427 The expression level of the lysozyme gene appeared up regulated in haemocytes after 1, 2 and 4h of
428 contact with the parasite. Similar results were observed in *Mercenaria mercenaria* infected with the
429 parasite QPX (Quahog parasite X) (Perrigault et al., 2009). Lysozyme is a lysosomal enzyme with
430 an important defence role as it can hydrolyse bacterial components. *O. edulis* lysozyme was
431 previously identified by Matsumoto et al., (2006). Lysozyme activity has been detected in the body
432 fluids and tissues of many bivalve molluscs and is believed to play a role in host defence and
433 digestion (Takahashi et al., 1986, Cronin et al., 2001). Lysozyme concentrations were unchanged in
434 clams *Ruditapes decussatus* infected by *Perkinsus atlanticus* (Ordás et al., 2000) and in oysters *C.*
435 *virginica* infected by *P. marinus* (Chu et al., 1993). However, a subsequent investigation in oysters
436 showed a slight decrease in lysozyme concentration in *P. marinus* infected oysters (La Peyre et al.,
437 1995).
438
439 Four genes involved in degradation and cellular protection (OGST, CYP450, Oe-SOD Cu/Zn and
440 Oe-EcSOD), were also studied by real time PCR. CYP450 and OGST were up regulated after 1h of
441 infection. This result can be related to parasite internalization and the production of toxic
442 components produced by the haemocyte and the parasite. Indeed, glutathione S-transferases play an
443 important role in the detoxification of toxic compounds of endogenous and exogenous origin
444 (Brophy and Pritchard, 1992 ; Brophy and Pritchard, 1994) and cytochrome p450 is involved in
445 various functions notably oxidative metabolism. Cytochrome p450 has been studied in other
446 bivalves subjected to parasite challenges (Tanguy et al., 2004, Perrigault et al., 2009). Perrigault et
447 al., (2009) reported an up regulation of this gene after 14 days post QPX infection with in *M.*
448 *mercenaria*.
449 Two genes (Oe-SOD Cu/Zn, Oe-EcSOD) involved in the oxidative stress were completely
450 characterized and their expression levels were studied. Oe-SOD Cu/Zn homologous genes have
451 been recently characterized in other oyster species including *C. gigas* (Huvet et al., 2004) and

452 *Crassostrea virginica* (Tanguy et al., 2004). Oe-EcSOD appeared similar to the permin or cavortin
453 previously identified in other bivalve species such as *Perna canalialus*, *C. gigas* and *C. virginica*
454 (Scotti et al., 2001 ; Scotti et al., 2007). Surprisingly, these two genes presented a different
455 expression pattern during the experiment. The cytoplasmic SOD appeared slightly up regulated
456 after 2h and 4h while the extracellular SOD was down regulated after 1h and not significantly
457 modulated at 2h, 4h and 8h. The down regulation observed after 1h of contact could be induced by
458 the parasite in order to facilitate its own internalization and to escape degradation before
459 internalization. The EC-SOD in *Saccostrea glomerata* was found up regulated in *Marteilia sydneyi*
460 resistant oyster (Green et al., 2009). Cytoplasmic SOD up regulation might reflect the cell response
461 to parasite internalization. ROS production showed a decrease after 1h, 2h and 4h. Decrease of ROS
462 production could be related to the up regulation of the cytoplasmic SOD at 2h and 4h of haemocyte
463 incubation with the parasite.
464
465 The present work is the first study combining cellular and molecular approaches to investigate the
466 interactions between haemocytes from *O. edulis* and the parasite *B. ostreae*. Results showed
467 internalisation of the parasite after 1h of contact and an increase of the number of the parasites
468 observed inside infected haemocytes during the time course of the experiment suggesting a
469 multiplication of the parasite especially after 2h of contact. Flow cytometry revealed a general
470 decrease of esterase activities, ROS production and phagocytosis capacity in infected cells. Real
471 time PCR measures showed an up regulation of some genes potentially involved in the entrance of
472 the parasite within host cells (e.g. OeGal) or involved in the detoxification and degradation like
473 lysozyme or cytochrome P450. Such results contribute to better understand how the flat oyster
474 reacts against the infection with *B. ostreae* and how the parasite may escape these defence
475 mechanisms.
476

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Figure captions

Figure 1 Mean number of parasites observed inside infected haemocytes and total number of binucleated cells observed inside haemocytes. Bars represent standard deviation of the mean number of parasites observed inside infected haemocytes.

