FINAL REPORT

PURIFICATION OF LYSOZYME FROM SHELL LIQUOR OF EASTERN OYSTER (*CRASSOSTREA VIRGINICA*) AND POTENTIAL COMMERCIAL USE

JEROME F. LA PEYRE

LOUISIANA STATE UNIVERSITY AGRICULTURAL CENTER,
BATON ROUGE, LA 70803

NA03NMF4270092

JANUARY 31, 2006

II ABSTRACT

The feasibility of purifying lysozyme from shell liquor collected during commercial processing (shucking) of oysters was demonstrated and the lysozyme yield in mg protein per liter of shell liquor was determined seasonally over one year. The purified oyster lysozyme was shown to have strong antimicrobial activity against several food spoilage bacteria (*i.e., Enterococcus faecalis*, *Enterococcus faecium, Lactobacillus plantarum, Pediococcus cerevisiae*) and bacteria causing food poisoning in humans (i.e., *Campylobacter coli*, *Campylobacter jejuni*, *Clostridium perfringens*). Purified oyster lysozyme was found not to be allergenic. Our results indicate oyster lysozyme potential for use as a food preservative and a pharmaceutical.

III EXECUTIVE SUMMARY

The development of new and unique products from oysters can help the expansion of the oyster industry which is currently limited to one product, oysters for consumption. There is increasing interest in the potential commercial use of lysozymes from bivalve molluscs including oysters. Lysozymes from bivalve molluscs are generally more active at higher salt concentrations and lower temperature than lysozyme from hen egg white (HEWL) and therefore may be better suited for use in the food and pharmaceutical industries. HEWL has been approved in several countries for use as a natural food preservative. High lysozyme activity is detected in fluid filling the shell cavity of eastern oysters, *Crassostrea virginica*. The combination of this fluid and plasma released when oysters are shucked is commonly referred to as 'shell liquor'. Shell liquor is generally discarded by the oyster industry. In order to evaluate the use of oyster shell liquor, a by-product of oyster processing, as a source of lysozyme to be used by the food processing and pharmaceutical industries we therefore determined 1) if lysozyme could be consistently purified from shell liquor, 2) the antimicrobial activities of purified oyster lysozyme against bacteria causing food spoilage or food poisoning in humans and 3) the allergenicity of purified oyster lysozyme.

Three batches of shell liquor were obtained during summer and fall 2003, and winter and spring 2004 from an oyster processor. Protein and lysozyme concentrations in each shell liquor batch were measured and lysozyme was purified from one batch of shell liquor from each season to calculate yield. Shell liquor protein and lysozyme concentrations were greatest in winter 2004 (i.e., 4.66 ± 1.26 g protein per l, 12.74 ± 2.74 mg lysozyme per l) and spring 2004 (5.94 ± 0.83 g protein per l, 15.45 ± 7.07 mg lysozyme per l). The major lysozyme purified from shell liquor was a 18 kDa enzyme first purified from oyster plasma and designated lysozyme 1. The purification yield was greatest in winter 2004 with 5.27 mg of lysozyme 1 being purified from 1 liter of shell liquor. A scaled-up procedure to purify lysozyme 1 from 300 liters of shell liquor was developed empirically yielding 205 mg of lysozyme 1 with a specific activity of 1.9×10^5 units per mg protein.

The antibacterial activity of purified lysozyme 1 was measured against 19 food spoilage bacteria and bacteria causing food poisoning. Out of the 19 bacteria tested, oyster lysozyme showed significant inhibition of *Clostridium perfringens* at a concentration of 2.5 µg/ml, and *Lactobacillus plantarum, Pediococcus cerevisiae, Campylobacter jejuni Campylobacter coli* at a concentration of 5 µg/ml. *Enterococcus faecium* and *Enterococcus faecalis* were inhibited at a concentration of 20 µg/ml (Table 6). The growth of *Listeria monocytogenes, Salmonella anatum and Lactobacillus viridescens* were inhibited at a concentration of 160 µg/ml. There was no growth inhibition of *Staphylococcus aureus, Bacillus cereus, Yersinia enterocolita, Aerococcus viridans, Pseudomonas fluorescens, Psedomonas aeruginosa, E.coli 0157:H7, Shigella spp* and *Salmonella enteriditis* at the highest lysozyme concentration tested of 160 µg/ml.

Finally, the allergenicity of oyster lysozyme was determined by measuring IgE concentration in sera of mice sensitized and feed purified oyster lysozyme by gavage for 28 days and in sera of control mice which were sham-fed with the amino acid lysine. No IgE was detected in sera of mice fed oyster lysozyme or in sera of control mice as measured by a sandwich enzyme-linked-immunosorbent assay (ELISA) and a radial immunodiffusion test (RID). Results showed that oyster lysozyme did not illicit production of IgE in mice and was therefore not allergenic to mice.

