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cDNA cloning and tissue expression of plasma lysozyme in the eastern oyster, *Crassostrea virginica*

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Abstract

The cDNA sequence of a 17,861 Da lysozyme first purified from plasma of eastern oysters (*Crassostrea virginica*) was identified and its complete amino acid sequence deduced. The amino acid sequence of the plasma lysozyme, designated cv-lysozyme 1, contained both a unique and a conserved region when compared to the amino acid sequences of other bivalve lysozymes. *In situ* hybridisation located cv-lysozyme 1 gene expression in mantle and gill cells in standard histological sections. Quantitative real-time RT-PCR detected cv-lysozyme 1 expression in all organs examined and circulating haemocytes. The number of cv-lysozyme 1 mRNA transcripts was particularly high in mantles and labial palps suggesting those organs are the main sites of cv-lysozyme 1 synthesis. Cv-lysozyme 1 enzyme activity measured by lysing *Micrococcus lysodeikticus* bacteria and expressed in units per gram tissue was highest in mantles, labial palps and gills. Most cv-lysozyme 1 enzyme activity in oysters was found in plasma. Cv-lysozyme 1 main organs of synthesis, its abundance in plasma and its strong antimicrobial properties suggest its main role is in oyster host defences.

Keywords: *i*-type lysozyme; cDNA; Oyster; Bivalve mollusc; *In situ* hybridisation; Quantitative real-time RT-PCR; Gene expression; Enzyme activity; Invertebrate immunity

1. Introduction

Lysozymes (EC 3.2.1.17) are well known antibacterial enzymes that cleave the glycosidic bonds between *N*-acetylmuramic acid and *N*-acetylglucosamine of peptidoglycan, a major cell wall component of Gram-positive bacteria [1,2]. Antifungal and antiprotozoal activities of lysozymes have also been reported and have been attributed to the hydrolysis of *N*-acetylglucosamine linkages in chitin that makes up the cell wall of fungus and some protozoa [3–5]. There is also increasing evidence that lysozymes kill Gram-positive and Gram-negative bacteria by non-enzymatic means [6,7]. While the antimicrobial activity of lysozymes has repeatedly been demonstrated *in vitro*, the contribution of lysozymes to host defences is only beginning to be unravelled *in vivo* [8,9].

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Lysozyme activity has been detected in the body fluids and tissues of various bivalve molluscs [10,11]. Lysozymes in cell-free haemolymph (plasma) and pallial and extrapallial fluids are generally believed to be involved in bivalves' host defences [12–15]. In many bivalves, lysozyme activity was found to be associated primarily with the digestive system and therefore ascribed a role in bacterial digestion [10,16]. While McHenery and Birkbeck [17] suggested that lysozymes from different organs and tissue fluids may have different properties and functions, it was only recently that Olsen et al. [18] demonstrated the presence of multiple lysozymes in blue mussels (*Mytilus edulis*) much like in other animals [19–21].

Most information on bivalve lysozymes' biochemical and antimicrobial properties is derived from enzymes purified from parts of the digestive system, such as the crystalline style and visceral mass [17,18,22–25]. A lysozyme was, however, recently purified from the plasma of eastern oysters (*Crassostrea virginica*) and characterised by Xue et al. [26]. This lysozyme, the first to be purified from the plasma of a bivalve, was unique in its N-terminal sequence, high ionic strength for optimal enzyme activity, and strong antimicrobial activity [26]. The identification of the gene for eastern oyster plasma lysozyme and characterisation of its expression is needed to assist in identifying the protein's function in eastern oysters.

The main objectives of the study were therefore to (1) isolate and characterise cDNA encoding a 17,861 Da lysozyme first purified from plasma of eastern oysters, (2) locate the tissue synthesising the plasma lysozyme, named cv-lysozyme 1, and (3) measure gene expression of cv-lysozyme 1 in oyster organs and haemocytes by quantitative real-time RT-PCR. An additional objective was to characterise the distribution of cv-lysozyme 1 activity in organs and plasma.

2. Materials and methods

2.1. Oyster organs and haemolymph collection

Several 5 mm^3 fragments of the digestive gland of eastern oysters (*Crassostrea virginica*) collected from Barataria Bay, Louisiana, were excised and immediately immersed in RNA*later* (QIAGEN, Valencia, CA). The samples were stored at −20 °C until used to determine cv-lysozyme 1 cDNA sequence.

The haemolymph and organs of five 1-year old eastern oysters (9.52 ± 2.07 g tissue wet weight) grown at the Louisiana Sea Grant oyster hatchery in Grand Isle, Louisiana, were collected in May 2005 to measure cv-lysozyme 1 gene expression and enzyme activity. The shells were notched and the shell cavities were drained of fluid. About 200 μl of haemolymph was withdrawn from the adductor muscle sinus of each oyster with a 1 ml syringe and 25-gauge needle and immediately centrifuged at 800 × *g* for 2 min at 4 °C. The supernatants were collected as plasma and frozen at −20 °C and haemocyte pellets were resuspended in RNA*later* and stored at −20 °C. Concurrently, the oysters were shucked and their whole tissue wet weights were measured. The mantle, gills, labial palps, digestive gland and crystalline style sac midgut of each oyster were excised, rinsed in saline and their wet weights measured. Small fragments (<100 mg) of each organ were stored in RNA*later* (QIAGEN) until used for quantitative real-time RT-PCR, and the remainder was frozen at −20 °C for later measurement of enzyme activity.

2.2. Identification of cv-lysozyme 1 cDNA sequence

Total RNA was extracted from digestive gland tissue fragments, using an RNeasy mini kit (QIAGEN). RNA quantity was measured using Gene Quant™ pro RNA/DNA calculator (Amersham Biosciences, Piscataway, NJ) and 400 ng of total RNA was used to synthesise cDNA with an Omniscript Reverse Transcript Kit (QIAGEN) with oligo $(dT)_{12-18}$ primer (Invitrogen, Carlsbad, CA) and RNase inhibitor (Invitrogen).

The sense primer, Cal-12F, and the reverse primer, Cv-m3R were designed based on the amino acid sequences, MVSQKCLRCIC and WIDCGSP, respectively (GenBank accession number P83673) (Table 1) to amplify the cDNA of plasma lysozyme [26] by polymerase chain reactions (PCR). For the PCR, 0.6 μl of 25 μM of each primer, 350 ng of synthesised cDNA in 15.1 μl of distilled water, 0.1 μl of Takara Ex Taq TM DNA polymerase, 2 μl of 10× PCR buffer and 1.6 μl of dNTP (TaKaRa, Kyoto, Japan) were mixed. The PCR conditions consisted of an initial denaturation at 96 °C for 3 min, followed by 35 cycles of denaturation at 96 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1 min, and a final extension at 72 °C for 7 min. The PCR products were separated by electrophoresis on a 2.0% agarose and visualised by staining the gel with ethidium bromide. The PCR product corresponding to the expected molecular size was extracted from the gel and purified using an Ultra Clean Gel Spin DNA