Research article

In vitro production of peroxynitrite by haemocytes from marine bivalves: C-ELISA determination of 3-nitrotyrosine level in plasma proteins from Mytilus galloprovincialis and Crassostrea gigas

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Abstract

Background: Peroxynitrite is increasingly proposed as a contributor to defence system in marine bivalve. It can be formed by combinaison of superoxide and nitric oxide, and can react with tyrosine residues of proteins giving rise to 3-nitrotyrosine.

Results: The present article describes a competitive ELISA for the measurement of 3-nitrotyrosine contents of plasma proteins from marine bivalves by means of a monoclonal anti 3-nitrotyrosine antibody mouse IgG.

Conclusions: This assay is sensitive enough to determine the amounts of 3-nitrotyrosine in plasma proteins from one animal only.

Using the C-ELISA, we have shown that the phagocytosis of zymosan particles increased the 3-nitrotyrosine levels of plasma proteins from mussel M. galloprovincialis and oyster C. gigas 5.8 and 7.5 times respectively.

Background

Bivalves, unlike vertebrates, do not have humoral antigen specific active compounds such as antibodies and their self-defence systems are based on non-specific defensive compounds and phagocytosis by haemocytes [1, 2].

During phagocytic burst or after *in vitro* stimulation with PMA or LPS, haemocytes produce superoxide anions, i.e. the initial species of reactive oxygen intermediates (ROI) and nitric oxide (NO).

ROI generation has been reported in *Patinopecten vessoensis* [3] *Crassostrea virginica* [4], *Crassostrea gigas* [5], *Ostrea edulis*, *Pecten maximus* [6], *Mytilus edulis* [7] and *Mytilus galloprovincialis* [8]. NO-synthase activity was detected in haemocytes of *M. edulis* [9] and *C. gigas* [10] and peroxynitrite production by *M. galloprovincialis* haemocytes has been recently reported [8, 11, 12].

In the presence of superoxide anions, nitric oxide generates peroxynitrite, a strong oxidant which kills bacteria [13] and parasitic protozoa [10, 14, 15]. Moreover, peroxynitrite is a nitrating agent, that converts tyrosine in 3-nitrotyrosine [16]. Such nitration has been observed in proteins from human polymorphonuclear cells [17] and 3-nitrotyrosine has been used as a marker to assess peroxynitrite involvement in pathological processes such as adult respiratory distress syndrome [18], rheumatoid arthritis [19] and celiac disease [15].

To determine levels of protein-associated 3-nitrotyrosine in human plasma or serum, Khan et al. [20] developed a competitive enzyme-linked immuno-assay (C-ELISA) for 3-nitrotyrosine using a polyclonal anti-3-nitrotyrosine rabbit IgG raised against nitrated KLH. In the present study, we slightly modified this C-ELISA assay to investigate 3-nitrotyrosine levels in plasma proteins from mussel *M. galloprovincialis* and oyster *C. gigas* before and after zymosan phagocytosis.

Results

ELISA standard curve construction

We developed a competitive ELISA to quantify 3-nitrotyrosine residues in plasma proteins from marine bivalves . A standard curve was constructed by determining the binding inhibition of the anti-3-nitrotyrosine antibody to a synthetic antigen (BSA^{NT}) immobilised on coated microtitration plates in the presence of serial dilutions of the free antigen BSA^{NT} in solution.

Figure 1 shows that the curve obtained was linear from 0.2 to 0.003 µM BSA^{NT} / assay. In contrast, unmodified BSA showed no significant binding inhibition of the 3-nitrotyrosine antibody and no significant cross-reaction of KLH, an invertebrate protein model was observed.