IV PURPOSE

A. Funding priority addressed

The oyster industry of the Gulf of Mexico produces about 50% of all domestic oysters sold in the United States in recent years. Louisiana generally leads in the number of oysters harvested with an average of 10-12 million pounds of shucked meat per year. In 1998, for example, Louisiana oyster production represented 54% of the United States eastern oyster production and 41% of the total United States production (eastern, Pacific, olympia oysters). Its commercial harvests totaled 5,831 metric tons and were valued at over 30 million dollars (NMFS, Fisheries statistics and economic division, www.st.nmf.gov). This multi-million dollar industry employs as many as 5,000 people in coastal Louisiana. As of February 2001, there were 8,731 leases designated for oyster production totaling 419,00 acres. While it is clear that the oyster industry of Louisiana is a valuable industry, it is however limited to one product, oysters for consumption. The development of new and unique products from oysters will help expansion of the oyster industry in Louisiana and other states. Growth of the Louisiana oyster industry which already leads the United States in oyster production can represent a model for growth of oyster industries in other States.

Lysozymes are antimicrobial proteins which are defined as 1,4-$-N-acetylmuramidases cleaving a glycosidic bound between N-acetylmuramic acid and N-acetylglucosamine of peptidoglycan, a major component of bacterial cell walls. Several types of lysozyme which differ in their amino acid composition and their biochemical and antimicrobial properties, have been identified in a wide range of organisms. Lysozyme from chicken egg-white which belong

to the c type (chicken or conventional) is by far the best studied lysozyme. It is used commercially in a variety of food products (e.g., cheeses, wines) as a natural preservative and in pharmaceutical products in most European countries and Japan. It is awaiting regulatory approval in the United States and has been tentatively granted GRAS (Generally Recognized as Safe) status by the FDA. There is however less chicken egg-white lysozyme available on the market than needed.

In contrast to the many examples of the commercial use of chicken egg white lysozyme there is little information on the potential for use of lysozymes from other sources. It has been hypothesized that lysozymes from aquatic species may have inhibitory activity against both gram-negative and gram-positive bacteria but little research has been carried out on these enzymes. Lysozymes of bivalve molluscs have been shown to generally be more enzymatically active at lower temperature, at lower pH and at higher salt concentrations and ionic strengths than chicken egg-white lysozyme and therefore may be better suited for use in the food and pharmaceutical industries. In addition, their specific activities are generally greater than the specific activity of chicken egg-white lysozyme. High lysozyme activity has been detected in the fluid filling the shell cavity of oysters. The combination of this fluid and plasma released when oysters are shucked is commonly referred as 'shell liquor'. Shell liquor is currently discarded by the oyster industry because oysters are washed after shucking to remove dirt and shell fragments before packing for sanitary and aesthetic reasons. Large volumes (i.e., millions of liters) can be collected from the several million liters of raw shucked oysters produced by Gulf oyster processors. This shell liquor could be an ideal source for oyster lysozyme and a potential new by-product for the oyster industry.

Although the United States maintains one of the world's safest food supplies, food-borne illness afflicts between 6.5 million and 33 million Americans every year. *Listeria monocytogenes* for example has been identified as one of the most serious pathogenic microorganism hazards in meat and dairy products along with *Salmonella* sp., *Campylobacter jejuni* and *E. coli* O157:H7 (Sveum, 1993). One of the President Food Safety Initiative's major programs involves the "readiness for new and emerging threats to the US food supply". Continued monitoring of food by the various federal, state, and local agencies should be accompanied by the search for safe compounds to keep microbial contamination of US food supply under check.

The proposed project responded to funding priority 'D. Optimum utilization of harvested resources under Federal or State management'. Specifically the proposed project was meant to develop a usable product from a byproduct of the oyster industry. The development of a novel product for the oyster industry can benefit oyster processors which could sell shell liquor to biotechnology companies to extract oyster lysozyme. This project could be especially beneficial to Louisiana because oyster processing in this state has been in decline since the 1980s. All participants of the oyster industry from fisherman and farmers to wholesalers and processors may eventually benefit if demand for oysters for the specific production of lysozyme is increased.

B. Objectives of the project

The goal of this project was therefore to evaluate the use of oyster shell liquor, a by-product of oyster processing, as a source of lysozyme to be used by the food processing and pharmaceutical industries. The objectives of this proposal were to:

1) Purify lysozyme from oyster shell liquor and determine yield.

2) Evaluate the antimicrobial activity of purified oyster lysozyme against food spoilage bacteria and bacteria causing food poisoning in humans.

3) Determine the allerginicity of oyster lysozyme.