Figure 2 Flow cytometry analysis for esterase's activities, ROS and phagocytosis activity.

2a Percentages of positive haemocytes for non specific esterase activities after 1h, 2 h, 4h and 8h of incubation with parasites at 5:1 ratio. Values are means of three replicates and bars represent standard deviation (n=6). * indicates a significant difference of positive cells compared to the control.

2b Percentages of positive haemocytes for ROS production after 1h, 2 h, 4h and 8h of incubation with parasites at 5:1 ratio. Values are means of three replicates and bars represent standard deviation (n=6). * indicates a significant difference of positive cells compared to the control

2c Percentages of positive haemocytes for phagocytosis after 1h, 2 h, 4h and 8h of incubation with parasites at 5:1 ratio. Values are means of three replicates and bars represent standard deviation (n=6). * indicates a significant difference of positive cells compared to the control

Figure 3 Complete ORF nucleotide and deduced amino acid sequences of the flat oyster *Oe-SOD Cu/Zn*. ORF contains some conserved (boxed) amino acids (C^{60}, C^{149}, H^{50}, H^{52}, H^{66}, H^{66}, H^{74}, $H^{83}$and H^{123} and D^{86}) and signature (surrounded) amino acids (GFHVHQFGDNT and GNAGGRLACGVI).

Figure 4 Complete ORF nucleotide and deduced amino acid sequences of the flat oyster *Oe-EcSOD*. ORF contains a signal peptide (MQSLIILVLAALFYVA^{20}) and a glycosylation site (NLS^{41}) boxed and surrounded respectively on the sequence. The polyadenylation signal (AATAAA) is underlined in grey.

Figure 5 Neighbour-joining (NJ) tree showing phylogenetic analysis of both SOD (cytoplasmic and extracellar) amino acid sequences from various species. The tree is based on 1000 bootstrap replications. The scale for branch length (0.1 substitutions/site) is shown below the tree.

Figure 6 Relative expression by quantitative PCR of selected genes (HSP 90, CYP450, OGST, Oe-SOD Cu/Zn, Oe-EcSOD, OeGal and lysozyme) after 1h, 2h, 4h and 8h of incubation with *Bonamia ostreae*. Expression levels were normalized to EF1-α and presented as relative expression to controls (mean ± SD, n = 4). Controls are arbitrarily assigned a value of 1. * indicates a significant difference of gene expression compared to controls

Table(s)

Name	Oligonucleotide sequence (5'-3')	Concentration of Forward primer (µM)	Concentration of Reverse primer (µM)
OGST	Forward: GGTCGTCAGGGGTCAGTTT Reverse: GGTTCCCGTTCTTGAGCA	3	3
CYTOP450	Forward:GTCATCAAGCGAATGCGATA Reverse: GGAGAGCTCCCTCATTTTCC	3	3
Hsp90	Forward: TTTGTGGAACGGGTCAAAA Reverse:AACGTCGAGCACAGTCGAG	3	3
OeGal	Forward: TCGGAGGTCGCCCTTAAT Reverse: TTGCCGTGAACAATCAACA	3	3
Lysozyme	Forward: TTGGAAATACCCGCAGGA Reverse: ATATCGGACGCCTGCTTG	3	3
Oe-EcSOD	Forward:GAGGAGGAAGAGGACCATCC Reverse: ATTTTCCTCCGCTTTGTGTG	2,5	2,5
Oe-SOD	Forward: TCGTCAATGTCAGCGTGAA Reverse: AAATGTTGGGGCTGGTGA	3	3
ElongPCRQ5m	Forward: GTCGCTCACAGAAGCTGTACC Reverse: CCAGGGTGGTTCAAGATGAT	3	3
Topo F	GACCATGATTACGCCAAGC		
Topo R	CCCAGTCACGACGTTG		

