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Evidence in oyster of a plasma extracellular superoxide dismutase which binds LPS

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Abstract

We have characterized in the oyster *Crassostrea gigas* an extracellular superoxide dismutase (*Cg*-EcSOD) which appears to bind lipopolysaccharides (LPS). The protein has been purified from the oyster plasma and identified as a Cu/ZnSOD according to its N-terminal sequencing and biological activity. *Cg*-EcSOD expression and synthesis are restricted to hemocytes as revealed by in situ hybridization and immunocytochemistry. *Cg*-EcSOD-expressing hemocytes were seen in blood circulation, in connective tissues, and closely associated to endothelium blood vessels. *Cg*-EcSOD presents in its amino acid sequence a LPS-binding motif found in the endotoxin receptor CD14 and we show that the protein displays an affinity to *Escherichia coli* bacteria and with LPS and Lipid A. Additionally, an RGD motif known to be implicated in the association to membrane integrin receptor is present in the amino acid sequence. The purified *Cg*-EcSOD was shown to bind to oyster hemocytes and to be immunocolocalized with a β-integrin-like receptor.

Keywords: Mollusc bivalve; Invertebrate; *Crassostrea gigas*; Hemocyte; Oxidative burst; β-Integrin

The superoxide dismutases (SODs) are metalloenzymes that represent one important line of defenses against reactive oxygen species (ROS) and in particular superoxide anion, resulting from aerobic metabolism. These enzymes catalyze the dismutation of superoxide (O_2^-) into molecular oxygen and hydrogen peroxide (H_2O_2) [1]. The production of ROS can be beneficial as killing mechanism against invading pathogens through the activation of the respiratory burst, a phenomenon present in both vertebrates and invertebrates, such as gastropods [2,3] and bivalve molluscs [4]. Additionally, it has been shown that ROS, and particularly H_2O_2, may serve as second messengers in signal transduction pathway by activating the NF-κ-B transcription factor [5]. However, elevated concentrations of ROS can be deleterious for the tissues. The SODs are present in both prokaryotic and eukaryotic organisms, invertebrates, and vertebrates. In animals, there are two distinct groups of SODs classified depending on the metal content in the active sites, i.e., manganese SOD (MnSOD) restricted to mitochondrial matrix [6]; copper/zinc SODs (Cu/ZnSOD) found primarily in intracellular cytoplasmic compartments or localized to extracellular elements. Cytosolic Cu/ZnSODs have a highly conserved structure, widespread in eukaryotes [7]. They are often constitutively expressed and composed of two 16 kDa subunits. In invertebrates, they have been characterized in insects [8] and in molluscs, the gastropod *Biomphalaria glabrata* [9], and the oyster *Crassostrea gigas* [10]. In the oyster, the expression of the cytosolic Cu/ZnSOD would be modulated upon hydrocarbon exposure [10]. The extracellular SOD (EcCu/ZnSODs) differs from the cytoplasmic Cu/ZnSOD by the presence of an N-terminal signal cleavage peptide that routes the molecule for secretion [11]. In invertebrates,

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EcCu/ZnSODs have been characterized in several species of parasitic nematodes such as *Brugia pahangi* [12], *Onchocerca volvulus* [13], and *Caenorhabditis elegans* [14]. In crustaceans, the blue crab *Callinectes sapidus* has an EcCu/ZnSOD present in the hemolymph and expressed in hemocytes [15]. Interestingly, in the crayfish *Pacifastacus leniusculus*, an EcCu/ZnSOD has been shown to interact with peroxinectin, a plasma cell-adhesive peroxidase which binds to the surface of the hemocytes. It was proposed that this interaction, SOD–peroxinectin, might mediate hemocyte reactions such as cell adhesion and phagocytosis [16]. In mollusc bivalves, Cu/ZnSOD have only been identified at the level of activity and protein in the digestive gland of mussel (*Mytilus edulis*) [17], whereas in the oyster *C. gigas*, Cu/ZnSOD sequence has been evidenced by suppression subtractive hybridization from the mantle–gonad tissue [18].

Here, we have purified the major protein from the oyster *C. gigas* plasma and identified it as being an extracellular Cu/ZnSOD (*Cg*-EcSOD) similar to the sequence previously identified in mantle–gonad, according to its N-terminal sequencing and biological activity. We show that the extracellular Cu/ZnSOD gene expression is restricted to hemocytes, located in the oyster blood vessels and connective tissues. Additionally, the plasma protein appears to bind bacteria, lipopolysaccharides (LPS), and Lipid A, a property which is reinforced by the presence in its amino acid sequence of a LPS-binding motif. Finally, because *Cg*-EcSOD sequence contains also an RGD motif, we have investigated the potential binding of this plasma protein to hemocyte through the presence of a β-integrin-like receptor that we evidence here by confocal microscopy immunolocalization. This is the first report of an EcSOD both acting as adhesion molecule and involved in LPS binding.

Materials and methods

Animals and hemolymph collection. Adult oysters, *C. gigas*, were purchased from a local oyster farm in Palavas-Les Flots (Gulf of Lion, France) and kept in seawater at 15 °C. Hemolymph was collected from the pericardial cavity through the adductor muscle under an equal volume of antiaggregant buffer modified Alsever solution [4] and immediately centrifuged at 1000*g* for 10 min (4 °C) for obtaining hemocytes.

