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Research Article

A new lysozyme from the eastern oyster (*Crassostrea virginica*) indicates adaptive evolution of *i*-type lysozymes

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Abstract. A new lysozyme (cv-lysozyme 2) with a MALDI molecular mass of 12 984.6 Da was purified from crystalline styles and digestive glands of eastern oysters (*Crassostrea virginica*) and its cDNA sequenced. Quantitative real time RT-PCR detected cv-lysozyme 2 gene expression primarily in digestive gland tissues, and *in situ* hybridization located cv-lysozyme 2 gene expression in basophil cells of digestive tubules. Cv-lysozyme 2 showed high amino acid sequence similarity to other bivalve mollusk lysozymes, including cv-lysozyme 1, a lysozyme recently purified from *C. virginica* plasma. Differences between cv-lysozyme 2 and cv-lysozyme 1 molecular characteristics, enzymatic properties, antibacterial activities, distribution in the oyster body and site of gene expression indicate that the main role of cv-lysozyme 2 is in digestion. While showing that a bivalve mollusk employs different lysozymes for different functions, findings in this study suggest adaptive evolution of *i* type lysozymes for nutrition.

Keywords. *i* type lysozyme, oyster, *Crassostrea virginica*, digestion, host defense, adaptive evolution, bivalve mollusk.

Introduction

Lysozymes (EC 3.2.1.17) are enzymes sharing the same specificity for cleaving the glycosidic bond between *N*-acetylmuramic acid and *N*-acetylglucosamine of peptidoglycan, but differing substantially in amino acid sequences [1]. Three families of lysozymes have been identified in animals: *c* (chicken) type, *g* (goose) type, and *i* (invertebrate) type [2, 3]. The major biological role ascribed to lysozymes is host defense, as these enzymes can act as antibacterial and immune-modulating agents [4, 5]. In addition, lysozymes can function as important digestive enzymes in some animals [6–11].

The first evidence of the role of lysozymes in digestion came from the discovery of these enzymes in the stomach of cows and other ruminant artiodactyls [6, 12]. In these animals, plant materials indigestible to the ruminants' conventional digestive enzymes are digested by symbiotic bacteria and other microorganisms growing in the foregut. The bacteria are then broken down by lysozymes as they enter the stomach and digested by conventional mammalian enzymes [13]. A similar use of lysozymes has been found in leaf-eating monkeys and birds [7, 9], and in fruit flies that feed on decomposing matter rich in bacteria [8]. Digestive lysozymes apparently evolved in parallel in these different species, acquiring the ability to function in highly acidic and protease-rich environments [7, 9, 11, 14, 15]. All digestive lysozymes described so far belong to the *c* type family of lysozymes.

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At the same time a digestive lysozyme was discovered in cow stomach, McHenery and Birkbeck [16] proposed that the role of a lysozyme purified from crystalline styles of blue mussels (*Mytilus edulis*) was to digest bacteria. The high lysozyme activity detected in the digestive systems of many bivalve mollusks [17–19] and the ability of some bivalve mollusks to use bacteria as food [20–22] support a digestive role of lysozymes in these animals. Jollès et al. [23] also suggested a similar role for lysozymes in deep-water bivalve mollusks that rely on symbiotic bacteria in gills for nutrition. Nearly all biochemical and molecular information about bivalve mollusk lysozymes have been obtained from enzymes purified from digestive systems [16, 23–27], and no evidence that is attributable to adaptive evolution was found in a phylogenetic analysis of their amino acid sequences [3]. Although Olsen et al. [27] demonstrated the presence of multiple lysozymes with different biochemical properties from the soft body and crystalline styles of blue mussels, their molecular characteristics, antibacterial activities, distribution in the mussel body and site of gene expression need to be described to better assess their physiological roles.

Recently, a lysozyme, now designated cv-lysozyme 1, was purified from the plasma of eastern oysters (*Crassostrea virginica*) [28]. Cv-lysozyme 1, the first lysozyme to be purified from the plasma of a bivalve mollusk, was unique in its N-terminal amino acid sequence and optimal activity at high ionic strength and relatively broad pH range [28]. The site of expression of cv-lysozyme 1, its abundance in plasma and strong antimicrobial activity suggests its main role is in host defense (Itoh et al., submitted). We now present evidence that a different lysozyme, designated cv-lysozyme 2, can be purified from the crystalline styles and digestive glands of eastern oysters. Cv-lysozyme 2 shows high amino acid sequence similarity to other bivalve mollusk lysozymes, including cv-lysozyme 1, and its biochemical and molecular properties, distribution in the oyster body and site of expression indicate its role is in digestion. While showing that a bivalve mollusk employs different lysozymes for different functions, the findings in this study also suggest adaptive evolution of *i* type lysozymes for nutrition.

Materials and methods

Sampling. Five hundred eastern oysters (*Crassostrea virginica*), 9–13 cm in shell length, were collected from Barataria Bay, Louisiana in July 2005. The oysters were shucked and their crystalline styles removed from the style sacs with forceps and scissors. The mantle, gills, labial palps, and digestive gland of each oyster were excised, pooled by organ type, and frozen on dry ice; the dark-colored tissue of visceral masses was sampled as digestive gland in this study. The samples were stored at –20 °C until used for lysozyme purification.

In addition, hemolymph was withdrawn from the abductor muscle sinuses of five oysters with a 1-ml syringe and a 25 G needle and immediately centrifuged at 800 *g* for 2 min at 4 °C. Supernatants were removed and the hemocyte pellets were transferred into RNAlater (Qiagen, Valencia, CA, USA). The right valve of each oyster was then removed and 5-mm^3 fragments of the digestive gland, mantle, gills, style sac-midgut, and labial palps were excised and immediately immersed in RNAlater. The samples were stored at 4 °C until used for quantitative real time RT-PCR.

Purification of cv-lysozyme 2 from crystalline style. The thawed crystalline styles, which liquefied at room temperature, were added to 250 ml of 1% (v/v) acetic acid and centrifuged at 4000 *g* for 30 min at 20 °C. The supernatant, designated as 'crystalline style sample', was desalted in a 5×80 cm Sephadex G-25 column equilibrated with 0.02 M acetic buffer, pH 5.0. The desalted sample was applied onto a 2.6×35 cm SP-Sepharose Fast Flow column equilibrated with 0.02 M acetic buffer, pH 5.0. The column was sequentially eluted with 0.1, 0.3, and 0.6 M NaCl solutions in 0.02 M acetic buffer, pH 5.0, at an elution rate of 5 ml/min. The 0.3 M NaCl solution eluted fraction, designated as 'cv-lysozyme 2-enriched proteins', was concentrated by freeze-drying and desalted in a 2.6×30 cm Sephadex G-25 column equilibrated with 0.1 M NaCl solution in 0.02 M acetic buffer, pH 5.0. Desalted cv-lysozyme 2-enriched proteins were then applied into a 1.0×20 cm CM-Sepharose Fast Flow column equilibrated with 0.1 M NaCl solution in 0.02 M acetic buffer, pH 5.0. The column was eluted with a linear gradient of 0.1–0.4 M NaCl solution in 0.02 M acetic buffer, pH 5.0 over 200 ml at an elution rate of 1 ml/min. The eluate was collected in 4-ml fractions and each fraction tested for lysozyme activity. Fractions with lysozyme activity were pooled as 'crude cv-lysozyme 2' and concentrated by freeze-drying. Concentrated crude cv-lysozyme 2 was further purified in a 1.6×70 cm Superdex 75 column by elution with 0.5 M NaCl in 0.02 M acetic buffer, pH 5.0, at an elution rate of 0.5 ml/min. The elution was monitored for absorbance at 280 nm with an Econo UV Monitor (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and collected in 3-ml fractions. Fractions were tested for lysozyme activity, and those with high lysozyme activity and in the same absorbance peak were pooled as 'purified cv-lysozyme 2'. After concentration by freeze-drying, the purified cv-lysozyme 2 protein was desalted in a Sephadex G-25 column equilibrated with deionized water and stored at –20 °C until use.

