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Purification and characterization of lysozyme from plasma of the eastern oyster (*Crassostrea virginica*)

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Abstract

Lysozyme was purified from the plasma of eastern oysters (*Crassostrea virginica*) using a combination of ion exchange and gel filtration chromatographies. The molecular mass of purified lysozyme was estimated at 18.4 kDa by SDS-PAGE, and its isoelectric point was greater than 10. Mass spectrometric analysis of the purified enzyme revealed a high-sequence homology with *i*-type lysozymes. No similarity was found however between the N-terminal sequence of oyster plasma lysozyme and N-terminal sequences of other *i*-type lysozymes, suggesting that the N-terminal sequences of the *i*-type lysozymes may vary to a greater extent between species than reported in earlier studies. The optimal ionic strength, pH, cation concentrations, sea salt concentrations, and temperature for activity of the purified lysozyme were determined, as well as its temperature and pH stability. Purified oyster plasma lysozyme inhibited the growth of Gram-positive bacteria (e.g., *Lactococcus garvieae*, *Enterococcus* sp.) and Gram-negative bacteria (e.g., *Escherichia coli*, *Vibrio vulnificus*). This is a first report of a lysozyme purified from an oyster species and from the plasma of a bivalve mollusc.

Keywords: Eastern oyster; Bivalve mollusc; Plasma protein; Lysozyme; Amino acid sequence; Biochemical properties; Antibacterial properties; Invertebrate immunity

1. Introduction

Lysozymes are a group of proteins defined as 1,4-β-*N*-acetylmuramidases (EC 3.2.1.17). These enzymes cleave the glycosidic bond between *N*-acetylmuramic acid and *N*-acetylglucosamine of peptidoglycan, a major component of bacterial cell walls (Salton, 1957; Jolles, 1969; Chipman and Sharon, 1969). Several types of lysozyme (e.g., *c*-, *g*-, *i*-types) which differ in their amino acid composition, biochemical and antimicrobial properties, and gene sequences have been identified in a wide range of organisms from bacteriophages to humans (Beintema and Terwisscha van Scheltinga, 1996; Fastrez, 1996; Holtje, 1996; Hultmark, 1996; Jolles, 1996; Jolles et al., 1996; Prager, 1996). There has been increasing interest in recent years in the distribution and characterization of invertebrate *i*-type lysozymes which include lysozymes of bivalve molluscs (Bachali et al., 2002; Olsen et al., 2003; Takeshita et al., 2003; Zavalova et al., 2003; Bachali et al., 2004).

Lysozyme activity has been detected in the body fluids and tissues of many bivalve molluscs and is believed to play a role in host defense and digestion (McDade and Tripp, 1967; Rodrick and Cheng, 1974; McHenery et al., 1986; Takahashi et al., 1986; Chu and La Peyre, 1989; Maginot et al., 1989; Allam et al., 2000; Cronin et al., 2001). Lysozymes of several bivalve molluscs have been purified mostly from parts of the digestive system, such as the crystalline style and visceral mass (McHenery and Birk-

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beck, 1979; Myrnes and Johansen, 1994; Jolles et al., 1996; Ito et al., 1999; Nilsen et al., 1999; Miyauchi et al., 2000; Montenegro-Ortega and Viana, 2000; Olsen et al., 2003). Their molecular masses range from 11 to 22 kDa, and the analysis of their N-terminal amino acid sequences, when determined, indicated that they belong to a distinct type of lysozyme, the *i*-type (Jolles et al., 1996; Ito et al., 1999; Nilsen et al., 1999; Miyauchi et al., 2000; Olsen et al., 2003). Their biochemical properties vary between species, and at least one lysozyme from the Manila clam, *Tapes japonica*, was reported to possess isopeptidase activity in addition to 1,4-β-*N*-acetylmuramidase and chitinase activity (McHenery and Birkbeck, 1982; Viana and Raa, 1992; Myrnes and Johansen, 1994; Ito et al., 1999; Nilsen et al., 1999; Miyauchi et al., 2000; Takeshita et al., 2003).

The presence of lysozyme activity in cell-free haemolymph (plasma) of the eastern oyster (*Crassostrea virginica*) was first demonstrated by McDade and Tripp (1967). The plasma contained an enzyme which (1) caused a reduction in the turbidity of bacterial cell walls, (2) liberated reducing sugars, and (3) liberated a complex containing glucosamine and muramic acid, therefore fulfilling the criteria of Salton (1957) and Jolles (1964) for the designation of the enzyme as a lysozyme. While the effects of pH and various salts on the lytic activity on *Micrococcus lysodeikteicus* as well as the temperature stability of the plasma lysozyme have been determined (McDade and Tripp, 1967; Rodrick and Cheng, 1974), basic knowledge of biochemical and antimicrobial properties of this enzyme in purified form is lacking. The eastern oyster is an important commercial bivalve species along the Atlantic and Gulf of Mexico coasts of North America and is threatened by diseases (Ford and Tripp, 1996). The development of a procedure to purify lysozyme from the plasma of eastern oysters will allow investigations of its potential role in the oyster host defense. To our knowledge, no lysozyme has been purified from the cell-free haemolymph (plasma) of any bivalve mollusc. Lysozyme(s) purified from plasma may differ from those purified from the digestive system in biochemical and antimicrobial properties because of their respective putative role in host defense and digestion. Lysozymes with different molecular weights and biochemical properties have for instance been recently purified from blue mussels (*Mytilus edulis*; Olsen et al., 2003).

The objectives of this study were to (1) purify lysozyme(s) from the plasma of eastern oysters, (2) estimate the molecular mass(es) and isoelectric point(s) of the purified lysozyme(s), (3) determine the N-terminal amino acid sequence(s) of the purified lysozyme(s) by automatic Edman degradation, (4) analyze the purified lysozyme(s) by mass spectrometry to compare primary amino acid sequence(s) with other proteins and to verify molecular mass(es), (5) determine the optimal ionic strength, pH, cation concentrations, sea salt concentrations, and temperature for activity of the purified lysozyme(s), (6) determine temperature and pH stability of the purified lysozyme(s), and (7) determine the antibacterial properties of the purified lysozyme(s).

2. Materials and methods

2.1. Chemicals

Sephadex G-25 (Superfine), CM-Sepharose Fast Flow, and Superdex G-75 media were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Chemicals used for sodium-dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), electroblotting and isoelectric were purchased from Sigma-Aldrich (Saint Louis, MO).

2.2. Oysters

Eastern oysters (*C. virginica*), 10–15 cm in shell length, were collected from the coast of Louisiana between November 2001 and March 2002. They were transported to Louisiana State University, Baton Rouge, and maintained in a 1000-l recirculating seawater system at a salinity of 15 ppt and a temperature of 15 °C. Haemolymph was sampled from individual oysters within a week after their transfer to the recirculating seawater system.

2.3. Haemolymph sampling

Oyster haemolymph was withdrawn from the adductor muscle sinus with a 3-ml syringe equipped with a 25 gauge needle through a notch on the dorsal side of the shell. Haemolymph from about 200 oysters were pooled and centrifuged at 500×*g* for 15 min at 4 °C. Supernatant (plasma, 800 ml) was collected and stored at −20 °C for lysozyme purification.

2.4. Lysozyme activity and protein concentration

Lysozyme activity was measured in 96-well plates by mixing in each well 20 μl of sample with 180 μl of *Micrococcus lysodeikticus* bacterial suspension prepared at a concentration of 0.8 mg/ml in appropriate buffer solutions. Buffer solutions were selected according to the requirements of the individual experiments described later in the text. Absorbance of wells was immediately measured at 450 nm with a microtiter plate reader (Dynatec, Chantilly, VA). Absorbance was measured 5 min after the initial reading, and the decrease in absorbance at 450 nm/min was calculated. Assays were performed at room temperature (RT, 20 °C) unless otherwise indicated. In this study, one unit of lysozyme was defined as that quantity which caused a decrease in absorbance of 0.001/min of *M. lysodeikticus* suspended in 0.18 M (*I*=0.180) acetate buffer at pH 5.5. All lysozyme measurements in 96-well plates were done in triplicates. Sample protein concentration was measured using the Micro BCA Protein Assay Reagent Kit from

Pierce Biotech (Rockford, IL). All protein measurements in 96-well plates were done in triplicates.