V APPROACH

A. Detailed description of the work performed

Task 1: Collection of shell liquor and determination of protein and lysozyme concentration

Shell liquor was collected at the P & J oyster company processing plant in New Orleans at three different times during summer and fall 2003, and winter and Spring 2004. The volume of shell liquor per sack of oysters was recorded. After centrifugation of the shell liquor from each batch, the supernatant was collected, pooled, and the protein and lysozyme concentrations were measured for statistical comparison. In addition, 300 liters of shell liquor were collected in early 2004 for bulk purification of lysozyme which was used to conduct the antimicrobial and antigenicity assays (i.e., tasks 4 and 5).

Task 2: Purification of oyster shell liquor lysozyme, determination of seasonal yields and bulk purification of lysozyme

Lysozyme was purified from 1 liter of shell liquor collected in summer and fall 2003 and winter and spring 2004. Protein and lysozyme concentrations were measured, at the beginning and the end of the process to calculate and compare seasonal lysozyme specific activities and purification yields. A scaled-up procedure for bulk purification of shell liquor was developed empirically.

Task 3: Acquiring and growing food spoilage bacteria and bacteria causing food poisoning

Bacteria species (i.e., *Bacillus cereus*, *Campylobacter coli*, *Campylobacter jejuni*, *Clostridium perfringens*, *E. coli* 0157:H7*, Listeria monocytogenes*, *Salmonella anatum*, *Salmonella enteriditis*, *Shigela* spp.*, Staphylococcus aureus*, *Yersinia enterocolita*,) which account for the majority of food poisoning cases in the United States and food spoilage bacteria

(*Aerococcus viridans*, *Enterococcus faecalis*, *Enterococcus faecium, Lactobacilus viridescens*, *Lactobacilus plantarum*, *Pediococcus cerevisiae, Pseudomonas fluorescens* and *Pseudomonas aeruginosa*) were acquired from various sources, cultured and used to test the activity of purified shell liquor lysozyme.

Task 4: Determine oyster lysozyme minimum antibacterial concentrations (MIC).

The antibacterial activity of the purified lysozyme was measured against 19 food spoilage bacteria and bacteria causing food poisoning and results were expressed as the minimum concentration of oyster lysozyme which significantly inhibited bacterial growth compared to control.

Task 5: Determine the allerginicity of oyster lysozyme

The allergenicity of oyster lysozyme was determined by measuring IgE concentration in sera of mice which were sensitized and feed purified oyster lysozyme for 28 days and in sera of control mice which were sham-fed with the amino acid lysine. Radial immunodiffusion (RID) was also used to qualitatively identify the presence of IgE in all mice sera.

B. Project management

Task 1: Collection of shell liquor and determination of seasonal shell liquor protein and lysozyme concentrations

Personnel: Task 1 was performed by personnel of the P&J oyster company processing plant, co-PI Dr. Xue and PI Dr. La Peyre, both from the Department of Veterinary Science at the Louisiana State Agricultural Center.

Collecting shell liquor: P&J Oyster Company plant personnel collected all fluids (i.e., shell cavity fluid and blood) released from oysters during shucking at three different times during summer and fall 2003, and winter and Spring 2004 (Table 1). The volume of the fluid, designated as shell liquor, collected from each sack (about 40 kg) of oysters was recorded. The shell liquor was placed in 4 L bottles in coolers filled with ice and transported to the department of Veterinary Science at LSU in Baton Rouge.

Table 1: Date of collection for determining seasonal shell liquor protein and lysozyme concentrations

Batch	Date collected
Summer-1	5/15/03
Summer-2	5/29/03
Summer-3	6/16/03
Fall-1	10/16/03
Fall-2	11/6/03
Fall-3	11/11/03
Winter-1	1/24/04
Winter-2	2/18/04
Winter-3	2/27/04
Spring-1	4/6/04
Spring-2	4/28/04
Spring-3	5/12 /04

Shell liquor processing: The shell liquor was then centrifuged at 4,000 g for 30 min to remove any shell fragments and pieces of tissues cut from the oyster meats during shucking. The supernatant was collected, pooled and the protein and lysozyme concentration were measured.

Measuring protein concentration: Shell liquor protein concentration was measured using the Micro BCA Protein Assay (Pierce Biotechnology, Rockford, IL). All measurements were carried out in triplicates.

Measuring lysozyme activity and concentration: Shell liquor lysozyme activity was measured spectrophotometrically according to the method of Xue et al. (2004). Lysozyme concentration was then calculated using oyster lysozyme purified from plasma as a standard (Plasma and shell liquor lysozymes amino acid sequences were identical). Briefly, 20 µl of oyster shell liquor sample was mixed with 180 µl of *Micrococcus lysodeikticus* suspended in 0.2 M acetate buffer at pH 5.8 in a 96-well microplate at room temperature. The absorbance of the mixture 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 per min was calculated. All measurements were done in triplicates. One unit of lysozyme was defined as that quantity which causes a decrease in absorbance of 0.001 per min of *Micrococcus Lysodeikticus* suspended in 0.2 M acetate buffer at pH 5.8. Lysozyme concentration in shell liquor was then calculated using purified plasma lysozyme with a specific activity of 1.5×10^5 U/mg to construct a standard curve.