Purification of cv-lysozyme 2 from other organs. Tissues from each organ type were homogenized in 1% ace-

tic acid at a ratio of 5 ml/g tissue with a Bio-Homogenizer (Biospec Products Inc. Bartlesville, OK, USA). Tissue homogenates were centrifuged at 4000 *g* for 30 min at 4 °C and the supernatants collected. The pellets were resuspended in one half of the original volume of 1% acetic acid and centrifuged under the same conditions. The two batches of supernatant were pooled and treated with ammonium sulfate to 80% saturation. Proteins were recovered by centrifugation at 4000 *g* for 30 min at room temperature and then dissolved in deionized water. The recovered proteins were dialyzed against 0.1 M NaCl in 0.02 M acetic buffer, pH 5.0 at 4 °C overnight and fractionated in a 2.6 × 20 cm SP-Sepharose Fast Flow column. After sample loading, the column was eluted with a linear gradient of 0.1–0.6 M NaCl in 0.02 M acetic buffer, pH 5.0 over 480 ml at an elution rate of 2 ml/min. The elution was monitored for absorbance at 280 nm and collected in 6-ml fractions. Lysozyme activity in each fraction was tested. Fractions with lysozyme activity were pooled, concentrated by freeze-drying, and further purified by size exclusion chromatography in a Superdex 75 column as described above.

All purifications were conducted at room temperature. Chromatography supplies were purchased from Amersham Biosciences (Piscataway, NJ, USA). Protein concentrations were measured using a Micro BCA kit (Pierce Biotechnology, IL, USA). Lysozyme purity was verified by SDS-PAGE using a 15% acrylamide/bis gel stained with silver nitrate.

Lysozyme activity assay. Lysozyme activity was determined by monitoring the decrease in turbidity of a *Micrococcus lysodeikticus* suspension (Sigma-Aldrich Corporation, St. Louis, MO, USA) in appropriate buffer solutions. Buffer solutions were selected according to the requirements of the individual experiments described later in the text. The assay was carried out in a 96-well microplate, in which 20 µl sample were mixed with 180 µl of a 0.8 mg/ml *M. lysodeikticus* suspension. The plate was measured at 25 °C for absorbance at 450 nm for 5 min using a Bio-Rad BenchMark Plus microplate reader (Bio-Rad Laboratories) with a built-in kinetic mode. The difference in absorbance between 1 and 5 min was used to calculate lysozyme activity. In this study, 1 U lysozyme was defined as the quantity that caused a decrease in absorbance of 0.001 U/min. In some experiments, the highest activity detected was defined as 100% when results were expressed as percent activity. All measurements of lysozyme activity were done in triplicate.

Determination of optimal conditions for lysozyme activity. Optimal pH and ionic strength for lysozyme activity were determined using procedures and buffers reported by Xue et al. [28]. An MES-NaOH buffer, 0.005 M (I = 0.005) and pH 6.0, was then selected as a base buffer in assessing the effects of ionic strength and cations on lysozyme activity. NaCl was added to the base buffer to test the effects of ionic strength on cv-lysozyme 2 activity and to compare it to cv-lysozyme 1 activity. The base buffer was supplemented with the chlorides of sodium, ammonium, calcium, magnesium, and zinc to measure the effects of cations on cv-lysozyme 2 activity.

The optimal temperature and the thermal stability of purified cv-lysozyme 2 were determined following the procedures of Xue et al. [28] except that the base buffer was changed to 0.01 M MES-NaOH at pH 6.0.

Chitinase and isopeptidase assays. Chitinase activities of purified cv-lysozyme 1 and 2 were measured using chitin azure suspensions prepared in two buffers: 0.1 M sodium acetate, pH 5.0 or 0.01 M MES-NaOH, pH 6.0. The release of Remazol Brilliant Blue R covalently linked to crab shell chitin was measured as an indication of chitinase activity. Assays were carried out in 96-well plates in which 50 µl lysozyme diluted in the buffers were mixed with 50 µl chitin azure suspension at 2.0 mg/ml. After a 48-h incubation at 37 °C, 100 µl ice-cold buffer were added to each well and the plates centrifuged at 4000 *g* for 30 min at 4 °C; 100 µl supernatant from each well was transferred to a new plate and the absorbance was measured at 545 nm. All measurements were done in triplicate and chitinase from *Streptomyces griseus* was used as a positive control.

Isopeptidase activity of purified cv-lysozyme 1 and 2 were tested following the procedure of Takashita et al. [29]. Measurements were carried out in triplicate in 0.5-ml Eppendorf tubes containing 50 µl lysozyme solution mixed with 50 µl L-γ-glutamine-*p*-nitroanilide (L-γ-Glu-pNA) solution in 0.10 M MOPS buffer containing 0.02 M NaCl and at pH 7.0. Following a 20-h incubation at 37 °C, the mixtures were transferred to a 96-well plate and absorbance measured at 405 nm. The final concentrations used in the study were 65 mM for lysozymes and 2.0 mM for the substrate.

Antibacterial activities. The concentrations of purified cv-lysozyme 1 and 2 inhibiting the growth of two Gram-negative bacteria, *Escherichia coli* and *Vibrio vulnificus* and a Gram-positive bacterium, *Pediococcus cerevisiae* were compared. Bacterial species were obtained from Dr. John Hawke or Dr. Marlene Janes at the Louisiana State University, Baton Rouge. The Gram-negative bacteria were grown in a nutrient broth containing 5 g beef extract, 2 g neopeptone, 0.1 g bactose dextrose, 1 g yeast extract and 10 g NaCl in 1 l water, while *Pediococcus cerevisiae* was grown in Difco™ APT broth. The bacteria were resuspended in phosphate-buffered saline (PBS) at a density of about 10^6 bacteria/ml and 20 µl were added to 20 µl of two-fold serially diluted lysozyme (200–6.25 µg/ml) in PBS or to 20 µl PBS alone (control) in 96-well plates. After 2-h in-

cubation at room temperature, 160 µl of appropriate broth were added to each well and the plates were incubated at 30 °C. The growth of the Gram-negative bacteria was measured at 570 nm after 12-h incubation and the growth of *P. cerevisiae* was measured at 640 nm after 24-h incubation with a Bio-Rad BenchMark Plus microplate reader. Results were expressed as the minimum concentration (MIC) of lysozymes significantly inhibiting bacterial growth. All measurements were done in triplicate and the experiment was repeated twice.

N-terminal sequencing. Purified cv-lysozyme 2 was separated by SDS-PAGE in a 15% acrylamide/bis gel under reduced conditions and transferred to a Sequiblot™ PVDF membrane (Bio-Rad Laboratories, Inc.) in 0.01 M CAPS-10% (v/v) methanol, pH 11, using a Mini Tran-Blot Electrophoretic Transfer Cell (Bio-Rad Laboratories, Inc.). The membrane was stained with 1% (w/v) Coomassie blue R-250 in 10% (v/v) acetic acid–40% (v/v) methanol and destained with 10% (v/v) acetic acid–40% (v/v) methanol solution. The PVDF membrane bearing the cv-lysozyme 2 was cut and washed six times in deionized water. N-terminal sequencing was carried out with automated Edman degradation using an Applied Biosystems Procise 494/HT protein sequencer (Applied Biosystems, Foster city, CA, USA).

Mass spectrometric determination of molecular mass and amino acid sequence. Purified cv-lysozyme 2 in sodium acetate buffer with 0.3 M NaCl was mixed 1 : 3 (v/v) with α-cyano-4-hydroxycinnamic acid (CHCA) MALDI matrix and spotted onto a sample plate. The dried spot was washed with 2 µl deionized water for 10 s, which was then removed. MALDI spectra were acquired on an Applied Biosystems Voyager STR instrument operated in linear mode using hen egg white lysozyme (MH+ 14 306.6) as an external standard.