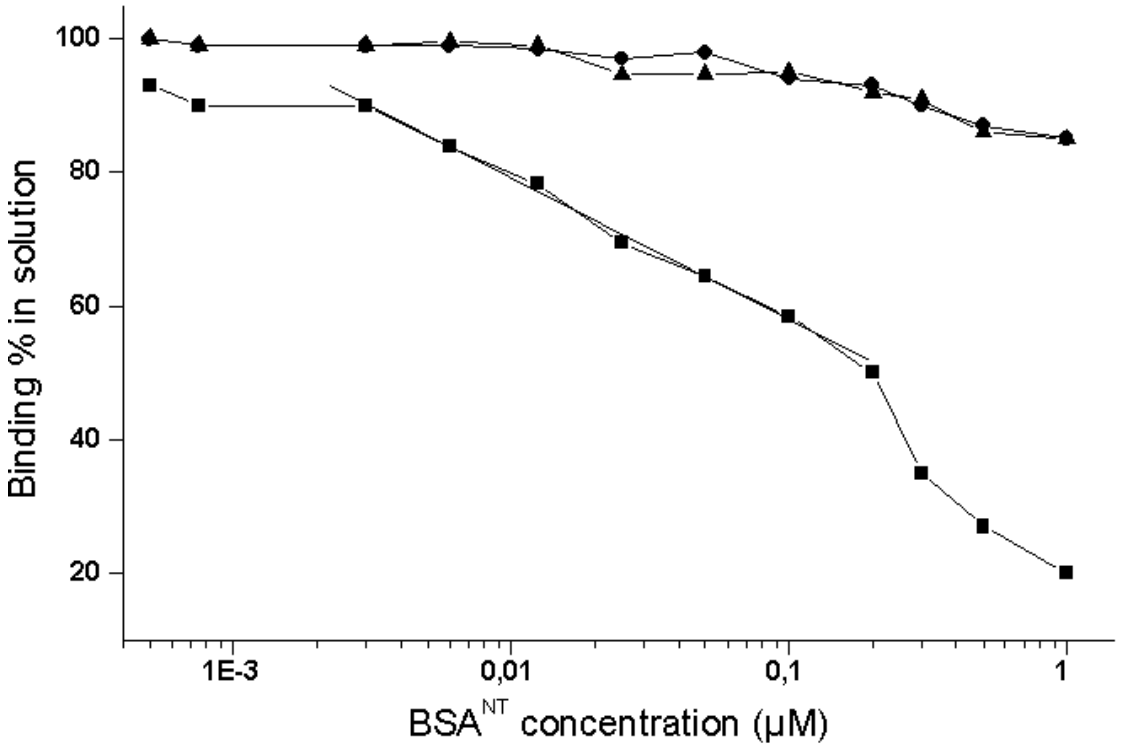

Figure 1
Comparison of standard curves for the inhibition of anti-3-nitrotyrosine antibody binding by various proteins in the C-ELISA. The curves show competition for the anti-3-nitrotyrosine antibody between the immobilized BSA^{NT} and competing free proteins in solution: BSA^{NT} (n = 3); BSA (n = 2); KLH (n = 2). The percentage inhibition of maximum antibody binding (absorbance at 490 nm in the absence of competition) is plotted against the competing free protein concentration.

Effect of stimulation of mussel hemocytes with PMA on 3-nitrotyrosine levels in plasma proteins:in vivo

The 3-nitrotyrosine concentration in proteins from *M. galloprovincialis* plasma samples was quantified by C-ELISA and expressed as BSA^{NT} equivalents using the standard curve of Fig. 1. As shown in table 1, marked individual variations were observed and a mean concentration (n = 20) of 0.037 ± 0.025 μM BSA^{NT} equivalents was estimated. After 1 h incubation of mussel hemocytes with PMA, the *in vitro* level of 3-nitrotyrosine in plasma increased to a mean value (n = 20) of 0.118 ± 0.024 μM BSA^{NT} equivalents (3.2-fold enhancement). To confirm that the increase of BSA^{NT} equivalents was dependent on NO production, we incubated mussel hemocytes with L-NIO, a NO-synthase inhibitor. When these hemocytes were stimulated with PMA, mean 3-nitrotyrosine concentrations of 0.082 ± 0.024 μM BSA^{NT} equivalents were obtained (Table 1). They corresponded to 69% inhibition when compared to hemocytes untreated with NO-synthase inhibitor.

Table 1: Mean 3-nitrotyrosine levels in plasma from mussels (n = 20) before and after PMA-stimulation. 3-nitrotyrosine contents of plasma proteins were determined in triplicate by C-ELISA as described in the Material and Methods and expressed as BSA^{NT} equivalents. "unstimulated": plasma from unstimulated haemolymph, "+ PMA": plasma 30 min after addition of 10 μL PMA (1 mg mL^{-1}) to haemolymph, "PMA + L-NIO": plasma after 5 min incubation of haemolymph with L-NIO and 30 min with PMA.