Statistical Analysis: Seasonal shell liquor protein and lysozyme concentration data were analyzed with a one factor analysis of variance (ANOVA) using SAS version 8.0 software (SAS Institute, Inc., Cary, North Carolina, USA). LSMeans with a Tukey adjustment was used following significant ANOVA results ($p<0.05$) to examine differences in protein or lysozyme

concentrations between seasons. All data are reported as mean ± standard deviation. Data were log transformed to achieve normality and homogeneity of variance.

Task 2: Seasonal and bulk purification yield of lysozyme from shell liquor

Personnel: Task 2 was performed by Dr. Xue and Shreya Datta, a graduate student in the department of Food Science at the Louisiana State Agricultural Center.

Determining seasonal yield of lysozyme purified from shell liquor: Lysozyme was purified from 1 liter of shell liquor collected in summer and fall 2003 and winter and spring 2004. The project's protocol to purify lysozyme from shell liquor was as originally described by Xue et al. (2004) except for a slight modification as described below. Oyster shell liquor was desalted by dialysis against distilled water for 6 hours, with two water changes. The sample was then dialyzed against 20 mM sodium acetate buffer pH 5.0 overnight, at 4^{o}C. Dialysis was used because it enabled larger volume of shell liquor to be processed in anticipation of bulk lysozyme purification. After centrifugation at 4000 x g for 30 min, 4^{o}C, the sample in sodium acetate buffer was directly loaded by continuous pumping into SP-Sepharose column (2.6 cm x 20 cm) and all subsequent steps in the purification protocol were identical to the original proposal's protocol as described by Xue et al. (2004). The protein and lysozyme concentrations of shell liquor before purification and of purified lysozyme, were measured as described earlier, to determine the specific activity, yield and percentage of lysozyme recovered from shell liquor collected during each season.

Determining molecular weight and purity of oyster lysozyme: The approximate molecular weight and purity of the purified oyster lysozyme was estimated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions using a 12.5% running gel and 4% stacking gel. A low range molecular weight markers (14.4-97.4 KDa) was used as standards to calculate the molecular weight of lysozyme. Protein separation was carried in a vertical slab unit (Bio-Rad, Richmond, CA).

Bulk purification of lysozyme from oyster shell liquor: Purification of lysozyme from oyster shell liquor collected each season indicated lower than expected specific activity of the purified enzyme. Dialysis of shell liquor prior to purification was found to be associated with a decrease of lysozyme specific activity. The dialysis was therefore replaced by desalting samples with a sephadex G-25 column after freeze-drying the shell liquor. This new steps allowed processing large volume of shell liquor for the bulk purification of lysozyme as described below.

Sample processing: Three hundred liters of oyster shell liquor collected in winter and spring 2004 were concentrated by freeze-drying using a Genesis 35 X L lyophilizer (Virtis Co. NY, NY). The dried powder was suspended in distilled water to one tenth of the original volume by stirring the solution overnight at at 4^{o}C. The suspension was centrifuged at 4000 x g for 30 min at 4^{o}C and the supernatant collected. Proteins, including lysozyme were precipitated from the supernatant by adding ammonium sulfate to 65% saturation. The sample was centrifuged at 4000 g for 30 min at 4^{o}C and the supernatant discarded. The pellet was dissolved in distilled

water to a protein concentration of 40 mg/ml and the proteins were desalted and transferred in 20 mM acetate buffer using a sephadex G-25 columm. Lysozyme in the sample was then purified by two rounds of ion exchange chromatography as described below and according to Xue et al (2004).

Strong cation exchange 'step-wise' chromatography: The samples were loaded at the rate of 6 ml/min onto a SP-Sepharose FF column (2.6 x 35 cm), equilibrated with 0.02 M sodium acetate buffer at 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 elution rate of 6 ml/min. The elution was monitored by measuring the absorbance of fractions at 280 nm. Fractions from the 0.6 M NaCl eluted peak were collected and designated as "lysozyme enriched sample". Lysozyme enriched sample from seven runs were pooled and concentrated by freeze drying. Final volume, protein concentration and lysozyme activity of the lysozyme enriched sample were determined as described above.