Aliquots of purified cv-lysozyme 2 (100–200 pmol.) were reduced with 50 µl 5 mg/ml dithiothreitol in 6 M guanidine-HCl, 1.5 M Tris, pH 8.4 (buffer A) at 37 °C for 35 min followed by alkylation with 50 µl 15 mg/ml iodoacetamide or iodoacetic acid in buffer A at 37 °C for 45 min. Excess reagents were removed by step elution over a 2.1 × 100 mm C_{18} Brownlee Aquapore column (Perkin-Elmer, Boston, MA, USA). After sample injection, the column was washed with 5% acetonitrile, 0.1% trifluoroacetic acid (TFA) for 5 min at a flow rate of 200 µl/min. Cv-lysozyme 2 was eluted with 100% acetonitrile, 0.1% TFA. Absorbance was monitored at 214 nm and collected fractions dried in a speed vac.

Reduced and alkylated cv-lysozyme 2 containing fraction was solubilized in 100 mM ammonium bicarbonate buffer, pH 7.8, 10% acetonitrile (20 µl). Trypsin (50 ng) was added and the digestion allowed to proceed for 18 h at 37 °C. Tryptic peptides were either injected directly into a nano-liquid chromatography tandem mas spectrometery (LC-MS/MS) instrument (LC Packings Ultimate HPLC and ThermoFinnigan LTQ) or were desalted using C_{18} ZipTips (Millipore) and eluted with CHCA MALDI matrix (saturated in 70% acetonitrile, 0.1% TFA) for direct MALDI MS/MS analysis (Applied Biosystems 4700 instrument).

In LC-MS/MS experiments, tryptic peptides were eluted from a 15-cm, 75-µm inner diameter C_{18} column (Microtech Scientific) using a gradient of 2% solvent A (98% water/2% acetonitrile/0.2% formic acid) to 70% solvent B (2% water/98% acetonitrile/0.2% formic acid) over 40 or 70 min into the LTQ ion trap mass spectrometer. The most abundant peptide signals were selected for MS/MS analysis to provide sequence information. Alternatively, MALDI MS/MS analysis was employed without peptide separation to directly obtain sequence information on peptides observed by MALDI MS. Tandem mass spectra were manually interpreted and compared to the predicted DNA sequence for assignment.

Cv-lysozyme 2 cDNA cloning and sequencing. Total RNA was extracted from digestive gland tissues of an eastern oyster using an RNeasy Mini Kit (Qiagen). cDNA was synthesized from 400 ng mRNA using the Omniscript Reverse Transcript Kit (Qiagen) supplemented with oligo-dT primer (Invitrogen, Carlsbad, CA, USA) and RNase inhibitor (Invitrogen) according to manufacturer's instructions.

Cv-lysozyme 2 cDNA was isolated by nested-PCR to increase specificity. The forward primers were designed from the Edman degradation-determined N-terminal sequence of purified cv-lysozyme 2, and reverse primers from the conserved sequence region of *i* type lysozymes (Table 1). For the first PCR, 350 ng synthesized cDNA, 0.1 µl Takara Ex Taq TM DNA polymerase, 2 µl 10× PCR buffer, 1.6 µl dNTP (TaKaRa, Kyoto, Japan) and 15 pmol of each primers were mixed in a total volume of 20 µl. Following the initial denaturation at 96 °C for 3 min, the PCR was performed for 35 cycles of denaturation at 94 °C for 30 s, annealing at 53 °C for 45 s and extension at 72 °C for 30 s, and a final extension at 72 °C for 7 min. The second PCR was carried out in a similar procedure except that 2 µl of the 5-fold diluted first PCR product was used as template and that 55 °C was used for annealing. The nested-PCR products were analyzed by electrophoresis in 2.0% agarose gel stained with ethidium bromide. An expected 134-bp fragment was purified with an Ultra Clean Gel Spin DNA Purification Kit (Mo Bio Laboratories, Solana Beach, CA, USA) and cloned in a plasmid vector, pCR 2.1-TOPO of the TOPO TA Cloning Kit (Invitrogen). The sequence of the nested-PCR product was determined by sequencing the recombinant plasmid in a 3130 Genetic Analyzer using Big Dye Terminator v.3.1 sequencing reagent (Applied Biosystems).

Table 1. Primers used in cv-lysozyme 2 cDNA cloning, real-time RT-PCR and *in situ* hybridization.

Primer	Usage	Direction	Sequence
Lyso2-F1[a]	Nested-PCR 1	Forward	Nucleic: 5′-TCN ATH TCN GAC CAG TG-3′ Peptidic: NH2-Ser Ile Ser Asp Gln Cys-COOH
Lyso2-R1[b]	Nested-PCR 1	Reverse	Nucleic: 5′-CTK GGW CCK CCR TTR TG-3′ Peptidic: NH_2-His Asn Gly Gly Pro Arg-COOH
Lyso2-F2[a]	Nested-PCR 2	Forward	Nucleic: 5′-TCN GAC CAG TGY CTN AGR TG-3′ Peptidic: NH_2-Ser Asp Gln Cys Leu Arg Cys-COOH
Cv-m3R[b]	Nested-PCR 2	Reverse	Nucleic: 5′-GGR CTS CCR CAR TCD ATC CA-3′ Peptidic: NH_2-Trp Ile Asp Cys Gly Ser Pro-COOH
Lyso2–3′RACE	3′-RACE	Forward	5′-GGC TAT CGG TTG CCA CTG GGA CGT GTA T-3′
Lyso2–5′RACE	5′-RACE	Reverse	5′-GTC AGC GCA GGA TTC CAT GCT ATG TC-3′
Lyso2-RT1-F	Real-time RT-PCR	Forward	5′-GCA GAG GGA GAT GAA TTT TCT GA-3′
Lyso2-R1-R	Real-time RT-PCR	Reverse	5′-TCG GAA ATG CTG CTG TAG ACA-3′
Lyso2-RT	Real-time RT-PCR (TaqMan probe)	Forward	5′-TGT TTT GCG TCG TTG CTT CAG CGT-3′
28S-RT-F	Real-time RT-PCR	Forward	5′-GTT GAC GCA ATG TGA TTT CTG C-3′
28S-RT-R	Real-time RT-PCR	Reverse	5′-TAG ATG ACG AGG CAT TTG GCT A-3′
28S-RT	Real-time RT-PCR (TaqMan probe)	Forward	5′-ATT CAA TCA AGC GCG GGT AAA CGG C-3′
Lyso2-ISH-F	*In situ* hybridization	Forward	5′-CTA CAG CAG CAT TTC CGA TCA G-3′
Lyso2-ISH-R	*In situ* hybridization	Reverse	5′-GTA ACT CCG AAC ACA CCC T-3′

[a]The primers were designed from cv-lysozyme 2 N-terminal amino acid sequence determined by Edman degradation. [b]The primers were designed at the conserved sequence regions of *i* type lysozymes.

Full-length cDNA was generated by 5′- and 3′-RACE using two intragenic oligonucleotides, Lyso-2-3′RACE and Lyso-2-5′RACE (Table 1), as primers. The reactions were carried out with the BD SMART™ RACE cDNA Amplification Kit (BD Biosciences, Palo Alto, CA, USA). The RACE products were purified, cloned and sequenced as described above. To ensure the authenticity of the cDNA sequence, three randomly selected clones were used for sequence determination.

Computational sequence analyses. Amino acid sequence deduction of cv-lysozyme 2 cDNA sequence was performed using the software Genetyx Mac Ver. 10.1.6 (Software Development, Tokyo, Japan). Signal peptide was predicted by both neural networks and hidden Markov models on SignalP 3.0 server [30]. Sequence similarity was determined using BLAST program on the server of National Center of Biotechnology Information (NCBI) [31]. Molecular weight, isoelectric point (p*I*), and protease cleavage sites were predicted using ProtParam and PeptideCutter on the ExPASY Server [32].