2.5. Lysozyme purification

2.5.1. Sample preparation

Oyster plasma (800 ml) was freeze-dried (FTS systems, Stone Ridge, NY) and resuspended in 80 ml of Millipore water. The preparation was clarified by centrifugation at 3000×*g* for 30 min at 4 °C. The supernatant was divided into two 40-ml samples, and these were applied onto Sephadex G-25 columns (2.6×30 cm) equilibrated with 0.02 M sodium acetate buffer, pH 5.0. The columns were then washed with 0.02 M sodium acetate buffer, pH 5.0, at a linear superficial flow rate (LSFR) of 60 cm/h. The elution was monitored for its absorbance at 280 nm with an Econo UV Monitor from Bio-Rad Laboratories. Fractions collected in the first peak were pooled. This pool contained plasma proteins including lysozyme in 0.02 M sodium acetate buffer, pH 5.0, and was designated as 'crude plasma lysozyme sample'.

2.5.2. Initial ion (IE) exchange chromatography

The crude plasma lysozyme sample was loaded onto a CM-Sepharose Fast Flow column (1.6×30 cm) equilibrated with 0.02 M sodium acetate buffer, pH 5.0. The column was successively washed with 0, 0.1, 0.3, and 0.6 M of NaCl in 0.02 M sodium acetate buffer, pH 5.0, at an LSFR of 60 cm/h. The elution was monitored for absorbance at 280 nm. Fractions from the 0.6 M NaCl eluted peak, which contained lysozyme activity, were pooled and concentrated by centrifugation at 2800×*g* at 4°C using Centriprep YM-10 filters (Millipore, Bedford, MA). The pooled sample was designated as 'lysozyme-enriched IE sample'.

2.5.3. Gel-filtration (GF) chromatography

The lysozyme-enriched IE sample was applied to a Superdex G-75 column (1.6×60 cm) equilibrated with 0.1 M sodium acetate buffer, pH 5.0. The column was eluted with the same buffer at an LSFR of 30 cm/h. The elution was monitored for its absorbance at 280 nm, and the fractions of the second peak with lysozyme activity were pooled. The buffer of the pooled fractions was changed to 0.02 M acetate buffer, pH 5.0, using Sephadex G-25 columns (2.6×30 cm), and the pooled fractions were designated as 'lysozyme GF-separated sample'.

2.5.4. Final ion-exchange chromatography

Lysozyme GF-separated sample was loaded onto a CM-Sepharose Fast Flow column (0.8×10 cm). The column was washed with a linear gradient of NaCl, 0.3 to 0.7 M in 0.02 M sodium acetate buffer, pH 5.0, at an LSFR of 60 cm/h. The elution was monitored for its absorbance at 280 nm. The lysozyme activity of each fraction was tested, and an aliquot from each fraction containing high lysozyme activity was subjected to SDS-PAGE as described below. Fractions showing a single protein band were pooled, and the salts in the solution were eliminated by gel filtration in a Sephadex G-25 column (2.6×30 cm) equilibrated with Millipore water. The protein obtained by washing the column with Millipore water was designated as 'purified oyster lysozyme'. It was concentrated to 2 mg/ml by ultrafiltration using a Centriprep YM-10 filter at 2800×*g*, 4 °C, and stored at −20 °C as stock solution until use.

2.6. Molecular mass by SDS-PAGE and isoelectric point determination

The purity and approximate molecular mass of the lysozyme sample were estimated by SDS-PAGE under reduced condition in a protean III vertical slab gel unit (Bio-Rad, Richmond, CA) with a 12.5% running gel and a 4% stacking gel. The low-range (14.4–97.4 kDa) protein molecular mass markers from Bio-Rad were used as standards to calculate molecular mass. The isoelectric point of the purified lysozyme was determined by isoelectric focusing in a MINI IEF Cell (Model 111, Bio-Rad) using isoelectric point standards (4.45–9.6) from Bio-Rad for reference.

2.7. N-terminal amino acid sequencing

The purified lysozyme in the solution was sent to the Protein Chemistry Laboratory of the University of Texas Medical Branch, Galveston, for N-terminal amino acid sequencing. The N-terminal amino acid sequence was analyzed by automatic Edman degradation using an Applied Biosystems Procise 494/HT protein sequencer (Applied Biosystems, Foster City, CA) after reduction and alkylation of the sample. The sample was reduced in 50 μl of 6 M guanidine–HCl, 0.25 M Tris buffer at pH 8.5, 1 mM EDTA in the presence of 2.5 μl of 10% β-mercaptoethanol (Hawke and Yuan, 1987). The reduction was allowed to proceed for 2 h at room temperature under argon in the dark. One microliters of 4-vinylpyridine was added, and incubation was continued for an additional 20 min in the dark at RT under argon (Hawke and Yuan, 1987; Andrews and Dixon, 1987; Tempst et al., 1990). The sample was cleaned prior to sequencing on a prosorb sample preparation cartridge (Applied Biosystems). Another sample of the purified lysozyme on PVDF membrane was also submitted for N-terminal amino acid sequencing. Following an SDS-PAGE as described earlier, the lysozyme was electroblotted onto Sequi-blot™ PVDF membrane (Bio-Rad) in CAPS buffer (0.01M CAPS, 10% methanol, pH 11) using a Bio-Rad Mini Transblot® Electrophoretic cell (Bio-Rad). The membrane was stained with Coomassie blue R-250 to locate lysozyme on the membrane. The PVDF membrane with the lysozyme was cut, washed six times in Millipore water, and sent to the Protein Chemistry Laboratory of the University of Texas Medical Branch. Sequence similarity between the N-

terminal amino acid sequence of oyster plasma lysozyme and proteins in the GenBank databases of the National Center for Biotechnology Information (NCBI) was investigated using the BLAST program.

2.8. Primary amino acid sequencing using tandem mass spectrometry and molecular mass determination by matrix-assisted laser desorption ionization (MALDI)

A 5-μl aliquot of purified lysozyme (555 pmol) was reduced with 50 μl of 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 of 15 mg/ml iodoacetamide 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). After sample injection, the column was washed with 5% acetonitrile, 0.1% trifluoroacetic acid for 5 min at a flow of 200 μl/min. Lysozyme was eluted with 85% acetonitrile, 0.1% TFA. Absorbance was monitored at 214 nm, and collected fractions dried in a speed vac.

The reduced and alkylated lysozyme-containing fraction was solubilized in 100 mM ammonium bicarbonate buffer, pH 7.8 (50 μl). Trypsin (100 ng) was added, and the digestion allowed to proceed for 18 h at 37 °C. Tryptic peptides were desalted using C_{18} ZipTips (Millipore) and eluted with 2 μl nanospray solvent [water/acetonitrile/acetic acid (49:49:2, v/v/v)].

Nanospray mass spectrometry was carried out on a tandem quadrupole/time-of-flight mass spectrometer (QSTAR, Applied Biosystems) equipped with a Protana nanospray source. Approximately 1.75 μl of the tryptic digest solution was loaded into custom-pulled, gold-coated quartz capillaries. Tryptic peptides were selected for sequencing by tandem mass spectrometry according to their observed molecular ions. Molecular ions were selected and subjected to collision-induced dissociation in the collision quadrupole filled with nitrogen. The product ion spectra were recorded with the high-resolution time-of-flight mass spectrometer. Typically 100 spectra were accumulated to produce data of high signal-to-noise ratio. In addition, tandem time-of flight-mass spectrometry (Applied Biosystems 4700 Proteomics Analyzer) was carried out to obtain confirmatory and additional sequence information. A 0.50-μl aliquot of the desalted tryptic digest was mixed (1:3, v/v) with matrix (α-cyano-4-hydroxycinnamic acid, 50 mM in 70% acetonitrile, 0.1% TFA). Matrix-assisted laser desorption ionization (MALDI) with an Nd-YAG laser was used to ionize tryptic peptides, and ions of interest were selected for collision-induced dissociation. Typically 200–5000 laser shots were used to generate the product ion spectra. Manual interpretation of both Q-TOF and TOF-TOF data was carried out. Purified lysozyme was prepared for MALDI analysis as described above to obtain the molecular mass of the intact protein. This analysis was accomplished on a Voyager-DE STR instrument (Applied Biosystems) operating in linear mode.

The resulting peptide sequences were subjected to BLAST searches of the National Center for Biotechnology Information (NCBI) GenBank database to ascertain sequence homology. The peptide sequences were then aligned with sequences of four bivalve mollusc lysozymes aligned according to Bachali et al. (2002). The four sequences were for lysozymes from the cold-seep clam, *Calyptogena* sp. (Bachali et al., 2002; GenBank accession no. AF334667), from the Mediterranean mussel, *Mytilus galloprovincialis* (Bachali et al., 2002; GenBank accession no. AF334665), from the Icelandic scallop, *Chlamys islandica* (Nilsen and Myrnes, 2001; GenBank accession no. CAC34834), and from the Manila clam, *T. japonica* (K. Takeshita et al., unpublished, GenBank accession no. AB091383).