Protein extraction and Cg-EcSOD purification. Plasma samples (cell-free hemolymph) collected without antiaggregant solution from 20 oysters were pooled and 50 μg of protein was applied to reverse-phase high performance liquid chromatography (C-18 RP-HPLC column, 2 × 150 mm, Waters Associates), previously equilibrated with 0.05% (v/v) trifluoroacetic acid acidified (TFA) water. A first purification step was performed with a linear gradient of 5–100% acetonitrile in TFA water, over 30 min at a flow rate of 1 ml/min. Absorbance was monitored at 225 nm. The active fraction was pooled, lyophilized, and reconstituted in sterilized water. Then, the second HPLC purification step was performed using the same column and *Cg*-EcSOD was eluted with a 5–100% acetonitrile gradient developed over 50 min.

Protein fractions containing *Cg*-EcSOD were identified by SDS–PAGE under reducing condition to evaluate the homogeneity of the preparation. Purified *Cg*-EcSOD was incubated without and with 25 mM DTT in 50 mM Tris–HCl, 50 mM NaCl, pH 7.5, buffer for 30 min at 25 °C. Then, *Cg*-EcSOD was alkylated with iodoacetamide for 30 min at room temperature and subjected to SDS–PAGE. Protein concentrations were quantified using micro BCA Protein Assay Reagent Kit (Pierce) and bovine serum albumin (BSA) was used as a standard. N-terminal sequence analysis of purified *Cg*-EcSOD, previously subjected to SDS–PAGE and transferred to polyvinylidene difluoride (PVDF) membrane, was performed by automated Edman degradation and detection of the phenylthiohydantoin (PTH) derivates on an automated Procise Applied Biosystems Sequencer.

Antibodies, immunodetection, and confocal microscopy. A Balb/C mouse was immunized by three subcutaneous injections of the purified *Cg*-EcSOD (20 μg) diluted in PBS and ascite was collected 2 weeks after intraperitoneal injection with 5×10^6 mice tumor cells in 500 μl RPM 1640 (Gibco). Immunoglobulins G (IgG) were purified from ascitic fluid on a Hitrap protein G–Sepharose column (Pharmacia). Antibodies directed against a peptide (sequence KLSDLREYRRFEKEKLKS) chosen in the cytoplasmic domain of the human β2-integrin chain were elicited in New Zealand rabbits as described [19]. Anti-peptide antibodies were purified by affinity chromatography on the related peptide as immunoadsorbent [20].

Native *Cg*-EcSOD was detected in hemocytes by immunocytochemistry according to the method previously described [21]. Briefly, hemocytes cytocentrifuged on slides were permeabilized with 0.1% Triton X-100 and successively incubated overnight with anti-*Cg*-EcSOD polyclonal antibody purified IgG (4 μg/ml). Alkaline phosphatase (PAL)-conjugated goat anti-mouse IgG (Jackson Immuno Research Laboratories, USA) was incubated for 1 h at room temperature, followed by a 1 h incubation in the dark in a solution of 100 mM Tris–HCl, 100 mM NaCl, and 50 mM $MgCl_2$, pH 9.5, containing 0.19 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP, Sigma), 0.4 mg/ml Nitro Blue Tetrazolium (NBT, Sigma), and 0.24 mg/ml levamisole (Sigma).

For colocalization of β-integrin and *Cg*-EcSOD, cytocentrifuged hemocytes were incubated for 1 h at room temperature with anti-β-integrin cytoplasmic domain IgG (4 μg/ml) and anti-*Cg*-EcSOD IgG, followed by goat anti-rabbit FITC and Texas red-conjugated goat anti-mouse IgG (Jackson Immunoresearch) diluted at 1:500 in PBS–Tween 20 (PBS-T) 0.1% containing 0.005% Evan's blue (Sigma Diagnostics), respectively. Then, the slides were washed and observed by confocal microscopy Leica TCS 4D. Percentages of hemocytes labelled, respectively, by anti-β-integrin IgG and anti-*Cg*-EcSOD IgG were deducted from a confocal observation of 480 hemocytes. For the visualization of colocalized pixels, the plugin of Image J software (NIH) has been used.

Binding of purified *Cg*-EcSOD on hemocytes was studied by immunodetection of the molecule on non-permeabilized cytocentrifuged hemocytes. After incubation of the cells with or without purified *Cg*-EcSOD (10 μg) for 30 min, the hemocytes were washed twice with PBS and incubated for 1 h with the mouse anti-*Cg*-EcSOD IgG (4 μg/ml), and then with (PAL)-conjugated goat anti-mouse IgG for immunodetection as described above. Percentage of positive cells was determined among 600 randomly chosen hemocytes per conditions. Statistical analyses were carried out using Student's *t* test and a *p* value less than 0.05 was considered as significant.

For all these experiments, controls were incubations of specific IgGs preabsorbed by purified protein and the absence of secondary antibodies cross-reactivity was controlled by omitting the primary antibodies.

SOD activity assay. SOD activity of the purified plasmatic protein in non-denaturating SDS–PAGE gels was first detected by staining with nitroblue tetrazolium (NBT) according to the method of Beauchamp and Fridovich [22]. Additionally, the SOD inhibitory activity on hemocyte respiratory burst was studied according to the NBT reduction assay as described by Munoz et al. [23]. Briefly, 100 μl of hemolymph containing 5×10^5 hemocytes was distributed per well, and 50 μl of zymosan A (1.43×10^8 particles/ml) was used at a 10:1 ratio (zymosan/hemocyte) as elicitor of respiratory burst. In some wells, purified *Cg*-EcSOD (10 μg) or plasma was added. NBT (0.3%) working solutions were immediately distributed to the wells. After 2 h incubation, the supernatants were removed and the hemocytes were fixed with methanol, washed with methanol 70%, and dried. The formazan deposits were solubilized in 2 M KOH and DMSO and, after homogenization, the OD at 620 nm was recorded in a microplate reader. Experiments were performed in triplicate and results are reported as mean ± standard error (SE) of the mean. Statistical

analysis was performed using one-way ANOVA with the Newman–Keuls post test (STATISTICA; StatSoft). A p value less than 0.05 was considered as significant.