Weak cation exchange 'linear' chromatography: The lysozyme enriched samples were loaded onto a CM-Sepharose Fast Flow column (1.6 x 35 cm) at the rate of 6 ml/minute. The column was washed with a linear gradient of NaCl using 0.3 M - 0.65 M NaCl in 0.02 M sodium acetate buffer, pH 5.0 at an elution rate of 6ml/min. The elution was monitored by measuring the absorbance of fractions at 280 nm. The lysozyme activity of each fraction was measured and an aliquot from each fraction containing high lysozyme activity was subjected to SDS-PAGE as described above. The fractions showing a single and similar size protein band by SDS-PAGE at about 18 kDa were pooled and desalted using a Sephadex G-25 column equilibrated with distilled water. The desalted preparations were designated as "purified lysozyme". The sample was lyophilized, reconstituted in distilled water, adjusted to 1.0 mg/ml, and stored at -20^{o}C as a stock solution until use.

Tasks 3 and 4: Acquiring bacteria and testing antimicrobial activity of oyster shell liquor lysozyme

Personnel: Tasks 3 and 4 were performed by Shreya Datta under the supervision of Dr. Janes from the department of Food Science at the Louisiana State Agricultural Center and a co-PI on the project

Acquiring and growing bacteria causing food poisoning and spoilage: The bacteria *Staphylococcus aureus*, *Clostridium perfringens*, *Listeria monocytogenes*, *Yersinia enterocolita*, *Bacillus cereus*, *Enterococcus faecalis Enterococcus faecium*, *Lactobacilus viridescens*, *Lactobacillus plantarum*, *Pediococcus cerevisiae*, *Aerococcus viridans*, *Pseudomonas fluorescens*, *Pseudomonas aeruginosa, Campylobacter jejuni*, *Campylobacter coli*, *Shigela* spp., *Salmonella enteriditis*, *Salmonella anatum* and *E. coli* 0157:H7 were obtained from a variety of sources (Table 2) . All bacteria were cultured in brain heart infusion (BHI) broth under aerobic or anaerobic conditions, except for *Lactobacillus viridescens*, *Lactobacillus plantarum* and *Pediococcus cerevisiae* which were grown in all purpose tween (APT) broth (Table 2).

Table 2: List of bacterial cultures, their sources, growth conditions, incubation periods for determining oyster shell liquor lysozyme minimum inhibitory concentration (MIC)

Bacteria	Source	Broth	Growth conditions	Incubation period
Staphylococcus aureus	Lindquist[1]	BHI[3]	Aerobic	12 h
Clostridium perfringens	Lindquist[1]	BHI	Anaerobic	48 h
Listeria monocytogenes	USDA	BHI	Aerobic	24 h
Yersinia enterocolita	ATCC 23715	BHI	Aerobic	12 h
Bacillus cereus	ATCC 11778	BHI	Aerobic	12 h
Enterococcus faecium	Lindquist[1]	BHI	Aerobic	12 h
Enterococcus faecalis	Lindquist[1]	BHI	Aerobic	12 h
Lactobacillus viridescens	Lindquist[1]	APT[4]	Aerobic	24 h
Lactobacillus plantarum	Lindquist[1]	APT	Aerobic	24 h
Pediococcus cerevisiae	Johnson[2]	APT	Aerobic	24 h
Aerococcus viridans	Johnson[2]	BHI	Aerobic	24 h
Pseudomonas fluorescens	Johnson[2]	BHI	Aerobic	24 h
Psedomonas aeruginosa	Johnson[2],	BHI	Aerobic	24 h
Campylobacter jejuni	ATCC 29428	BHI	Anaerobic	48 h
Campylobacter coli	ATCC 43480	BHI	Anaerobic	36 h
E.coli 0157:H7	ATCC (Salvik) 43889	BHI	Aerobic	12 h
Shigella spp	Johnson[2]	BHI	Aerobic	12 h
Salmonella anatum	ATCC 27869	BHI	Aerobic	12 h
Salmonella enteriditis	Lindquist[1]	BHI	Aerobic	12 h

[1]University of Wisconsin, Madison, [2]University of Arkansas
[3]Brain heart infusion broth, [4]All purpose tween broth

Preparing bacterial cultures: About 10 µl of each bacterial culture maintained on slants was transferred to 10 ml of BHI or APT broths (Table 2). The cultures were grown overnight and transferred to 10 ml BHI or APT broth the next day. On the third day (after two transfers), 1 ml of culture was collected and centrifuged at 4,000 x g for 2 min. The pellet was suspended in 1 ml phosphate buffered saline (PBS) and centrifuged twice at 4,000 x g for 2 min. To verify the bacterial concentration before carrying out the experiment, plate counts were carried out and colony forming units were calculated. The bacteria suspension was serially diluted in PBS to a concentration of 2×10^5 bacteria/ml for use in determining oyster lysozyme's MICs.