2.9. Determination of pH and ionic strength optima and pH stability

The lytic activity of purified lysozyme on *M. lysodeikticus* was measured at 12 pHs (3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.5, 9.5, and 10.5) and 10 ionic strengths (0.005, 0.010, 0.020, 0.040, 0.070, 0.100, 0.140, 0.180, 0.220, 0.260, and 0.280). A total of 120 buffer solutions were prepared with sodium hydroxide–acetic acid (pH 3.5–5.5), Na_2HPO_4–$NaH_2PO_4H_2O$ (pH 6.0–8.5), and boric acid–NaOH (9.5–10.5). The pHs of all buffers were measured and adjusted to the expected pH whenever slight pH deviation (<0.1) was found from the calculated pH. The buffers were used to dilute *M. lysodeikticus* stock suspension (16 mg/ml in water) to 0.8 mg/ml, and the activity of

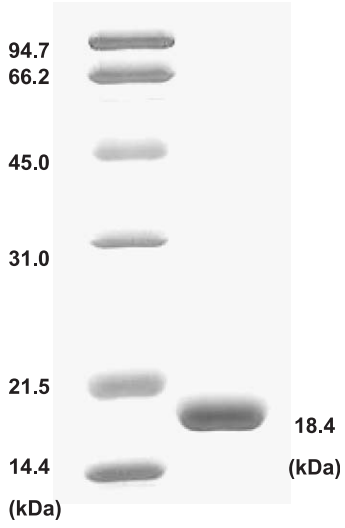

Fig. 1. SDS-PAGE of purified oyster (*C. virginica*) plasma lysozyme and protein molecular markers after staining with Coomassie blue. The molecular mass of oyster lysozyme under reducing conditions was estimated at 18.4 kDa. Both lanes contained about 2.5 μg of protein.

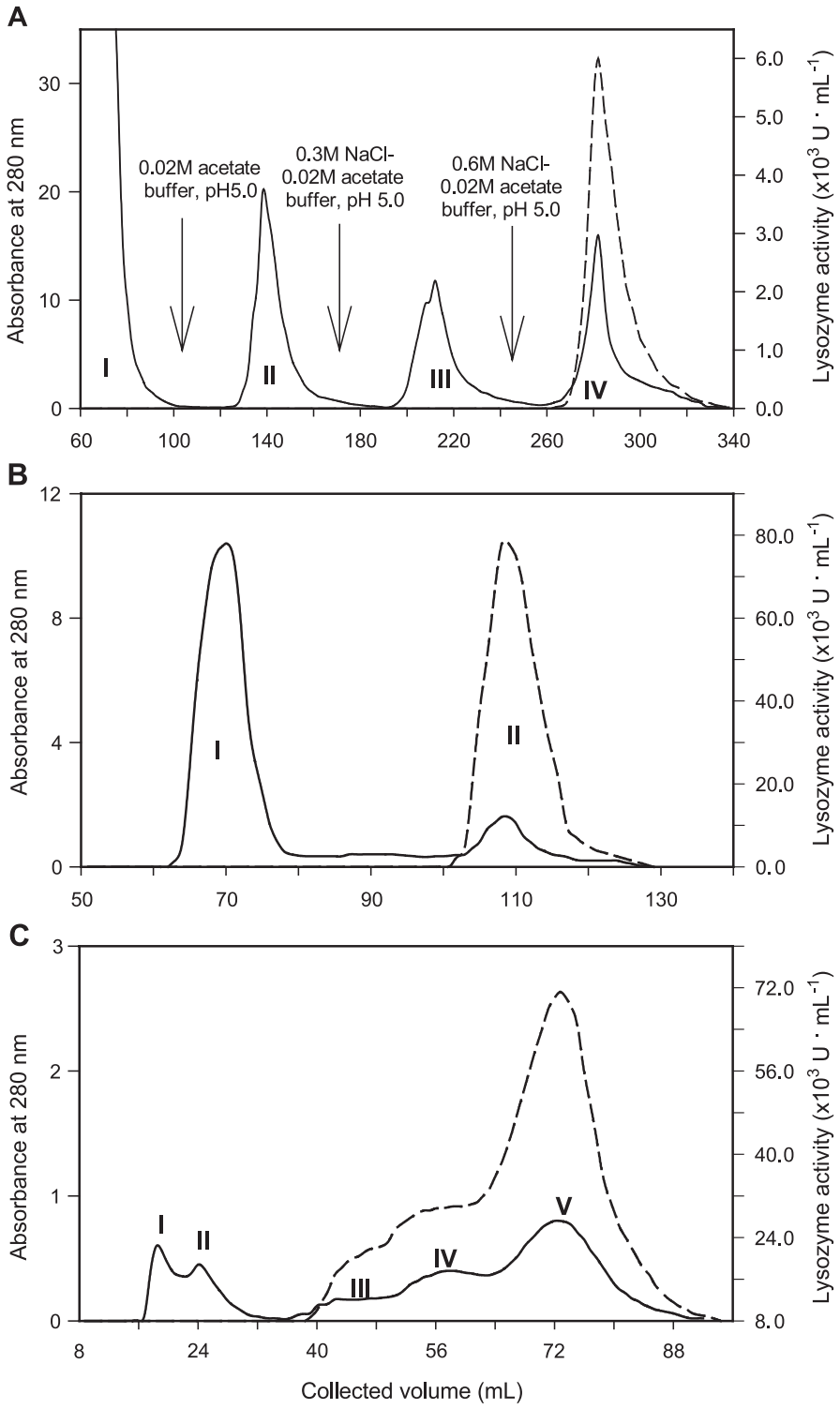

Fig. 2. Purification of plasma lysozyme from eastern oysters (*C. virginica*) by ion exchange and gel filtration chromatographies. (A) Ion exchange chromatography of crude plasma lysozyme sample on CM-Sepharose FF column after a stepwise elution with NaCl. Fractions covered by peak IV were recovered and pooled as 'lysozyme-enriched IE sample'. (B) Gel filtration chromatography of lysozyme-enriched IE sample on Superdex G-75 column. Fractions in peak II were collected and pooled as 'lysozyme GF-separated sample'. (C) Ion exchange chromatography of lysozyme GF-separated sample on CM-Sepharose FF column. The column was eluted with a linear gradient of NaCl from 0.3 M to 0.7 M in 0.02 M acetate buffer at pH 5.0. Fractions from peaks IV and V were pooled and designated 'purified lysozyme', because they showed a single protein band with the same molecular weight by SDS-PAGE and Coomassie blue staining. Eluted proteins (solid line) were monitored at an absorbance of 280 and lysozyme activity (broken line) was measured against *M. lysodeikticus* suspension.

Table 1
Summary of the oyster (*C. virginica*) lysozyme purification

Sample	Total protein (mg)	Total activity (U)	Specific activity (U/mg protein)	Yield (%)
Crude plasma lysozyme sample	4240.0	7.55×10^5	1.78×10^2	
Lysozyme-enriched IE sample	28.8	5.16×10^5	1.79×10^4	68.34
Lysozyme GF-separated sample	6.4	4.00×10^5	6.25×10^4	52.98
Purified oyster lysozyme	1.0	1.52×10^5	1.52×10^5	20.13

lysozyme at 5 μg/ml was measured as described earlier. Results were expressed as percent activity of the highest activity measured in the experiment. All measurements in microplates were done in triplicates.

The pH stability was tested by diluting the lysozyme stock solution to 50 μg/ml in buffers of pH 2.0 to 13.0 in increments of one pH unit. All buffers had ionic strength of I=0.180. Following incubation for 10 and 30 min, respectively, at each pH, the lysozyme was diluted to 5 μg/ml in 0.18 M ammonium acetate buffer, pH 5.5, and the activity measured as described earlier. Results were expressed as percent activity with the activity from lysozyme diluted directly to 5 μg/ml with 0.18 M ammonium acetate buffer, pH 5.5, as 100%. All microplate measurements were done in triplicates.