Unstimulated	+ PMA	+ PMA + L-NIO
0.037 ± 0.025 (n = 20)	0.118 ± 0.024 (n = 20)	0.082 ± 0.024 (n = 20)

Measurement of 3-nitrotyrosine content in plasma proteins from marine bivalve before and after in vitro phagocytosis of zymosan particles:

As shown in table 2, the phagocytosis of zymosan particles by *M. galloprovincialis* haemocytes promoted a marked increase in the 3-nitrotyrosine concentration in mussel plasma, reaching mean concentrations (n = 20) of 0.214 ± 0.023 μM BSA^{NT} equivalents (5.8-fold enhancement). The addition of DPI, a strong inhibitor of both NO-synthase and NADPH-oxidase, before phagocytosis lowered the 3-nitrotyrosine contents of plasma proteins to a mean concentration (n = 20) of 0.117 ± 0.036 μM BSA^{NT} equivalents (54.6 % inhibition).

As with mussel (*M. galloprovincialis*), the concentration of 3-nitrotyrosylated proteins in plasma samples from oysters (*C. gigas*) was quantified by ELISA and expressed as BSA^{NT} equivalents using the standard curve of Fig. 1. Substantial individual variations were observed and a mean concentration (n = 18) of 0.023 ± 0.017 μM BSA^{NT} equivalents was estimated (Table 2). *In vitro* Phagocytosis of zymosan particles by hemocytes, increased the mean 3-nitrotyrosine content of plasma to 0.176 ± 0.022 μM BSA^{NT} equivalents (7.5-fold enhancement).

Table 2: Mean 3-nitrotyrosine levels in plasma from mussels (n = 20) and oysters (n = 18) before and after phagocytosis of zymosan particles. 3-nitrotyrosine contents of plasma proteins were determined in triplicate by C-ELISA as described in the Material and Methods and expressed as BSA^{NT} equivalents. "unstimulated": plasma from unstimulated haemolymph, "zymosan": plasma 30 min after addition of 50 μL zymosan (40 mg mL^{-1}) to haemolymph, "zymosan + DPI": plasma after 5 min incubation of haemolymph with DPI and 30 min with zymosan particles.

	Unstimulated	Zymosan	Zymosan + DPI
Mussels	0.037 ± 0.025 (n = 20)	0.214 ± 0.023 (n = 20)	0.117 ± 0.036 (n = 20)
Oysters	0.023 ± 0.017 (n = 18)	0.176 ± 0.022 (n = 18)	0.127 ± 0.037 (n = 18)

As in the mussel experiments, the increase in the 3-nitrotyrosine level was strongly inhibited by incubation of haemolymph with DPI (72 % inhibition), before the addition of zymosan particles (Table 2).

Discussion

This study describes a C-ELISA for the detection of 3-nitrotyrosine residues in proteins from marine bivalve plasma.

Concentrations of 3-nitrotyrosine residues in the plasma proteins from untreated mussels and oysters varied between animals but they always increased markedly after *in vitro* PMA-stimulation of haemocytes or after phagocytosis of zymosan particles. These increases were inhibited by haemolymph preincubation with DPI or L-NIO.

The low levels of endogenous 3-nitrotyrosine residues in the plasma of untreated animals could have been due to peroxynitrite, a reactive nitrogen species that can modify tyrosine residues of proteins into 3-nitrotyrosine, as shown by Beckman and Koppenol [14] when studying human blood cells. Other reactive nitrogen species such as nitric oxide and nitrite anion could also be involved [21]. The 3-nitrotyrosine levels detected in proteins from untreated mussel and oyster haemolymph could thus reflect the exposure of proteins to all the nitrating agents produced by cells and thus confirm the generation of nitric oxide by these species.

The increased 3-nitrotyrosine levels observed after PMA-stimulation of haemocytes and zymosan particle phagocytosis, and inhibition with DPI and L-NIO, confirmed our previous results [8, 11, 12] and showed that peroxynitrite is generated by haemocytes in response to activation of both NADPH-oxidase and NO-synthase metabolic pathways.

Conclusion

The C-ELISA method we developed is sensitive enough to determine the amounts of 3-nitrotyrosine in plasma proteins of a single animal and to measure variations in 3-nitrotyrosine contents promoted by haemocyte stimulation or zymosan particle phagocytosis.

