ABSTRACT

Title of Dissertation: MOLECULAR AND FUNCTIONAL CHARACTERIZATION OF A 15 KDA GALECTIN FROM STRIPED BASS (*MORONE SAXATILIS*)

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I have employed biochemical and molecular techniques to investigate the role of galectin during bacterial challenge in teleost fish, using striped bass as an experimental model. Striped bass (*Morone saxatilis*) possesses a 15 kiloDalton β-galactoside binding protein, here after called MS15. It is a single polypeptide with an experimental mass of 15,000 Daltons. *In vivo*, MS15 exists as a non-covalently linked dimer, with two identical carbohydrate binding sites per protein. The organization of the gene coding for MS15 was the same as other vertebrate proto type galectins, with four exons the same size between organisms and three introns varying greatly in length. These investigations have shown that galectin is localized to the dermis and lamina propia (loose connective tissue) throughout the body, to smooth vascular muscle (veins, arteries), to large circulatory cells (heterophils, monocytes), to peripheral leukocytes (tissue resident macrophages, heterophils), to rodlet cells (possible immune cell in alimentary canal), and to leukocytes throughout the gills but not to mucus-producing cells. This is similar to what is found in mammals, but differs greatly from what has been observed in other teleosts. This finding led to the hypothesis that proto type

galectin in striped bass was involved in innate immunity. To test this hypothesis, galectin was characterized based on stability, carbohydrate binding specificity, and native structure. Interactions between galectin and mucus, and galectin and bacteria were then tested. Results revealed galectin binds skin mucus and agglutinates selected bacteria. Striped bass were challenged with bacteria by intradermal and intramuscular injections. Results suggest an increase in the number of galectin-positive leukocytes observed in injection sites, independent of the presence of bacteria. Also, a novel observation was the strong galectin-positive nature of rodlet cells in the esophagus, stomach, and pyloric caeca of the striped bass. The function of rodlet cells is disputed, but in striped bass, galectin was detected most strongly in rodlet cells and both circulatory and peripheral leukocytes. Collectively, these data provide the foundation for three models to explain the biochemical events involved in bacterial clearance and/or wound healing in teleost fish, with potential relevance to innate immunity in other organisms.

MOLECULAR AND FUNCTIONAL CHARACTERIZATION OF A 15 KDA GALECTIN FROM STRIPED BASS (*MORONE SAXATILIS*)

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This dissertation is gratefully dedicated to:

my wife, Susan, whose love, support and tenacity,
gave me the strength to complete this;

my son, Leif, and my daughters, Hannah and Sophia,
for their patience and understanding;

my father, Donald, for teaching me about
research with his curiosity and intellectual engagement;

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LIST OF ABBREVIATIONS

2-D	twɵ dimensional
AB	Alcian Blue
Ab	antibody
APES	3-aminopropyltriethoxysilane
ASF	asialofetuin
β-ME	β-mercaptoethanol
BL	basal lamina
BSA	bovine serum albumin
CAM	carboxamidomethylation
cDNA	complementary deoxyribonucleic acid
Cer	ceramide
DAB	3,3'-diaminobenzidine
dNTP	deoxynucleotides
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EM	electron microscopy
EST	expressed sequence tags
Fuc, F	L-Fucose
Gal	D-Galactose
Glc	D-Glucose
GlcA	D-Glucuronic acid
GlcNAc, GN	N-Acetyl-D-glucosamine
GM3	Sialyl-lactosylceramide
GTC	guanidine thiocyanate
H&E	hematoxylin-eosin
HEV	high endothelial venules
HPLC	high performance (pressure) liquid chromatography
HRP	horseradish peroxidase
IdoA	L-Idouronic acid
IEF	isoelectric focusing
iMET	initiator methionine
IPG	immobilized pH gradient
IPTG	isopropyl-beta-D-thiogalactopyranoside
kDa	kiloDalton
L	liter
Lac	lactose, Galβ1-4Glc
LacdiNAc	N-acetyllactosdiamine, GalNAcβ1-4GlcNAc
LacNAc, LN	N-acetyllactosamine, Galβ1-4GlcNAc
Lea Lewis a	Galβ1-3(Fucα1-4)GlcNAc
Leb Lewis b	Fucα1-2Galβ1-3(Fucα1-4)GlcNAc
Lex Lewis x	Galβ1-4(Fucα1-3)GlcNAc
LexNAc	GalNAcβ1-4(Fucα1-3)GlcNAc

Ley Lewis y,	Fucα1-2Galβ1-4(Fucα1-3)GlcNAc
M	molar (moles/liter)
m/z	Mass to charge ratio
MALDI-TOF MS	Matrix assisted laser desorption/ionization time-of-flight mass spectrometry
Man	D-Mannose
ManNAc	N-acetyl-D-mannosamine
MS15	15 kDa galectin, *Morone saxatilis*
NBF	neutral buffered formalin
NBT	nitro blue tetrazolium
NeuNAc/Neu5Ac	N-acetyl-D-neuraminic acid
NMR	nuclear magnetic resonance
PAS	periodic acid-Schiff
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
pI	isoelectric point
ppt	parts per thousand
PSGL-1	P-selectin glycoprotein ligand-1
PVDF	polyvinylidene fluoride
RACE	rapid amplification of cDNA ends
rEK	recombinant enterokinase
RNA	ribonucleic acid
SSC	sodium chloride-sodium citrate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SE	sulfate ester
UV	ultraviolet
TBE	Tris-Borate-EDTA
TMB	tetrazolium blue
v/v	volume to volume
w/v	weight to volume
w/w	weight to weight
WGA	wheat germ agglutinin
ZP	zona pellucida