2.10. Determination of the effects of cations and seawater

The effects of Na^+, Ca^{2+}, and Mg^{2+} on oyster lysozyme activity were determined by adding NaCl, $CaCl_2$, or $MgCl_2$ at different concentrations to *M. lysodeikticus* in 0.1 M ammonium acetate buffer at pH 5.5 and an ionic strength of 0.100. The selection of a buffer at suboptimal ionic strength (0.100) allowed the demonstration of changes in activity due to salts, above or below an intermediary level of activity. NaCl was added to the final concentrations of 0.025, 0.050, 0.075, 0.100, 0.125, 0.150, 0.200, 0.250, 0.300, 0.350, and 0.400 M, and $CaCl_2$ or $MgCl_2$ were added to the final concentrations of 0.005, 0.010, 0.015, 0.020, 0.025, 0.030, 0.035, 0.040, 0.045, 0.050, and 0.055 M. Two sets of ammonium acetate buffer at pH 5.5 were also prepared with increasing concentrations of ammonium acetate to be used as reference buffers for the increase in ionic strength. One set of buffers with ionic strengths of 0.125, 0.150, 0.175, 0.200, 0.225, 0.250, 0.400, 0.450, and 0.500 was used as a reference for changes in ionic strength caused by the addition of NaCl. Another set of buffers with ionic strength of 0.115, 0.130, 0.145, 0.160, 0.175, 0.190, 0.205, 0.220, 0.235, 0.250, and 0.265 was used as reference for changes in ionic strength caused by the addition of $CaCl_2$ or $MgCl_2$. All of these solutions were used to prepare *M. lysodeikticus* suspension. The lytic activity of lysozyme at a final concentration of 5 μg/ml on *M. lysodeikticus* suspensions was measured in microplate in triplicates as described above. Results were expressed as the percent of the activity of lysozyme on *M. lysodeikticus* in 0.1 M ammonium acetate buffer at pH 5.5 and an ionic strength of 0.100 which was taken to represent 100%.

The effects of seawater were tested by measuring the lytic activity of purified oyster lysozyme (5 μg/ml in water) on *M. lysodeikticus* in artificial seawater diluted to 2, 4, 6, 8, 10, 15, 20, 25, or 30 parts per thousand (ppt). The salinity of full-strength seawater is about 35 ppt (i.e., 35 g of sea salts per kilogram of water). Eastern oysters thrive in estuarine waters between 5 and 15 ppt and have a wide tolerance to salinity ranging from 5 to 40 ppt (Galtsoff, 1964; Berrigan et al., 1991). Artificial seawater (pH 8.5) was prepared with *hw* Marinemix *Professional* sea salts (Hawaiian Marine Imports, Houston, TX), and the salinities adjusted with a refractometer (Aquatic Ecosystems, Apopka, FL). The measurements were performed in microplates and results expressed as percent of the activity of lysozyme on *M. lysodeikticus* in 0.18 M ammonium acetate buffer at pH 5.5. All measurements were performed in triplicates.

2.11. Determination of temperature optimum, thermal stability, and comparison with chicken egg white lysozyme

The effects of temperature on oyster lysozyme were determined using 1.5-ml-capacity disposable microcuvettes with 1 cm light path, and absorbance was measured with a Shimadzu UV 600 spectrophotometer (Shimadzu, Kyoto, Japan). This larger volume was used to minimize temperature variation during measurements of lysozyme activity

Fig. 3. Amino acid sequence of purified oyster (*C. virginica*) plasma lysozyme. (A) N-terminal sequence determined by automatic Edman degradation. Purified lysozyme was submitted to Edman degradation analysis both in liquid form and after being electroblotted onto PVDF membrane. The resulting sequences from the two preparations were identical. N-terminal sequences of bivalve species other than *C. virginica* were according to references of Jolles (1996) for *Calyptogena* sp. and *M. galloprovincialis*, Nilsen et al. (1999) for *C. islandica*, and Ito et al. (1999) for *T. japonica*. (B) Sequences of oyster lysozyme tryptic peptides determined by tandem mass spectrometry. (C) Alignment of amino acid sequences of purified plasma lysozyme with that of four bivalve mollusc species. Peptide sequences were subjected to BLAST searches of the National Center for Biotechnology Information (NCBI) GenBank database. Seven oyster lysozyme peptide sequences were aligned with the sequences of four bivalve mollusc lysozymes obtained from GenBank, from *Calyptogna* sp. (Bachali et al., 2002—GenBank accession no. AF334667), from the Mediterranean mussel, *M. galloprovincialis* (Bachali et al., 2002—GenBank accession no. AF334665), from the Iceland scallop, *C. islandica* (Nilsen et al., 1999—GenBank accession no. CAC34834), and from the Manila clam, *T. japonica* (K. Takeshita et al., unpublished—GenBank accession no. AB091383) aligned by Bachali et al. (2002). The boxed region encompasses the aligned sequences. Identical residues shared by all sequences are in boldface. The first three N-terminal amino acids determined by Edman degradation are shaded.

at different temperatures. The enzyme temperature optimum was determined by diluting *M. lysodeikticus* stock suspension to an absorbance of 0.7 at 450 nm with 0.18 M ammonium acetate buffer at pH 5.5 and equilibrated to temperatures of 0 to 70 °C in increment of 5 °C. The reaction was initiated by mixing 0.1 ml of purified lysozyme (2 μg/ml in water) with 1.4 ml of *M. lysodeikticus* suspension at each temperature and measuring the absorbance at 450 nm every 20 s for 2 min. The decrease in absorbance per minute was calculated and used

A. N-terminal sequences of lysozyme from *C. virginica* and other bivalve species by Edman degradation:

C. virginica	SDAPCLRAGGRCQHDSITCSGRTRTGLCSGGVRRRCCVPSSSN
Calyptogena sp.[1]	FAQGMVSQACLRCICLRESKPV
C. islandica[2]	AHNFATGIVPQ(/G)S(/A)C(/Y)LEC(/N)ICR(/K)TEGG(/S)CRAIGDK
M. galloprovincialis[1]	GLVSDKCMRCICMVES
T. japonica[3]	FAPGMVSQKCLLCMCKLESGGCKPIGCRMDVGS

B. Trypsin-cleaved fragment sequences of *C. virginica* lysozyme:

Peptide MW	Sequence
817.4	SDAPCLR
1319.8	CQHDSITCSGR
2536.6	CCVPSSSNSGSFSTGMVSQKCLR
1253.4	CICNVES (partial)
1610.6	AYWIDCGSPGGDWK
2845.8	AYWIDCGSPGGDWKTCANNIACSSR
997.5	CVQAYMAR
1360.8	SGCSNSCESFAR
1125.0	NSNTEGYWR

C. Amino acid sequence alignment

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C. virginica                    SDAPCLRAGGRCQHDSITC SGRTRTGLCS GGVRRRCCVP
Calyptogena sp.                                     LVYKQNVSSIG KVFESWYEDI
C. islandica                                                MMYFVLLCLLATG
M. galloprovincialis      MMTELKMSVALFFALLC GLNVCCGLKE IVESYKVEFE QREVDVESEG
T. japonica                                                           METV

C. virginica        SSSNSGSFST GMVSQKCLRC ICNVES
Calyptogena sp.     SDDEGFTFAQ GMVSQACLRC ICMRES*SC* MPIGCVMDVG SLSCGYFQIK
C. islandica        TTYGAHNFAT GIVPQSCLEC ICKTES*GC* RAIGCKFDVY SDSCGYFQLK
M. galloprovincialis LVSGDLNESN GLVSDKCMRC ICMVES*HCN NNIGCRMDVG SLSCGPFQIK
T. japonica         SVEEGLDFAP GMVSQKCLLC MCKLESGGC* KPIGCRMDVG SLSCGYFQIK

C. virginica         AYWIDCGSPGGDWKTCANN IACSSRCVQA YMAR    SG CSNSCESFAR
Calyptogena sp.     KAYWIDCGL* **DWKTCAND ITCSSTCVQN YMKRYAVHYR CPLICEGFAR
C. islandica        QAYWEDCGRP GGSLTSCADD IHCSSQCVQH YMSRYIGHTS CSRTCESYAR
M. galloprovincialis KAYWIDCGQP KGDYKACAND YACAYNCIET YMARYIGHSG CPKGCESYAR
T. japonica         QPYWIDCGKP GKDWKSCSND INCSSKCVQQ YMKRYATHYR CPLNCEGFAR

C. virginica                NSNTWNYW R
Calyptogena sp.     EHNGGPRGCH NSNTWNYW LAVQRVPGCK GVQ        147
C. islandica        LHNGGPHGCE HGSTLGYW GHVQGHGC              137
M. galloprovincialis IHNGGPRGCT NPNTIGYW NKIKQQGCTI YS        176
T. japonica         EHNGGPNGCH SSRTLKYW ELLQKIPGCK GVKFS      136
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to compare activities at different temperatures. Results were expressed as percent activity relating to the highest activity in the experiment. All measurements were performed in triplicates.