LPS-binding properties. *Cg*-EcSOD LPS-binding properties were investigated as described previously by Mannion et al. [24]. Briefly, *Escherichia coli* K 1/r [25] bacteria were resuspended in 1 ml of 10 mM sodium acetate/acetic acid (NaAc/HAc) buffer, pH 4.0, and incubated for 10 min with 100 μg protein under gentle agitation (4 °C). *E. coli* cells were washed twice by centrifugation with 500 μl NaAc/HAc buffer and bound proteins were subsequently eluted with 30 μl of 200 mM $MgCl_2$ in NaAc/HAc buffer, pH 4.0, and further subjected to SDS–PAGE and HPLC analyses as described above.

Alternatively, LPS-binding properties of purified *Cg*-EcSOD were verified by ELISA using mouse anti-*Cg*-EcSOD polyclonal antibody. *E. coli* 026:B6 LPS or Lipid A (2.5 μg/well) (Sigma) were coated on a microtiter plate for 2 h at 60 °C. After 1 h blocking with PBS buffer containing 5% BSA, the solid phase was incubated for 60 min with increasing amounts of purified *Cg*-EcSOD (from 0 to 200 μg/well). After washing with PBS buffer, the ELISA was developed by successive incubation with mouse anti-*Cg*-EcSOD polyclonal antibody (10 μg/ml) for 2 h and with peroxidase-conjugated anti-mouse IgG (0.4 μg/ml). Controls consisted in the omission of specific antibodies. The colorimetric reaction was initiated by adding 50 μl per well of orthophenylenediamine chromogen (0.4 mg/ml) in substrate buffer (0.1 M citric acid, 0.1 M sodium acetate, pH 5.4, in 0.33% H_2O_2). The reaction was stopped after 15 min by adding 25 μl H_2SO_4 to the wells. Optical density (OD) was recorded in a microplate reader at 492 nm. All steps were performed at room temperature and two washes with PBS–T were performed between each step. Experiments were performed in triplicate for statistical analyses. The binding parameters, apparent dissociation constant K_d, and the maximum binding (A_{max}), were determined by non-linearly fitting as $A = A_{max}[L]/(K_d + [L])$, where A is the absorbance at 492 nm and [L] is the ligand concentration by using the CURVE FIT software developed by K. Raner Software (Victoria, Australia) [26].

RT-PCR and molecular cloning. Total RNA was extracted from *C. gigas* hemocytes using the Trizol reagent according to manufacturer's instructions (Invitrogen) and treated with DNAse Turbo (Ambion). Following heat denaturation (70 °C for 5 min), reverse transcriptions were performed using 1 μg of total RNA prepared with 50 ng/μl oligo$(dT)_{12–18}$ in a 50 μl reaction volume containing 1 mM dNTPs, 1 U/μl RnaseOUT (Invitrogen), and 200 U/μl M-MLV reverse transcriptase in reverse transcriptase buffer. The cDNAs were amplified using primers: EcSODfw, 5′ AGAGAATCCTGAGCTACAGC 3′ and EcSODrev, 5′ TGAGCAA AACTCTCTACAAGC 3′ designed in the untranslated region of the cDNA sequence (AY551094). The amplification program consisted of a 5 min at 94 °C, followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min, and a final elongation step of 72 °C for 10 min. Amplified products were analyzed on 1% agarose gels, cloned into pCR 2.1 TOPO TA cloning vector (Invitrogen), and sequenced from both directions with T7 and T3 primers.

In situ hybridization. Tissues and hemocytes from *C. gigas* oyster were prepared for histology and in situ hybridization analyses as described by Munoz et al. [21]. The plasmid containing *Cg*-EcSOD cDNA (GenBank Accession No. DQ010420) was used as template for the preparation of the probes. Digoxigenin (DIG)-UTP-labelled antisense and sense riboprobes were generated from linearized cDNA plasmids by in vitro transcription using RNA labelling kits, T3 RNA polymerase (Roche). DIG-labelled riboprobes were hybridized both to oyster tissues and to cytocentrifuged hemocyte preparations as described previously [21] for determination of the percentage of *Cg*-EcSOD-expressing circulating hemocytes. Control consisted in replacing antisense riboprobe with sense riboprobe.

Sequence analysis. Homology searches were performed with the BLAST software on the NCBI home page (http://www.ncbi.gov./Blast). Deduced amino acid sequences were aligned by ClustalX (http://www.ch.embnet.org/software/ClustalW.html).

Results and discussion

Isolation of a plasma extracellular Cu/ZnSOD

As observed on SDS–PAGE analysis, a protein appears to greatly predominate in the plasma of the oyster *C. gigas* (Fig. 1). In an attempt to characterize this major protein, we have performed its purification by reverse-phase HPLC from oyster plasma. In SDS–PAGE under non-reducing

