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Role of Apolipoprotein A-1 in Defense Against Bacteria by Striped Bass (Morone saxatilis)

L. Danielle Johnston

College of William and Mary - Virginia Institute of Marine Science

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ROLE OF APOLIPOPROTEIN A-I IN DEFENSE AGAINST BACTERIA
BY STRIPED BASS (MORONE SAXATILIS)

A Thesis
Presented to

The Faculty of the School of Marine Science
The College of William and Mary in Virginia

In Partial Fulfillment
Of the Requirements for the Degree of
Master of Science

by
L. Danielle Johnston
2006
This thesis is submitted in partial fulfillment of
the requirements for the degree of

Master of Science

L. Danielle Johnston

Approved November, 2006

Peter A. Van Veld, Ph.D.
Advisor

Howard Kator, Ph.D.

Kimberly S. Reece, Ph.D.

Patty Zwollo, Ph.D.
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ABSTRACT

Apolipoprotein A-I (ApoA-I) is associated with high-density lipoprotein (HDL). ApoA-I is best known for its role in reverse cholesterol transport. Recent studies in mammals and fishes have reported antibacterial, antiviral, and anti-inflammatory activities of HDL and ApoA-I. The goals of the present study were: to purify ApoA-I from striped bass plasma, obtain sequence information from the striped bass ApoA-I gene, investigate the antibacterial activity of striped bass ApoA-I against one gram-positive bacteria (*Streptococcus* sp.), one species of gram-negative bacteria (*E. coli*), and one species of mycobacteria (*Mycobacterium marinum*) *in vitro*, and finally evaluate the role of diet in regulation of ApoA-I *in vivo*. Striped bass ApoA-I was purified from plasma using Affi-gel Blue affinity chromatography to isolate HDL, followed by delipidation to remove HDL associated lipoproteins from HDL lipid, and then ApoA-I was separated from other proteins using gel filtration chromatography. Two-dimensional electrophoresis was used to help characterize the purified protein as approximately 25kD in size with an acidic pI of approximately 5.0. The monoclonal antibody 6D6 recognized the 25kD protein and N-terminal sequencing generated a 25 amino acid sequence highly similar to other fish ApoA-I sequences. The ApoA-I gene was sequenced from both genomic striped bass DNA isolated from spleen, and liver cDNA. The resulting sequence containing the coding region of the gene is approximately 1240 bp. The translated protein is 275 amino acids long and is similar to other ApoA-I fish sequences. The predicted secondary and tertiary structure is highly conserved in ApoA-I sequences of both mammals and fishes and contains characteristic proline kinks spaced 22 and 11 amino acids apart as well as -helical characteristics. Purified ApoA-I exhibited antibacterial activity against *E. coli*, *Streptococcus* sp., and *M. marinum* using a microtiter broth dilution assay. Concentrations as low as 125 µg/ml for *E. coli*, 250 µg/ml for *Streptococcus* sp., and 250 µg/ml for *M. marinum*, inhibited bacterial growth by 50% compared to control. ApoA-I plasma concentrations in experimental fish and wild fish tested ranged from 0.5-5 mg/ml or more, which is greater than *in vitro* concentrations used in assays.

ApoA-I is regulated by fat obtained in the diet of mammals. In mammals, low-fat diets and starvation diets result in reduced levels of HDL and associated ApoA-I. We studied two groups (22 fish each) of juvenile striped bass fed either a high fat diet (8% menhaden oil) or a low fat diet (0% menhaden oil) over a period of 120 days. However, no significant differences in growth or ApoA-I plasma concentrations were observed between the high fat and low fat groups.

Results of our studies indicate that striped bass ApoA-I is an effective antibacterial agent against *E. coli*, *Streptococcus* sp., and *M. marinum* *in vitro* at concentrations below the range of physiological concentrations in striped bass plasma. Therefore we speculate that ApoA-I could play a role in defense against infections with these bacteria in striped bass. Further studies are needed to isolate the mechanism of antibacterial activity, and determine how the protein may act *in vivo*. 
GENERAL INTRODUCTION

Striped Bass Diet

Striped bass are generalist, carnivorous fish, consuming small fishes, crustaceans, squid, shellfish, and worms (Raney 1952). Larger striped bass prefer a piscivorous diet, consisting mainly of silversides (*Menidia menidia*), Atlantic menhaden (*Brevoortia tyrannus*), bay anchovy (*Anchoa mitchilli*), spot (*Leiostomus xanthurus*), Atlantic croaker (*Micropogonias undulates*), and killifishes (*Fundulus* spp.) (Raney 1952). Between 1990 and 1992, Chesapeake Bay striped bass diet was dominated by a few prey species, and the relative dominance of these species changed with predator age and size (Hartman and Brandt 1995). Bay anchovy were the dominant prey found in stomach contents of young fish (age 1) while older individuals (age 2+) fed on Atlantic menhaden, spot, and Atlantic croakers (Hartman and Brandt 1995). Atlantic menhaden became increasingly important in the diet of older fish and contributed up to 60% of the diet from September to December, when much of the annual growth of striped bass occurs (Hartman and Brandt 1995).

As the Chesapeake Bay stocks of striped bass began to rebound after a moratorium on fishing in the late 1980s (Figure 1 and 2), the demand for Atlantic menhaden by striped bass increased. By the late 1990s the ratio of striped bass to menhaden reached their lowest levels at 49 menhaden per kg of striped bass, from previous levels of 1700 menhaden per kg of striped bass in the early 1980s (Uphoff 2003). Analysis of 1997 diet data of large striped bass (458-1151mm, 0.91-17.6 kg) indicates menhaden accounted for 44% by weight and occurred in 18% of all stomachs (Walter and Austin 2003). Recent
studies, however, have suggested a more significant absence of menhaden in the diet of Chesapeake Bay striped bass (McNamee personal communication).

**Infectious Diseases of Striped Bass**

A variety of pathogens affect the health of striped bass (*Morone saxatilis*) in the wild and in aquaculture. Common bacterial diseases include vibriosis, aeromonad infections, pasteurellosis, edwarshiellosis, flavobacteriosis, streptococcosis, myxobacterial infection, bacterial kidney disease, and mycobacteriosis (Frerichs 1989; Hughes et al. 1990; Plumb 1991). Most of these infections have been reported in cultured populations of striped bass; however several of these bacteria species including *Pasteurella piscicida*, *Mycobacterium marinum*, *Streptococcus* spp., and *Edwardsiella* sp. have been associated with epizootics in wild populations (Baya et al. 1990, 1997; Hughes et al. 1990; Sakanari et al. 1983; Sniezsko et al. 1964; Vogelbein et al. 1999). Outbreaks of bacterial infections have recently occurred in the Chesapeake Bay striped bass. Mortalities of striped bass in Maryland in 1988 were associated with *Streptococcus* sp. (Baya et al. 1990) and in 1994, *Edwardsiella tarda* was associated with lesions of the skin and internal organs (Baya et al. 1997).

In 1997, *Mycobacterium* sp. was identified in Chesapeake Bay striped bass by histology in association with skin and visceral lesions (Vogelbein et al. 1999; Cardinal 2001). Visceral and dermal lesions prevalence of 62.7% and 28.8%, respectively, have been recorded from Virginia tributaries of the Chesapeake Bay (Cardinal, 2001). A survey of striped bass in the Maryland portion of the Chesapeake Bay has indicated a high prevalence of splenic granulomas associated with mycobacteria (Overton et al. 2003).
The only other previously reported outbreak of mycobacteriosis in wild striped bass occurred in fish from four areas in northern California and in Coos Bay, Oregon, in 1983 (Sakanari et al. 1983). These fish exhibited granulomatous lesions in spleen, kidney, and/or liver.

**Mycobacteria**

Mycobacteria are gram-positive staining, acid-fast, aerobic, non-motile, rod-shaped bacteria. They are distinct from other gram-positive aerobes because of the high content of mycolic acids and free lipids as well as waxy fatty acids contained in the cell wall (Pfyffer et al. 2003). Species of mycobacteria are classified as either slow-growers, or fast growers requiring more or less than seven days to form colonies on agar (Pfyffer et al. 2003). The majority of pathogenic mycobacteria are intracellular parasites of macrophages.

**Mycobacteriosis in fishes**

External clinical signs of mycobacteriosis in fishes include scale loss, dermal ulceration, emaciation, exophthalmia, pigmentation changes and spinal defects (Nigrelli and Vogel 1963; Gomez et al. 1993). Internal gross pathologies include enlargement of the spleen, kidney, and liver with grey or white nodules throughout the affected organs (Chinabut 1999).

As of 1963, 150 species of fish have been described as being affected by mycobacteriosis (Nigrelli and Vogel, 1963) and the list is still growing. *Mycobacterium marinum, fortuitum, and cheloneae* are species of mycobacteria that infect both freshwater and saltwater fish worldwide (Herbst et al. 2001; Lansdell et al. 1993; Pfyffer et al. 2003; Tortoli et al. 1996). Recently two additional novel species of slow-growing mycobacteria
have been described in association with striped bass mycobacteriosis in the Chesapeake Bay (Rhodes et al. 2001, 2003).