Thermal stability of the purified lysozyme was tested by incubating lysozyme (2 μg/ml in water) at 20, 40, 60, 80, or 100 °C for 10 and 30 min separately. The lytic activity on *M. lysodeikticus* was then measured at 20 °C. The results were expressed as the percent of the activity of lysozyme maintained on ice during the duration of the experiment and measured at 20 °C. All measurements were performed in triplicates.

The lytic activities of purified oyster lysozyme (2 μg/ml in water) and chicken egg white lysozyme (HEWL, 10 μg/ml in water) on *M. lysodeikticus* were compared at 10, 15, 20, 25, 30, 35, and 40 °C using 1.5-ml-capacity disposable microcuvettes as described above. The enzyme concentrations were selected to give the same activity for oyster lysozyme and HEWL at 20 °C. Results were expressed as percent activity relating to the activity of each enzyme at 20 °C.

2.12. Antibacterial activities

The concentrations of oyster lysozyme inhibiting the growth of three Gram-positive bacteria (*Lactococcus garvieae*, *Streptococcus iniae*, *Enterococcus* sp.) and four Gram-negative bacteria (*Escherichia coli*, *Vibrio vulnificus*, *Aeromonas hydrophila*, *Edwarsiella ictaluri*) were determined. Bacterial species were obtained from Dr. John Hawke, Dr. Richard Cooper, or the late Dr. Ronald Siebeling at the Louisiana State University, Baton Rouge. All bacteria were grown in nutrient broth containing 5 g beef extract, 2 g neopeptone, 0.1 g bactose dextrose, 1 g yeast extract, and 10 g NaCl per liter of water and were harvested in log phase. The bacteria were resuspended in phosphate buffer saline (PBS) to a density of about 10^7 bacteria/ml, and 20 μl were added to 20 μl of twofold serially diluted lysozyme (400–0.4 μg/ml) in PBS or to 20 μl of PBS alone (control) in 96-well plates in duplicate wells. After 2 h of incubation at RT, 160 μl of nutrient broth were added to each well, and the plates were incubated at 28 °C. Bacterial growth was measured at 640 nm with a microtiter plate reader (Chantilly, VA) at 12 h for *E. coli* and *V. vulnificus*, 24 h for *A. hydrophila* and *E. ictaluri*, and 36 h for the slower growing Gram-positive *L. garvieae*, *S. iniae*, *and Enterococcus* sp. Results were expressed as the minimum concentration of lysozyme which significantly inhibited bacterial growth compared to control (PBS only). The experiment was repeated twice.

2.13. Statistical analysis

Data on the effects of each cation on lysozyme activity compared to the activity of lysozyme in appropriate control buffer were analyzed by paired *t* tests. All other data were analyzed by one- and two-factor analysis of variance, followed by SNK's multiple comparison of means when significant differences ($p<0.05$) were found.

3. Results

3.1. Lysozyme purification, molecular mass by SDS-PAGE and isoelectric point

A protein with high lytic activity against *M. lysodeikticus* was purified from oyster plasma by a combination of ion exchange and gel filtration chromatographies. The protein appeared as a single band with a molecular mass of 18.4 kDa determined by SDS-PAGE and Coomassie blue staining (Fig. 1). Sixty eight percent of the lysozyme from the crude plasma lysozyme sample was recovered following the first ion exchange chromatography, and the specific activity of the lysozyme enriched IE sample (peak IV, Fig. 2A) was 100-fold greater than that of the crude plasma lysozyme sample (Table 1). This first ion exchange chromatography was quite effective as a first purification step, because the bulk of the nonlysozyme plasma proteins was eluted during sample loading and column washing with 0.02 M sodium acetate buffer at pH 5.0 (peak I, Fig. 2A; whole peak range was not shown). Some proteins bound to CM-Sepharose Fast Flow were washed down with 0.1 and 0.3 M NaCl, but no lysozyme activity was detected in these fractions (peak II and III, Fig. 2A). Gel filtration chromatography of the lysozyme-enriched IE sample yielded lysozyme GF-separated sample with a 3.5 greater specific activity (Table 1, peak II, Fig. 2B). Finally, several peaks were observed when proteins of the lysozyme GF-separated sample were purified by ion exchange using a linear NaCl gradient elution (Fig. 2C). Lysozyme activity was detected in peaks III–V (Fig. 2C). Fractions from peaks IV and V were pooled and designated purified oyster lysozyme, because they showed a single protein band with the same molecular mass by SDS-PAGE and Coomassie blue staining. Fractions from peak III showed three proteins and were not used. The purified lysozyme had a specific activity 854 times greater than the specific activity of the crude oyster plasma preparation (Table 1). About 1.0 mg of purified lysozyme was obtained from 800 ml of oyster plasma which represented 20% of lysozyme from the original plasma sample (Table 1). The isoelectric point of purified lysozyme was greater than 10, the highest isoelectric point that could be measured the ampholytes used.

3.2. N-terminal amino acid sequence

The N-terminal sequence of the purified oyster plasma lysozyme was analyzed to the 43rd amino acid residue by

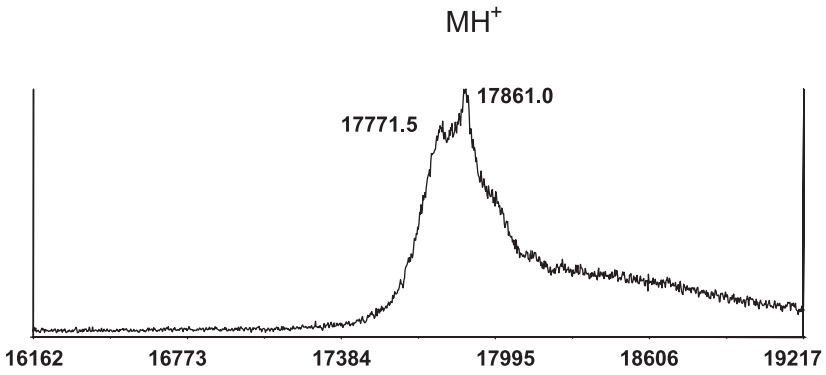

Fig. 4. Molecular mass determination of purified oyster (*C. virginica*) lysozyme by MALDI-MS. Purified lysozyme was mixed with matrix and molecular mass measurements made by MALDI-MS. Two signals, *m*/*z* 17771.5 and *m*/*z* 17861.0, were observed in a broad peak indicating possibly two forms of the protein.

automatic Edman degradation (Fig. 3A). Both the liquid sample and the PVDF membrane sample showed the same amino acid sequence. No similarity was found between the N-terminal sequence of oyster plasma lysozyme and other sequences from GenBank.

3.3. Primary amino acid sequence using tandem mass spectrometry

Mass spectrometric analysis yielded high-quality sequence information on nine tryptic peptides covering approximately 60% of the lysozyme sequence based on the number of residues in homologous species (Fig. 3B). Three tryptic peptides contained sequences identified by Edman degradation, and when the Edman sequence is combined with the tandem mass spectrometry sequence, approximately 70% of the sequence is covered. The protein sequence data reported in this paper will appear in the SWISS-PROT and TrEMBL knowledgebase under the accession no. P83673. The high-sequence homology observed allowed positive identification of a lysozyme via BLAST searching of interpreted sequences (Fig. 3C). Note