List of Organisms

Genus/Species	**Common name**
Anguilla japonica	Japanese eel
Arius thalassinus	Giant seacatfish
Bos taurus	Domestic cow
Bufo marinus	Marine toad, Giant toad
Clavelina picta	Painted tunicate
Conger myriaster	Conger eel
Danio rerio	Zebrafish
Dicentrarchus labrax	Sea bass
Drosophila melanogaster	Fruitfly
Electrophorus electricus	Electric eel
Gallus gallus	Chicken
Gasterostreus aculeatus	Stickleback
Genypterus blacodes	Pink ling,Kingklip
Haplochromis sp	Lake Victoria cichlid
Homo sapiens	Human
Ictalurus punctatus	Catfish
Morone saxatilis	Striped bass
Mus musculus	Mouse
Onchorhynchus mykiss	Rainbow trout
Oryzias latipus	Medaka
Osmerus eperlanus mordax	North American smelt
Ovis aries	Domestic sheep
Paralichthys olivaceus	Bastard flounder
Parasilurus asotus	Chinese catfish
Rattus norvigicus	Norway rat
Salmo salar	Atlantic salmon
Takifugu rubripes	Japanese pufferfish
Tetraodon nigroviridis	Green spotted pufferfish
Xenopus laevis	African clawed frog

Bacteria	
Vibrio mimicus	*Edwardsiella tarda,*
Vibrio parahemolyticus	*Pseudomonas aeruginosa*
Vibrio anguillarum	*Plesiomonas shigelloides*
Escherichia coli	*Photobacterium damselae*
Aeromonas hydrophila	*V. vulnificus*
Staphylococcus aureus	*Streptococcus faecalis*
Aeromonas veronii	*Carnobacterium piscicola*
Aeromonas trota	
Bacillus subtilis	

CHAPTER I. GENERAL INTRODUCTION

The purpose of this project is to examine the 15 kDa galectin present in the skin and mucus of the striped bass, *Morone saxatilis* Specifically, the main interest is to understand possible interactions between the galectin, mucus, and bacteria, and to gain insight into potential-role(s) of the galectin in innate immunity and wound healing. The following text describes adaptive and innate immunity in fish, focusing on constituents of mucus. It summarizes the status of knowledge about biochemical, molecular and biological aspects of galectins and their proposed roles in animals, with particular focus on fish. It will also give background in the species selected for the proposed studies, the striped bass (*Morone saxatilis*), and will conclude with an outline of the organization of this project.

Background in galectins

Lectins- Lectin has been defined as carbohydrate-binding proteins, other than enzymes and antibodies, which agglutinate cells and /or precipitate glycoconjugates (Sharon and Lis, 1972; Barondes, 1988). Based on amino acid sequence similarities in the carbohydrate binding domain, and other properties such as divalent cation dependence lectin "families" are grouped as C-type (calcium-dependent; includes selectins, collectins, Sastry and Ezekowitz, 1993); P-type (recognize phosphorylated mannose residues) and I-type (subset of the immunoglobulin superfamily) as reviewed in Rini and Lobsanov, 1999; F-type (fucose-binding; Bianchet, *et al*., 2002), pentraxins

(C-reactive protein, serum amyloid protein; Osmand, *et al*, 1977) and galectins (β-galactoside binding; Drickamer and Taylor, 1993; Barondes, *et al*, 1994). Lectins isolated and characterized in fish have been found in mucus, eggs, serum, skin, and macrophages; some types are ubiquitous in the animal, others are very tissue- and/ developmental stage specific. Most known animal lectins are found to exist internally, but some are also found in the skin mucus of several animal species, especially in fish. However, knowledge concerning the structure and functioning of such molecules remains limited. Purification of skin mucus lectins has been performed for many species of fish, including the windowpane flounder *Lophopsetta maculata* (Kamiya and Shimizu,1980), the Arabian Gulf catfish *Arius thalassinus* (Al-Hassan, *et al.*, 1986), the conger eel *Conger myriaster* (Kamiya, *et al*, 1988; Shiomi, *et al*,1989), the loach *Misgurnus anguillicaudatus* (Goto-Nance, *et al*, 1995), and the kingklip *Genypterus capensis* (Toda, *et al*, 1996).

Biological roles of lectins- The roles of lectins include pattern recognition, such as carbohydrate patterns of self and pathogen-associated molecular patterns. Following the binding of its carbohydrate ligand, lectin-carbohydrate interaction can lead to pathogen recognition (mannose-binding lectins; Sastry and Ezekowitz 1993), immune cell trafficking (P-selectins, E-selectins; Austrup, *et al*.,1997), and immune cell activation (C-type, galectins; reviewed in Vasta, *et al*. 1999). Lectin-carbohydrate interactions are in different processes, but immune regulation is probably a primary role (Rabinovich, *et al*, 2002; Weis, *et al*, 19 98).