**Polyunsaturated Fatty Acids**

Menhaden are an excellent source of long chain n-3 polyunsaturated fatty acids (PUFA), specifically eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Ackman et al. 1976; Morris and Culkin 1989). PUFAs are essential for normal growth, food utilization, health, and reproductive viability of fishes (Higgs and Dong 2000). The fatty acid composition of fish diets strongly influences the fatty acid composition of cell membrane phospholipids, signal transductions, and plasma lipid levels, which in turn can have a profound effect on the immune system (Balfry and Higgs 2001). For example, high levels of dietary n-3 PUFA increased the strength of the blood cell membrane in Atlantic salmon suffering from cold-water vibriosis (*Vibrio salmonicida*) (Salte et al 1988). Macrophages are a prime producer of byproducts of fatty acids (eicosanoids), which have a wide array of actions modulating immune responses (Johnston 1988). DHA, along with arachidonic acid (AA) makes up one third of brain lipid and also plays a key role in retinal, neural, and synaptic membranes, specifically the myelin sheath (Gurr et al. 2002). Marine fish lack Δ5 fatty acid desaturase, which converts lower fatty acids to PUFAs, therefore these fish must obtain PUFAs in their diet (Owen et al. 1972, Kanazawa et al. 1979, Higgs and Dong 2000). Studies involving varying levels of PUFA in feed for a variety of aquaculture-raised fish indicated increased growth rate and immune resistance in feed with good sources of PUFAs (Erdal et al. 1991, Fair et al. 1993, Lie et al. 1993).

**Innate Immunity and Nutrition**
Humoral innate immunity involves a variety of substances in the serum, mucus, and eggs that nonspecifically inhibit growth of microorganisms. These antimicrobial substances include lysozyme, complement, interferon, C-reactive protein, transferrin, lectin, and many others (Yano 1996). Many studies have demonstrated the importance of micronutrients such as vitamins C and E in disease resistance. Vitamin C in the form of ascorbic acid is a necessary factor in steroid synthesis, helps maintain activity of glutathione peroxidase and superoxide dismutase, and because of its role in collagen synthesis is essential for normal wound healing (Halver 1972, Halver 1989, Sato et al. 1978). Vitamin C has also been shown to be important in complement activity. Many groups have demonstrated a positive relationship between ascorbic acid and complement level, in a variety of fishes (Lin and Shiau 2005, Ai et al. 2004, Ortuno et al. 1999, and Li and Lovell 1985). In a study by Li and Lovell (1985) channel catfish fed the diet with ascorbic acid had higher complement activity and phagocytic activity. In addition, when experimentally infected with Edwardsiella ictaluri fish fed the diet without ascorbic acid showed 100% mortality and fish fed 300 mg/kg and 3000 mg/kg showed 15% and 0% mortality respectively. Similar studies with Vitamin E added to the diet have shown increased complement activity, lysozyme, and reduced mortality when challenged with bacteria (Wang et al. 2006, Lin and Shiau 2005).

Macronutrients also play an important role in the humoral innate immune system of fishes. Many groups have evaluated lipid supplementation, in the form of n-3 polyunsaturated fatty acids (PUFAs) (Blazer et al. 1989; Sheldon and Blazer 1991, Erdal et al. 1991, Kiron et al. 1995). Menhaden oil is a commonly used supplement because of its high level of PUFAs. Several studies report a significant increase in macrophage
bacterial killing activity and antibody titers in fish fed diets high in n-3 PUFAs or menhaden oil (Blazer et al. 1989, Sheldon and Blazer 1991, Erdal et al. 1991; Kiron et al. 1995). In addition Kiron et al. (1995) found lower mortality rates (10-15%) in rainbow trout hatchlings challenged with IHN virus when fed diets high in n-3 PUFAs compared to diets high in n-6 PUFAs. The recent depletion of Chesapeake Bay menhaden stocks (Uphoff 2003), and thus a reduced contribution to striped bass diet, may affect their growth, nutritional intake, and immune function.

Diet and HDL/ApoA-I Production

High-density lipoprotein (HDL) is a small, dense association of lipids containing a cholesterol ester core surrounded by a phospholipid monolayer. HDL is produced in the liver as a nascent particle consisting of a phospholipid monolayer without a lipid core. Apolipoprotein A-I (ApoA-I) readily associates with the HDL particle and activates the enzyme lecithin:cholesterol acyl transferase (LCAT) which catalyzes the conversion of triglycerides on the surface of HDL into cholesterol esters, which are incorporated into the core of the HDL particle. In humans and other mammals, ingested levels of dietary fat, especially polyunsaturated fats, modulate levels of HDL and ApoA-I secretion (Velez-Carrasco et al. 1999, Calleja et al. 2000, Shige et al. 2000, Wang et al. 2001, Rong et al. 2002, Hatahet et al. 2003). Cultured intestinal cells secrete ApoA-I into the cell media when incubated with linoleic acid (n-3 PUFA) (Rong et al. 2002). In addition, a diet high in n-3 PUFAs stimulated production of hepatic ApoA-I mRNA and plasma ApoA-I in rats (Calleja et al. 2000). Patients with chronic renal failure experienced an 8% increase in HDL levels after eating a diet with 2.4g of n-3 PUFAs per day for eight weeks (Svensson et al. 2004). Studies in fishes suggest the importance of a diet high in
n-3 PUFAs for growth and immune health. Fish diets are often supplemented with menhaden oil (Fair et al., 1993, Lingenfelser et al. 1995, Sheldon and Blazer 1991), but specific changes in HDL or ApoA-I secretion have not been investigated.

**Apolipoprotein A-I**

ApoA-I is an alpha-helical, amphipathic protein which comprises approximately 70% of total HDL associated proteins in humans and salmonids (Babin and Vernier 1989, Brouillette et al. 2001). ApoA-I is synthesized in the liver as a preprolipoprotein cleaved in hepatocytes before subsequent release into the plasma as a prolipoprotein. The “pro” segment is cleaved by a specific protease and converted into the mature ApoA-I (Hospattankar et al. 1987). Two molecules of ApoA-I associate with each other in an antiparallel fashion, forming an elliptical-shaped dimer. Specific contacts are made, including the interdigitation of side chains and salt bridges (Figure 3). The alpha helices are oriented in such a way as to allow a contiguous hydrophobic strip that runs along the length of the protein, making it well suited for binding lipids. In the absence of lipids, two dimers can bind to form a tetramer (Brouillette et al. 2001).

ApoA-I has a variety of functions; however the primary role is reverse cholesterol transport. ApoA-I is the most potent activator of LCAT, which catalyzes the esterification of cholesterol. This results in a more soluble cholesterol-HDL complex, which increases the cholesterol transport capacity of the HDL particle for subsequent removal by the liver. In addition, ApoA-I mediates cholesterol efflux from cells to HDL (Oram & Yokoyama 1996).

ApoA-I expression has not been fully investigated in fishes, however ApoA-I mRNA has been found in epidermal cells and liver of carp (Concha et al. 2003). Other
studies have discovered many sites of synthesis of ApoA-I in mammals (Elshourbagy et al. 1985, Haddad et al. 1986, Lenich et al. 1988) such as skeletal muscle, liver, intestine, brain, testes, and heart, however the main sites of synthesis are liver and intestine. It is possible there could be many different sites of synthesis of ApoA-I in fishes.

**Antimicrobial Action and Mechanisms of Apolipoprotein A-I**


Human ApoA-I also has antiviral activity against Herpes simplex virus (HSV), human immunodeficiency virus (HIV), and xenotropic murine virus (Srinivas et al 1990, 1991, Alonso-Villaverde et al. 2003, Kane et al. 1979). HSV and HIV entry into host cells involves fusion of the viral membrane with the host cell membrane. ApoA-I and amphipathic synthetic peptide analogs inhibit both virus-induced cell fusion and penetration of HSV and HIV (Srinivas et al. 1990 and 1991, Owens 1990). The focus of our study is the antibacterial action of ApoA-I.

Recent studies involving ApoA-I have been conducted on non-mammalian vertebrates. ApoA-I isolated from carp (*Cyprinus carpio*) demonstrated *in vitro* antibacterial activity (Concha et al. 2004). ApoA-I was co-incubated with *Escherichia coli*, *Yersinia ruckeri*, or *Pseudomonas* sp. The EC$_{50}$s observed after 24 hours were 5.2, 2.6, and 2.6 µM respectively. ApoA-I has also been isolated from cod, rainbow trout, sea bream, skate, brown bullhead, and sea turtle (Manadottir and Lange 2004, Duggan et al. 2004).
However Concha et al. (2004) were the first to demonstrate the antimicrobial activity of ApoA-I and implicate it as a protein possibly involved in innate immunity in fish.

The actual mechanism of antimicrobial activity by ApoA-I has not yet been identified; however, several mechanisms have been suggested. One hypothesis is that the high affinity of ApoA-I for cholesterol and lipids allows it to remove cholesterol from a cell, which could affect a pathogen's membrane integrity. In addition, bacterial growth may be inhibited by the insertion of lipids or proteins into the cell membrane (Tada et al. 1993). Synthetic amphipathic -helical model peptides can form ion channels in phospholipid bilayers of both Gram-positive and Gram-negative bacteria (Agawa et al. 1991). Thus the inherent structure of the ApoA-I protein may confer its antimicrobial activity.