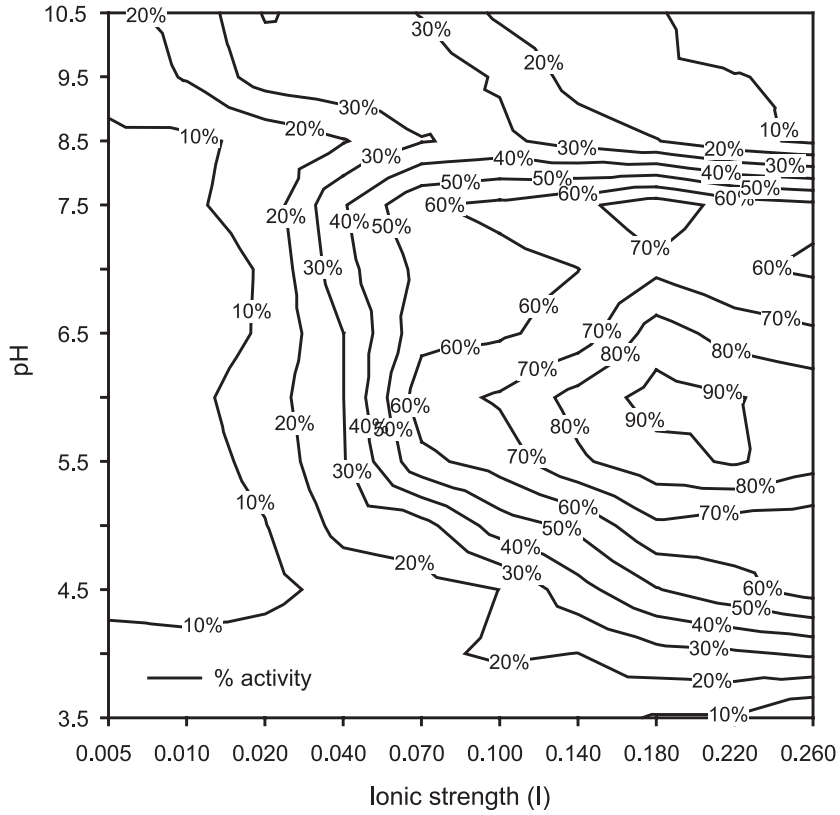

Fig. 5. Activity of purified oyster (*C. virginica*) plasma lysozyme as a function of pH and ionic strength. Data were obtained on the activity of purified lysozyme in 120 buffers covering a pH ranging from 3.5 to 10.5 and ionic strength ranging from I=0.005 to 0.280. A maximum lysozyme specific activity of 1.76×10^5 U/mg protein was observed at a combination of pH 5.9 and I=0.180. Activities were expressed as a percentage of that observed at maximum activity and a contour plot of lysozyme activity in 10% increment was generated.

that tandem mass spectrometry, as performed in this study, cannot distinguish between isoleucine/leucine and lysine/glutamine residues; therefore, assignments were made on sequence homology. Two molecular ions 17771.5 and 17861.0 Da were observed by MALDI for purified lysozyme, indicating two potential forms of the enzyme (Fig. 4).

3.4. pH and ionic strength optima

Optimal pH and ionic conditions for the purified lysozyme were observed at pHs between 5.5 and 6.0 and ionic strengths between I=0.180 and 0.200. Within this range of pHs and ionic strengths, the purified lysozyme expressed more than 90% of its maximum activity (Fig. 5). Lysozyme activity remained relatively high within a much broader pH and ionic strength ranges. Lysozyme retained 60% of its maximum activity within the pH range of 5.0–7.5 and ionic strength range of I=0.070–0.260 (Fig. 5). Preincubation of purified lysozyme in buffers of pH 2–13 for 10 or 30 min had no effect on its activity.

3.5. Effects of cations and seawater

Lysozyme activity increased as the NaCl concentration was increased from 0 to 0.1 M and then decreased from its maximum activity at 0.1 M NaCl with further increase in NaCl concentrations (Fig. 6A). The lowest activity was detected at NaCl concentrations greater than 0.25 M (Fig. 6A). The same trend was observed when lysozyme activity was measured in the reference ammonium acetate

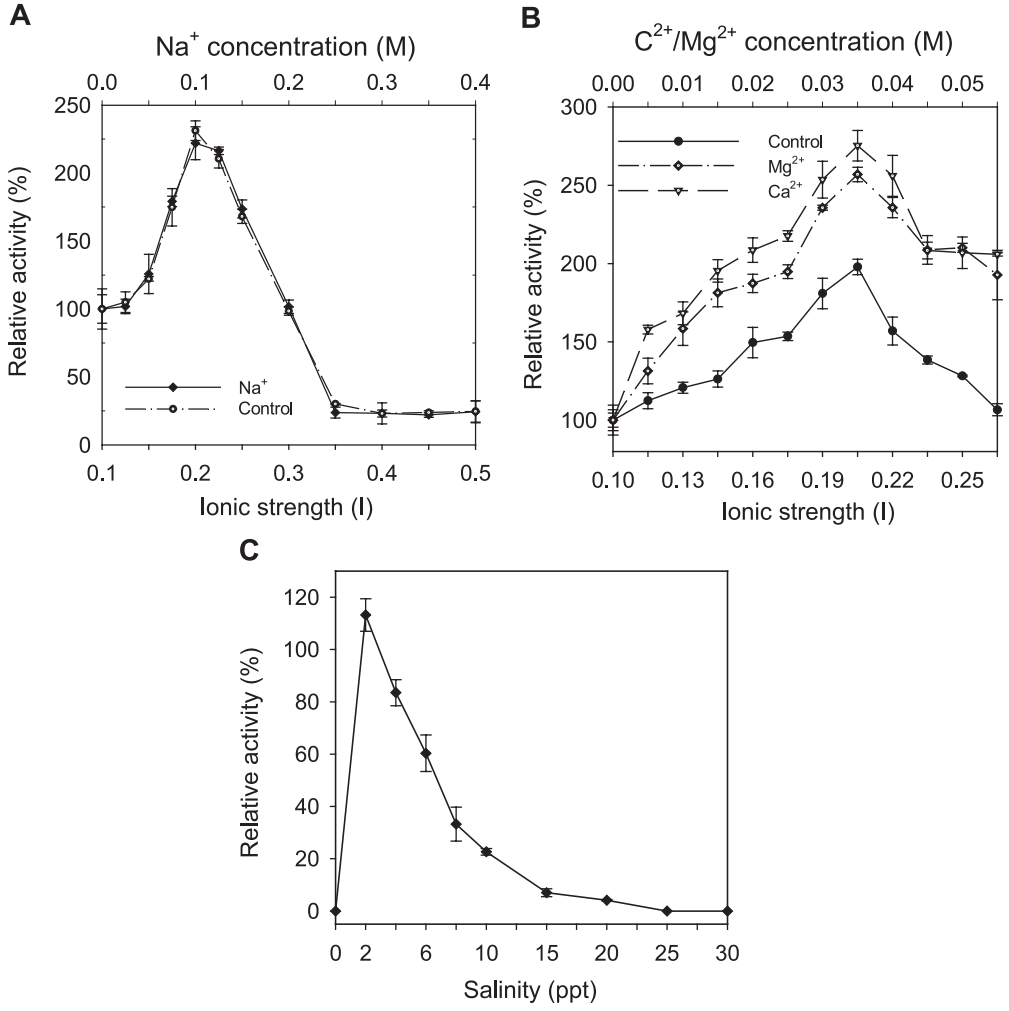

Fig. 6. Effects of cations and seawater on the activity of the purified oyster (*C. virginica*) plasma lysozyme. (A) Effects of Na^+ and (B) effects of Ca^{2+} and Mg^{2+}: in both cases, effects were observed by measuring the activity of lysozyme in 0.1 M ammonium acetate buffer at pH 5.5 supplemented with NaCl, $CaCl_2$, or $MgCl_2$ at different concentrations. Controls were two sets of ammonium acetate buffer at pH 5.5 with the ionic strengths corresponding to the buffers after addition of Na^+, Ca^{2+}, or Mg^{2+}. The 100% activity in both experiments represented a specific lysozyme activity of 7.4×10^4 U/mg protein in 0.1 M ammonium acetate buffer at pH 5.5. (C) Effects of diluted seawater at different salinities; full-strength seawater has a salinity of 35 ppt. The activity of lysozyme in 0.18 M ammonium acetate buffer at pH 5.5 was used to represent 100% and had a specific activity of 1.42×10^5 U/mg protein.

buffers. The activity of purified lysozyme measured in reference buffers was the same as the activity of lysozyme measured in buffers containing NaCl at each ionic strength (Fig. 6A). In contrast, the activity of lysozyme in buffers containing $CaCl_2$ and $MgCl_2$ were significantly higher than the activity of lysozyme in the reference buffers at each ionic strength ($p<0.01$; Fig. 6B). Lysozyme activity in $CaCl_2$-containing buffers increased by 45.38–99.22% relative to lysozyme activity in reference buffers. The activity of lysozyme in $MgCl_2$-containing buffers increased by 19.01–86.05% relative to the activity of lysozyme in reference buffers. Maximum lysozyme activity was measured in buffers with an ionic strength of I=0.205 regardless of the presence or absence of $CaCl_2$ or $MgCl_2$ (Fig. 6B).

No lytic activity was detected when the purified lysozyme was in distilled water (Fig. 6C). Lysozyme activity was highest when measured in seawater diluted to 2 ppt. At 2 ppt, lysozyme activity was about 20% higher than lysozyme activity in the reference buffer (0.18 M ammonium acetate, pH 5.5). Lysozyme activity decreased rapidly with further increase in seawater salinity. Lysozyme activity measured at 10 ppt was reduced to 20%, and activity was not detected at 25 ppt (Fig. 6C).