Galectins- Galectins have two essential biochemical properties: one, characteristic amino acid homologous sequences; and two, affinity for β-galactoside

sugars, *i.e.* a carbohydrate binding domain. Galectins require a reducing environment but do not require divalent cations for their binding activity (Barondes, *et al*., 1994). Their locations are intracellular (Sanford, *et al*, 1982; Vyakarnam, *et al*, 1998) and extracellular, and all known galectins lack a signal peptide, and are secreted as soluble proteins by a nonclassical secretory pathway (reviewed in Hughes, 1999). These galectins exhibit considerable similarities in primary structure and exhibit an invariant residue pattern in the carbohydrate recognition domain (Hirabiyashi and Kasai, 1993). Resolution of galectins co-precipitated with their ligands has helped to identify the amino acid residues that interact with the ligand and those that participate in maintaining the architecture of the binding site (Lobsanov, *et al*, 1993; Liao, *et al.*, 1994; Varela, *et al*, 1999; Seetharaman, *et al*, 1998; Shirai, *et al*, 2002). The three structural types of galectins were described in Hirabiyashi and Kasai, 1993 (Figure 1.1 *A*). Galectins have been isolated in many different species of animals, from insects to mammals. In particular, galectins have been isolated and characterized from several teleost (Ahmed, *et al*., 2004; Kamiya, *et al*, 1988; Shiomi, *et al*,1989; Tasumi, *et al*, 2004).

Among fish lectins, the primary structures for mucus galectins were determined in the conger eel, *Conger myriaster*, congerins I and II (Muramoto and Kamiya, 1992; Muramoto *et al*, 1999), Japanese eel, *Anguilla japonica*, AJL-1 (Tasumi, *et al.*, 2004). The structural sequences for skin mucus lectins in animal groups other than fish are also limited and have been reported in only two species, the land slug *Incilaria fruhstorferi* (Yuasa, *et al*, 1998) and the African clawed frog *Xenopus laevis* (Marschal, *et al*, 1992). Galectin repertoire in fish appears reduced as compared to

mammals. To date, protein and /or genomic sequence have been found for one chimera galectin and multiple proto and tandem repeat galectins in mammals. To the contrary, fish to date may have several prototypes, one chimera, and one tandem repeat. The recent completion of several genome projects reveal several putative galectins, but still the complexity of the fish galectin repertoire does not compare with mammalian galectins.

Biological roles of galectins- The biological role(s) of galectins remain unclear but experimental evidence suggests that they mediate cell-cell and cell-extracellular matrix interactions that occur in tissue development (Oda and Kasai, 1983; Levi and Teichberg, 1984; Ahmed, *et al.*, 2004), inflammation (Rabinovich, *et al*, 1999), apoptosis (Perillo, *et al.*, 1995), and tumor metastasis (Raz *et al*, 1989). The several potential mechanisms used to effect interactions involve crosslinking of ligands (Figure 1.1, *B-D*). Galectin may facilitate colonization of bacteria, and defense against parasites in fish mucus (Kamiya *et al.*, 1988; Tasumi *et al*, 2002). Teleost skin and mucus contains putative ligands for galectins, in particular glycosaminoglycans in the form of mucins and laminin. In addition, bacteria may possess ligands for galectins in their cell walls. Galectin-1 and -3 trigger oxidative burst in neutrophils, leading to release of reactive oxygen intermediates that potentially kill invading pathogens (Almkvist *et al*., 2002) and galectin-3 activates neutrophils primed by extravasation (Karlsson *et al*., 1998). Cytokine production is also modulated or triggered by galectins (Allione *et al*., 1998). For example, by inhibiting Th1 cytokines such as TNF-, IL-2, IL-12, and interferon, galectin-1 appears to bias immune responses towards Th2 responses, which preferentially induce humoral immunity. When galectin lattices are present on

leukocyte surfaces, the lattices appear to suppress T-cell receptor-mediating immune responses (Demetriou *et al*., 2001). Another major role for galectins is the homeostatic regulation of cells, particularly immune cells (Hsu and Liu, 2004).

At the beginning of an immune responses or inflammation, macrophage and heterophils are mobilized to the affected regions. These leukocytes phagocytose invading or dead cells, and secrete several factors such as microbicidal factors and cytokines. During or after infection/inflammation, the wound healing process and angiogenesis are promoted partly to restore the affected regions (Pettet *et al*., 1996) Recent works have demonstrated that galectin-3 and -7 accelerate re-epithelialization in the wound healing process (Cao *et al*, 2002). Galectin -3 could stimulate capillary tube formation, which could induce angiogenesis (Nangia-Makker *et al*, 2000). Galectins are implicated in leukocyte homeostasis through induction of cell death by apoptosis. For example, extracellular galectin-1 induces apoptosis in subsets of T-cell populations (such as immature thymocytes or activated T-cells; Perillo *et al*, 1995), and B -cells as well as macrophages, while galectin-9 induces apoptosis of eosinophils and some T-cell lineages. Therefore, immune responses and the subsequent healing processes could involve galectins.