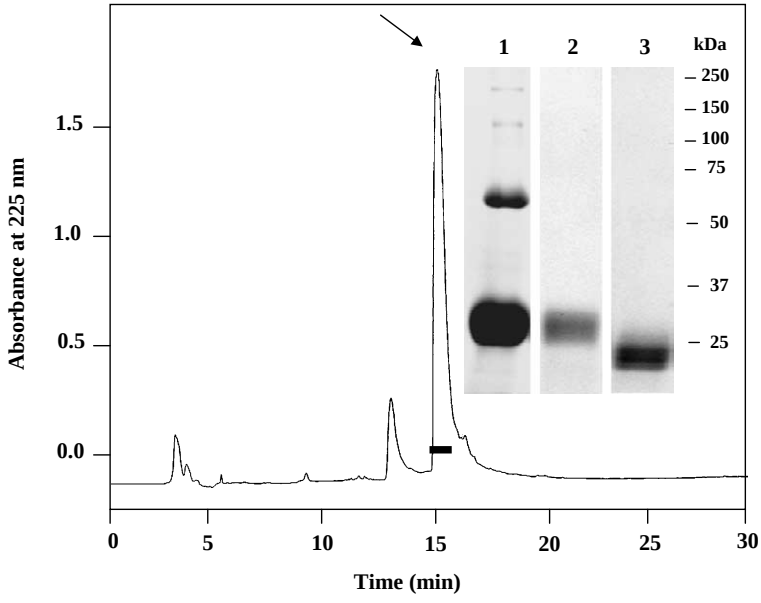

Fig. 1. *Cg*-EcSOD purification from *C. gigas* plasma. Reverse-phase HPLC profile of *C. gigas* plasma shows one major peak (arrow). Black rectangle means hand fraction collected and analyzed by SDS–PAGE and Coomassie blue staining. Lane 1, SDS–PAGE profile of crude oyster plasma revealing a major protein at around 30 kDa under reducing conditions; lane 2, eluted protein after RP-HPLC purification under reducing conditions appears as a unique band at 30 kDa; lane 3, under non-reducing conditions (-DTT) the eluted protein appears as a band corresponding to 20 kDa. Molecular mass markers are included on the right.

conditions, the purified protein appears as a single band with an apparent molecular mass of 20 kDa. However, following reduction and alkylation treatment, the linearized protein migrates as around a 30 kDa band, suggesting that the *Cg*-EcSOD is a monomer with intramolecular disulfide bonds (Fig. 1).

The purified protein was further electrotransferred on PVDF membrane for N-terminal sequencing by Edman degradation and a first 10 amino acid sequence was obtained -TARNEANVNI. Searching protein sequence databases, we showed that this plasma protein unequivocally matches with deduced amino acid sequence of SOD (AY551094) previously identified and cloned from mantle–gonad cDNA library [18] (Fig. 2). Oyster SOD consists of 174 amino acids and its sequence appears to be significantly similar to those of proteins from the extracellular SOD family while it shows lower identity with the oyster cytosolic SOD (14% identity) [10]. The oyster SOD we have isolated shares 20% amino acid sequence identity with the extracellular human and nematode SODs (Fig. 2) and it was consequently named *Cg*-EcSOD. *Cg*-EcSOD is also very similar (94% identity) to previously described oyster sequences named cavortin isolated from *C. gigas* oyster from a New Zealand farm (AY256853) but which would be an SOD.

Concordant with sequence similarity to SODs, we demonstrated a SOD activity for the protein purified from the oyster plasma. SOD activity was first detected in non-denaturing gel by the NBT reduction assay according to the method of Beauchamp and Fridovich [22]. Activity appeared located on a unique band both in the plasma and for the purified protein (data not shown). SODs are known to catalyze the dismutation of superoxide anion into molecular oxygen and hydrogen peroxide [1]. This antioxidant activity has also been evidenced for the purified *Cg*-EcSOD by an inhibitory effect on the ROS production from oyster hemocytes. The capacity of oyster hemocytes to generate respiratory burst upon phagocytic stimulation is well documented and various methods have been developed to study this phenomenon [4,27]. Here, we have used a colorimetric assay based on the detection of formazan deposit resulting from the reduction of NBT by ROS and previously described for shrimp hemocytes [23]. In our experiments, addition of purified *Cg*-EcSOD to oyster hemocytes phagocytosing zymosan particles resulted in an inhibition of ROS production which was measured as the optical density (OD) of solubilized formazan deposit (Fig. 3). While, the phagocytosis of zymosan by *C. gigas* hemocytes induced the production of superoxide anion corresponding to an OD of 0.25 compared to unstimulated hemocytes (base activity of 0.17 OD), hemocytes incubated with *Cg*-EcSOD displayed a significant reduced reaction with an OD of 0.20 ($p < 0.05$) which revealed a dismutation of the superoxide