**Apolipoprotein A-I and Inflammation**

ApoA-I inhibits excessive inflammatory responses involved in diseases such as rheumatoid arthritis, atherosclerosis, and lupus. Both HDL and ApoA-I bind and inactivate lipopolysaccharides (LPS) or endotoxin secreted from gram-negative bacteria (Munford et al. 1981; Cavaillon et al. 1990; Emancipator et al. 1992; Flegel et al. 1993; Massamiri et al. 1997). LPS initiates inflammatory reactions, which can lead to endotoxic shock. LPS-binding protein transfers LPS to ApoA-I, which partially prevents both acute and chronic inflammatory reactions (Burger and Dayer 2002).
GOALS AND OBJECTIVES

The goals of these studies were first to isolate and identify ApoA-I in striped bass and show that it exhibits the antibacterial activity seen in mammals and carp. The second goal of these studies was to determine if a diet with or without menhaden oil could regulate production of ApoA-I in the blood of striped bass. In order to achieve these goals, experiments were designed to address the following objectives:

1. Purify ApoA-I from striped bass plasma using Affi-Gel Blue gel affinity chromatography, delipidation, and molecular weight fractionation.
2. Amplify ApoA-I gene from striped bass genomic DNA using degenerate primers and sequence the DNA.
3. Use 5’ and 3’ RACE to obtain the entire coding region of the ApoA-I gene from cDNA.
4. Obtain N-terminal amino acid sequence of striped bass ApoA-I.
5. Perform antibacterial assays with purified ApoA-I and *E. coli*, *Streptococcus* sp., and *Mycobacterium marinum*.
6. Perform a diet study with juvenile striped bass fed diets supplemented with either 0% menhaden oil or 8% menhaden oil to evaluate regulation of ApoA-I in response to high-fat diet.
7. Use a specific striped bass monoclonal antibody to quantify ApoA-I in striped bass serum samples from the diet study.
MATERIALS AND METHODS

Sample Collection.

Striped bass blood samples were collected from wild-caught fish from the Chesapeake Bay, VA, as part of the Chesapeake Bay Multi-species Monitoring and Assessment Program (ChesMMAP). In addition, blood samples were collected from wild-caught fish in the York River, VA (Virginia Marine Resources permit # 05-76 and VIMS IACUC # 2005-0706-1). Blood was collected from the caudal vein using a sterile needle and syringe and put in sterile 4 mL vacutainers containing EDTA (Becton Dickinson, Franklin Lakes NJ). Samples were kept on ice and transported to the lab where they were centrifuged at 2000 x g to separate plasma from blood cells. Plasma was frozen at -80°C until use.

ApoA-1 purification, characterization and detection

Studies involving purification, characterization and determining antimicrobial properties of ApoA-I were performed on 2005 archival plasma samples obtained during ChesMMAP surveys, as well as additional samples collected from wild striped bass in the Chesapeake Bay. HDL containing ApoA-I was purified from plasma using affi-gel blue gel affinity chromatography (Concha et al. 2004). Affi-gel blue affinity gel (BioRad, Hercules CA) is a beaded, crosslinked agarose gel with covalently attached Cibacron Blue F3GA dye. The blue dye functions as an ionic, hydrophobic, aromatic or sterically active binding site in various applications. The plasma samples were first dialyzed against equilibration buffer (50 mM sodium citrate, 50 mM sodium chloride, pH 6.5).
After the plasma was applied to the column, the column was washed with 50 mL of wash 1 buffer (50 mM sodium citrate, 350 mM sodium chloride, pH 6.5) to remove bound albumin. The column was then washed with 50 mL of wash 2 buffer (50 mM sodium citrate, 50 mM sodium chloride, 300 mM sodium thiocyanate, pH 6.5) to further desorb albumin. HDL was selectively eluted by 5mL stepwise washes with anilinonapthalenesulfonate (ANS) buffer (50 mM sodium citrate, 50 mM sodium chloride, 2 mM ANS pH 6.5). The fractions were electrophoresed on SDS-PAGE gels and stained with ProteoSilver Silver stain (Sigma, St. Louis MO) or Sypro Ruby total protein stain (Invitrogen Corp., Carlsbad CA). Fractions containing a protein at 25kD with little or no contaminating proteins were pooled.

ApoA-I was separated from HDL-lipids by ethanol-ether delipidation (Scanu and Edelstein 1971) for use in antimicrobial assays. The pooled HDL from the Affigel blue column was first dialyzed in 150 mM sodium chloride, 1 mM EDTA pH 7.0 and concentrated using 15 mL centrifugal concentrators (Millipore, Billerica MA or Pierce, Rockford, IL). Approximately 2 mL of the samples was then mixed with 50 mL of 3:2 ethanol:ethyl ether and kept at -20°C for four to six hours. The precipitate was then centrifuged at 2000 x g for 15 min to pellet precipitated protein. The protein pellet was suspended in 50 mL of ice-cold ether and stored overnight at -20°C to remove any remaining lipids. The suspension was centrifuged to pellet precipitated proteins and dried under a stream of nitrogen.

Total lipid extractions were performed on striped bass serum, delipidated serum samples, and lipid soluble fractions to determine the efficacy of the delipidation protocol. The serum sample was mixed with chlorform:methanol:serum (2:2:1 by volume)
according to Folch (1957). A two-phase system was created in which the upper phase (aqueous) contained polar components. The lower phase (chloroform containing lipids) was removed and dried under a stream of nitrogen. The lipid soluble fractions (solubilized in 4:1 ether:ethanol) and the delipidated proteins were also dried under nitrogen. Lipids were then re-suspended in 1ml of 1:1 chloroform:methanol. Next each sample was transesterified with boron trifluoride ($\text{BF}_3$) and hexane and heated for 15 min at 100°C (Metcalfe and Schmitz 1961). Samples were extracted with carbon disulfide, and the organic phase was dried under nitrogen and re-suspended in hexane (Marty et al. 1992). The $\text{BF}_3$ attaches a methyl group to each fatty acid and then the fatty acid methyl esters (FAMEs) were analyzed according to Chu and Ozkizilcik (1995) using gas/liquid chromatography and identified by comparing their retention times with a $C_{23}$ standard (Sigma, St. Louis MO).

For the final purification, delipidated ApoA-I pellets were dissolved in 50 mM tris-buffered saline with 1 mM EDTA (pH 7.5) (TBSE) and then applied to an XK 1.6 x 70cm column (Amersham, Piscataway NJ) with S-300 Sephacryl media (Amersham, Piscataway NJ) using the buffer listed above. Fractions were collected using a fraction collector model 2110 (BioRad, Hercules CA) and electrophoresed on SDS-PAGE gels. They were then stained with ProteoSilver Silver stain (Sigma, St. Louis MO), Coomassie blue stain (Sigma, St. Louis MO), or Sypro Ruby total protein stain (Invitrogen Corp., Carlsbad CA) to determine which fractions contained a 25kD band with as few other contaminants as possible. These fractions were pooled and dialyzed in 1mM sodium citrate, pH 6.5. The protein concentration was analyzed via Bradford
method (BioRad, Hercules CA) or bicinchoninic acid (BCA) method for protein quantification before use in antibacterial assays.

**Development of Primers.**

Degenerate primers were derived from consensus teleost ApoA-I sequences (*Fundulus heteroclitus* (AAU50536), *Pseudopleuronectes americanus* (AAT01918), *Gadus morhua* (AAU87042), *Sparus aurata* (AAC78689), *Salmo salar* (CAA36942), and *Cyprinus carpio* (CAC34942)) from GenBank following ClustalW alignment (Thompson, 1997). The forward primer (29F) used was 5'-ATGCARGCNGAYGCNCCNTCNCARYTNGMNCA-3' and the reverse primer (222R) used was 5'-NADYTGNTCYTTRTAYTCYTCNRGRTANGG-3' (Invitrogen Corporation, Carlsbad CA).

**Amplification of striped bass ApoA-I gene segment using PCR**

PCR reactions contained final concentrations of 2.5 U of Platinum Taq DNA polymerase High Fidelity, 0.5 mM dNTPs, 3.5 mM MgCl2, 1x PCR Buffer (Invitrogen, Carlsbad, CA), 4 μM for each primer, and 1 μl (25ng) genomic striped bass DNA in a final volume of 50 μl. The genomic striped bass DNA was a gift from Dr. David Gauthier and was extracted from striped bass spleen using a Qiagen Dneasy kit. PCR parameters consisted of an initial denaturation of 2 min at 94°C, 2 cycles of 2 min denaturation at 94°C, 2 min annealing at 30°C, and 3-5 min extension at 72°C; 35 cycles of 2 min denaturation at 94°C, 2 min annealing at 55°C, and 3-5 min extension at 72°C, followed by a final extension for 10 min at 72°C. All PCR amplifications were performed on MJ Research, Inc. thermocyclers model PTC-200 (Watertown, MA). PCR products were electrophoresed on a 1% agarose gel, stained with ethidium bromide and
visualized using an ultraviolet-light transiluminator (Alpha Innotech Corp., San Leandro CA) to visualize bands for excision. Individual bands were excised from the gel and DNA extracted using QIAquick gel extraction kit (Qiagen, Inc., Valencia, CA). Prior to cloning A’s were added to PCR products after gel extraction using a protocol from Applied Biosystems. The reaction used 10 µl of PCR product 1.5 µl 10x buffer 1 without MgCl₂, 0.2 µl Taq polymerase, 0.3 µl dATPs, 2.55 µl water, and 0.45 µl 50mM MgCl₂ and was run at 72°C for 8-10 minutes.