Determining oyster lysozyme minimum inhibitory concentration: Twenty microliters of bacterial suspension were added to 20 µl of two fold serially diluted lysozyme (320 - 5 µg/ml) or 20 µl of distilled water alone (control) in 96 well plates. Sixty µl of BHI or APT broth was added to each well. The plates were incubated at 37° C for the required incubation period (Table 2). Bacterial growth was determined by measuring turbidity at 640 nm with a microtiter plate reader. The results were expressed as the minimum concentration of lysozyme which significantly inhibited the bacterial growth compared to the control.

Statistical analysis: Bacterial growth data was analyzed with a one factor analysis of variance (ANOVA) using SAS version 8.0 software (SAS Institute, Inc., Cary, North Carolina, USA). LSMeans with a Tukey adjustment was used following significant ANOVA results ($p<0.05$) to examine differences in the growth of each bacterium species between concentrations. All data are reported as mean ± standard deviation. Data were log transformed to achieve normality and homogeneity of variance.

Task 5: Determining the allergenicity of purified oyster shell liquor lysozyme

Personnel: This last task was performed by Dr. Losso from the Department of Food Science at the Louisiana State University Agricultural Center and a co-PI of the project.

Mice exposure to oyster shell liquor lysozyme: Seven Balb/c mice were sensitized with 10 µg of lysozyme mixed with 2 mg/ml of aluminum hydroxide by i.p. injection. Seven control mice did not receive an injection. The day of sensitization were refereed to as day 0 and oral feeding were given relative to day 0. Mice were fed by gavage using a 20-gauge needle. Each mouse was exposed to 0.1 mg of oyster lysozyme antigen on day 3, 7, 11, 14, 17, 21, 24, and 28. Seven control mice were sham-fed with lysine, an amino acid, alone. At the end of the experiment, mice were anesthetized with isoflurane and bled by cardiac puncture using a 22-gauge needle. The mice were sacrificed using CO_2 euthanasia

Measurement of mice serum IgE levels following exposure to oyster shell liquor lysozyme: Microplates were coated with 100 µl of 2 µg/ml of anti-mouse IgE capture mAb (BioLegend, San Diego, CA). The plates were incubated for 1 h at 37 °C followed by three washings with PBS/Tween. The wells were blocked with PBS buffer containing 1% BSA. Blanks, purified mouse IgE standards, in a series of dilutions between 0.05 µg/ml to 1 µg/ml in PBS containing 1% BSA were added in volumes of 100 µl to wells in triplicates. Mice serum samples diluted (1:1; 1:10; 1:50; 1:100; 1:250; 1:500; and 1:1,000) were added in a volume of 100 µl in triplicates to wells and the plates were incubated at room temperature for 1h. The plates were washed with PBS/Tween. One hundred µl of biotin anti-mouse IgE (1:1,000) in 1% blocking buffer was added to each well and the plates were incubated at room temperature for 1 h. Following washing with PBS/Tween, 100 µl of Extr-Avidin-AP (Sigma) (1:3000) in PBS was added to each well. The plates were incubated at room temperature for 30 min. After washing with PBS/Tween, 100 µl of substrate (pNPP in 0.1 M diethanolamine, pH 9.8) was added to each well and the plates were incubated at room temperature for 25 min. Color development was stopped by addition of 25 µl of 3 M NaOH. The plates were read at 405 nm using a plate reader. Radial immunodiffusion (RID) was also used to qualitatively identify the presence of IgE in the sera of mice exposed to lysozyme for 28 days.

VI FINDINGS

A. Accomplishment and findings

Task 1: Collection of shell liquor and shell liquor seasonal protein and lysozyme concentrations

The volume of shell liquor per sack (about 40 kg) of oysters averaged about 8 liters. Mean protein concentration of three batches (Table 3) of oyster shell liquor collected in winter 2004 (4.66 ± 1.26 mg/ml) and spring 2004 (5.94 ± 0.83 mg/ml) were significantly greater than the mean protein concentration of three batches (Table 3) of oyster shell liquor collected in summer 2003 (1.98 ± 0.90 mg/ml). Similar findings were obtained for lysozyme concentration as shell liquor collected in winter 2004 (12.74 ± 2.74 µg/ml) and spring 2004 (15.45 ± 7.07 µg/ml) had significantly greater lysozyme concentrations than shell liquor collected in summer 2003 (2.10 ± 0.18 µg/ml). No significant differences in protein and lysozyme concentrations could be shown between in shell liquor collected in fall 2003 and shell liquor collected during the other seasons.