However, this method remains semi-quantitative since the 3-nitrotyrosine antibody may not bind all 3-nitrotyrosine residues in a sample containing a mixture of proteins due to inaccessibility to some 3-nitrotyrosine residues because of the influence of adjacent aminoacids on antibody binding. We used the C-ELISA method to detect and quantify the stress of mussels and oysters exposed to environmental variations.

Materials and methods

Chemicals and buffers

Phorbol myristate acetate (PMA), bovine serum albumin (BSA), keyhole limpet haemocyanin (KLH) and zymosan were purchased from Sigma Chemical Co. (St. Louis, USA). L-N5-(1-iminoethyl)-ornithine (L-NIO) and diphenylene iodonium chloride (DPI) were products of Alexis Corporation (Switzerland). Phosphate buffered saline was prepared using Dulbecco PBS salt using HPLC-grade distilled water. The pH and osmolarity were adjusted to 8.3 and 1100 mOsm, respectively. All other chemicals and reagents used were analytical grade.

Anti-nitrotyrosine antibody (clone 1A6: mouse monoclonal IgG) was from Upstate Biotechnology Inc. (Lake Placid, USA) and anti-mouse IgG (H + L), peroxidase-conjugate antibody was from Jackson Immunochemical Laboratory, Inc. (Baltimore, USA).

Experimental animals

Two year old mussels (*M. galloprovincialis*) and oysters (*C. gigas*) raised at Palavas (France), were maintained in laboratory in a running sea water system (salinity: 33??, 17°C) with continuous aeration.

Haemolymph collection

One ml haemolymph from each animal was withdrawn (from the posterior adductor muscle of mussels and from the pericardial cavity of oysters) in disposable plastic syringes (2 ml, 21G needle) and transferred to polypropylene test tubes kept on ice.

Haemocyte stimulation

Haemocytes were stimulated by the addition of 50 µl of the zymosan suspension (40 mg mL^{-1}, final pH 7.1) or by 10 µl of PMA (1 mg mL^{-1}). In inhibition experiments, haemolymph was preincubated for 5 min with DPI, L-NIO or filtrated sea water (control). The haemolymph was centrifuged (180 g, 5 min., 4°C) and the supernatant (plasma) collected by carefull pipetting using Pasteur pipettes. The viability of cells in the presence of various added agents was tested before the experiments by trypan blue exclusion assay.

Synthesis of nitrosylated BSA (BSA^{NT})

3-nitrotyrosine was coupled to carboxylic groups of BSA using N-hydroxysuccinimide/N-ethyl-N'-(dimethylaminopropyl)-carbodiimide reagent at pH 8.3. After incubation overnight under stirring at 4°C, the solution was extensively dialysed against PBS. The 3-nitrotyrosine content of BSA^{NT} was determined by absorbance at 438 nm (pH 9.0) using a molar extinction coefficient of 4300 M^{-1} cm^{-1}. It was in the 2-3 mol nitrotyrosine / mol BSA range.

C-ELISA immunoassays

Each well from the 96-well plates was coated with 50 µL BSA^{NT} (10 µg mL^{-1} in PBS pH = 7.2), incubated overnight at 4°C, washed with 250 µL PBS / 0.1% Tween buffer and then blocked for 1 hr at 37°C with 5% skimmed milk to prevent nonspecific binding.

Serial dilutions of BSA^{NT} (0.2 to 0.003 µM) in PBS (for standard curve determination) or plasma sample from individual marine bivalves were incubated v/v overnight at 4°C under stirring with monoclonal anti 3-nitrotyrosine mouse IgG (1:2000). These solutions were then poured into coated wells (50 µL / well). After 2 h incubation at 37°C, 3-nitrotyrosine antibody bound to BSA^{NT} was labelled by the addition of anti-mouse IgG peroxidase-conjugate (1: 4000, 50 µL / well) and incubated for 90 min at 37°C. Then colour development was initiated by the addition of peroxidase substrate (orthophenylene diamine), allowed to develop for up to 15 min at room temperature and terminated by the addition of 4 N sulphuric acid. Antibody bound to BSA^{NT} was determined from the absorbance at 490 nm and expressed as BSA^{NT} equivalents.

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