**Cloning.**

Excised PCR fragments were cloned and transformed into OneShot TOP10 chemically competent *E. coli*, following manufacturers protocol (Invitrogen, Carlsbad, CA). Colonies were restreaked onto fresh agar-ampicillin plates and allowed to grow at 37°C for 8 hours. Restreaked colonies were picked with a toothpick, added to 70 µl of sterile water, and boiled for 5 min at 100°C. Insert amplification was performed using M13 forward and reverse primers (Invitrogen, Carlsbad CA). Reactions contained final concentrations of: 0.625 U of Taq DNA polymerase, 0.2 nM dNTPs, 1.5 mM MgCl₂, 1x PCR Buffer (Invitrogen, Carlsbad, CA), 25 pM for each primer, and 1µl from the boil prep in a final volume of 25 µl. The parameters consisted of an initial denaturation of 2 min at 94°C, followed by 30 cycles of 1min at 94°C, 1min at 54°C, and 1.5min at 72°C followed by a additional extension step at 72°C for 4 min. M13 amps were run on 2% agarose gels and colonies with an approximate 800 bp insert were chosen for sequencing. PCR products were purified using EXOSAP (USB Scientific, Cleveland OH). In addition, colonies were added to YT broth and grown overnight at 37°C. Plasmid DNA
was isolated from the bacteria using the Qiaprep Spin miniprep kit (Qiagen, Inc., Valencia, CA).

**Sequencing and Sequence Analysis.**

Samples were sequenced using BigDye Terminator v3.1 Cycle Sequencing Reagents (Applied Biosystems, Warrington, UK) with minor modifications of the manufacturer’s recommendations. Sequencing reactions were composed of 10-50 ng template DNA, 0.32 μl M13F or M13R primer (10 μM), 0.25 μl BigDye master mix, 0.875 μl 5x reaction mix and sterile milli-Q water to a final volume of 5 μl. Cycle sequencing conditions consisted of an initial denaturation of 1 min at 96°C, followed by 25 cycles of 10 s at 96°C, 5 s at 50°C, and 4 min at 60°C. Amplification products were electrophoresed using an ABI 3130 DNA sequencer equipped with an 80 cm capillary loaded with POP4 gel matrix. Results were analyzed using Sequencing Analysis v.5.1.1 software (Applied Biosystems, Warrington, UK). The resulting sequence was compared to those deposited in GenBank using BLAST (Altschul et al. 1990) searches of the NCBI database. Alignments were generated with these sequences using the ClustalW algorithm (Thompson, 1997) in MacVector 8.0 (Accelrys Inc., San Diego, CA) with default parameters.

**RNA extraction**

RNA was extracted from striped bass liver and reverse transcribed to cDNA. One striped bass was euthanized by an overdose of MS-222 and then the liver was dissected using sterile techniques. Pieces of liver (approximately 60 mg each) were snap frozen in liquid nitrogen and RNA was extracted using an RNeasy RNA extraction kit according to manufacturers protocol (Qiagen, Valencia CA). RNA quality and concentration were
determined by UV spectrophotometry at 260/280 nm, with background correction for protein contamination at 320 nm.

**RNA-ligase mediated rapid amplification of cDNA ends (RLM-RACE)**

Using the GeneRacer kit (Invitrogen, Carlsbad, CA) reverse transcription of 1 µg RNA was accomplished with Superscript III reverse transcriptase and GeneRacer RNA oligo according to the manufacturer’s protocol.

All gene specific primers (GSP) used are listed in Table 1. The primers were designed from the sequence of an initial 750 bp putative striped bass ApoA-I sequence fragment obtained using degenerate primers 29F and 222R and genomic DNA. Primer 20F was used in combination with the GeneRacer 3' primer to obtain the 3' segment and 28R was used in combination with the GeneRacer 5' primer to obtain the 5' segment. In both sets of reactions 1 µl of cDNA was added to a mastermix containing; 0.6 µM of appropriate GeneRacer primer, 0.2 µM GSP 20F or 28R, and 45 µl Platinum PCR SuperMix High Fidelity (Invitrogen, Carlsbad, CA). Cycling parameters consisted of a touchdown, hotstart PCR using the following conditions: 94°C for 2 min; 94°C for 0.5 min and 72°C for 1 min, for 5 cycles; 94°C for 0.5 min and 70°C for 1 min, for 5 cycles; 94°C for 0.5 min, 60°C for 0.5 min, and 68°C for 1.5 min, for 25 cycles; 68°C for 10 min. PCR products were electrophoresed on a 2% agarose gel and appropriate sized bands were excised. PCR products were extracted from the gel using SNAP columns according to manufacturer’s protocol (Invitrogen, Carlsbad, CA). A’s addition, cloning, and sequencing were performed as described previously.
2D Electrophoresis and N-terminal Sequencing

ApoA-I was separated by two-dimensional electrophoresis (2DE) using an Invitrogen IPG Zoom Runner System according to manufacturer’s instructions. Striped bass plasma (30 mg) was subjected to isoelectric focusing (first dimension) on pH 3-10 IPG strips (Invitrogen, Carlsbad, CA.) IPG strips were electrophoresed on NuPAGE 4-12% gradient Bis-Tris Zoom gels (Invitrogen, Carlsbad, CA.). Proteins were transferred to Immobilon-FL (Millipore) and stained with Coomassie Blue R250 (BioRad). The spot corresponding to ApoA-I and recognized by the monoclonal antibody 6D6 against striped bass ApoA-I (supplied by Dr. Erin Bromage, VIMS) was sent to the Microchemical Facility at Emory University, in Atlanta, GA for N-terminal sequencing via Edman degradation.

Monoclonal Antibody Production

Monoclonal antibody production was performed by Dr. Erin Bromage (VIMS) according to previously published procedures (Bromage 2004). A brief description of the methods used for specific antibody production against striped bass ApoA-I are listed below.

Six week-old female Balb/c mice were utilised for the production of monoclones against APOA1. The mice (3) were intraperitoneally (IP) immunized with an oil-in-water emulsion of striped bass HDL purified using Affi-gel Blue (50 μg) and Freunds complete adjuvant (FCA), followed by booster injections at week 8 and week 12 of delipidated ApoA-I (5 μg) in Freunds incomplete adjuvant (FIA). At regular intervals, 50 μl of blood was collected from the tail vein of immunised mice so that titres could be monitored during the vaccination process, and specificity monitored via Western blotting.
Four days before the fusion (week 16) the mouse with the highest titre was injected intravenously (IV) with 10 µg of delipidated ApoA-1. The fusion technique used was derived from the methods of Zola (Zola 2000) and Harlow and Lane (1988).

Following fusion the process of limiting dilution was conducted to ensure single cell cloning of hybridomas. This was performed by resuspending cells in RPMI supplemented with 20% NCS, to a cell density of 20 cells/ml. A 96 well tissue culture plate (Corning) was divided into 4 groups containing 3 columns each. To the first group 200 µl of this solution was added, 100 µl to the second, and 50 µl and 25 µl to the final 2 groups. Each group was then filled to 200 µl total with growth medium containing 20% NCS. This process ensured that 4, 2, 1 and 0.5 cells were present in the respective groups 1 through 4. The cells were allowed to grow for 10 days before being screened by enzyme-linked immunosorbent assay (ELISA). Cell supernate was screened against purified HDL and delipidated, molecular weight fractioned ApoA-I. Wells testing positive for specific antibody were examined via a microscope, and those thought to contain a single colony were re-cloned, following the above procedure. Cloning was considered complete when 100% of the wells containing cells tested positive in ELISA. In addition to ELISA, western blots were performed to ensure immunoreactivity against a single 25kD band believed to be ApoA-I.

**ApoA-I Quantification**

ApoA-I quantification in striped bass serum was performed by Western blot using the monoclonal antibody 6D6 (prepared by Dr. Erin Bromage, VIMS). Samples for SDS-PAGE were prepared using a modified version of the Laemmli protocol (Nature, 1970). Briefly, plasma samples were diluted in SDS reducing sample buffer (with 2-
mercaptoethanol) and boiled for 3 min. On each gel, four striped bass purified ApoAl standards (100 ng, 200 ng, 300 ng, and 500 ng) were loaded along with equal volumes of each sample (approximately 0.1μl of plasma in 5μl of sample buffer) in duplicate on 15 well 10-20% gradient Tris-HCl gels (BioRad, Hercules CA) and electrophoresed at 175V for 1h (BioRad protean III system). Serum proteins were transferred to Immobilon-FL (Millipore, Bedford, MA) in tris-glycine transfer buffer with 20% methanol for 1 h at 100V (200-400 mA). After transfer the membranes were incubated for 1 h in 0.5% casein in tris-buffered saline (TBS) pH 8.0. The 6D6 antibody was diluted 1:1000 in 0.5% casein-TBS and incubated at room temperature for 1 hour. The blot was then washed three times for ten min in TBS + 0.1% Tween 20 (TTBS) and incubated in goat anti-mouseIR 700 (Molecular Probes) secondary antibody diluted in 0.5% casein-TBS for 1 hour in the dark. The blot was then washed three times in TTBS and visualized using the Li-cor Odyssey infrared imaging system (Lincoln, NE) and quantified densitometrically according to the manufacturer’s protocol.