Table 1 Combinations of primers used in quantitative PCR assays

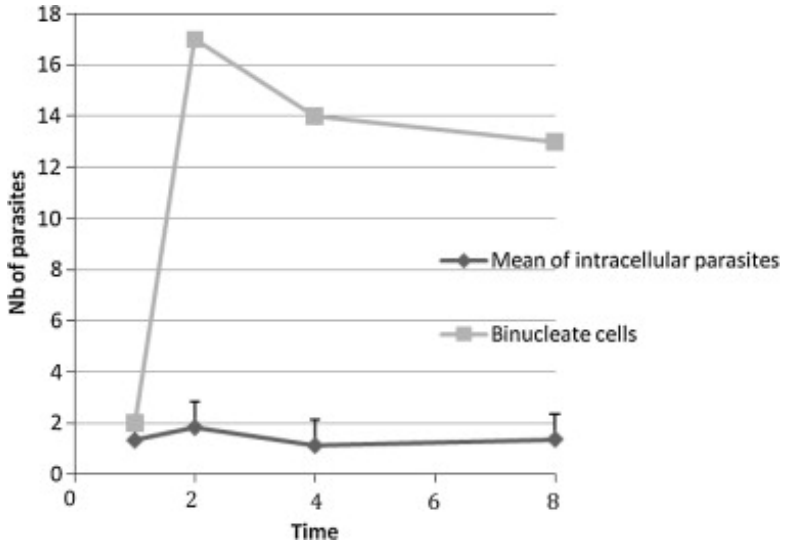
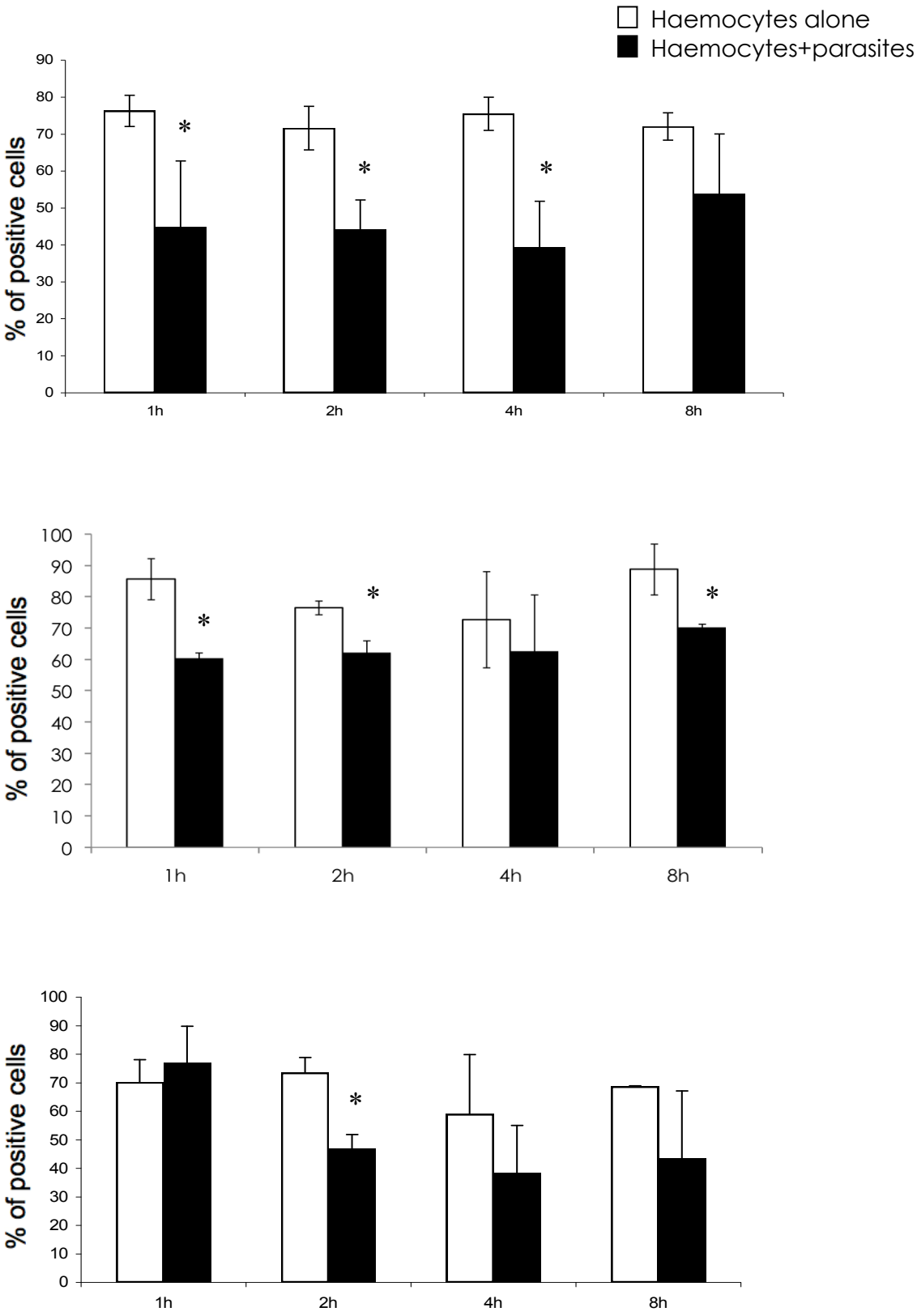