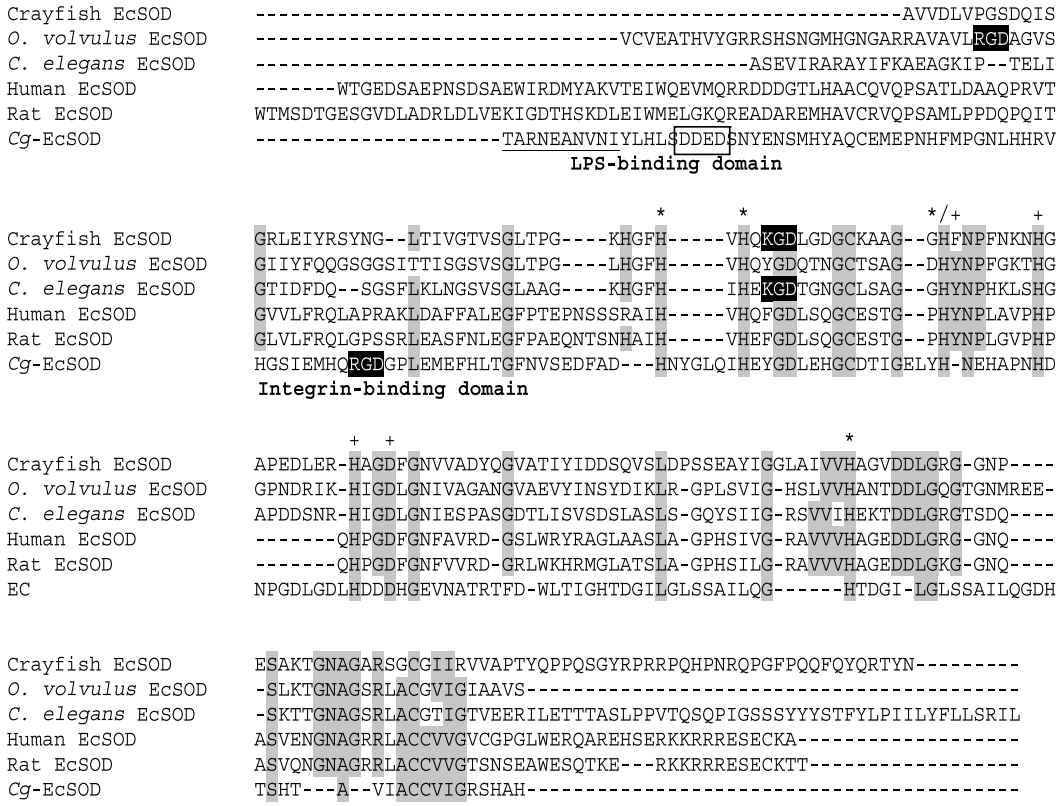

Fig. 2. Alignment of the oyster superoxide dismutase (*Cg*-EcSOD) with extracellular superoxide dismutases from some other animals. The accession numbers for these sequences are AF122900 (crayfish EcSOD), Q07449 (*O. volvulus* EcSOD), P34461 (*C. elegans* EcSOD), P08294 (human EcSOD), and P07509 (rat EcSOD). Highlighted in gray, conserved residues; (*) conserved amino acid residues responsible for Cu binding; (+) conserved amino acid residues responsible for Zn binding. Putative RGD or KGD integrin-binding motifs are highlighted in dark. The LPS-binding motif is enclosed in a box. The N-terminal sequence obtained from plasmatic purified *Cg*-EcSOD is underlined.

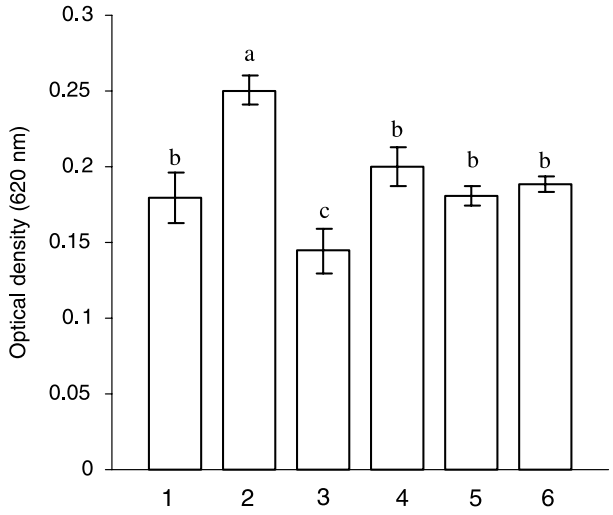

Fig. 3. Inhibitory effect of purified *Cg*-EcSOD and plasma on oyster hemocyte respiratory burst. *Cg*-EcSOD activity has been evidenced by its capacity to inhibit the oyster hemocyte production of ROS using a colorimetric NBT reduction assay. Absorbances are optical density of solubilized formazan deposits resulting from NBT reduction. Lane 1, base activity of unstimulated hemocytes incubated with buffer; lane 2, ROS production of hemocytes stimulated with zymosan at a ratio of 10 to 1 hemocyte (10/1); lane 3, unstimulated hemocytes incubated only with purified *Cg*-EcSOD (10 μg); lane 4, ROS production of hemocytes stimulated with zymosan (10/1) in the presence of purified *Cg*-EcSOD (10 μg); lane 5, unstimulated hemocytes incubated with oyster crude plasma; lane 6, ROS production of hemocytes stimulated with zymosan (10/1) in the presence of oyster crude plasma. The values represent means and SE for three independent experiments. Letters a, b and c for each lanes indicate significantly different mean values ($P < 0.05$).

anion. Similar effects have been shown in the assay following the addition of oyster plasma (0.18 OD; $p < 0.05$) which contains a huge amount of *Cg*-EcSOD (Fig. 3). In bivalve, as in other invertebrates, phagocytosis activity is known as an important component of defense reactions, but which may contribute to inflammatory reactions and tissue damage due to the massive release of ROS into extracellular fluids. The oyster has an open circulatory system and the hemolymph is in contact direct and bath connective tissues. It can be assumed that *Cg*-EcSOD which is the major plasma protein could play a protective role against inflammatory reaction as established for human phagocytic cells [28]. In other respect, SOD contributes to the production of H_2O_2 which in turn is dismutated by catalase. H_2O_2 is thought to be an important second messenger [29] and regulator of proliferation of mammalian cells. Low concentrations of H_2O_2 stimulate proliferation or enhanced survival of a wide variety of cell types [30,31]. Nothing is known about the similar role of H_2O_2 and SOD in the activation of hemocyte proliferation which would be a major element of the immune response in invertebrates such as the oyster [32].

Gene expression and production of Cg-EcSOD is restricted to hemocytes

We have produced a specific polyclonal antibody for the immunodetection and localization in oyster tissues of *Cg*-EcSOD, previously identified from the gonad and mantle of the oyster [18]. Surprisingly, an immunoreactivity was observed by Western blot mainly with circulating hemocytes and to a lesser extent on the tissues cited above (data not shown), questioning about the tissue origin of the plasma *Cg*-EcSOD. Consequently, we decided to look at the potential expression of *Cg*-EcSOD mRNA in hemocytes.