**Bacterial culture and maintenance.**

Three bacterial cultures were used in antibacterial assays; *Mycobacterium marinum*, *Escherichia coli*, and *Streptococcus* sp. *M. marinum* was previously isolated from infected striped bass spleen and maintained by Dr. Howard Kator (VIMS). *E. coli* was obtained from ATCC culture # 25922. The *Streptococcus* sp. was isolated by Dr. Ron Thune, Gulf Coast Research Laboratories. *E. coli* was grown and maintained in sterile Mueller Hinton broth (Becton Dickinson) at 37°C (Concha et al. 2004), *M. marinum* was grown and maintained in Middlebrook broth (Becton Dickinson) at room temperature, and *Streptococcus* sp. was grown and maintained in brain-heart infusion.
broth (Becton Dickinson) at room temperature. Back-up cultures of all bacteria were kept on agar slants at 4°C and also frozen in 20% glycerol and stored at −20°C.

**Antibacterial assays**

Initially, standard curves for cell concentration and growth time were generated using 96-well plates. Each bacterium was serially diluted five-fold across the plate from an optical density of 0.1 at 590 nm to a dilution of $10^{-6}$ and allowed to grow in the dark at the ideal temperature and media until log phase was reached. Optical density (OD) readings were recorded at several intervals to establish a growth curve. Aliquots were enumerated by plating on agar plates to correlate corresponding optical density readings with cell number. This process allowed determination of characteristic growth time and starting concentration for subsequent antibacterial assays.

Antibacterial activity assays were performed using delipidated ApoA-1 in the microtiter broth dilution assay previously described (Friedrich et al. 1999). Assays were performed in sterile flat-bottom 96-well plates (Becton Dickinson). Purified ApoA-I was diluted in 0.2% bovine serum albumin to a concentration of 2 mg/ml and sterile filtered. Concentrations of ApoA-I ranged from 1 mg/ml to 0 mg/ml for *Streptococcus* and *E. coli* and 1.5 mg/ml to 0 mg/ml for *M. marinum*. Each concentration was tested in triplicate on each plate. Each well was then inoculated with 100 μl of $1 \times 10^4$ or $10^5$ CFU/ml in triplicate rows in the appropriate broth (1,000 cells and 10,000 cells per well respectively). Two rows of control wells containing bacteria with a substitute protein (BSA) corresponding to ApoA-I concentrations were set up in duplicate on each plate (Figure 4). At time zero, OD measurements were taken using a microplate reader at 595
nm (Molecular Devices) and then measurements were taken at selected intervals until the bacteria reached plateau phase.

**Dietary study**

Approximately 50 juvenile (phase 2) striped bass (30-60 gm) were obtained in November 2005 from the Edenton National Fish Hatchery (Edenton, NC), transferred to one 2,600 L tank containing flow-through well water at VIMS, and allowed to acclimate to the aquaria over a ten-day period. Rock salt was added to all tanks to approximately 1 ppt when fish were disturbed and during transport in order to reduce stress effects. All fish were held in well water at the ambient room temperature (18-20°C). During the acclimation period, fish were fed to satiation the same dry pellets used at the fish hatchery (Zeigler Brothers). After the initial 10 day period all fish were fed the experimental high-fat diet for 50 days. On day 0 of the dietary study, six fish were sacrificed and exsanguinated for ApoA-I quantification. Remaining fish were weighed, measured for length, and distributed into two 2,600-liter tanks (22 fish per tank).

Fish were fed either the high-fat diet or the low-fat diet identical to those used in a previous study of the effects of dietary menhaden oil on growth and fatty acid composition of hybrid striped bass (Fair et al. 1993). These highly defined diets were custom prepared by Zeigler Brothers (Gardner, PA) and varied only in the amount of menhaden oil added (0% menhaden oil in low-fat diet, 8% menhaden oil in high-fat diet) (Table 2). Fish were fed up to 2 percent of their body weight per day for a period of four months.

Flow-through well water was used throughout the study and the temperature was maintained at 18-20 °C. This temperature range is equivalent to that found in the lower
Chesapeake Bay from October to November when striped bass are known to be abundant and feeding. Fish in each group were reared in identical water conditions. Water quality parameters such as nitrite and ammonia were measured periodically to help maintain the fish in a healthy environment.

Fish were sampled on days 0, 45, and 120. On day 0 nine six were exsanguinated for ApoA-I quantification and on days 45 and 120, 11 fish from each treatment were exsanguinated for ApoA-I quantification. Length and weight was measured before euthanasia for each time-point. All plasma samples were frozen at -80°C for later quantification of ApoA-I concentration.

**Statistical Analysis**

GraphPad Prism and Microsoft Excel were used for all statistical analyses. For detection of significant differences between time-points and diets in the diet study the Student's T-test was used at an alpha value of 0.05. In order to determine significant bacterial growth inhibition compared to control, a One-way ANOVA followed by Dunnett's multiple comparison test were used also at alpha values of 0.05.
Figure 1

Chesapeake Bay striped bass juvenile index in Maryland and Virginia waters from 1958-2004. The indices are presented as Arithmetic Mean Catch per Haul. Indicated with arrows are the years when moratoriums on fishing of striped bass existed in Maryland (1985-1990) and Virginia (1989-1990). The graph was taken from Chesapeake Bay Program website http://www.chesapeakebay.net/baybio.htm.
Figure 2

Estimated Atlantic coast menhaden population from the National Marine Fisheries Service population dynamics team data. This chart represents the estimated Atlantic Coast Menhaden population, ages 2-8. The population in 1999 is 66% below the ten year, historic average from 1955 to 1964.

Chart prepared by Chesapeake Bay Ecological Foundation, Inc.
ESTIMATED ATLANTIC COAST MENHADEN POPULATION

![Bar chart showing estimated Atlantic coast menhaden population from 1955 to 1999. The x-axis represents the years, and the y-axis represents population size in billions. The legend indicates that the bars represent total population ages 2-8.](image-url)
Figure 3.
ApoA-I structure when bound to HDL. Top figure shows an ApoA-I dimer binding to a molecule of HDL (Kumar et al., 2002). The bottom figure shows two ApoA-I monomers held together with salt bridges (Segrest et al. 1999).
ApoA-I Structure
Figure 4

Antibacterial assay setup design of 96-well plate. First BSA was added to the top two rows as an inert protein control. Concentrations are shown in μg/ml of 0.2% BSA. Row A shows concentrations used in assays using *E. coli* and *Streptococcus* sp. and row B were concentrations used in assays using *M. marinum*. Purified ApoA-I was diluted in 0.2% BSA and added to the wells using concentrations shown in row A and B. To row 1 and rows 3-5, 100 μl of a $10^5$ cell/ml suspension of bacteria was added to each well. To row 2 and rows 6-8 100 μl of a $10^4$ cell/ml suspension of bacteria was added to each well. After incubation high turbidity or optical density was seen in wells with the darkest coloring.
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- 10,000 cells/well
- 1,000 cells/well
Table 1

List of Primers

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<td>222R</td>
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Table 2

Artificial Diet Composition

Ingredient composition and proximate analysis of experimental diets (dry weight basis)

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<td>Fish oil (menhaden)</td>
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<td>Wheat (ground whole)</td>
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Proximate analysis (%)

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Figure taken from Fair et al. 1993
Results

ApoA-I Purification

ApoA-I was purified from striped bass serum using a three step process: Affinity chromatography using Affi-gel Blue (BioRad) and elution of HDL using 8-anilinonaphthalene sulfonate (ANS) (Figure 5), ethanol: ether delipidation removing lipid associated proteins from HDL, and gel filtration chromatography to isolate ApoA-I from other HDL associated proteins including apolipoprotein A-II (Figure 6). After Affi-gel Blue purification and delipidation steps, GC analysis of delipidated HDL confirmed removal of all lipids. Fractions from the gel filtration column were visualized on SDS-PAGE gels stained with ProteoSilver silver stain to identify the purest samples, which were subsequently pooled.

In this study, delipidated ApoA-I was used for monoclonal antibody development, antibacterial assays, and western blot standards. ANS has a very high affinity for ApoA-I (Amathauer et al. 1988) and could not be separated from ApoA-I by dialysis. Because of this the ApoA-I samples containing any ANS were not used for antibacterial assays. However, ApoA-I samples containing trace amounts of ANS were used as ApoA-I western blot standards. Proteins washed off the Affi-gel Blue column before ANS washes (specifically wash 2) were delipidated and ApoA-I was purified using gel filtration chromatography. This purified ApoA-I without ANS was used in antibacterial assays.
N-terminal Amino Acid Sequencing

Striped bass plasma was subjected to 2DE in order to obtain an isolated ApoA-I spot to be used for N-terminal amino acid sequencing. Monoclonal antibody 6D6 recognized a single 25kD spot with a pI of approximately 5.0 (Figure 7). The protein spot was subsequently sent to Emory University Microchemical Facility (Atlanta, GA) for N-terminal sequencing via Edman degradation. The resulting sequence 5' - ASLQADAPSQLAHVRAAMDVYLTQ-3' was compared to those deposited in GenBank using BLAST (Altschul et al. 1990) searches of the NCBI database. Results of the BLAST searches match other ApoA-I sequences of six other fish species with E-values as low as 10^-7 (Table 3). In addition, the N-terminal sequence matched the translated ApoA-1 sequences we obtained from striped bass genomic DNA and cDNA.