Multiple sequence alignments of cv-lysozyme 2 and other *i* type lysozymes were carried out with Clustal X [33] and modified by eye and hand. Neighbor-Joining analysis of distances with aligned sequences was used to create a phylogenetic tree with the gap-free model of the software PAUP*4.0b [34]. Bootstrap analysis was performed using 1000 replicates. The *i* type lysozymes compared to cv-lysozyme 2 were from the bivalve mollusks *Bathymodiolus azoricus* (AAN16208), *Bathymodiolu thermophilus* (AAN16209), *Crassostrea gigas* (BAD19059), *Crassostrea virginica* cv-lysozyme 1 (BAE47520), *Calyptogena* sp. SB2001_1 (AAN16211), *Calyptogena* sp. SB2001_2 (AAN16212), *Chlamys islandica* (CAC34834), *Mytilus edulis* 1 (AAN16207) and 2 (ABB76765), *Mytilus galloprovincialis* (AAN16210), *Ostrea edulis* (BAD19060), and *Tapes japonica* (BAC15553); from the nematode *Caenorhabditis elegans* (AAC19181); from the medicinal leech *Hirudo medicinalis* (AAA96144); from the shrimps *Litopenaeus vannamei* 1 (BF023863) and 2 (BF024192) and *Litopenaeus setiferus* (BF024309); from the mosquitoes *Anopheles gambiae* (AAT51799) and *Armigeres subalbatus* (AY439906); from the fruit fly *Drosophila melanogaster* 1 (CAA21317), 2 (AAF57940), and 3 (AAF57939); and from the starfish *Asterias rubens* (AAR29291).

Measurement of cv-lysozyme 2 gene expression by quantitative real-time RT-PCR. Total RNA was extracted from tissue fragments of organs sampled from five individual oysters, treated with DNase to prevent DNA contamination and 1 μg used to synthesize cDNA as described above. Quantitative real-time RT-PCR was carried out in an iCycler iQ Real-Time PCR Detection

System (Bio-Rad) using TaqMan TAMRA probe (Applied Biosystems). PCR probes (Table 1) were designed with the computer program Primer Express (Applied Biosystems). PCR amplifications were performed by mixing 2.5 µl of tenfold diluted cDNA, 12.5 µl TaqMan Universal PCR Master Mix (Applied Biosystems), 0.4 µM of each primer and 0.08 µM TaqMan TAMRA probe in a total volume of 25 µl. The reaction conditions were as follows: 50 °C for 2 min, 95 °C for 10 min, 40 cycles of 15 s at 95 °C and 1 min at 60 °C. The amplification efficiency was determined from a serial dilution of cDNA prepared from digestive glands, which showed high cv-lysozyme 2 gene expression in a preliminary experiment. The 28S rRNA expression was assayed under the same conditions with the primers indicated in Table 1, and used as reference to depict the expression level of cv-lysozyme 2 with the method developed by Liu and Saint [35]. The expression data was log transformed and analyzed by a one factor analysis of variance (ANOVA) using the software SAS (SAS Institute Inc., Cary, NC, USA). A Duncan test was used following significant ANOVA results ($p < 0.05$) to examine difference among organs. Expression data are reported as means ± SD.

Localization of cv-lysozyme 2 gene expression by *in situ* hybridization. A 210-bp product of the PCR using cDNA synthesized from digestive gland total RNA as template and Lyso2-ISH-F and Lyso2-ISH-R as primers (Table 1) was cloned into TOPO pCR II-TOPO plasmid (Invitrogen). The recombinant plasmid was used as template for the preparation of probes. Digoxigenin (DIG)-labeled sense and antisense riboprobes were prepared using a DIG RNA Labeling Kit (SP6/T7) from Roche Applied Science (Penzberg, Germany).

Oysters collected in January 2005 were shucked and standard cross-sections were cut transversely through the gills and digestive gland [36]. The tissue sections were immediately fixed in neutral buffered formalin (Richard-Allan Scientific, Kalamazoo MI, USA) for 24 h at 4 °C. After dehydration and embedment in paraffin wax, tissue blocks were sectioned to a thickness of 5 µm and mounted on poly-L-lysine-coated glass slides. Several continuous sections were prepared for each tissue block and were either stained with hematoxylin-eosin (H & E) or stored at 4 °C until used for hybridization.

Tissue sections for *in situ* hybridization were deparaffinized, rehydrated, and treated with boiled Vector Antigen Unmasking Solution (Vector Laboratories Inc., Burlingame, CA, USA) for 20 min. The sections were then washed sequentially with phosphate buffer (150 mM NaCl, 10 mM KCl, 10 mM Na_2HPO_4 and 10 mM KH_2PO_4, pH 7.4), phosphate buffer containing 0.15% (v/v) Triton X-100, phosphate buffer, and twice with a standard saline citrate solution (SSC) (Roche Applied Science), 10 min per wash. Pre-hybridization was performed in hybridization buffer (50% formamide, 4× SSC, 1% Denhardt's solution, 10% dextran sulfate, 0.2 mM EDTA, 50 µg/ml denatured herring sperm DNA and 50 µg/ml *E. coli* tRNA) for 1 h at 45 °C. Hybridization was performed by incubating the tissue sections with DIG-labeled sense or antisense riboprobe, 40 ng per section in 200 µl hybridization buffer, overnight at 45 °C. After hybridization, the sections were sequentially washed twice in 2× SSC, twice in 1× SSC and twice in 0.1× SSC, 20 min each at 45 °C, followed by a wash in 100 mM Tris-HCl and 150 mM NaCl, pH 7.6 (buffer 1) for 5 min at room temperature. The tissue sections were then blocked with 1% (w/v) blocking reagent from Roche Applied Science in buffer 1 (blocking buffer) for 30 min and incubated with alkaline phosphatase-conjugated sheep anti-DIG antibody (Roche Applied Science) 1 : 1000 diluted in blocking solution for 2 h at room temperature. Following two washes of 15 min in buffer 1 and a wash of 5 min in 100 mM Tris-HCl, 100 mM NaCl and 50 mM $MgCl_2$, pH 9.5 (buffer 2), antibodies bound to the tissues were detected by incubation of the sections with a NBT/BCIP (nitroblue tetrazodium chloride/5-bromo-4chloro-3-indonyl-phosphate) solution at 375 µg/ml in buffer 2 overnight in dark at room temperature. The sections were counterstained with 5% Bismarck Brown Y(G) (Sigma-Aldrich) and examined under light microscope. Hybridization with sense riboprobes was used as control.

Results

Cv-lysozyme 2 purification. A protein with high *M. lysodeikticus* lytic activity was purified from eastern oyster crystalline styles using a combination of ion exchange and size exclusion chromatographies. The purified protein, designated cv-lysozyme 2, appeared as a single band with a molecular mass of 13.2 kDa determined by SDS-PAGE under reduced conditions (Fig. 1). MALDI MS revealed an ion of 12 984.6 Da (Fig. 2). A total of 2.2 mg protein was purified from 5500 mg crystalline style proteins. Purified cv-lysozyme 2 had a specific activity 647 times higher than in the initial crystalline style sample (Table 2).

Proteins from digestive gland lysates were fractionated into two lysozyme activity peaks by ion exchange chromatography (I and II, Fig. 3a). Size exclusion purification revealed a lysozyme molecule identical to cv-lysozyme 2 by SDS-PAGE from peak I, but a protein identical to cv-lysozyme 1 from peak II (data not shown). A total of 0.8 mg cv-lysozyme 2 and 0.5 mg cv-lysozyme 1 were purified from 865 mg of proteins extracted from 210 g digestive gland tissues. Only one activity peak was detected in fractions of gill lysates and mantle lysates (Fig. 3b, c), from which a lysozyme identical to cv-lysozyme 1 was purified (data not shown). A total of 0.9 mg of cv-ly-

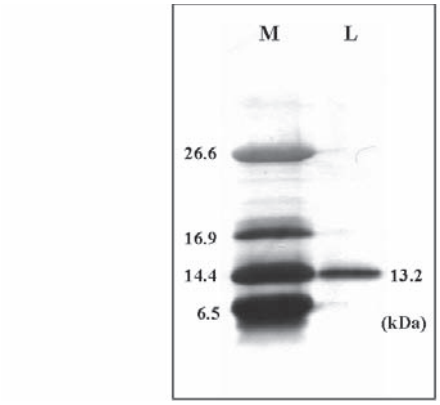

Figure 1. SDS-PAGE of cv-lysozyme 2 purified from crystalline styles of eastern oysters. Electrophoresis was carried out in a 15% acrylamide/bis gel under reduced conditions and the gel was then silver stained. M: Protein standards with the molecular sizes indicated; L: purified cv-lysozyme 2 detected as a single band with a molecular mass of around 13.2 kDa.