18
16
14
12
10
8
6
4
2
0
Nb of parasites
0
2
4
6
8
Time
Mean of intracellular parasites
Binucleate cells

Fig1


Haemocytes alone
Haemocytes+parasites
% of positive cells
0
10
20
30
40
50
60
70
80
90
100
1h
2h
4h
8h
*

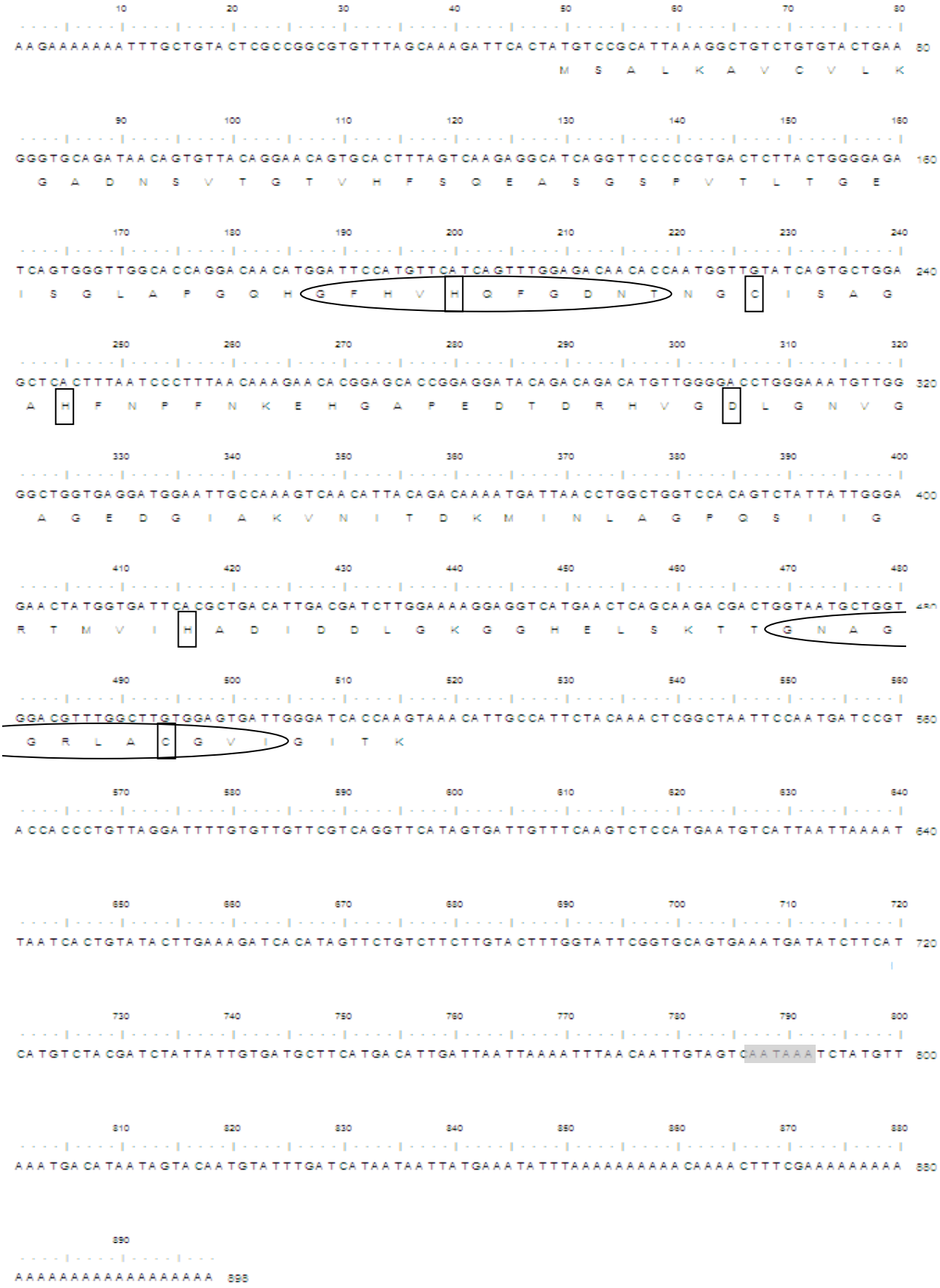


Fig 3

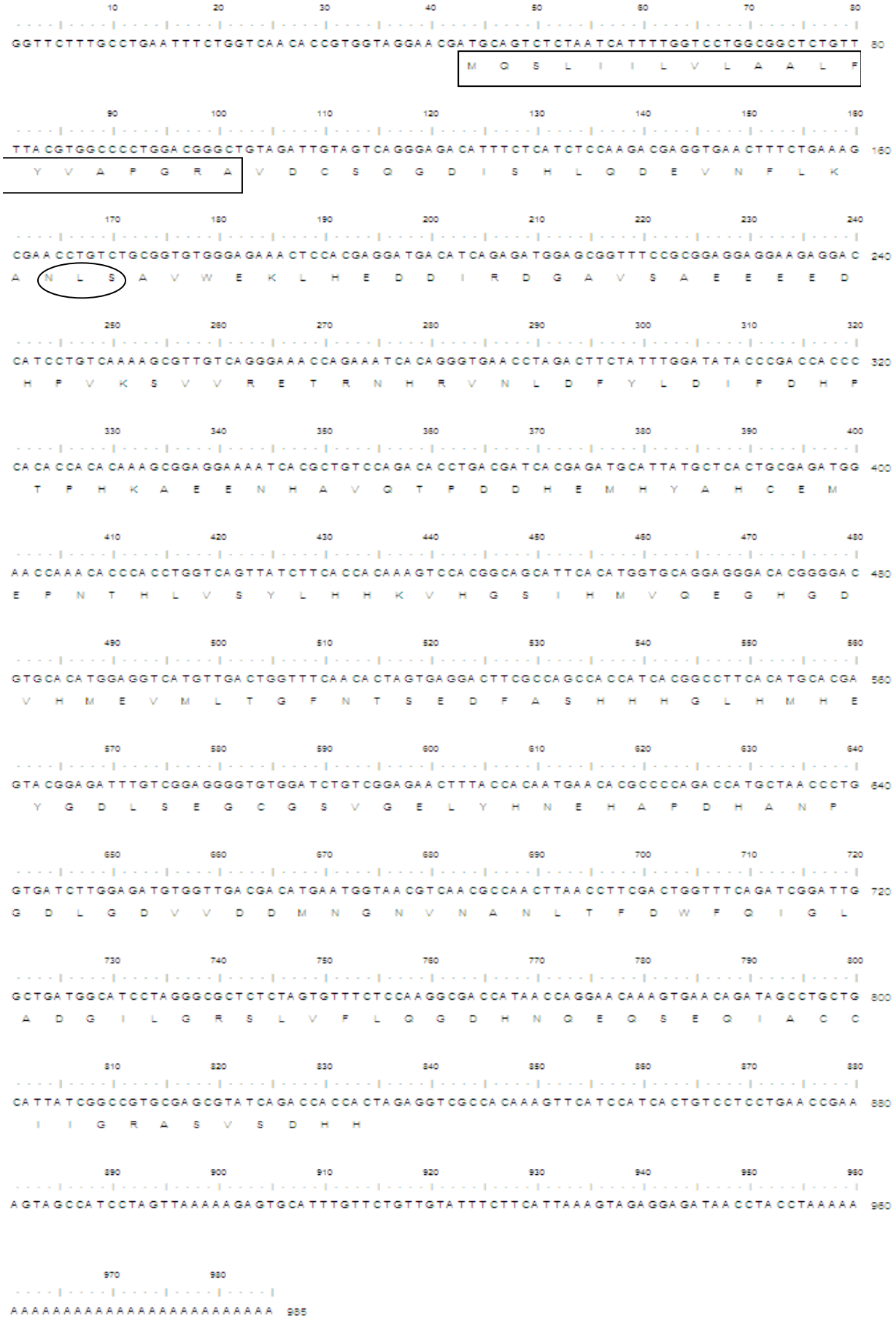

10 20 30 40 50 60 70 80
GGTTCTTTGCCTGAATTTCTGGTCAACACCGTGGTAGGAACGATGCAGTCTCTAATCATTTTGGTCCTGGCGGCTCTGTT 80
M Q S L I I L V L A A L F
90 100 110 120 130 140 150 160
TTACGTGGCCCCTGGACGGGCTGTAGATTGTAGTCAGGGAGACATTTCTCATCTCCAAGACGAGGTGAACTTTCTGAAAG 160
Y V A P G R A V D C S Q G D I S H L Q D E V N F L K
170 180 190 200 210 220 230 240
CGAACCTGTCTGCGGTGTGGGAGAAACTCCACGAGGATGACATCAGAGATGGAGCGGTTTCCGCGGAGGAGGAAGAGGAC 240