ApoA-I Sequence Analysis

An approximately 750bp fragment was amplified by PCR from genomic striped bass DNA using degenerate primers designed from an alignment of several teleost ApoA-I sequences. The amplicon was cloned and 40 clones were sequenced. The resulting sequence of clone 3_1 was compared to those deposited in GenBank using BLAST (Altschul et al. 1990) searches of the NCBI database. Results of the BLAST searches indicated a match to other ApoA-I fish sequences with E-values as low as 10^-19 (Table 4). When the translated amino acid sequence of clone 3_1 was aligned to other ApoA-I sequences a possible 179 bp intron was found. The sequence contains the universally conserved nucleotides 5' - GT and the 3' - AG indicating splice sites as well as a pyrimidine-rich region upstream from the 3' end, consistent with introns in higher organisms (Lodish et al. 1995)(Figure 8). When the intron was removed from the
sequence it aligned with other intronless fish ApoA-I sequences. Additional data that helped to confirm the identity of the isolated clone was the match between the last 10 amino acids of the N-terminal sequence data and the first 10 amino acids of the putative amino acid sequence of clone 3_1 translated from the DNA sequence (Figure 9).

In addition to cloned segment 3_1, 5' and 3' RACE was performed to obtain sequence information of the 5' and 3' ends of the striped bass ApoA-I gene. A 537 bp segment was sequenced using primer 28R and the GeneRacer 5' primer. The DNA sequence of this segment when translated aligns exactly with the N-terminal sequence of the protein and with the translated DNA sequence of clone 3_1. In addition, a 858 bp segment was sequenced using primer 20F and the GeneRacer 3' primer. Primers 20F and 28R were designed to produce overlapping sequences (130 bp) and the resulting 5' and 3' sequences do overlap in the 400 bp region. In addition the 5' and 3' sequenced segments aligned with the previous sequence of clone 3_1. (Figure 10) When 5' and 3' sequences were combined and translated the resultant amino acid sequence was 275 amino acids long.

The various characteristics of different segments of the human ApoA-I nucleotide and amino acid sequences were discussed by Shoulders et al. (1983). When the striped bass ApoA-I amino acid was aligned to human ApoA-I, regions previously characterized in the human sequence can be identified in the striped bass sequence. First the human ApoA-I gene contains an intron at the same point in the sequence as striped bass ApoA-I does. Second, the pre and pro segments of human ApoA-I are highly conserved at the sequence level in striped bass ApoA-I. Finally, the different helices have been identified
in human ApoA-I (Brouillette et al. 2001) and those corresponding regions have been highlighted in the striped bass amino acid sequence (Figure 10).

ApoA-I is modestly conserved among species, both at the level of the nucleotide and amino acid sequence, however there are features that are quite conserved such as its all α-helical secondary structure, predominantly amphipathic in nature and characterized by the presence of several 22 and 11-residue amphipathic α-helices punctuated by proline residues (Koppaka 2001). Using the PHDsec program (http://cubic.bioc.columbia.edu/predictprotein) a prediction of 95.6% of α-helical content for the combined sequence of 5’ and 3’ segments was obtained. The sequence also contains several conserved repeats and proline residues close to the carboxy-terminal end of the protein consistent with other ApoA-I sequences.

**Antibacterial Assays**

An inhibition in growth up to approximately 80% compared to control was seen in *E. coli* incubated with 500.0-1000.0 μg/ml ApoA-I for assay 2. The observed growth inhibition was seen after 5.5 hours of incubation at 37°C (as shown in assay 1). Concentrations of 750.0 and 1000.0 μg/ml completely inhibited growth of *E. coli* in assay 2. Inhibition of growth (at least 20%) was seen at concentrations of approximately 3.9 μg/ml using a starting concentration of 5x10³ cells/ml and 31.3 μg/ml using a starting concentration of 5x10⁴ cells/ml for assay 2 (Figure 12).

There was also significant reduction in growth (One-way ANOVA, Dunnett's multiple comparison) compared to control seen in *Streptococcus* sp. incubated with greater than 250.0 μg/ml ApoA-I (Figure 13). At both starting concentrations (5x10³
and 5x10⁴ cells/ml) of *Streptococcus* sp. there was no observed growth (100% inhibited) at concentrations higher than 500 µg/ml (Figure 14).

*M. marinum* was more sensitive to ApoA-I than either *E. coli* or *Streptococcus* sp. There was significant reduction in *M. marinum* growth compared to control (80-100% inhibition) at concentrations higher than 125.0 µg/ml and at concentrations higher than 375.0 µg/ml there was no significant growth compared to control (One-way ANOVA, Dunnetts multiple comparison) (Figure 14). Growth inhibition was seen after 12 hours of incubation at room temperature. In addition, there was significant stimulation of *M. marinum* growth compared to control at low levels of ApoA-I for all time points measured (15.6, 31.3, and 62.5 µg/ml for assay 1; 62.5, 125.0, and 250.0 µg/ml for assay 2) (Figure 15). Less relative growth inhibition was seen in all assays with starting concentrations of 5x10⁴ cells/ml versus 5x10³ cells/ml, however similar trends were seen in assays with the higher starting concentration of bacteria. For simplicity, data from assays using starting concentrations of 10³ cells/ml are shown in figures.

**Regulation of ApoA-I levels by diet**

Two groups of 22 juvenile striped bass were fed a diet varying in menhaden oil (MO) (0% MO = low fat; 8% MO = high fat) (Fair et al. 1993) to determine if a diet varying in lipid content altered levels of ApoA-I in striped bass plasma. There was no significant difference in ApoA-I concentration over time (One-way ANOVA) or between the high fat and low fat groups (Student’s T-test) (Figure 16). There was no significant difference (Student’s T-test) in weight between the two groups at any time point (Figure 17) even though the high-fat group ate slightly more on average than the low-fat group (1.5 ± 0.5 % BW/day, 1.0 ± 0.4% BW/day respectively).
There was no significant relationship between diet and ApoA-I levels found in this diet study. However, plasma samples of wild striped bass do show significant variation in ApoA-I levels (Figure 18). Random samples of striped bass plasma were selected from ChesMMAP archives (2005) and screened for ApoA-I. Concentrations ranged from 1 mg/ml up to greater than the highest ApoA-I standard of 5 mg/ml. Differences greater than 10-fold were found between samples (Figure 20). We have not identified a relationship between high levels of ApoA-I and diet in the wild striped bass samples, however this figure shows that there can be a wide variation in plasma ApoA-I levels.
Figure 5

SDS-PAGE of striped bass plasma proteins eluted from Affi-gel Blue column. 
Lane 1 (Wash 1); Lane 2 (Wash 2); Lane 3 (anilinonapthalenesulfonate (ANS) wash). 
Wash 1 contains all proteins not bound to the gel matrix. Wash 2 elutes albumin as well as some HDL molecules. The ANS wash selectively elutes HDL molecules containing ApoA-I as well as other HDL associated proteins. Approximately 4 μg of total protein from each wash was electrophoresed on a gradient SDS-PAGE gel (4-12%) stained with Sypro Ruby total protein stain (Invitrogen Corp., Carlsbad CA).
Figure 6

Gel filtration of delipidated wash 2 fraction obtained from the Affi-gel Blue column (Figure 1). Five μg of fractions containing ≥ 0.1 mg/ml of total protein were electrophoresed by SDS-PAGE and visualized with Coomassie blue stain. ApoA-I enriched fractions were pooled for antibacterial assays and for western blot quantification standards.
Two-dimensional electrophoresis of striped bass plasma using the Zoom-IPG system (Invitrogen Corp., Carlsbad CA). Plasma was electrophoresed on an IPG strip (pH 3-10) followed by 4-12% SDS-PAGE. A) 30 µg of striped bass plasma stained with Proteosilver silver stain (Sigma-Aldrich Co., St. Louis MO). B) Protein spot recognized using monoclonal antibody 6D6. The protein corresponding to this spot was subsequently used for N-terminal amino acid sequencing.
Table 3

Result of BLAST search of the NCBI database for the striped bass N-terminal sequence received from Emory.

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<th>Protein</th>
<th>Species</th>
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<td><em>Sparus aurata</em> (gilthead seabream)</td>
<td>4.00E-07</td>
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Table 4
Result of BLAST search of the NCBI database for the partial striped bass ApoA-I sequenced from clone 3_1.