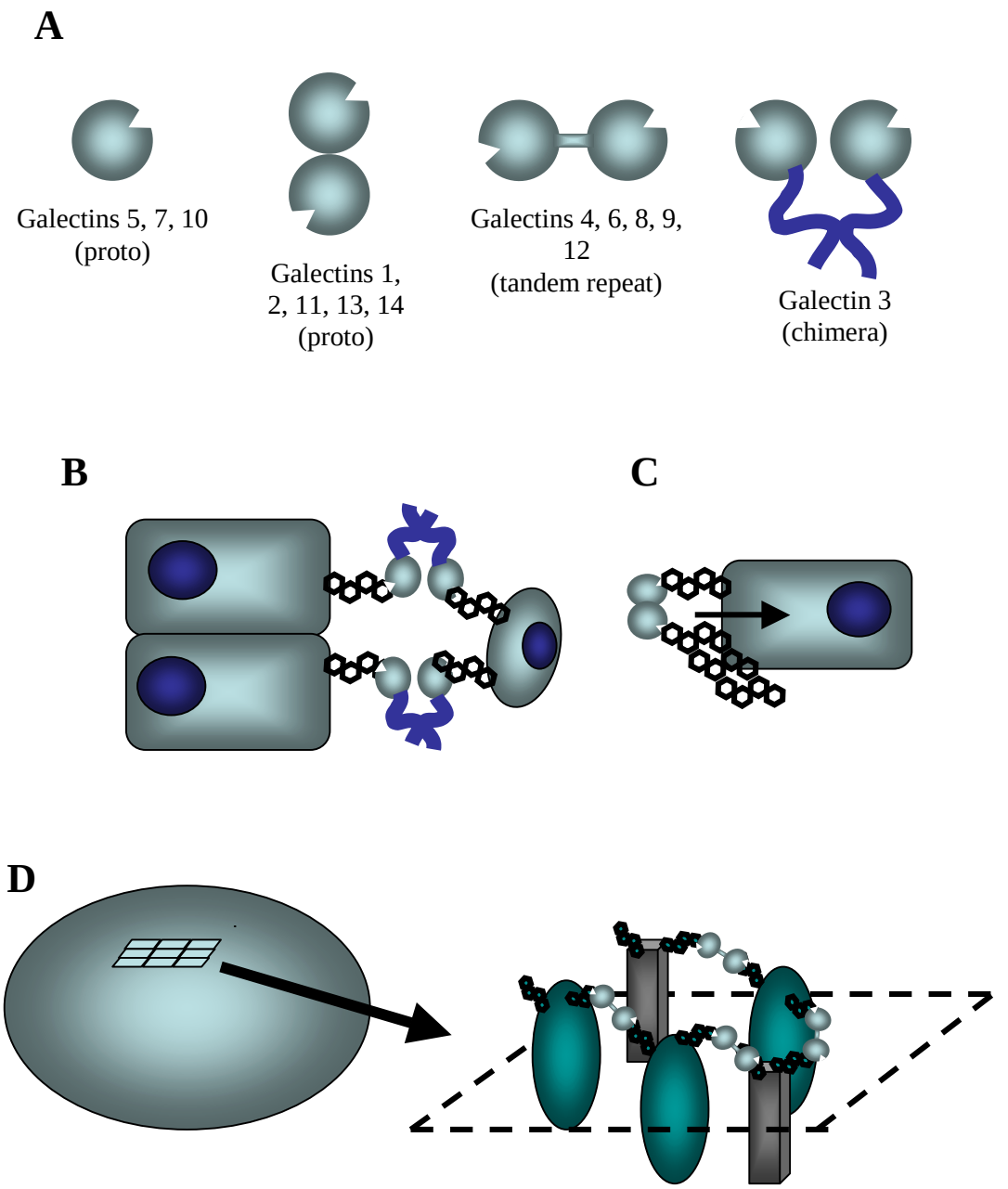


Figure 1.1. Galectin structure and ligand cross-linking: *A*) There are four quaternary structures of galectin identified in mammalian galectins. Proto type galectins are single polypeptide strands possessing a single carbohydrate-binding domain, found as monomers or non-covalent dimers. Tandem repeat galectins are a single polypeptide strand possessing two distinct carbohydrate-binding domains attached through a linker peptide. Chimera galectin, of which galectin-3 is the only member, is a single polypeptide strand having a carbohydrate binding domain and a collagen tail. The proposed mechanisms of interactions of galectins are *B*) cell-cell interactions; *C*) signal transduction by crosslinking of receptors; or *D*) lipid rafts, where several receptors of the same or different types can form large membrane complexes leading to signal transduction, or stabilization of the cell membrane.

Background in immunity

Immunity in Fish- Bacteria are not unchallenged in their colonization of fish mucus and skin. As with all animals, fish possess a wide array of defense mechanisms to protect themselves against disease-causing organisms and parasites. To combat these organisms, jawed vertebrates including teleosts possess two general processes in their immune response, the innate and the adaptive immune response. The adaptive immune response may take days or weeks after an initial infection to have an effect. However, fish are under constant assault from pathogens, such as viruses, bacteria, fungi, water molds, and dinoflagellates, which must be kept in check by the faster-acting innate immune response. Innate immunity fights pathogens using defenses that are quickly mobilized and triggered by receptors that recognize a broad spectrum of pathogens (Watts *et al*., 2001). Plants and many lower animals do not possess an adaptive immune response and instead rely on innate immunity.

The immunology of teleosts has been extensively studied due to the economic importance of many species, and has been found to be very similar to mammals in many respects. As previously mentioned, microbial pathogenesis involves numerous cellular and molecular interactions between microbes and their host organisms. Animals have developed various defense mechanisms against pathogens and, conversely, pathogenic microorganisms have evolved strategies to overcome these barriers to the infection process. For example, epithelial cells in mammalian digestive tracts are covered with a protective mucus layer, which plays a crucial role in protecting these cells (Forstner *et al*., 1994). This function of the mucosal layer relies on a major

component that is a heavily glycoprotein, known as mucin (Strous and Dekker, 1992). Mucins are reported to play an active role in preventing bacterial, viral, and other pathogens from interacting with vertebrate intestinal epithelia (Drumm *et al.*, 1988; Mantle *et al*., 1989). Investigation into the immune systems of teleosts suggests that though they possess an adaptive immune response, the innate immune response in much more developed. This is particularly true for cold-water fish because the rate of development of specific immune responses is temperature dependent (Bly and Clem, 1991). Both systems use cellular and humoral mechanisms to provide protection against infection.