In a first time, a PCR approach was used to identify and clone the *Cg*-EcSOD from hemocytes, considering primers designed from the sequence (AY551094) isolated from the mantle–gonad. A single band of around 600 pb was obtained, cloned, and sequenced. The sequence encompassed a 576-bp open reading coding for a 193-amino acid protein, which was shown to be homologous (96%) to the mantle–gonad SOD sequence. The complete *Cg*-EcSOD (DQ010420) cDNA encodes a 174-residue protein with the same N-terminal sequence as the purified plasma protein and with an estimated molecular mass of 19.465 Da and a p*I* of 4.84. This estimated mass corresponds to the apparent mass of 20 kDa seen in SDS–PAGE under non-reducing condition. As shown for the EcSOD identified in mantle–gonad, the sequence contains an 18-residue signal peptide

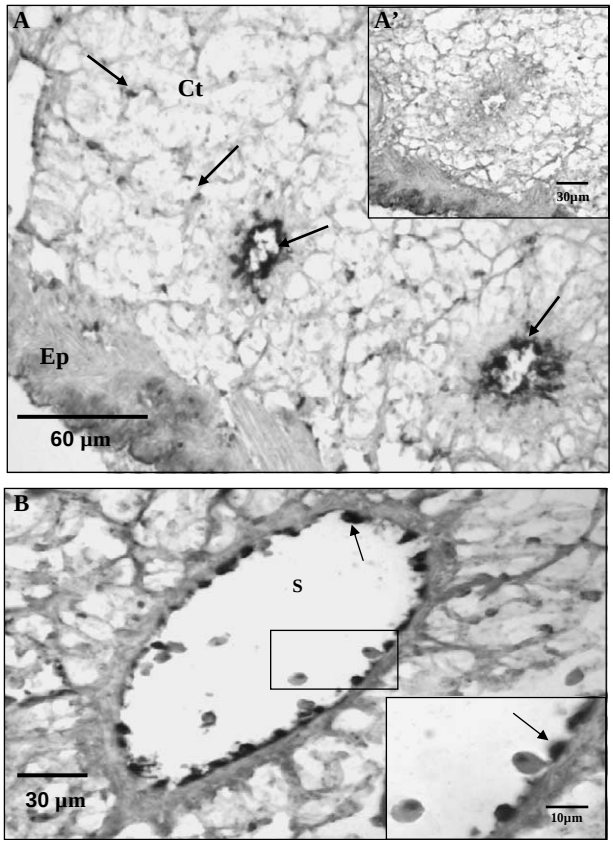

Fig. 4. Detection of *Cg*-EcSOD mRNA in oyster tissues by in situ hybridization. (A) In the mantle, hybridization positive signals were located to hemocytes associated with hemolymphatic sinus and infiltrating connective tissues (arrows). (A′) Negative control consisted of hybridization with *Cg*-EcSOD sense riboprobe. (B) In the hemolymphatic sinus, labelled hemocytes (arrows) are seen associated with the wall blood vessel while free hemocytes observed in the lumen are not labelled. Ct, means connective tissue; Ep, epithelium; and S, sinus.

followed by a mature protein domain as predicted by the Signal P program (http://www.cbs.dtu.dk/services/SignalP). Interestingly, *Cg*-EcSOD possesses in its sequence an RGD integrin-binding motif and has a LPS-binding motif as observed in mammalian CD14 protein [33] (Fig. 2).

Cg-EcSOD gene expression was studied by in situ hybridization for tissue localization. Tissue sections of oysters were probed with DIG-labelled antisense riboprobes and detected with alkaline phosphatase-conjugated anti-DIG antibodies, whereas sense riboprobes were used as control of specificity (Fig. 4A′). *Cg*-EcSOD expression was shown to be unequivocally restricted to hemocytes circulating and infiltrating connective tissues (Fig. 4A). Expressing hemocytes were also observed to be closely associated to the endothelium of blood vessel in the mantle (Fig. 4B). With respect to in situ hybridization analyses on circulating hemocytes, *Cg*-EcSOD transcripts were detected in a few populations of hemocytes with only 7.0% of positive cells (out of 650 hemocytes observed) (data not shown). These results reveal that the *Cg*-EcSOD cDNA sequence previously isolated by the genomic approach from the gonad–mantle of *C. gigas* [18] originated from hemocytes infiltrating this tissue. Additionally, immunolocalization of *Cg*-EcSOD with specific antibody revealed that around 12.0% of the total circulating hemocytes (out of 730 observed) were positive when the cell preparations have been membrane-permeabilized before incubation with the specific antibody (data not shown). Altogether, these results suggested that plasma *Cg*-EcSOD could also bind at the hemocyte membrane surface.