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Figure 8

Full 734 bp nucleotide sequence of clone 3_1 including the intron and aligned amino acid sequence shown in two open reading frames. Splice sites for the intron are boxed in and the intron is highlighted in dark grey. In light grey the amino acid sequence begins in open reading frame B and then continues after the intron in open reading frame C.
**Figure 9**

Aligned amino acid sequences using MacVector ClustalW. N-term = N-terminal sequence of 25kD protein recognized by mAB 6D6, 5’ = 5’ sequence from 5’ RACE, 3’ = sequence from 3’ RACE, clone 3_1 {no intron} = sequence of clone 3_1 obtained using genomic striped bass DNA without the intron, ApoA1 5 + 3 = composite sequence using 5’ and 3’ sequences from RACE.
**Figure 10**

Aligned amino acid sequences using MacVector ClustalW. ApoA1 5+3 = striped bass combined sequences from 5’ and 3’ RACE, wtflounder = *Pseudopleuronectes americanus* (winter flounder) ApoA-I, cod = *Gadus morhua*, puffer = *Takifugu rubripes*, carp = *Cyprinus carpio*, b.trout = *Salmo trutta trutta*, r.trout = *Oncorhynchus mykiss*, Salmon = *Salmo salar*, seabream = *Sparus aurata*, zebrafish = *Danio rerio*, gorilla = *Gorilla gorilla*, dog = *Canis familiaris*, baboon = *Papio hamadryas*, and human = *Homo sapiens*. Dark grey highlighted = 100% identity, light grey = consensus match, and white = mismatch (color key from MacVector program). Notice highly-conserved proline residue indicated with arrows. The large dark arrow pointing to the right denotes the beginning of the coding region for the mature form of human ApoA-I. The pre and pro sequences are shown under the bold line and dotted line respectively. The sequence before the intron is called exon A and after the intron is exon B (Shoulders et al. 1983). Areas highlighted in blue are regions corresponding to human ApoA-I lipid binding regions. Areas highlighted in pink are regions corresponding to human ApoA-I LCAT binding regions (Frank and Marcel 2000).
Growth curves for *E. coli*, *Streptococcus* sp., and *M. marinum*. Each species was diluted to 10,000 cells/ml in the appropriate medium and 100 μl in triplicate was added to wells of a 96 well plate. Normalized optical density is shown in the figure for several different time points.
Figure 12

Antibacterial activity of purified ApoA-I against *E. coli* using a range of ApoA-I concentrations. Growth inhibition was observed up to 90% of control at the highest concentration of ApoA-I in assay 1 with initial concentration of \(5 \times 10^4\) cells/ml. Growth of treated wells is significantly different from control at concentrations higher than 7.8 \(\mu g/ml\) (One-way ANOVA \(p < 0.05\), Dunnetts multiple comparison \(p < 0.05\)). Growth inhibition was observed up to 100% of control at concentrations of 1000.0 and 75.0 \(\mu g/ml\) ApoA-I with initial concentration of \(5 \times 10^3\) cells/ml. Growth of treated wells is significantly different from control at concentrations higher than 31.3 \(\mu g/ml\) ApoA-I (One-way ANOVA \(p < 0.05\), Dunnetts multiple comparison \(p < 0.05\)).
Figure 13

Growth (OD increase) over time for antibacterial assay 2 using *E. coli* at a starting concentration of 5x10³ cells/ml. Optical Density (normalized) of *E. coli* incubated with ≥250.0 µg/ml ApoA-I was significantly reduced compared to 0 µg/ml ApoA-I control. No increase in optical density occurred at concentrations of 1000.0, 750.0, and 500.0 µg/ml, therefore lines are superimposed on top of each other along the x-axis.
Figure 14

Antibacterial activity of purified striped bass ApoA-I against *Streptococcus* sp. using a range of ApoA-I concentrations and bacteria at an initial concentration of $5 \times 10^3$ cells/ml.

The bold line at 1 represents 100% growth. *Streptococcus* sp. growth was significantly inhibited compared to control at concentrations of ApoA-I greater than 250.0 μg/ml.

Growth was 100% inhibited at concentrations of 1000.0, 750.0, and 500.0 μg/ml.
Figure 15

Growth (OD increase) over time for antibacterial assay 2 using *Streptococcus* sp.

Starting concentration of the bacterium were $5 \times 10^3$ cells/ml. Optical density (normalized) of *Streptococcus* sp. incubated with $\geq 250.0 \, \mu g/ml$ ApoA-I was significantly reduced compared to $0 \, \mu g/ml$ ApoA-I control. No increase in optical density occurred at concentrations of 1000.0, 750.0, and 500.0 $\mu g/ml$, therefore lines are superimposed on top of each other along the x-axis.
Figure 16

Antibacterial activity of purified ApoA-I against *M. marinum* using a range of ApoA-I concentrations measured at different time points. Both assays had an initial concentration of $5 \times 10^3$ cells/ml. There was significant inhibition (One-way ANOVA, $p < 0.05$; Dunnett's multiple comparison, $p < 0.05$) of growth compared to control at concentrations higher than 250.0 µg/ml for assay 1 and 500.0 µg/ml for assay 2. *M. marinum* growth was 100% inhibited in assay 1 at concentrations higher than 375.0 µg/ml and in assay 2 at concentrations higher than 1250.0 µg/ml.
Assay 1

Assay 2

Optical Density % of Control

ApoA1 μg/ml

Hours
- 24
- 36
- 48

Optical Density % of Control

ApoA1 μg/ml

Hour
- 24
- 36
- 48
Figure 17

Growth (OD increase) over time for antibacterial assays 1 and 2 using *M. marinum*.

Optical density (normalized) of *M. marinum* incubated with ≥ 31.3 μg/ml ApoA-I and 62.5 μg/ml ApoA-I was significantly reduced compared to 0 μg/ml ApoA-I control in assays 1 and 2 respectively. No increase in optical density occurred at concentrations greater than 375.0 μg/ml ApoA-I for assay 1, therefore lines are superimposed on top of each other along the x-axis.
Assay 1

Optical Density (590 nm)

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<tr>
<th>ApoA-I µg/ml</th>
<th>1500</th>
<th>1250</th>
<th>1000</th>
<th>750</th>
<th>500</th>
<th>375</th>
<th>250</th>
<th>125</th>
<th>62.5</th>
<th>31.25</th>
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<tbody>
<tr>
<td>375 to 1500</td>
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Assay 2

Optical Density (590 nm)

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<th>1250</th>
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<th>750</th>
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<th>62.5</th>
<th>31.25</th>
<th>15.63</th>
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time (hours)
Figure 18

Striped bass weight measured over time. Striped bass juveniles obtained from the
Edenton national fish hatchery for the diet study, were weighed at four different times.
Day –53 was the day they arrived at VIMS, day 1 is the first day of the study, day 45 is
the 45th day of the study and day 120 is the last day of the diet study. There were no
significant differences in growth between the high fat and low fat groups at any time-
point. Error bars represent 2 times the standard error.
Figure 19

ApoA-I levels (mg/ml) of high fat and low fat groups of striped bass at three time points. There were no significant differences between groups or over time (Student’s T-test, ANOVA, p < 0.05). Error bars represent 2 times the standard error.
Figure 20

(A) ApoA-I western blot of striped bass plasma from the diet study. The four lanes to the left of the line are ApoA-I standards (100, 200, 300, and 500 ng ApoA-I) and to the right are plasma samples (0.1 μl) loaded in duplicate from the high fat group on day 120.

(B) ApoA-I western blot of striped bass plasma samples randomly selected from the ChesMMAP archives (2005). The four lanes to the left of the line are ApoA-I standards (100, 200, 300, and 500 ng ApoA-I) and to the right are plasma samples (0.1 μl) from ten different fish. Some values cannot be accurately quantified because they were above the highest standard, however using the standard curve ApoA-I concentrations as high as 12.0 mg/ml and as low as 0.5 mg/ml were calculated.
Discussion

These studies present evidence of the *in vitro* antibacterial activity of striped bass ApoA-I against *E. coli*, *Streptococcus* sp., and *M. marinum*. Our findings support the findings of Concha et al. (2004). In addition, we provide the first sequence information of striped bass ApoA-I and a method for screening ApoA-I plasma levels in striped bass via western blot.

The 275 amino acid striped bass ApoA-I sequence obtained from amplification by degenerate primers and RACE contains amino acid sequences highly conserved among teleost ApoA-I. In addition, it contains highly conserved proline residues spaced 22 and 11 amino acids apart near the C-terminal end which are a characteristic of all ApoA-I sequences, mammalian and teleost (Koppaka 2001). These highly conserved regions of ApoA-I sequences are responsible for generating the α-helical structure of ApoA-I, which is necessary for its function (Frank and Marcel 2000; Segrest et al. 2000). While the striped bass ApoA-I nucleotide or amino acid sequence does not show much sequence similarity with mammals it does share the characteristic proline residues. These prolines cause the protein to kink into a curved shape allowing binding to lipid (Borhani et al. 1997; Brouillette et al. 2001). To further confirm the isolation of the ApoA-I protein, an N-terminal sequence matching other ApoA-I sequences (E value = 10^-7) was obtained from the immunoreactive protein recognized by striped bass monoclonal 6D6.

The striped bass ApoA-I protein sequence is not highly conserved at the primary structural level when compared to mammalian sequences, however it does resemble
ApoA-I proteins at the secondary level. The 275 amino acid striped bass ApoA-I sequence was submitted to the Protein Prediction site (using the PHDsec program (http://cubic.bioc.columbia.edu/predictprotein). The striped bass ApoA-I sequence submitted was predicted to have 96.5% alpha-helical content, consistent with ApoA-I proteins from humans of 60-90% (Koppaka 2001).