Adaptive immunity in fish-The adaptive immune response in fish is induced by an initial interaction between antigen (foreign substance from the environment such as chemicals, bacteria, viruses, or pollen) and the T and B lymphocytes, which bear unique receptors, and circulate throughout the body in search of antigens. Differing from the innate immunity found in all multicellular organisms, this response is "specific, selective, remembered, and regulated" (Laird *et al*., 2000). B cell receptors, called immunoglobulins (Ig), recognize intact macromolecular complexes on the invaders; the cell progeny of B cells secrete massive amounts of the specific Igs, known as antibodies, which permeate the intercellular milieu (Janeway *et al*., 2001). Antibodies (specific cellular response) are molecules specifically formed to fight invading proteins or organisms. By contrast, T cell receptors (TCRs) recognize small pieces of antigen presented in the major histocompatibility complex (MHC) receptor found on nearly every cell in the body (Laird *et al*., 2000). TCR and Ig are encoded by V, D, and J genes that are combinatorially rearranged during lymphocyte development

(Janeway *et al*., 2001); rearrangement is initiated by a unique DNA splicing enzyme complex, the recombinase activating genes (RAGs). This results in an initial production of antibodies against the antigen and the development of a memory response. The memory response enables a rapid secondary antibody response upon re-exposure to the antigen. The first time the fish is exposed to an invader, antibodies are formed which will protect the fish from future infection by the same organism. Exposure to sublethal concentrations of pathogens is extremely important for a fish to develop a competent immune system. An animal raised in a sterile environment will have little protection from disease. Young animals do not have an immune response that works as efficiently as the immune response in older animals and therefore, may be susceptible to disease. The rate and duration of the adaptive immune response in fish may be determined by the point of exposure, whether systemic or mucosal (skin or gut) (dos Santos *et al*., 2001). This adaptive immune system, with MHC and recombinant antigen receptors (Ig, TCR) has been found in all jawed bony fish and sharks (reviewed in Flajnik, 1996), but not in jawless fish (hagfish, lamprey, etc.) Recently, though, a new system of variable lymphocyte receptors has been identified in these fishes (Pancer *et al*., 2004), so there may be much more than the "classical" adaptive immune system to be discovered.

Innate immunity in fish- The innate immune system is a universal and ancient form of host defense against infection (Janeway and Medzhitov, 2002). The innate immune response in teleost can be characterized in three main components- non-specific humoral defense mechanisms, non-specific cellular defense mechanisms, and

physical barriers. The non-specific humoral defense mechanisms in fish are numerous and include lysozyme, trypsin, complement, C-reactive protein, lectins, transferrin, interferons, hemolysin, proteinases, α2-macroglobulin, chitinase, α-precipitins, and defensins (Ellis, 2001). Some of these mechanisms bridge the adaptive and innate immunity (Sunyer *et al*, 2003). The non-specific cellular defense mechanisms include inflammation and phagocytosis by macrophages and heterophils. Inflammation is a cellular response characterized by pain, swelling, redness, heat, and loss of function. Inflammation is a protective response and is an attempt by the body to wall off and destroy an invader. Phagocytosis is a defense mechanism against infection common to all metazoans, including invertebrates. The phagocytic system consists of resident macrophages or macrophage-like cells present throughout the body and plays a very important role in the initial stages of infections, as they are the first phagocytes to encounter the invading microorganisms. Recent results show that in *Dicentrarchus labrax* fish species, in addition to macrophages, heterophils (neutrophils) are important phagocytic cells that are quickly mobilized to infected territories and have a high capacity of phagocytosis (do Vale *et al*, 2002). In that study, resident macrophages were the first to begin to phagocytose bacteria, due to proximity to the infection. Heterophils, however, were rapidly attracted to the infected area by microbial products and chemotactic substances produced by immune cells. Heterophils, as compared to the macrophages, are armed with more potent antimicrobial molecules, and are present under normal conditions in reserve pools in the haemopoietic organs and blood, but in much lower numbers in the tissues and body cavities. That is, while macrophages are important in the initial steps of infectious processes, heterophils are called into play

later, when extracellular growth or shedding from infected macrophages produce high numbers of extracellular bacteria.

The physical barriers include the epithelial layer and the mucus layer. The epithelial layer, including the integument and the surface of the gut, of all vertebrate animals is made up of two main layers: a relatively thin outer layer, the epidermis; and a thicker, tougher inner layer, the dermis. The skin of teleosts consists of these distinct layers of tissue covered externally by a mucus layer. The innermost layer is the dermis, a fibrous connective tissue consisting of regular collagen layers at 45 degree angles to body axis. The arrangement of the dermis resists forces that deform skin, and may aid in locomotion. The dermis is composed of connective tissues in which are nerve endings, muscle cells, and blood vessels, and it provides nourishment for the epidermis. Arising from the dermis in most modern teleosts are the scales, which are thin acellular, fibrous bone covering the body but absent or nearly absent in some fish. The primary function of scales appears to be protection. The closely packed cells of the epidermis provide a barrier against desiccation, microbes, radiation (Ahmed and Setlow, 1993), and chemicals. The epidermis of fish is of two or more layers (Hawkes, 1974). The deepest is the close-packed germinal layer called the *stratum germinativum*. The outer cell layer is formed by its daughter cells. There is much variation in the outer cells, dependent on the group of fishes. Body mucus is the product of the daughter cells and their degradation products and, as such, is continually replaced. The old materials are sloughed off and replaced by those underneath. Fish, while possessing keratinocytes, do not have a keratin layer over the epidermis (Baden and Kubilus, 1983).