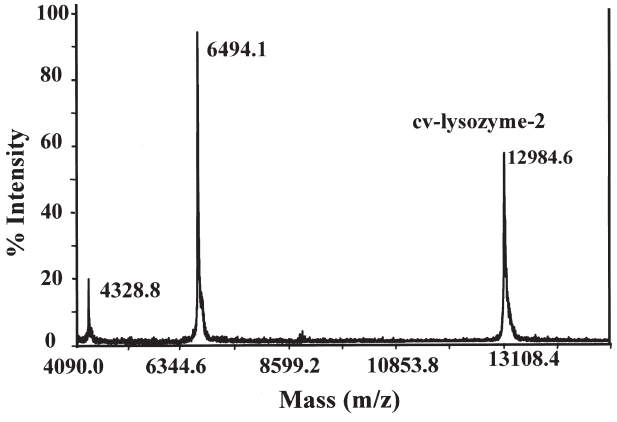

Figure 2. MALDI mass spectrum of purified cv-lysozyme 2. Cv-lysozyme 2 was detected as an MH+ ion at *m/z* 12 985.6. The signals at *m/z* 4328.9 and 6494.1, respectively, indicate triply and doubly charged intact cv-lysozyme 2 molecules.

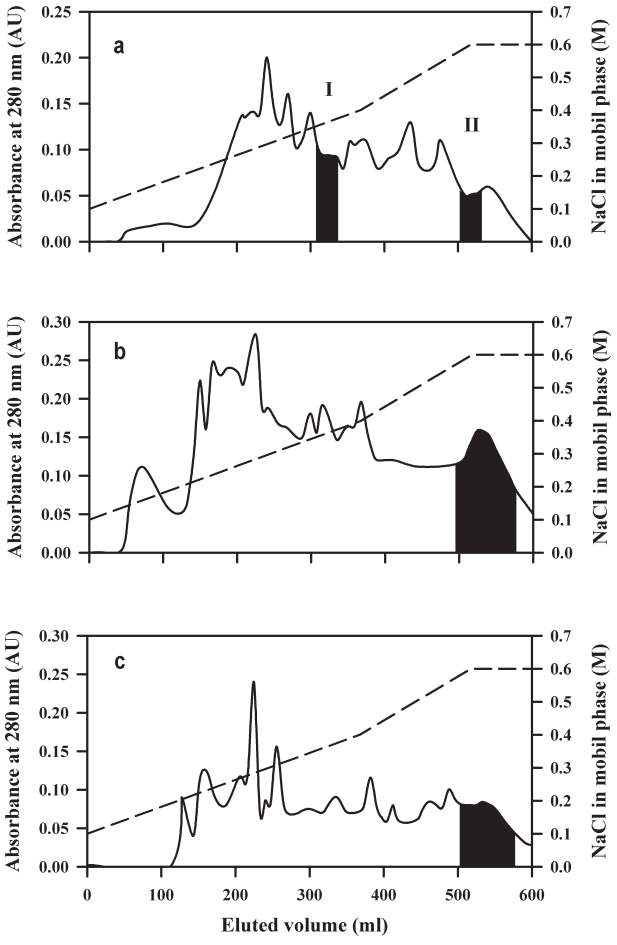

Figure 3. Initial ion exchange chromatographies for purifying lysozymes from digestive gland tissue lysate (*a*), gill tissue lysate (*b*) and mantle tissue lysate (*c*). Ion exchange chromatographies were performed in a 2.6 × 20 cm SP-Sepharose High Performance column eluted with a linear gradient of 0.1–0.6 M NaCl solution in 0.02 M acetic buffer, pH 5.0 over 480 ml of elution volume at an rate of 2 ml/min. Shadowed regions of the graphs indicate fractions containing lysozyme activity.

sozyme 1 was purified from 750 mg proteins extracted from 110 g gill tissues and 0.7 mg cv-lysozyme 1 from 950 mg proteins extracted from 160 g mantle tissues.

Lysozyme activity and optimal conditions. Purified cv-lysozyme 2 expressed more than 90% of its maximum activity in the pH range of 5.4–6.4 and the ionic strength range of I = 0.005–0.01 (Fig. 4a). In buffers with I = 0.005, cv-lysozyme 2 retained more than 75% of its maximum activity in a pH range of 5.3–7.5. When I = 0.02, however, more than 75% of its maximum activity was detected only in a pH range of 5.3–6.5. Moreover, cv-lysozyme 2 expressed more than 75% of its maximum activity in the ionic strength range of I = 0.005–0.08 at pH 5.5 but only in the range of I = 0.005–0.02 at pH 6.5 (Fig. 4a).

Table 2. Summary of digestive lysozyme purification from crystalline style.

Sample	Total protein (mg)	Specific activity (U/mg)	Total activity (U)	Yield (%)
Crystalline style	5500.0	1.20×10^3	6.60×10^6	100.0
Enriched sample	82.5	5.11×10^4	4.22×10^6	63.9
Crude co-lysozyme 2	14.5	1.97×10^5	2.86×10^6	43.3
Purified co-lysozyme 2	2.2	7.77×10^5	1.71×10^6	25.9