A N L S A V W E K L H E D D I R D G A V S A E E E E D
250 260 270 280 290 300 310 320
CATCCTGTCAAAAGCGTTGTCAGGGAAACCAGAAATCACAGGGTGAACCTAGACTTCTATTTGGATATACCCGACCACCC 320
H P V K S V V R E T R N H R V N L D F Y L D I P D H P
330 340 350 360 370 380 390 400
CACACCACAAAGCGGAGGAAAATCACGCTGTCCAGACACCTGACGATCACGAGATGCATTATGCTCACTGCGAGATGG 400
T P H K A E E N H A V Q T P D D H E M H Y A H C E M
410 420 430 440 450 460 470 480
AACCAAACACCCACCTGGTCAGTTATCTTCACCACAAAGTCCACGGCAGCATTCACATGGTGCAGGAGGGACACGGGGAC 480
E P N T H L V S Y L H H K V H G S I H M V Q E G H G D
490 500 510 520 530 540 550 560
GTGCACATGGAGGTCATGTTGACTGGTTTCAACACTAGTGAGGACTTCGCCAGCCACCATCACGGCCTTCACATGCACGA 560
V H M E V M L T G F N T S E D F A S H H H G L H M H E
570 580 590 600 610 620 630 640
GTACGGAGATTTGTCGGAGGGGTGTGGATCTGTCGGAGAACTTTACCACAATGAACACGCCCCAGACCATGCTAACCCTG 640
Y G D L S E G C G S V G E L Y H N E H A P D H A N P
650 660 670 680 690 700 710 720
GTGATCTTGGAGATGTGGTTGACGACATGAATGGTAACGTCAACGCCAACTTAACCTTCGACTGGTTTCAGATCGGATTG 720
G D L G D V V D D M N G N V N A N L T F D W F Q I G L
730 740 750 760 770 780 790 800
GCTGATGGCATCCTAGGGCGCTCTCTAGTGTTTCTCCAAGGCGACCATAACCAGGAACAAAGTGAACAGATAGCCTGCTG 800
A D G I L G R S L V F L Q G D H N Q E Q S E Q I A C C
810 820 830 840 850 860 870 880
CATTATCGGCCGTGCGAGCGTATCAGACCACCACTAGAGGTCGCCACAAAGTTCATCCATCACTGTCCTCCTGAACCGAA 880
I I G R A S V S D H H
890 900 910 920 930 940 950 960
AGTAGCCATCCTAGTTAAAAAGAGTGCATTTGTTCTGTTGTATTTCTTCATTAAAGTAGAGGAGATAACCTACCTAAAAA 960
970 980
AAAAAAAAAAAAAAAAAAAAAAAAA 985