3.6. Temperature optimum, thermal stability, and temperature effects

Activity of the purified lysozyme increased with increasing temperature from 0 to 45 °C and decreased markedly at temperatures greater than 55 °C (Fig. 7A). No decrease in activity was measured after incubation of lysozyme at 20 °C for 10 and 30 min, and no activity was detected after a 30 min incubation of lysozyme at 100 °C (Fig. 7B). In the temperature range of 10 to 40 °C, the activity of oyster lysozyme was significantly less affected by temperature change than the activity of hen egg white lysozyme (Fig. 7C). The activity of oyster lysozyme at 10 °C was 68.3%±2.7% of the activity at 20 °C, and at 40 °C, 138.9%±2.2% of its activity at 20°C (Fig. 7C). In contrast, the activity of hen egg white lysozyme at 10 and 40 °C was 51.7%±3.5% and 170.2%±5.5%, respectively, of the activity at 20 °C.

3.7. Antibacterial activities

Purified lysozyme at concentrations of 0.8 and 3.1 μg/ml significantly inhibited the growth of two Gram-positive bacteria, *L. garvieae* and *Enterococcus* sp., respectively. The purified lysozyme at concentrations of 6.3 and 25 μg/ml significantly inhibited the growth of two Gram-negative bacteria, *E. coli* and *V. vulnificus*, respectively. The growth of *Aeromona hydrophila* was inhibited only at a high concentration of 400 μg/ml. No growth inhibition of *S. iniae* and *Edwardsiella ictaluri* by purified lysozyme was

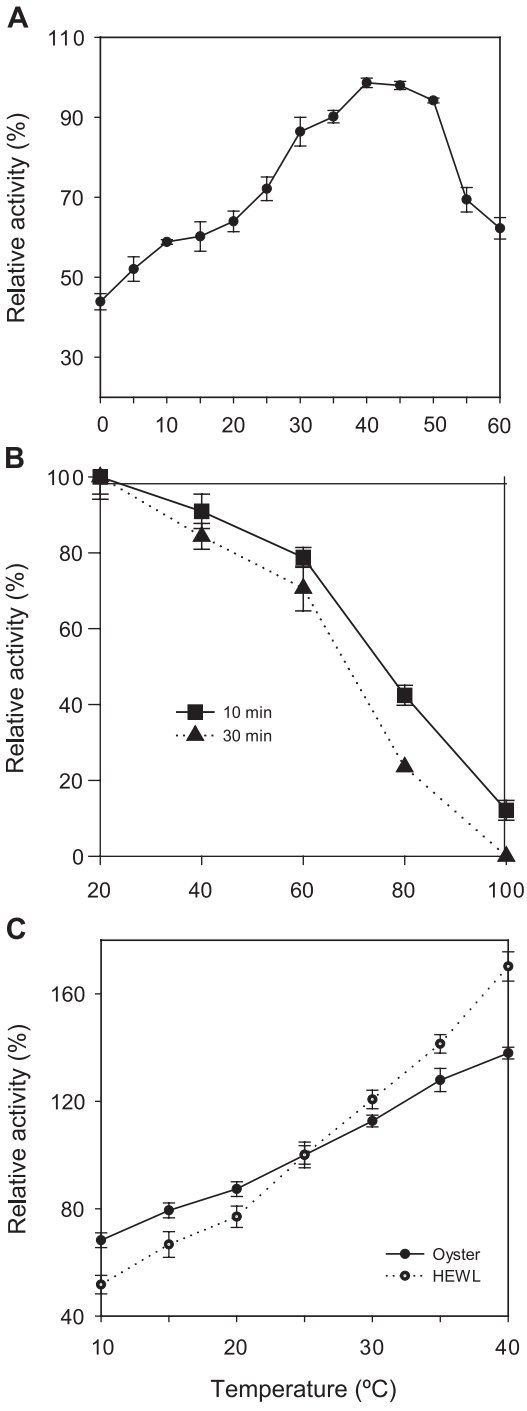

Fig. 7. Effects of temperature on the activity of purified oyster (*C. virginica*) plasma lysozyme. Assays were carried out using 1.5-ml-capacity disposable microcuvettes with a 1 cm light path in a Shimadzu UV 600 spectrophotometer. *M. lysodeikticus* stock suspension was diluted to an absorbance of 0.7 at 450 nm in ammonium acetate buffer at 0.18 M and pH 5.5 to measure lysozyme activity. (A) Optimal temperature: the maximum activity was observed at 40 °C, which represented 100% activity. (B) Thermal stability: lysozyme activity at 20 °C was used to represent 100% activity. (C) Comparison of activities of purified oyster lysozyme and hen egg white lysozyme (HEWL) between 10 and 40 °C; activity level at 20 °C for both lysozymes were used to represent 100% activity.

Table 2
Antibacterial activities of purified oyster (*C. virginica*) lysozyme

	Minimum inhibitory concentration (μg/ml)
Gram-positive	
Lactococcus garvieae	0.8
Enterococcus sp.	3.1
Streptococcus iniae	>400
Gram-negative	
Escherichia coli	6.3
Vibrio vulnificus	25
Aeromona hydrophila	400
Edwardsiella ictaluri	>400

noted at the maximum concentration tested (400 μg/ml; Table 2).

4. Discussion

A protein with high lytic activity against *M. lysodeikticus* was purified from the plasma of eastern oysters by a combination of ion exchange and gel filtration chromatographies as indicated by SDS-PAGE and N-terminal sequencing. The purified protein lytic activity against *M. lysodeikticus*, low molecular mass, high isoelectric point, and heat stability suggested the protein was a lysozyme (Jolles, 1969). Mass spectrometric sequence analysis confirmed that the purified protein was a lysozyme and that it belonged to the invertebrate type of lysozymes (*i*-type). This is a first report of a lysozyme purified from an oyster species and from the plasma of a bivalve mollusc.

No similarity was found between the N-terminal amino acid sequence of purified oyster plasma lysozyme and N-terminal amino acid sequences of other purified *i*-type lysozymes (Jolles and Jolles, 1975; Jolles et al., 1996; Fradkov et al., 1996; Ito et al., 1999; Nilsen et al., 1999). Primary amino acid sequencing of the purified protein using tandem mass spectrometry, however, confirmed the N-terminal amino acid sequence obtained by Edman degradation. Moreover, the sequence of a 2536.6-MW tryptic peptide (i.e., CCVPSSSNSGSFSTGMVSQKCLR) we obtained, overlapped with the N-terminal amino acid sequence of our purified protein determined by Edman degradation (i.e., CCVPSSSN–) and showed sequence homology with *i*-type lysozymes (i.e.,–GMVSQKCLR). Our study suggests the N-terminal amino acid sequence of *i*-type lysozymes may vary to a greater extent between species than reported in earlier studies and may not always be a reliable criterion to identify *i*-type lysozymes. It is noteworthy that the N-terminal sequences of *i*-type lysozymes described in earlier studies have all been for proteins about a third smaller (~ 13 kDa) than oyster plasma lysozyme (~ 18 kDa; Jolles et al., 1996; Ito et al., 1999; Nilsen et al., 1999; Zavalova et al., 2003). Amino acid sequencing of purified oyster lysozyme by Edman degradation and mass spectrometry identified 49 amino acids preceding the sequence—GMVSQKCLRCICNVES—that shows a strong similarity to the N-terminal amino acid sequences of other *i*-type lysozymes. The 49 amino acids account for about 30% of the molecular mass of oyster plasma lysozyme and explains its higher molecular mass compared to all other *i*-type lysozymes except for some of the multiple forms of blue mussel (*M. edulis*) lysozymes. It will be interesting to compare the N-terminal amino acid sequence of oyster plasma lysozyme to that of the largest (18, 22 kDa) purified blue mussel lysozymes when their N-terminal sequences are eventually determined (McHenery and Birkbeck, 1979; Olsen et al., 2003).

Mass spectrometry (i.e., MALDI-TOF) analysis of oyster lysozyme indicated two potential forms of the enzyme with molecular masses of 17771.5 and 17861 Da. Multiple forms of lysozymes which showed different biochemical properties have been recently identified in blue mussels and the medicinal leech, *Hirudo medicinalis* (Olsen et al., 2003; Zavalova et al., 2003). The consistent N-terminal amino acid sequence of our sample and the absence of secondary activity optimum under a wide range of conditions, however, suggest the two forms of the enzyme are similar in biochemical properties and differ in sequence by only one or a few amino acids. This is in contrast to secondary pH and ionic strength optima observed for lysozymes isolated from blue mussels and the Manila clam, *Ruditapes philippinarum* (*T. japonica* synonym; McHenery and Birkbeck, 1982; Maginot et al., 1989). Future research is needed to separate the two potential forms of lysozyme to test our hypothesis.