Except for the Chondrichthyes, all known living fishes have skin with mucus, released from dispersed glandular cells in the epidermis. The mucus of the fish usually consists of two layers. One layer, called the cuticle, is made up of neutral and sulphated mucosubstances and is associated with the cell membranes of the epidermis (Saxena and Kulshrestha, 1981). The second layer, the mucus, contains sulpho- and sialomucins, as well as immunoglobulins (Fletcher and Grant, 1969), complement (Sunyer and Lambris, 1998), carbonic anhydrase (Lacy, 1983), lectins (Shiomi, *et al*, 1988, Nakamura, *et al.*, 2001), crinotoxins (Cameron *et al*, 1981), C- reactive protein (Jones, 2001), antimicrobial peptides (Cho, *et al*, 2002), proteolytic enzymes (Aranishi and Nakane, 1997), and free amino acids (Saglio and Fauconneau, 1985), all substances with biostatic or biocidal activity. A single goblet cell contained either neutral or acid glycoproteins alone or in combination, and at each site in the skin, the goblet cell population can be mixed, with cells producing each type of glycoprotein (Fletcher *et al.*, 1976). Mucus, once secreted, becomes a hydrated gel network of mucopolysaccharides and mucins that coats the lumenal surfaces of epithelia throughout the body. Mucins are the "structural" component of mucus. Mucins are a complex family of high molecular weight glycosylated proteins. They can be soluble (Perez-Vilar and Hill, 1999) or membrane-bound (Lan *et al*, 1990) and their most characteristic feature is the presence of tandemly repeated peptide domains rich in serine and/or threonine and proline (Figure 1.2). Typically, more than 60% of their mass is accounted for by carbohydrate, predominantly O-linked oligosaccharides with some N-linked oligosaccharides. Membrane anchored mucins may have additional roles concerned with protein interactions at the cell surface.

Mucus serves four main general functions for all fishes: osmoregulation, external protection, reducing turbulence (Videler, 1995), and defense from parasitism. In osmoregulation, mucus provides a selective interface to maintain internal - external ionic balance. As external protection, the mucus protects by covering over a wound caused by infection or mechanical injury. Besides species-specific uses such as skin toxin (Fusetani and Hashimoto, 1987), cocoon formation in lungfish; food source (Bremer and Walter, 1986), and alarm substance (Wisenden, 2000) a fourth function of mucus on fish skin is defense from disease causing organisms and parasites. These parasites include cestodes, nematodes, fungi, dinoflagellates, bacteria, and viruses. One route of entry for parasites is to colonize and invade fish through the mucus of the fish skin. However, mucus as a physical barrier makes it energetically unfavorable to a parasite to penetrate to the skin, which is also sloughing off. It has been demonstrated that there is a significantly negative correlation between the density of mucous cells and that of monogeneans on the surface of rainbow trout (*Oncorhynchus mykiss*) (Buchmann and Bresciani, 1998). They also play a role in the regeneration of epithelial layers that have been damaged by toxins, ulceration, inflammation or mechanical trauma. In malignant tumors, mucins can sometimes protect tumor cells from host immune killer cells, with the consequence that metastases are favored.

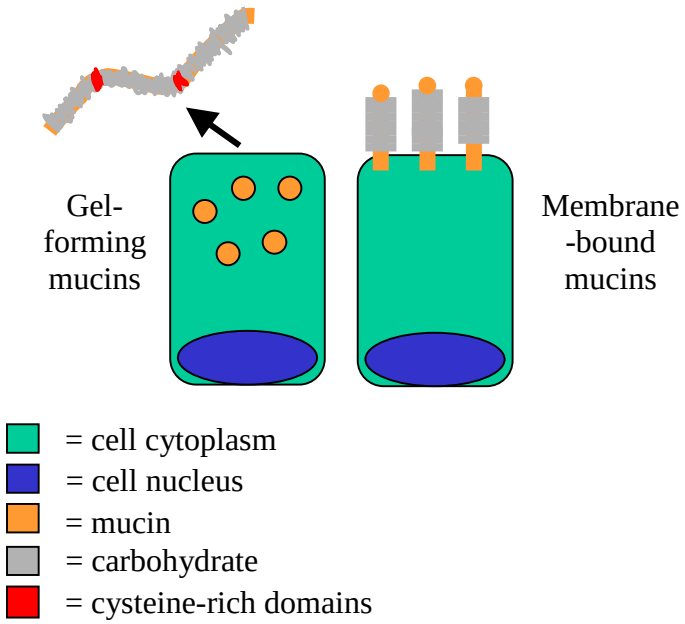


Figure 1.2. Soluble and Membrane-bound Mucins: Gel forming mucins expand when hydrated freely coat the epithelial surface. Membrane-bound mucins remain attached to the cell that produced them, and create a "cuticle" to protect epithelium.

Background in teleost model striped bass and disease-

Fish- Fish are a diverse group of vertebrate organisms that have adapted to complex and highly variable aquatic environments. There are about 29,000 species of fishes described that live in marine, estuarine and freshwater environments all over the world, with 200-300 new species being described each year. Fishes are divided into three Classes: Agnatha (jawless fish), Chondricthyes (cartilaginous fish), and Osteoichthyes (bony fish). Ninety percent of known fish belong to the Class

Osteoichthyes, and almost all of them are in the Order Teleostei, or the teleosts.