Table 3: Shell liquor protein and lysozyme concentrations

Batch	Date collected	Protein concentration	Lysozyme concentration*	mg lysozyme per g plasma
Summer-1	5/15/03	2.94 g/L	2.30 mg/L	0.77 mg/g
Summer-2	5/29/03	1.63 g/L	2.05 mg/L	1.26 mg/g
Summer-3	6/16/03	1.31 g/L	1.95 mg/L	1.49 mg/g
Fall-1	10/16/03	1.72 g/L	3.24 mg/L	1.88 mg/g
Fall-2	11/6/03	6.41 g/L	11.10 mg/L	1.73 mg/g
Fall-3	11/11/03	5.06 g/L	8.22 mg/L	1.63 mg/g
Winter-1	1/24/04	5.02 g/L	15.88 mg/L	3.16 mg/g
Winter-2	2/18/04	5.71 g/L	11.43 mg/L	1.99 mg/g
Winter-3	2/27/04	3.27 g/L	10.90 mg/L	3.33 mg/g
Spring-1	4/6/04	6.68 g/L	13.96 mg/L	2.09 mg/g
Spring-2	4/28/04	6.11 g/L	23.14 mg/L	3.79 mg/g
Spring-3	5/12 /04	5.04 g/L	9.24 mg/L	1.85 mg/g

* Lysozyme concentration was measured using a stock solution of purified oyster plasma lysozyme with a specific activity of 1.5×10^5 U/mg, as a standard.

Task 2: Seasonal and bulk purification yield of lysozyme from shell liquor

The greatest yield of purified lysozyme, 5.27 mg per liter shell liquor, was obtained from shell liquor collected in winter 2004 (2/27/04) (Table 4). Shell liquor collected in summer 2003 (5/15/03) yielded the lowest weigh of purified lysozyme, 0.54 mg per liter shell liquor. Shell

liquor collected in fall 2003 (11/6/03) and spring 2004 (5/12/04) yielded intermediate weighs of purified lysozyme, 4.33 mg and 2.18 mg per liter of shell liquor, respectively.

The percentage of lysozyme recovered from shell liquor following purification ranged from a high of 39.0% in fall 2003 to a low of 9.4% in spring 2004 (Table 4). The percentage of lysozyme recovered from shell liquor following purification in summer 2003 (23.6%) and winter 2004 (33.2%) were intermediate in values. These yields varied but were comparable to the yield of 20.1% reported for lysozyme purified from oyster plasma (Xue et al., 2004).

The specific activities of purified lysozyme ranged from 1.13×10^4 to 2.52×10^4 units per mg protein (Table 4) and were lower than the specific activity of 1.52×10^5 units per mg protein reported for lysozyme purified from oyster plasma (Xue et al. 2004). Dialysis of shell liquor prior to purification most likely caused a decrease of lysozyme activity as a dialysis step was not used by Xue et al. (2004). Moreover, when dialysis of shell liquor was replaced by desalting samples with a sephadex G-25 column after freeze-drying the shell liquor, the specific activity of the lysozyme purified from shell liquor was comparable to that of lysozyme purified from oyster plasma.

Table 4: Seasonal yield of purified lysozyme from 1 liter of oyster shell liquor

Batch	Lysozyme yield (mg)	% Recovery	Specific activity (U/mg)
Summer 2003	0.54	23.6	2.11×10^4
Fall 2003	4.33	39.0	1.13×10^4
Winter 2004	5.27	33.2	1.19×10^4
Spring 2004	2.18	9.4	2.52×10^4

Our empirically-derived protocol to purify lysozyme in bulk from shell liquor yielded 205 mg of purified lysozyme from 300 liters of shell liquor collected in winter 2004. The purified lysozyme appeared as a single protein band of about 18 kDa after SDS-PAGE under reducing conditions and Coomassie blue staining. Specific activity of the purified lysozyme was 1.9×10^5 units per mg protein and 922 times greater than the specific activity of lysoyme in shell liquor prior to purification (Table 5). About 11% of the shell liquor lysozyme was recovered as purified lysozyme. This purified lysozyme was used for the antimicrobial and antigenicity assays.

Table 5: Summary of oyster lysozyme purification in bulk from shell liquor

Sample	Total protein (mg)	Total activity (U)	Specific activity (U/mg protein)	Recovery (%)
Crude shell liquor	1.73×10^6	3.5×10^8	2.06×10^2	
Lysozyme enriched sample*	6.70×10^3	9.8×10^7	1.47×10^4	28
Purified oyster lysozyme	2.05×10^2	3.9×10^7	1.90×10^5	11.1

* after first ion-exchange chromatography

Tasks 3 and 4: Antimicrobial activity of oyster shell liquor lysozyme

Out of the 19 food borne pathogens tested, oyster lysozyme showed significant inhibition of *Clostridium perfringens* at a concentration of 2.5 µg/ml, and *Lactobacillus plantarum, Pediococcus cerevisiae, Campylobacter jejuni Campylobacter coli* at a concentration of 5 µg/ml. *Enterococcus faecium* and *Enterococcus faecalis* was inhibited at a concentration of 20 µg/ml (Table 6). The growth of *Listeria monocytogenes, Salmonella anatum and Lactobacillus viridescens* were inhibited at a concentration of 160 µg/ml. There was no growth inhibition of *Staphylococcus aureus, Bacillus cereus, Yersinia enterocolita, Aerococcus viridans, Pseudomonas fluorescens, Psedomonas aeruginosa, E.coli 0157:H7, Shigella spp* and *Salmonella enteriditis* at the highest lysozyme concentration tested of 160 µg/ml.