Fig 4

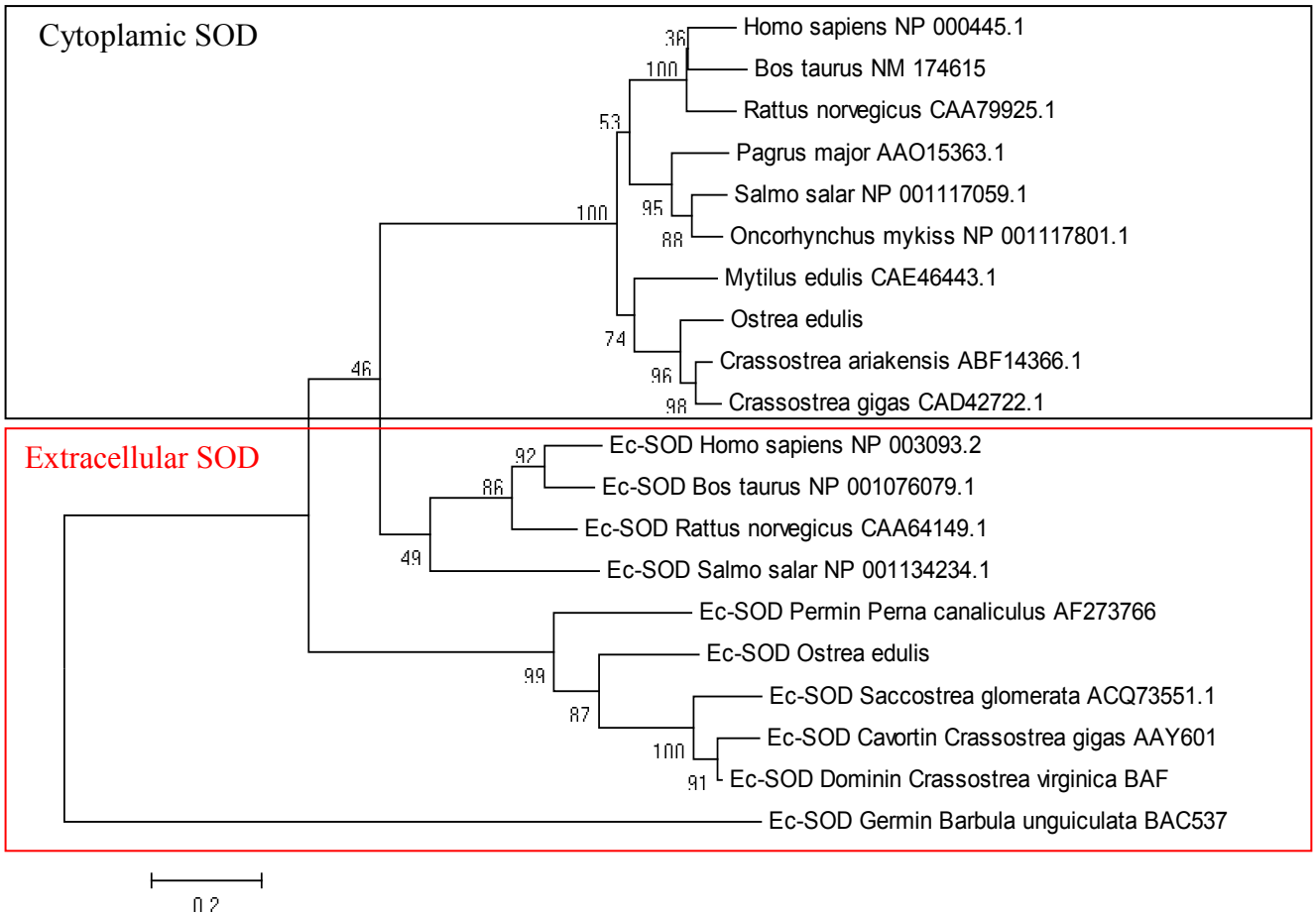

Cytoplamic SOD
Homo sapiens NP 000445.1
Bos taurus NM 174615
Rattus norvegicus CAA79925.1
Pagrus major AAO15363.1
Salmo salar NP 001117059.1
Oncorhynchus mykiss NP 001117801.1
Mytilus edulis CAE46443.1
Ostrea edulis
Crassostrea ariakensis ABF14366.1
Crassostrea gigas CAD42722.1
36
100
53
100
95
88
74
46
96
98
Extracellular SOD
Ec-SOD Homo sapiens NP 003093.2
Ec-SOD Bos taurus NP 001076079.1
Ec-SOD Rattus norvegicus CAA64149.1