One species of teleost of environmental and economic importance is *Morone saxatilis* (Walbaum 1792), also known as the striped bass or rockfish (Figure 1.3). *M. saxatilis* is the largest member of the family Percichthydae, order Perciformes. *M. saxatilis* is an anadramous, euryhaline fish, found marine environments as adults and rivers and estuaries as juveniles or as migrating adults. *M. saxatilis* is distributed from the St. Lawrence River to northern Florida and western Florida (northern Gulf of Mexico) to Louisiana. *M. saxatilis* has also been introduced to the Pacific Coast of the United States *M. saxatilis* has been an important resource along the Atlantic coast of the United States since colonial times, and continues to be one of the most sought-after

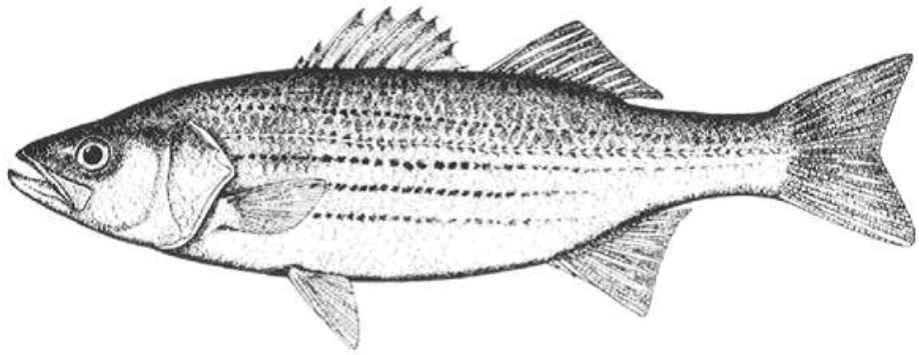


Figure 1.3. The striped bass, *Morone saxatilis* (from Atlantic Fishes of Canada, W.B. Scott and M.G. Scott)

commercial and recreational finfish in the Chesapeake Bay. *M. saxatilis* can be bred in captivity for the purpose of aquaculture. The recurring problem of disease in Chesapeake Bay and in aquaculture has serious impact on quantity and quality of fish.

Environment- In the Chesapeake Bay on the east coast of the United States, there is an ecosystem that is rapidly changing due to man's impact on the marine

environment (Llanso *et al.,* 2003). Non-point-source and point-source pollution is an increasing source of stress to aquatic, estuarine, and marine ecosystems (Mason *et al*, 2000; Preston, 2002). In addition, the average temperature of the bay has increased during the 1990's, and the bay has been experiencing record levels of hypoxia. These stressors have lead to not only increase bacterial loads, but to sick fish. The problem extends beyond the diseased organism. Increased bacterial loads can be passed on to humans that consume fish from the bay. Currently, 37% of all food borne illness in the United States is caused by seafood borne disease (Diaz, 2004). Many types of bacteria can live in and on the fish, but are non-pathogenic to fish or humans. However, increased stress in the fish may lead to conditions where bacteria may cause disease (Noga *et al*., 1998). The host (fish) must be in a susceptible state, and certain environmental conditions must be present for a disease outbreak to occur. This state and these conditions are prevalent in the Chesapeake Bay, and are common in aquaculture (Noga *et al*., 1998).

Several diseases in fish are initially observed as sores on the fish's skin. The appearance of sores on wild fish is not uncommon (May and Sindermann, 1999), and may be increasing. Striped bass of the Wicomico River (western tributary of the Potomac) of Maryland exhibiting sores in the mid 1990's, revealed isolates of *Edwardsiella tarda* (Baya *et al.,* 1997). This bacterium had never been linked to diseases in wild fish and the Baya *et al*, 1997 report was the first to suggest that *E. tarda* could affect wild populations. By later summer through fall of 1997, 10% of striped bass sampled in the Chesapeake Bay had lesions and nearly 13% through October 1998 (Overton *et al*., 2003). Prior to 1997, varieties of other gram-negative

enteric bacteria were isolated including *Aeromonas, Pseudomonas*, and *Vibrio* sp. (MDNR, 1997; 1998). Since 1997, most of bacterial isolates identified from symptomatic striped bass have been *Mycobacterium* sp. It is believed that *Mycobacterium* was responsible for many of the sores identified in the striped bass taken from the Potomac in 1997 and 1998 (Vogelbien unpublished). In aquaculture, examples of bacterial diseases that are most frequently encountered are bacterial Vibriosis, caused by various *Vibrio* species, and motile *Aeromonas* septicemia, caused by *Aeromonas hydrophila*. *V. anguillarum* typically causes a hemorrhagic septicemia. It appears that most infections with *V. anguillarum* begin with the colonization of the fish gastrointestinal tract. The bacteria are strongly attracted to intestinal mucus (Ascencio *et al*. 1998).

Bacterial Adhesion- Bacterial adhesion to host cells and tissues is a prerequisite for colonization by both pathogenic and commensal bacteria (Carpetier and Cerf, 1993). It begins by reversible attachment of the bacterium to the eukaryotic surface, called “docking”, followed by nonreversible permanent attachment of the microorganism to the surface involving permanent formation of many specific bonds between complementary molecules on each cell surface, called “anchoring” (An *et al*, 2000).