Plasma Cg-EcSOD binds to hemocyte membrane

To further investigate the putative binding of plasma *Cg*-EcSOD on the hemocyte surface, purified protein has been incubated with non-permeabilized hemocytes previously to its immunodetection. Significant increase in the number of positive hemocytes (32.9%, $p < 0.05$) (Figs. 5B

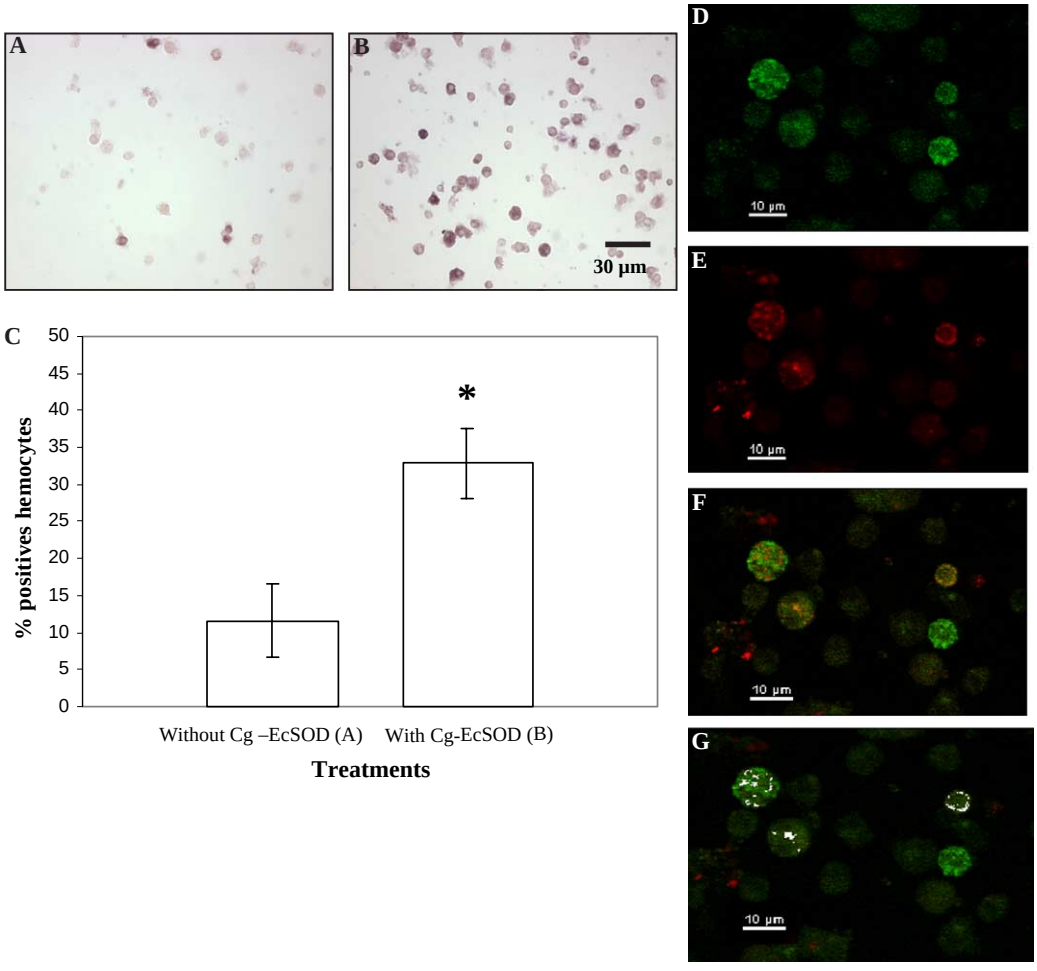

Fig. 5. Confocal microscopic images of *Cg*-EcSOD (red) and β-integrin (green) double immune labelling in oyster circulating hemocytes. Immunolocalization of *Cg*-EcSOD on hemocyte cell surface has been determined by incubating non-permeabilized cells without (A) and with purified *Cg*-EcSOD (10 µg protein) (B); percentage of *Cg*-EcSOD-immunoreactive hemocytes has been determined from 600 hemocytes. (*) Statistical difference with respect to incubation with purified *Cg*-EcSOD protein, $P < 0.05$ (C). The values represent means and SE for three independent experiments. Merged confocal microscope images suggest that β-integrin (green) and *Cg*-EcSOD (red) are present in different hemocytes (D,E, respectively), but also colocalized inside the same hemocytes (F). The colocalized pixels shown in white were determined by Image J software (G). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

and C) was obtained compared to conditions where hemocytes were not incubated with purified *Cg*-EcSOD (11.6% of positive cells) (Figs. 5A and C). In the crayfish, *P. leniusculus*, an EcSOD was also shown to be localized at the hemocyte surface and it was additionally recognized as a peroxidase-binding protein [16]. The authors proposed that the EcSOD could be maintained at the cell surface through ionic interactions. In oyster, as previously mentioned, *Cg*-EcSOD does not have transmembrane region but it presents in its sequence a RGD motif (Fig. 2) which is known to be recognized by integrins that function as cell–cell or cell–substrate adhesion and in signal transduction [34]. These proteins are ubiquitous in vertebrates and invertebrates. They are involved in several immune functions, including cell adhesion, motility, and cell proliferation [35,36]. In invertebrates, hemocyte β-integrins have been shown to be involved in phagocytosis processes [37]. The presence of a RGD motif in the *Cg*-EcSOD sequence prompted us to investigate the presence of β-integrin in oyster hemocytes and its potential colocalization with the plasma *Cg*-EcSOD. This has been approached using a specific antibody directed against a peptidic sequence chosen in the cytoplasmic domain of β-integrin which is conserved among vertebrates [38]. In our experiments, merged confocal microscope images revealed that β-integrin and *Cg*-EcSOD were detected in different circulating hemocytes with 1.8% of cells immunoreactive to β-integrin (Fig. 5D) and 7.1% for *Cg*-EcSOD (Fig. 5E), respectively. However, for 2.9% of the cell populations, β-integrin and *Cg*-EcSOD were shown to be closely associated within same hemocytes as shown by merged confocal images (Fig. 5F). To facilitate the visualization of the merged image, the colocalized pixels appear in white and the pixel colocalization was estimated with a threshold of 5% (Fig. 5G). Additionally, to reinforce this result of an interaction of *Cg*-EcSOD on hemocyte via a β-integrin receptor, we evidenced in our *C. gigas* hemocyte Expression Sequence Tag (EST) database (http://www.ifremer.fr/GigasBase), two clones (GenBank Accession Nos. BQ42637; BQ426447) which present sequence homologies with β-integrins from *Mus musculus* and the jellyfish *Podocoryne carnea*. Altogether, these findings argue in favor of a binding of the plasma *Cg*-EcSOD on some hemocyte population via a β-integrin–RGD interaction for further activating cellular reactions or immune processes.