Ec-SOD Salmo salar NP 001134234.1
Ec-SOD Permin Perna canaliculus AF273766
Ec-SOD Ostrea edulis
Ec-SOD Saccostrea glomerata ACQ73551.1
Ec-SOD Cavortin Crassostrea gigas AAY601
Ec-SOD Dominin Crassostrea virginica BAF
Ec-SOD Germin Barbula unguiculata BAC537
92
86
49
99
87
100
91
0.2

Fig 5

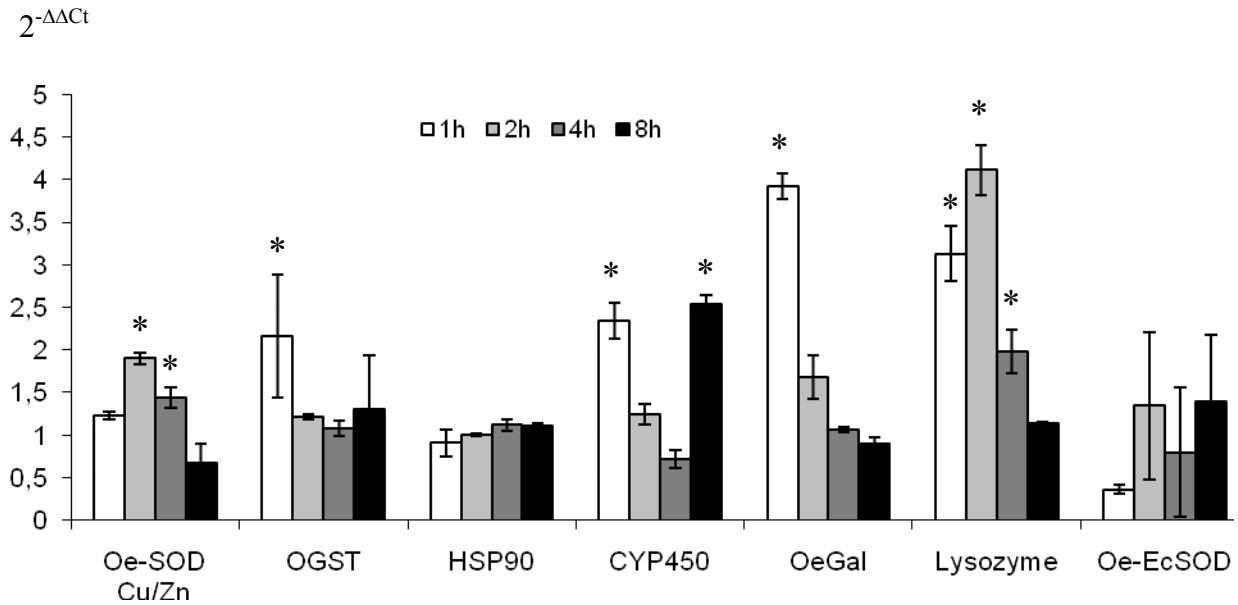

2-ΔΔCt
5
4,5
4
3,5
3
2,5
2
1,5
1
0,5
0
1h 2h 4h 8h
Oe-SOD Cu/Zn
OGST
HSP90
CYP450
OeGal
Lysozyme
Oe-EcSOD

Fig 6