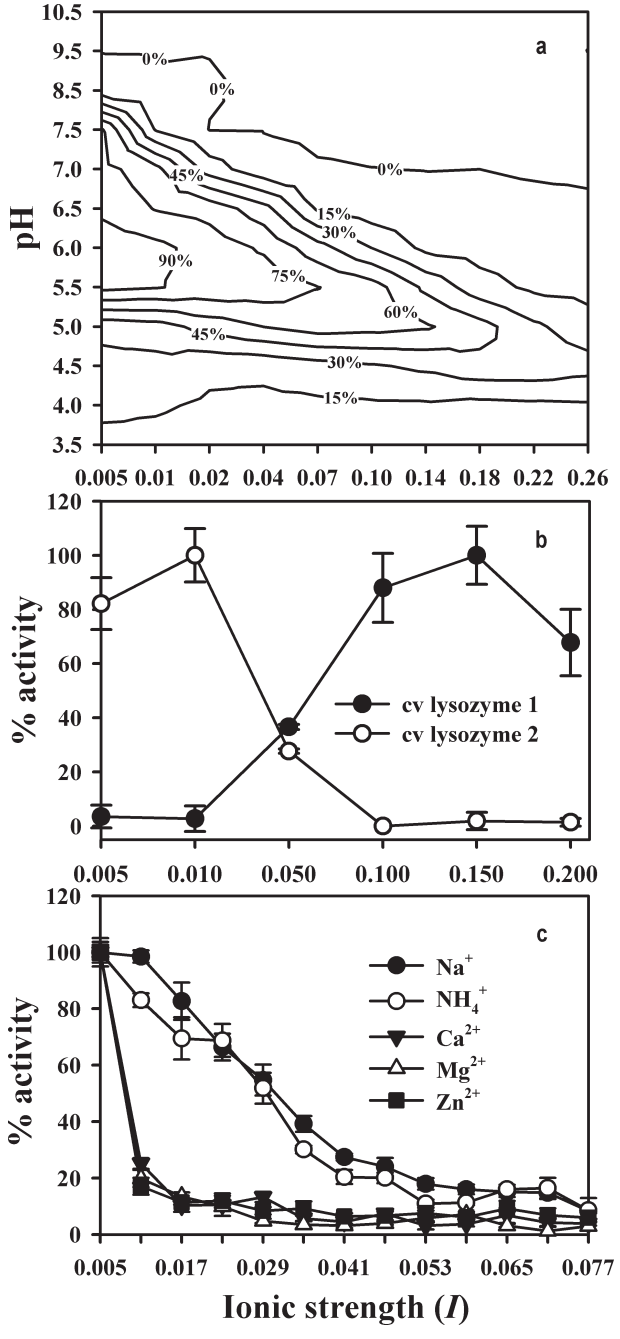

Figure 4. Optimal pH and ionic strength and effects of ionic strength and cations on the lytic activity of purified cv-lysozyme 2. Optimal pH and ionic strength were determined by measuring cv-lysozyme 2 lytic activity against a *Micrococcus lysodeikticus* suspended in 120 buffers covering a pH range of 3.5-10.5 and ionic strength range of I = 0.005–0.260 at 25 °C. The effects of ionic strengths on cv-lysozyme 2 and cv-lysozyme 1 were compared by measuring their lytic activities in a 0.005 M MES-NaOH, pH 6.0 buffer supplemented with NaCl to the ionic strengths indicated. The effects of cations on cv-lysozyme 2 was assessed by measuring its lytic activity in a 0.005 M MES-NaOH, pH 6.0 buffer supplemented with chlorides of Na^+, NH_4^+, Ca^{2+}, Mg^{2+}, and Zn^{2+} to the ionic strengths indicated. (*a*) Optimal pH and ionic strength of cv-lysozyme 2. (*b*) Effects of ionic strength on the lytic activity of cv-lysozyme 1 and cv-lysozyme 2. (*c*) Effects of cations on the lytic activity of cv-lysozyme 2.

At pH 6.0, the activity of the cv-lysozyme 2 reached its maximum at I = 0.01 and decreased markedly with increasing ionic strengths (Fig. 4b). In contrast, cv-lysozyme 1 activity was detected only after the ionic strength was increased to 0.05 and the enzyme reached its maximum activity at I = 0.15 (Fig. 4b).

Cv-lysozyme 2 activity was decreased by 80% when Ca^{2+}, Mg^{2+}, and Zn^{2+} were added to the base buffer at a concentration of 0.01 M, resulting in an ionic strength increase from I = 0.005 to I = 0.035 (Fig. 4c). In contrast, cv-lysozyme 2 activity was only decreased by 2% and 17%, respectively, when Na^+ and NH_4^+ were added to the base buffer, resulting in the same ionic strength increase as after addition of 0.01 M Ca^{2+}, Ma^{2+}, and Zn^{2+} (Fig. 4c).

The optimal temperature for cv-lysozyme 2 activity was about 55 °C. No activity decrease was detected when the enzyme was incubated at 30 °C for 30 min. Cv-lysozyme 2 activity, however, could no longer be detected after an incubation at 90 °C for 30 min or 100 °C for 10 min.

Other enzyme activities. Cv-lysozyme 2 expressed chitinase activity. The minimum enzyme concentrations for detection of chitinase activity were 0.1 mg/ml when measured in 0.2 M sodium acetic buffer, pH 5.8, and 0.5 mg/ml when measured in 0.01 M MES-NaOH buffer, pH 6.0. Cv-lysozyme 1 expressed similar chitinase activity. No isopeptidase activity was detected at the concentration of lysozymes used in this study.

Antibacterial activities. Purified cv-lysozyme 2 at concentrations of 50 µg/ml and 100 µg/ml significantly inhibited the growth of *E. coli* and *V. vulnificus*, respectively. Cv-lysozyme 2 was significantly less effective than cv-lysozyme 1, which inhibited the growth of *E. coli* at a concentration of 1.56 µg/ml and *V. vulnificus* at a concentration of 12.5 µg/ml when both were tested concurrently in the same 96-well plate. Similarly, purified cv-lysozyme 2 significantly inhibited the growth of *P. cerevisiae* at a concentration of 100 µg/ml, while cv-lysozyme 1 inhibited *P. cerevisiae* growth at a concentration of 25 µg/ml.

Amino acid sequence of cv-lysozyme 2. The 15 N-terminal amino acid residues of cv-lysozyme 2 determined by Edman degradation were Ser-Ile-Ser-Asp-Gln-Cys-Leu-Arg-Cys-Ile-Cys-Glu-Val-Glu-Ser. The amino acid sequences of ten peptides derived from trypsin treatments of purified cv-lysozyme 2 were determined by MS/MS. The MS determined sequences identified 107 of the 117 amino acid residues deduced from cv-lysozyme 2 cDNA sequence, with amino acids 88–97 being unidentified (Fig. 5). The protein sequence data reported in this paper will appear in the Uniprot Knowledgebase under the accession no. Q1XG90.

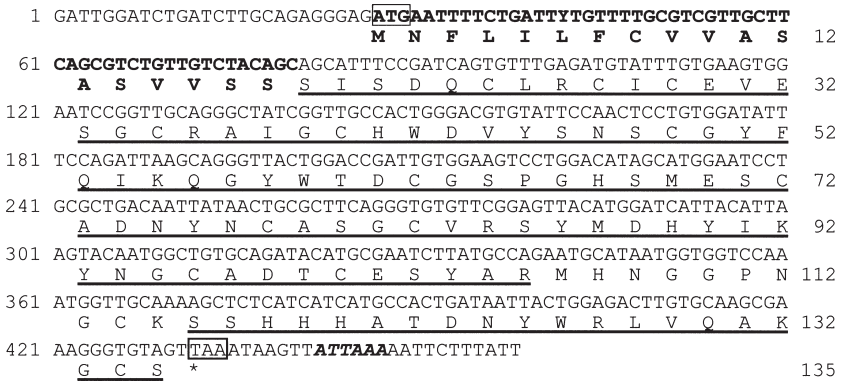

Figure 5. Nucleotide sequence (upper) and predicted amino acid sequence (one letter code, below) of cv-lysozyme 2 cDNA. Numbering of the nucleotide and amino acid sequence is shown on the left and right, respectively. Assigned initial and terminal codons are shown bordered; the predicted polyadenylation signal is shown in italics. The predicted nucleotide sequence encoding the signal peptide and the signal peptide sequence are shown in bold. The underlined amino acid sequence indicates the regions corresponding to the sequence determined by tandem mass spectrometry of purified cv-lysozyme 2. The cDNA sequence was registered in NCBI GenBank under accession number AB252064.

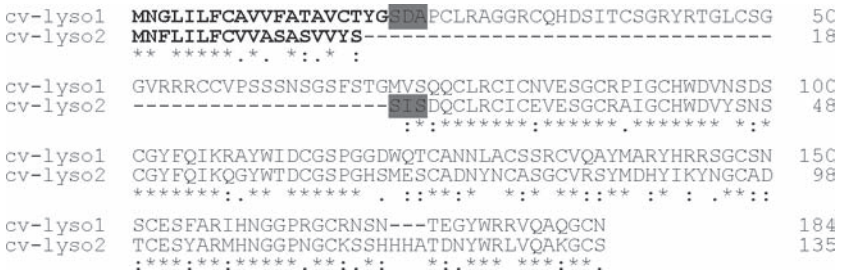

Figure 6. Comparison of the amino acid sequences of cv-lysozyme 2 and cv-lysozyme 1. Sequence alignments were carried out with Clustal X and then modified by eye and hand. The predicted signal peptides are shown in bold. The first three N-terminal amino acid residues are shadowed.

cDNA sequence of cv-lysozyme 2. A 458-bp cDNA sequence was obtained after the nested-PCR and RACE reactions (Fig. 5). Nucleotide BLAST searches revealed no identical DNA sequence in GenBank databases. The first ATG codon of the sequence at position 27–29 was assigned as the translational initiation codon and the TAA codon at position 432–434 as the termination codon. A putative polyadenylation signal, ATTAAA, was observed at position, 442–447. Based on this assignment, an open reading frame of 408 bp with the deduced amino acid sequence of 135 residues was determined. SignalP 3.0 predicted that the N-terminal 18 amino acid residues of the deduced sequence constituted a signal peptide. With this prediction, the N terminus of the predicted mature protein was identical to that of the purified lysozyme determined by Edman degradation. In addition, the deduced amino acid sequence covered all the sequences determined by MS/MS (Fig. 5). The calculated average molecular mass of the predicted mature protein was 13 004.2 Da with a p*I* of 6.33. The cDNA sequence was registered in NCBI GenBank under the accession no. AB252064.