Table 6: Antibacterial activities of lysozyme purified from oyster shell liquor against bacteria causing food spoilage and food poisoning reported as the minimum concentration inhibiting bacterial growth (MIC).

Food spoilage causing bacteria	MIC (µg/ml)
Aerococcus viridans	>160
Enterococcus faecalis	20
Enterococcus faecium	20
Lactobacillus viridescens	160
Lactobacillus plantarum	5
Pediococcus cerevisiae	5
Pseudomonas fluorescens	>160
Pseudomonas aeruginosa	>160
Food poisoning causing bacteria	
Bacillus cereus	>160
Campylobacter coli	5
Campylobacter jejuni	5
Clostridium perfringens	2.5
Escheria coli 0157:H7	>160
Listeria monocytogenes	160
Salmonella anatum	160
Salmonella enteriditis	>160
Shigella spp	>160
Staphylococcus aureus	>160
Yersinia enterocolita	>160

Task 5: Determining the allergenicity of purified oyster shell liquor lysozyme

Results from both sandwich ELISA and RID showed that oyster lysozyme did not illicit production of IgE in mice. No IgE was detected in serum of mice fed oyster lysozyme for 28 days or in serum of control mice. Results of the radial immunodiffusion test were also negative.

B. Negative results

None

C. Need for additional work

All tasks of the project were completed. Follow-up projects should evaluate the specific use of oyster lysozyme to enhance food preservation and in pharmaceutical products.

VII EVALUATION

A. Achievement of project goals and objectives

All project objectives have been met. As a result of this project the feasibility of purifying lysozyme from shell liquor, a by-product of the oyster collected during commercial processing (shucking) of oysters was demonstrated and the lysozyme yield in mg protein per liter of shell liquor was determined seasonally over one year. The purified oyster lysozyme was shown to have strong antimicrobial activity against several food spoilage bacteria (*i.e., Enterococcus faecalis*, *Enterococcus faecium, Lactobacillus plantarum, Pediococcus cerevisiae*) and bacteria causing food poisoning in humans (i.e., *Campylobacter coli*, *Campylobacter jejuni*, *Clostridium perfringens*). Purified oyster lysozyme was found not to be allergenic. Our results indicate oyster lysozyme potential for use as a food preservative and a pharmaceutical

B. Dissemination of Project results

1) The project resulted in two presentations at international meetings in 2005. An abstract was also submitted to the Institute of Food Technologists to present our results at their Annual Meeting June 2006 in Orlando, Florida. Titles of the abstracts are as follows:

Datta S, Xue Q, Janes ME, Losso JN and La Peyre JF. 2005. Potential use of lysozyme from shell liquor of eastern oysters against bacteria causing food poisoning and food spoilage. 97th Annual Meeting of the National Shellfisheries Association, Philadelphia, Pennsylvania, April 10-14.

Datta S, Xue Q, Janes ME, Losso JN and La Peyre JF. 2005. Purification of lysozyme from shell liquor of eastern oysters (*Crassostrea virginica*). Institute of Food Technologists Annual Meeting, New Orleans, Louisiana, July 15-20.

Datta S, Janes ME, Xue Q, Losso JN, Beverly RL and La Peyre JF. Submitted. Control of *Listeria monocytogenes* and *Salmonella anatum* on the surface of smoked salmon coated with edible films containing oyster lysozyme and nisin. Institute of Food Technologists Annual Meeting. Orlando, Florida, June 24-28, 2006.

2) One abstract was published in the Journal of Shellfish Research

Datta S., Xue Q.G., Janes M.E., Losso J.N. and La Peyre J.F. 2005. Potential use of lysozyme from shell liquor of eastern oysters against bacteria causing food poisoning and food spoilage. Journal of Shellfish Research 24:650.

3) The project supported one Master student Shreya Datta from the Department of Food Science, Louisiana State University Agricultural Center. Shreya Datta successfully defended her thesis in July 2005 and graduated in August 2005. Her thesis can be cited as:

Datta S. 2005. Purification of lysozyme from oyster shell liquor and potential commercial use against foodborne pathogens. Master thesis, Louisiana State University. Baton Rouge, LA 70803.

4) Finally two manuscripts are in preparation and will be submitted for publication in peer-reviewed journals (i.e., Journal of Food Protection, Journal Shellfish Research) in 2006.