The oyster Cg-EcSOD binds to bacteria, LPS, and Lipid A

As previously mentioned, *Cg*-EcSOD displays in its N-terminal sequence a LPS-binding motif, DDED (Fig. 2), which is known to be involved in the fixation of LPS on the endotoxin receptor CD14 [33,39]. To approach the role of this motif in the oyster plasma protein, we have investigated the potential property of both the plasma and the purified *Cg*-EcSOD to bind LPS. For that, *E. coli* bacteria were incubated with *C. gigas* plasma and, after several washes, two bounded proteins were recovered by magnesium chloride elution as revealed by RP-HPLC and SDS–PAGE analysis. Besides a minor undetermined plasma protein of a molecular mass of around 60 kDa, *Cg*-EcSOD was evidenced to be eluted (data not shown). This property of purified *Cg*-EcSOD was further verified against soluble LPS and Lipid A. Increasing amounts of purified *Cg*-EcSOD have been added to immobilized LPS or Lipid A on a microplate and, after several washes, bound protein has been detected by ELISA using the specific anti-*Cg*-EcSOD antibody. From these assays made in triplicates, we evidenced an increase in absorbance according to the concentration of *Cg*-EcSOD that revealed the binding of the protein for both LPS and Lipid A. In wells without LPS or Lipid A, no binding of *Cg*-EcSOD has been detected. The apparent constant of dissociation of *Cg*-EcSOD for LPS ($K_d = 1.50 \times 10^{-5}$ (±0.25) M) was almost the same as that obtained for Lipid A ($K_d = 1.25 \times 10^{-5}$ (±0.23) M) (Fig. 6). Compared to well-known LPS-binding proteins such as the Limulus endotoxin-binding protein-protease inhibitor, the affinity of the purified *Cg*-EcSOD for LPS appears to be 1000-fold weaker [40]. However, considering its great abundance in the oyster hemolymph, this property appears to be relevant with a potential role of this circulating protein in LPS or bacteria binding.

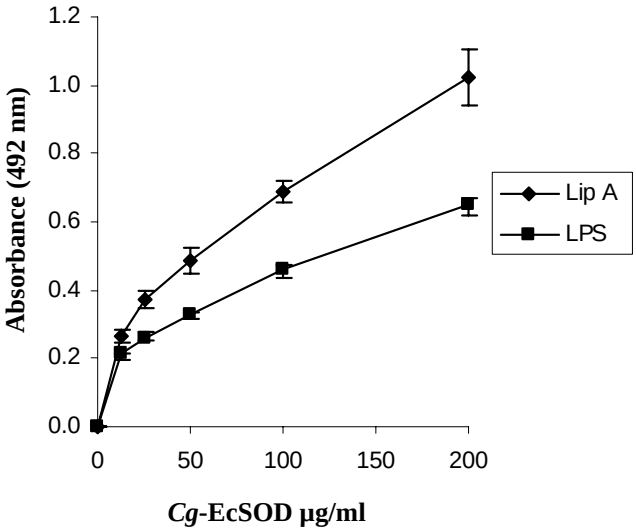

Fig. 6. Binding properties of *Cg*-EcSOD to LPS and Lipid A. Increasing concentrations of purified *Cg*-EcSOD (0–200 µg/ml) have been incubated with lipopolysaccharides (LPS) and Lipid A (Lip A) immobilized on a microplate and bound protein has been detected by ELISA using specific antibody. The values represent means and SE for three independent experiments.

Conclusion

Numerous proteins are known with LPS-binding properties [41]. To our knowledge, this is the first evidence of a SOD which appears to display a LPS-binding property. Further work must be addressed for characterizing this property and how *Cg*-EcSOD may interact with Lipid A, LPS or the bacteria. It is known that recognition of micro-

bial products leads to cell activation and production of a large array of mediators necessary for the establishment of inflammatory processes. LPS recognition mechanisms can be provided by several actors which can interplay such as plasma LBP-binding protein (LBP), membrane-bound or soluble forms of CD14 and integrins [42]. In vertebrates, LBP would accelerate the binding of LPS to CD14 [43,44]. In contrast, β2-integrin has been shown to only participate in the recognition of particulate form of LPS [42]. This has also been evidenced for insect hemocytes in which *E. coli* is internalized through a β_3-integrin-dependent process whereas cell-free LPS is internalized through a receptor-mediated endocytosis [37]. As we observed in our experiments the formation of aggregation with the purified *Cg*-EcSOD, we can assume that aggregated *Cg*-EcSOD-LPS particles or *Cg*-EcSOD bound bacteria may interact with β-integrin for further activation and internalization within the oyster hemocytes. For the oyster *C. gigas* which is permanently in contact with a rich microflora in its environment, it is likely that the major plasma protein may display different functions related to the elimination of microorganisms including antioxidant effect and LPS-binding property. *Cg*-EcSOD may have an opsonic activity by promoting recognition and elimination of bacteria through the binding to hemocyte integrins. However, through the SOD activity, the molecule may participate in the regulation of inflammatory response induced by sepsis by detoxifying internal oyster tissues or by improving the ability of the hemocytes to resist oxidant injury.

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