ApoA-I enriched samples purified from striped bass plasma inhibit growth of *E. coli*, *Streptococcus* sp. and *M. marinum* in vitro. In previous work studying the antibacterial activity of carp ApoA-I, a concentration of 143 μg/ml of ApoA-I inhibited growth of *E. coli* (at an initial concentration of 5 x 10⁴ cells/ml) by 50% (Concha et al. 2004). Our studies were consistent with this carp study in that concentrations of ApoA-I greater than or equal to 125.0 μg/ml inhibited growth of *E. coli* (also at an initial concentration of 5 x 10⁴ cells/ml) by 50%. Therefore, striped bass ApoA-I inhibits growth of *E. coli* at similar concentrations as carp ApoA-I. In addition, *E. coli* growth was significantly reduced from control when incubated with concentrations of ApoA-I greater than 31.3 (after 5.5 hours) and 3.9 μg/ml (after 10 hours) for assays 1 and 2 respectively. *E. coli* growth was not completely inhibited except at concentrations of 1000.0 and 750.0 μg/ml ApoA-I in assay 2, whereas concentrations of greater than 500.0 μg/ml and 250.0 μg/ml completely inhibited growth of *Streptococcus* sp. and *M. marinum*, respectively. The concentration where growth (OD) was inhibited by 50% for *Streptococcus* sp. were between 250-375 μg/ml. The concentrations where growth (OD) was inhibited by 50% for *M. marinum* assay 1 was between 125-250 μg/ml and for assay 2 was between 375-500 μg/ml.
Concentrations of ApoA-I used in these assays were well below concentrations seen in the plasma of captive (1-3 mg/ml) and wild (0.5 to greater than 5 mg/ml) striped bass used in this study. ApoA-I concentrations of striped bass samples ranged from roughly 0.5 to greater than 5.0 mg/ml in plasma whereas concentrations used in our assays were 1.5 mg/ml or less. Thus, concentrations used in these assays were lower than in vivo concentrations and produced dramatic bacterial growth inhibition in these three species, which included both gram positive and gram negative.

ApoA-I has been detected in epidermal cells of several species. Tarugi et al. (1991) determined that chick skin can synthesize and secrete ApoA-I. In addition, ApoA-I was detected in carp epidermis and epidermal mucus via immunohistochemistry as well as small amounts of ApoA-I mRNA in the epidermal cells (Concha et al. 2003, 2004). This suggests that ApoA-I could be synthesized and secreted by the epidermis either as nascent HDL particles or in a lipid-free form. The ApoA-I located in the mucus and epidermis could possibly play more of a role as a first line of defense against bacteria than ApoA-I in the plasma. Antimicrobial peptides play an important role in defense of epithelia against invasion and colonization by pathogens (Schroeder 1999). The epithelia is the first line of defense against pathogens and therefore plays an important role in innate immunity.

While there has been much research indicating the possible role of ApoA-I in innate immune resistance to microbes (Concha et al. 2003, 2004; Kane et al. 1979; Owens et al. 1990; Srinivas et al. 1990, 1991; Tada et al. 1993), no evidence has been provided for a mechanism of antimicrobial activity. Srinivas et al. (1991) suggests that the lipid-binding properties of ApoA-I prevent cell fusion of the Herpes Simplex Virus.
Only ApoA-I peptides not bound to lipids, but containing lipid-binding sites inhibited viral cell fusion. Owens et al. (1989 & 1990) found similar results with Human Immunodeficiency Virus. Agawa et al (1991) have suggested that a synthesized basic amphipathic alpha-helical model peptide has antimicrobial activity against both Gram-positive and -negative bacteria due to formation of ion channels in the bacterial membrane. The designed peptide 46 was long enough to span a lipid bilayer with transmembrane bundles of α-helices, had potent antimicrobial activities, and channel-forming abilities. Thus, the antimicrobial activity of ApoA-I could be solely due to its amphipathic nature and high affinity for lipids and its α-helical structure.

Additional evidence that suggests amphipathic, alpha-helical, proteins have pore-forming properties is the lysis of the lysosomal membrane in the parasite Trypanosoma brucei, by human apolipoprotein L-I. This results in lysis of the trypansome (ApoL-I) (Perez-Morga et al. 2005). ApoL-I is an amphipathic alpha-helical lipoprotein associated with ApoA-I containing HDL particles (Duchateau et al. 1997, 2001; Monajemi et al 2002; Page et al. 2001; Poelvoorde et al. 2004). Lysis of the trypansome involved uptake of the HDL particle (with ApoL-I) into the lysosome of the parasite. After this ApoL-I was co localized with the lysosomal membrane, forming an ion channel causing an influx of Cl⁻ ions into the lysosome, which in turn caused a compensatory movement of extracellular Cl⁻ across the plasma membrane into the cytoplasm. The swelling of the lysosome compromised the physical integrity of the trypansome, which preceded lysis of the trypanosome.

The antibacterial activity of ApoA-I fragments has been investigated and Concha et al. (2004) have demonstrated the antibacterial activity in vitro of a peptide analog to
the C-terminus of Apo A-I. An interesting feature of this peptide is its ability to synergize with lysozyme to enhance growth inhibition (Concha et al. 2004). Lysozyme alone does not have an inhibitory effect at concentrations below 10.0 µg/ml, however when combined with the peptide at less than 1mM concentration significant inhibition occurred (Concha et al. 2004). Several other antimicrobial peptides previously described also synergize with lysozymes (Singh et al. 2000, Patrzykat 2001). Therefore, ApoA-I may not act alone. Many lysozymes are present in the epidermis of fish (Fast et al. 2002) and Concha et al. (2003) has shown that ApoA-I is also produced in the skin of carp. The potential synergistic effect of lysozyme and ApoA-I could be involved in innate immunity against microbes. Concha et al. (2004) also determined that the protease chymotrypsin could degrade ApoA-I into several stable fragments. Chymotrypsin used in this study has the same specificity as chymase, a protease released by mast cells after an insult (Lee et al. 2003, Eberini et al. 2002). Therefore it is possible that intact ApoA-I and peptide fragments generated by physiologically relevant proteases could play a role in innate immunity.

No significant differences in ApoA-I levels were found between juvenile striped bass fed a low-fat (0% menhaden oil) diet versus a high-fat (8% menhaden oil) diet. Lipoprotein patterns of fishes are influenced by a number of factors, among them: diet, time from last feeding, temperature, season and sexual stage (Kayama and Iijima 1976; Fremont et al., 1981; Fremont and Marion, 1982; Iijima et al., 1985; Leger 1985; Rogie and Skinner, 1985; Nakagawa et al., 1986; White et al., 1986; Hidalgo and Alliot, 1988; Santulli et al., 1988; MacFarlane et al., 1990). In this study, it is possible the diet did not differ enough in fat content, the fish did not feed well or were under stress, or fish
differed in their stage of maturity. Wild striped bass demonstrated greater than 10-fold differences in ApoA-I concentrations therefore there is great variation in ApoA-I among striped bass.

There have been many studies of ApoA-I regulation in mammals. In humans, a high fat diet increases levels of ApoA-I and HDL (Mensink et al. 2003), however a diet high in polyunsaturated fat compared to saturated fat actually can actually lower plasma concentrations of ApoA-I and HDL (Vessby et al. 1980). In studies with mice, increased ApoA-I production in response to dietary fat was not associated with any increase in hepatic or intestinal ApoA-I mRNA (Hayek et al. 1993). This suggests that the mechanism of the dietary fat effect was post-transcriptional. Possibly reduced degradation of the ApoA-I protein or increased translatability of ApoA-I mRNA was the cause of the elevated ApoA-I levels in plasma.

At this point we cannot ensure that the antibacterial activity of striped bass ApoA-I is physiologically relevant. Once the mechanism of antibacterial activity is ascertained it would be interesting to evaluate antibacterial activity in vivo.
GENERAL CONCLUSIONS

Significance of this Thesis

This thesis demonstrates for the first time the *in vitro* antibacterial activity of purified striped bass ApoA-I. These results suggest that striped bass ApoA-I may play a role in striped bass resistance to bacteria. Additionally, this work supports findings of Concha et al. (2003) that carp ApoA-I has antibacterial activity *in vitro*.

Conclusions

1. Purified striped bass ApoA-I inhibits growth *in vitro* of *E. coli*, *Streptococcus* sp., and *Mycobacterium marinum*.

2. The striped bass (*Morone saxatilis*) genome contained an ApoA-I DNA sequence for the ApoA-I gene. The coding segment (1240 bp) of the gene was sequenced and encodes a 275 amino acid protein.

3. Striped bass ApoA-I shows highly conserved characteristics seen in all ApoA-I sequences (mammalian, and teleost) and is highly similar to that of other teleost species.
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VITA

Lisa Danielle Johnston

Born in Houston, Texas on May 10, 1979 to Dr. and Mrs Daniel Johnston. Earned B.S.E in Biomedical Engineering at Tulane University in May 2001. Worked as a research scientist at Baylor College of Medicine in the departments of Pediatrics and Neurology. Entered the masters program at the Virginia Institute of Marine Science in August of 2003 within the department of Environmental and Aquatic Animal Health and completed the degree in December 2006. In September 2006, entered the School of Public Health at the University of Washington for a doctoral program in Nutritional Sciences.