Computational analysis of the amino acid sequence of cv-lysozyme 2. Protein-protein BLAST search in the GenBank revealed that the cDNA deduced amino acid sequence of cv-lysozyme 2 has a high similarity with other *i* type lysozymes and is particularly homologous to bivalve lysozymes. The least closely related lysozyme was a protein from *D. melanogaster* (AAF57940) with a similarity of 46% and an E-value of 5×10^{-6}. The most closely related protein was cv-lysozyme 1 from the eastern oyster with an identity of 62%, a similarity of 81% and an E-value of 3×10^{-43} (Fig. 6).

Multiple alignments of *i* type lysozymes were performed and 93 amino acid residues were chosen by the computer program to construct a Neighbor-Joining phylogenic tree (Fig. 7). Bivalve lysozymes formed a cluster that was independent of lysozymes from other phyla. Within the bivalve cluster, cv-lysozyme 2 was found in the group composed of lysozymes from the Iceland scallop (*C. islandica*), the blue mussel (*M. edulis*), the Pacific oyster (*C. gigas*) and the flat oyster (*O. edulis*), whereas cv-lysozyme 1 appeared in the group which included lysozymes from vesicomyas (*Calyptogena* sp.) and the manila clam

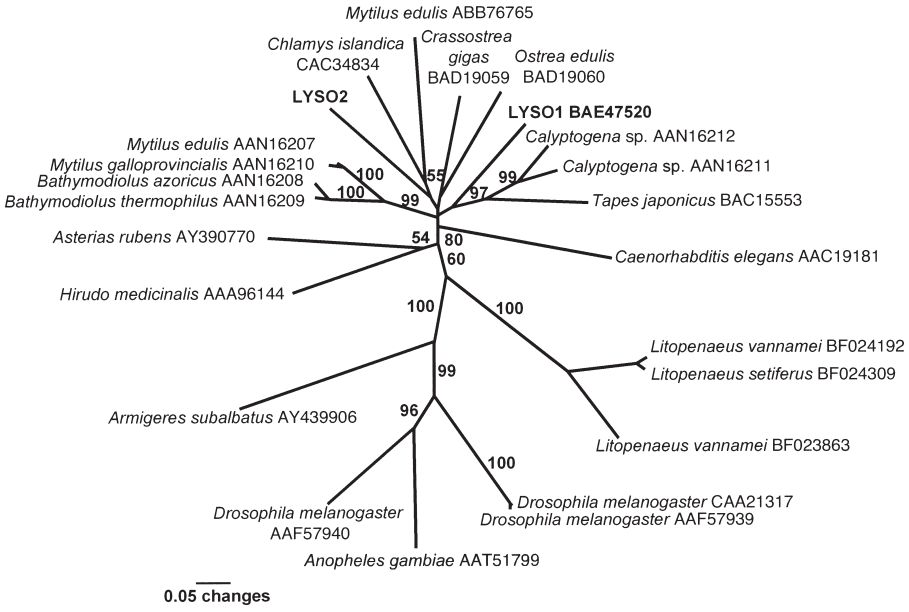

Figure 7. Phylogenetic analysis of *i* type lysozymes. In addition to cv-lysozyme 2 (LYSO2), the sequences included in the phylogenetic tree are those of the bivalve mollusks *Bathymodiolus azoricus* (AAN16208), *Bathymodiolus thermophilus* (AAN16209), *Crassostrea gigas* (BAD19059), *Crassostrea virginica* cv-lysozyme 1 (LYSO1, BAE47520), *Calyptogena* sp. SB2001_1 (AAN16211), *Calyptogena* sp. SB2001_2 (AAN16212), *Chlamys islandica* (CAC34834), *Mytilus edulis* 1 (AAN16207) and 2 (ABB76765), *Mytilus galloprovincialis* (AAN16210), *Ostrea edulis* (BAD19060), and *Tapes japonica* (BAC15553); the nematode *Caenorhabditis elegans* (AAC19181); the medicinal leech *Hirudo medicinalis* (AAA96144); the shrimps *Litopenaeus vannamei* 1 (BF023863) and (BF024192) and *Litopenaeus setiferus* (BF024309); the mosquitoes *Anopheles gambiae* (AAT51799) and *Armigeres subalbatus* (AY439906); the fruit fly *Drosophila melanogaster* 1 (CAA21317), 2 (AAF57940), and 3 (AAF57939); and starfish *Asterias rubens* (AAR29291). The phylogenetic tree was constructed using Neighbor-Joining analysis of distances on the aligned sequences in a gap-free model; 93-amino acids were automatically chosen for tree creation. The bootstrap analysis was performed using 1000 replicates.

(*T. japonica*). Maximum parsimony analysis also yielded trees with the same topology (data not shown).

Measurement of cv-lysozyme 2 gene expression by quantitative real time RT-PCR. High numbers of cv-lysozyme 2 mRNA transcripts were measured in digestive gland tissues of all five oysters examined, with a gene expression level of $6.51 \times 10^{-4} \pm 6.66 \times 10^{-4}$ relative to 28S rRNA gene expression. Cv-lysozyme 2 mRNA was also detected in the style sac-midgut tissues of two of the five oysters examined but at a relative expression level 10 000 times lower than in digestive gland tissues. No cv-lysozyme 2 gene expression was detected in the mantle, gills, labial palps, or hemocytes. The specificity of quantitative RT-PCR was confirmed by sequencing the PCR products. The probes did not cross-react with cv-lysozyme 1 cDNA (data not shown). The efficiencies of the reactions were 82.6% for cv-lysozyme 2 mRNA and 95.3% for 28S rRNA.

Localization of cv-lysozyme 2 gene expression by *in situ* hybridization. *In situ* hybridization using DIG-labeled antisense riboprobes and alkaline phosphatase-conjugated anti-DIG antibodies revealed that cv-lysozyme 2 mRNA is expressed in the epithelia of the digestive tubules (Fig. 8a). Comparison with the H & E-stained con-

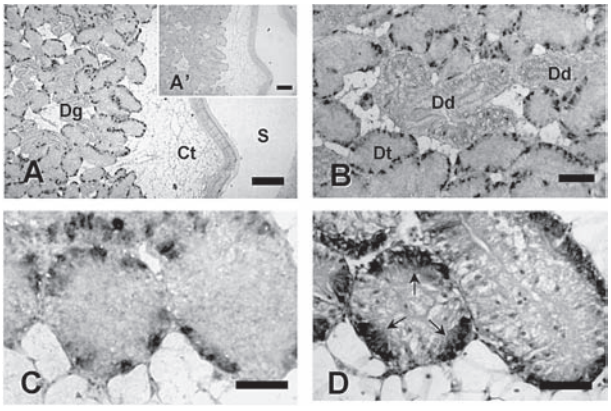

Figure 8. Localization of cv-lysozyme 2 gene expression in eastern oyster tissues by *in situ* hybridization. Hybridization was performed in standard paraffin-embedded tissue sections using digoxigenin (DIG)-labeled sense and antisense cv-lysozyme 2 riboprobes and alkaline phosphatase-conjugated sheep anti-DIG antibody. Antisense riboprobes were used to verify the specificity of the hybridization. A continuous section next to the hybridized one was stained with hematoxylin-eosin (H & E) to determine the cell types. (*a*) Tissue section hybridized with antisense or sense (A') riboprobes, indicating cv-lysozyme 2 mRNA was expressed in the epithelia of the digestive tubules; S: stomach, Ct: connective tissue, Dg: digestive gland; bars = 200 μm. (*b*) Epithelia lining the digestive duct (Dd) did not show positive reaction; bar = 100 μm. (*c*) Antisense riboprobes hybridized digestive tubules; bar = 50 μm. (*d*) H & E-stained section, indicating (arrows) *in situ* hybridization positive regions in (*c*) corresponded to basophil cells of digestive tubules. Bar = 50 μm.

tinuous sections indicated that the expression was limited to basophil cells (Fig. 8c, d). Cv-lysozyme 2 mRNA was not detected in epithelial cells lining the digestive ducts and stomach (Fig. 8a, b), nor in any other tissues in standard cross-sections. Sense riboprobes did not show reactivity with the tissue sections.