The isoelectric point of our purified lysozyme was greater than 10. The high isoelectric point is typical for most lysozymes which are usually basic proteins (Jolles and Jolles, 1984). Data on the isoelectric points of bivalve mollusc lysozymes are limited. The isoelectric point of lysozyme purified from blue mussels was 9.2 (McHenery and Birkbeck, 1979) and that of lysozyme purified from pismo clams (*Tivela stutorum*) was 7.7 (Montenegro-Ortega and Viana, 2000). In contrast to our results, Feng (1974) reported that lysozyme-like activity from the plasma of eastern oysters was mostly associated with acidic proteins of different electrophoretic mobilities. It is likely that oyster plasma lysozyme, which has a very high isoelectric point and constitutes a fraction of oyster plasma proteins as determined in our study, may have associated with plasma acidic proteins in Feng's study and as a result carried an overall negative charge (McHenery and Birkbeck, 1979). Hen egg white lysozyme, for example, is known to strongly associate with proteins with low isoelectric points (Essink et al., 1985). Alternatively, an acid form of oyster lysozyme may exist as reported for some *i*-type lysozymes. The isoelectric point predicted from the amino acid composition of lysozyme purified from the Icelandic scallop *C. islandica* was 6.9 (Nilsen et al., 1999). Moreover, Zavalova et al. (2003) recently purified a new acid form of destabilase lysozyme from medicinal leeches, while other forms of

destabilase lysozyme were basic proteins. The isoelectric points of *i*-type lysozymes are therefore quite variable and may be related to different functions of the protein (e.g., host defense, digestion).

Optimal pH and ionic conditions for the purified lysozyme were observed at pHs between 5.5 and 6.0 and ionic strengths between I=0.180 and 0.200. This range falls between the pH optima of 6.5 and 5.0 of eastern oyster plasma lysozyme activity reported in two earlier studies (McDade and Tripp, 1967; Cheng and Rodrick, 1974). Differences in the type and molarity of buffers used in the earlier studies likely accounted for the variation in optimal pH. In our study, the optimal activity of the purified oyster lysozyme at each pH tested varied markedly with the ionic strength of the buffer used. The activity of purified lysozyme at low pHs was highest at the highest ionic strengths tested, while the activity of purified lysozyme at high pHs was highest at the lower ionic strengths. A similar phenomenon was observed with hen egg white lysozyme activity (Davies et al., 1969). The optimal pH for hen egg white lytic activity moved from approximately pH 5 to 9 as the ionic strength decreased from I=0.2 to 0.02, and it was hypothesized that binding of lysozyme to the cell wall of *M .lysodeikticus* requires a certain electrostatic condition (Davies et al., 1969). This might be a general phenomenon for lysozymes (Saint-Blancard et al., 1970; Jensen and Kleppe, 1972).

A unique feature of the purified oyster lysozyme activity was its relatively high optimal ionic strength compared to hen egg white lysozyme (Davies et al., 1969). There is, however, limited information to compare our results to the ionic strengths optima of purified lysozymes of other bivalve molluscs. In most studies, in which the effects of ionic strengths or molarities were determined, measurements were not systematically made at different pHs (Ito et al., 1999; Nilsen et al., 1999; Montenegro-Ortega and Viana, 2000; Viana and Raa, 1992). It is important to note that the activity of purified oyster lysozyme remained relatively high within relatively broad pH and ionic strength ranges. Purified oyster lysozyme retained about 60% of its maximum activity within an ionic strength range of I=0.070–0.260 and pH range of 5.5–7.5. Activity of the purified lysozyme in these ranges of ionic strength and pH would be advantageous for eastern oysters because (1) oyster plasma ionic strength can be expected to vary considerably, because oysters thrive in estuaries between 5 and 15 ppt and are osmoconformers (Berrigan et al., 1991; Shumway, 1996); and (2) oyster haemolymph pH has been reported in the range of 6.8 to 8.0 (Jones et al., 1995; Boyd and Burnett, 1999).

The effects of a wide range of concentrations of Na^+, Ca^{2+}, and Mg^{2+}, which are the major cations in oyster plasma, were tested on purified oyster lysozyme activity. Lysozyme activity in the presence of the divalent cations Ca^{2+} or Mg^{2+} (0.005–0.055 M) was always greater than in reference ammonium acetate buffer at each ionic strength tested (I=0.1–0.265). No such increase in lysozyme activity over the reference ammonium acetate buffer was measured in the presence of Na^+ (0.025–0.4 M). In either the presence or absence of the cations tested in ammonium acetate buffer (pH 5.5), the activity of purified oyster lysozyme was always greatest when the final ionic strength was 0.2, thereby confirming our earlier results. Seawater diluted to 10 ppt, which has a similar ionic strength, markedly inhibited the activity of purified lysozyme. A large part of this inhibition was likely due to the high pH of the freshly made seawater used (i.e., pH 8.5). At pH 8.5, the optimal ionic strength for purified lysozyme was about 0.07 and much closer to the ionic strength of diluted seawater at 2 ppt, a salinity at which the activity of purified lysozyme was greatest. Some inhibition of purified lysozyme by seawater components may also have occurred. Lysozyme purified from the crystalline styles of blue mussels was also reported to be very sensitive to seawater (McHenery and Birkbeck, 1982). The activity of lysozyme in oysters, however, would be greater than in seawater, because oyster hemolymph has a lower pH than seawater.

The activity of purified oyster lysozyme was optimal at about 45 °C and in the range reported for other bivalve mollusc lysozymes (McHenery and Birkbeck, 1982; Ito et al., 1999; Nilsen et al., 1999; Miyauchi et al., 2000; Montenegro-Ortega and Viana, 2000). This range varied greatly from a low optimal temperature of 20 °C for lysozyme purified from Icelandic scallops (Nilsen et al., 1999) to a high optimal temperature of 75 °C for lysozyme purified from Manila clams (Ito et al., 1999). An interesting feature of purified oyster lysozyme is that at 5 °C it retained about 50% of its activity observed at 45 °C, indicating a low Q_{10} value for this enzyme. This feature is similar to that observed for lysozyme of the Icelandic scallop (Nilsen et al., 1999). Change in temperature, for example, between 10 and 40 °C, had a much greater effect on the activity of hen egg white lysozyme than on the activity of lysozymes from either eastern oyster (this study) or from the Icelandic scallop (Viana and Raa, 1992).

Purified oyster lysozyme had antibacterial activities against both Gram-positive and Gram-negative bacteria. Information on the antimicrobial properties of lysozymes purified from bivalve mollusks is limited to only two studies, but both reported antibacterial activities against Gram-positive and Gram-negative bacteria (Nilsen et al., 1999; Montenegro-Ortega and Viana, 2000). Lysozyme purified from the Icelandic scallop at moderate concentrations (2–10 μM) inhibited the growth of several Gram-positive bacteria (*Listeria monocytogenes*, *Bacilus cereus*, *Staphylococcus epidermis*, *Enterococcus faecalis*) and Gram-negative bacteria (*E. coli*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Vibrio salmonicida*), which are associated with infection diseases in humans and animals (Nilsen et al., 1999). Montenegro-Ortega and Viana (2000) tested partially purified lysozyme of the pismo clam (*Tivela stultorum*) against the Gram-positive bacteria *Micrococcus luteus*,

Staphylococcus aureus, and *Streptococcus alfa*, and the Gram-negative bacteria *E. coli*, *Pseudomonas putrefaciens*, and *Vibrio parahaemolyticus*, but only found antimicrobial activity against *M. luteus*, *S. alfa*, and *E. coli*. While lysozymes are generally considered more active against Gram-positive bacteria because their cell walls are largely made of peptidoglycan (i.e., 90%), there is increasing evidence that lysozymes are also active against Gram-negative bacteria through mechanisms not related to their enzymatic activity (Pelligrini et al., 1992; During et al., 1999; Ibrahim et al., 2001). It remains to be determined whether oyster lysozyme antibacterial activity is related to its enzymatic activity or to other properties such as its significant positive charge as proposed by Pelligrini et al. (1992) for hen egg white lysozyme.

In summary, an *i*-type lysozyme was purified from the plasma of eastern oysters, and some of its biochemical and antibacterial properties were characterized. This study is a first step towards elucidating the role this enzyme plays in the eastern oyster's host defense or digestion. The antibacterial properties of the purified oyster lysozyme observed in our study imply a possible role of plasma lysozyme in the oyster host defense, but further research is needed to identify its antibacterial mechanism(s) of action. Future identification of the lysozyme gene and characterization of its expression will assist in identifying the protein function in eastern oysters.

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