Discussion

A 12984.6 Da lysozyme was purified from crystalline styles and digestive glands of eastern oysters. The purified lysozyme, named cv-lysozyme 2, shared similar biochemical and enzymatic properties (*i.e.* optimal pH, ionic strength and temperature) with most bivalve mollusk lysozymes that have been characterized [24, 25, 27, 29, 37, 38]. The amino acid sequence deduced from the sequence of cloned cDNA showed high similarity to all other known *i* types lysozymes, including the paradigmatic *i* type lysozyme from the European starfish *A. rubens* [39, 40].

Significant differences in the molecular and biochemical characteristics between cv-lysozyme 2 and cv-lysozyme 1 were found, suggesting the enzymes have different function in the eastern oyster (Table 3). First, cv-lysozyme 2 lytic activity was inhibited by high ionic strengths and the presence of divalent cations, in contrast to cv-lysozyme 1. Cv-lysozyme 1, which shows relatively high activity within a broad range of pH and ionic strengths, is therefore better suited than cv-lysozyme 2 to function in the hemolymph where pH and salt concentrations are usually high [41–44]. Secondly, the lower p*I* of cv-lysozyme 2 compared to cv-lysozyme 1 favors the function of cv-lysozyme 2 in an acidic environment [6, 13, 45]. Moreover, cv-lysozyme 2 lytic activity was largely maintained at variable ionic strengths only when the pH was low. Thirdly, the amino acid sequence of cv-lysozyme 2 contains fewer protease cutting sites than that of cv-lysozyme 1, suggesting an increased resistance to proteolytic digestive enzymes [45]. The reduction of arginine residues and replacement by lysine may contribute to this resistance as lysyl bonds are less sensitive to hydrolysis

Table 3. Comparison between cv-lysozyme 2 and cv-lysozyme 1 from the eastern oyster.

Characteristic	cv-lysozyme-2	cv-lysozyme-1
Lysozyme activity		
Optimal pH	5.4–6.4	5.5–6.5*
Optimal ionic strength (I)	0.005–0.01	0.18–0.20*
Bivalent cation effects	Inhibitory	Enhancing*,[a]
Optimal temperature (°C)	50	45*
Other enzyme activity		
Chitinase activity	+	+
Isopeptidase activity	ND	ND
Antibacterial activity (MIC)		
E. coli	50 µg/ml	1.56 µg/ml
V. vulnificus	100 µg/ml	12.5 µg/ml
P. cerevisiae	100 µg/ml	25 µg/ml
Molecular properties		
MW (Dalton)[b]	12984.6	17861.0*
Predicted pI	6.33	8.95**
Amino acid sequence		
Total amino acid residues	117	164**
Arginine residues number	5	19/12[c]
Protease cutting sites		
Trypsin	9	18/12[c]
Thermolysin	20	28/21[c]
Pepsin (pH > 2.0)	5	12/8[c]
Distribution		
Major sites of gene expression		
Real-time RT-PCR	Digestive glands	Labial palps, mantle
In situ hybridization	Basophil cells of digestive tubules	Non-vesiculated cells of mantle connective tissue and cells of interlamelar junctions and epithelia surrounding the water tubes of the gills**
Major sites of enzymic activity	Crystalline style, digestive glands	~90% in plasma; <1% in digestive tissues**

[a] When ionic strength is lower than $I = 0.2$. [b] MALDI-determined mature protein molecule. [c] Between residue 51 and 164 (covering the length of digestive lysozyme). * Cited from [28]. ** Cited from Itoh, N., Xue, Q. G., Li, Y., Cooper, R. K. and La Peyre, J. F. (2006). cDNA cloning and tissue expression of plasma lysozyme in the eastern oyster, *Crassostrea virginica*. Submitted.

by trypsin than are arginyl bonds [45]. Cv-lysozyme 2 therefore appears much better suited than cv-lysozyme 1 to function as a digestive enzyme.

Cv-lysozyme 2 mRNA and protein distribution in oyster tissues further support its role in digestion. Production in stomach or midgut is one of the important characteristics of digestive lysozymes in ruminants [6] and fruit flies [15]. In our study, *in situ* hybridization showed that cv-lysozyme 2 mRNA is expressed in basophil cells of the digestive tubules. Moreover, cv-lysozyme 2 protein could only be purified from crystalline styles and digestive glands. Cv-lysozyme 2 secreted by basophil cells of digestive tubules apparently enters the stomach posterior chamber where the rotating crystalline style protruding from the style sac mixes food particles and gastric juices [46]. Cv-lysozyme 2 mRNA detected in the style sac-midgut of two out of five oysters may have been the result of contamination by digestive gland tissues as the style sac-midgut is adjacent to the digestive gland [47]. While cv-lysozyme 1 was also purified from the digestive glands, it is likely it was from hemolymph retained in the tissues. Hemolymph is abundant in all bivalve mollusks tissues constituting 47–70% of their tissue weights [48–50]. Moreover, most (>90%) cv-lysozyme 1 activity is found in plasma with less than 1% contained in digestive gland tissues (Table 3). Although lysozymes of bivalve mollusks have been hypothesized to function in host defense and digestion, our findings show for the first time that these functions are provided by different lysozymes with distinct molecular and biochemical characteristics.

Noteworthy is the finding that eastern oyster cv-lysozyme 2 possesses molecular and biochemical characteristics comparable to those acquired by digestive lysozymes of other animals through adaptive evolution [11]. However, the evolution mechanisms for *c* type lysozyme might be different from that for *i* type lysozymes of eastern oysters. Cv-lysozyme 2 lacks the 47-residue N-terminal regions of cv-lysozyme 1, suggesting that the evolutionary relationship between these two lysozymes should be more complicated than simple amino acid replacements as found in *c*-type digestive lysozymes in other animals [7, 11, 51]. The finding that cv-lysozyme 1 and cv-lysozyme 2 were found in different groups on the phylogenetic tree supports that notion. One possibility would be that genes encoding the two functionally different lysozymes split before the speciation of bivalve mollusks and then evolved independently under different constraints. For example, cv-lysozyme 1 could have evolved under the pressure of highly variable salinity and pH conditions of the hemolymph, while cv-lysozyme 2 adapted to the acidic and proteolytic environment of the digestive system. As multiple lysozymes have recently been reported in blue mussels [27], it will be important to see if lysozymes with different functions are also present in blue mussels and other bivalve mollusks.

In our study, purified cv-lysozyme 2 was 19.6 Da smaller than the cDNA predicted protein molecule. It is likely that the 14 cysteine residues in cv-lysozyme 2 form seven disulfide bridges as all the 8 cysteines in hen egg white lysozyme form intra-chain disulfide bridges [52]. When all these cysteines form intra-molecule disulfide bridges, the difference between the calculated molecular mass and the MALDI determined molecular mass is 5.6 Da, a discrepancy that can be from the accuracy errors of predication algorithm and/or MS measurement. The molecular mass difference may also reflect the presence of cv-lysozyme 2 isozymes as isozymes of digestive lysozymes are commonly found in ruminants and fruit flies [6, 53–55]. Future studies on identifying the number of genes and genomic organization of lysozymes in the eastern oyster should provide valuable information on isozymes and evolution mechanisms of lysozymes in bivalve mollusks. Molecular tools such as quantitative real time RT-PCR developed in this study will also need to be used to measure the level of cv-lysozyme 2 expression in response to feeding to further support cv-lysozyme 2 functions in digestion.

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