Docking reactions include hydrophobic interactions, electrostatic attractions, atomic and molecular vibrations resulting from fluctuating dipoles of similar frequencies, Brownian movement, and recruitment and trapping by biofilm polymers interacting with the bacterial glycocalyx (capsule) (Carpentier and Cerf 1993; An *et al*, 2000). Anchoring involves specific ligand-receptor interactions that mediate adhesion

(An *et al*, 2000). Complementary receptor and adhesin molecules must be accessible and arranged in such a way that many bonds form over the area of contact between the two cells. Once the bonds are formed, attachment under physiological conditions becomes virtually irreversible. Many bonds are protein-carbohydrate interactions, examples of which are a cell-bound protein on *Streptococcus pneumoniae* and N-acetylhexosamine-galactose disaccharide (Beuth *et al*., 1987), cell-bound protein on *Staphylococcus aureus* and amino terminus of fibronectin (Sinha *et al*., 2000), N-methylphenylalanine pili of *Neisseria gonorrhoeae* and glucosamine-galactose carbohydrate (Merz and So, 2000), fimbriae ("filamentous hemagglutinin") of *Bordetella pertussis* and galactose on sulfated glycolipids (Bassinet *et al*., 2000), N-methylphenylalanine pili of *Vibrio cholerae* fucose and mannose carbohydrate (Jonson *et al*., 1991), and a membrane protein on *Mycoplasma* and sialic acid (Chandler, 1982).

Specific aims of research- The specific aims of this research consisted of the testing of a series of hypotheses on the interactions of striped bass galectin with striped bass mucus and environmentally relevant bacteria. Hypothesis 1 is that a β-galactoside binding protein purified from the skin and mucus of *M. saxatilis* has a role in interactions of the mucus and bacteria. Hypothesis 2 is that when skin and/or muscle are damaged, galectin present in the wound can interact with bacteria and with various components to help reduce infection and facilitate repair. A literature review established that, at the time, nothing was known about the function of galectins in fish. While preparing this dissertation, some studies have been completed which support hypotheses in which galectins play roles in teleost development and possibly defense (Ahmed *et al*, 2004; Kamiya *et al*, 1988; Tasumi *et al*, 2002). The significance of this

study would be to help elucidate some possible function(s) of galectin in fish. The study of pathogen/host interactions is important in commercially significant animals such as the striped bass. Galectins have been implicated in innate and acquired immunity, and since fish appear to have a less complex repertoire of galectins as compared with mammals, studies about immune function of galectin(s) may be simplified using fish as the experimental model. To test these hypotheses, galectin from striped bass will first be biochemically and molecularly characterized. Next, interactions with mucus and bacteria will be investigated, and followed by localization of endogenous galectin in striped bass. Finally, several models will be proposed based on the data collected to date.

CHAPTER II: BIOCHEMICAL CHARACTERIZATION OF THE STRIPED BASS (*MORONE SAXATILIS)* 15 KDA GALECTIN

II.A. Introduction

Morone saxatilis (Walbaum 1792), commonly known as "striped bass" or "rockfish", is a teleost fish of considerable environmental and economic importance in the Chesapeake Bay. *M. saxatilis* is the largest member of the family Percichthydae, order Perciformes and are an anadramous, euryhaline fish, which are usually found in rivers and bays as juvenile and young adult, and in coastal waters as mature adults, except during migrations. *M. saxatilis* is distributed from the St. Lawrence River to northern Florida and western Florida (northern Gulf of Mexico) to Louisiana. *M. saxatilis* has also been introduced to the Pacific Coast of the United States, and due to both intentional and non-intentional stocking, there are some landlocked populations. *M. saxatilis* has been an important resource along the Atlantic coast of the United States since colonial times, and continues to be one of the most sought-after commercial and recreational finfish in the Chesapeake Bay. As most fish, striped bass must defend against the constant pressure of parasitic and commensal organisms that can potentially cause disease. This defense begins with the environmental interface of the fish, which are the epidermal layers and the mucus coating these layers produce. Numerous defense molecules have been isolated from the mucus, with one type being carbohydrate-binding proteins, called lectins. To date, lectins isolated from fish mucus include congerins (Muramoto, *et al.*, 1992), *Anguilla japonica* lectins 1 and 2 (Tasumi, *et al*, 2002), pufflectins (Tsutsui, *et al*., 2003), kingklip mucus lectin (Toda, *et al*., 1996),

Genypterus sp. lectin (Oda, *et al*., 1984). It was the possibility of lectins performing a function in defense that led to the investigation of galectins in fish. Galectins have been biochemically characterized in many animals (zebrafish, Ahmed, *et al*., 2004; African clawed frog, Shoji*et al*.,. 2003; fruit fly, Pace, *et al*., 2002; marine toad, Ahmed, *et al*., 1996; cow, Ahmed *et al*., 1996; human, Sharma *et al*., 1990; Ackerman, *et al.*, 1992; sheep, Dunphy, *et al*., 2002) and these established techniques were used to purify and characterize galectin from striped bass. This family of proteins possesses characteristics such as a single-stranded polypeptide, affinity to lactose, small globular structure, acidic pI, and ubiquitous presence in animals.

This chapter describes the purification and biochemical characterization of a lactose-binding protein present in the skin and muscle of striped bass *M. saxatilis*. Biochemical characterization of *M. saxatilis* 15 kDa galectin began by using the parameters determined in other galectins as a starting point for purification and analysis. Ascertaining these biochemical properties has helped determine that this protein is similar to previously described galectins in other animals, gave insight into the possible physiological conditions in which the protein would be functional, and determined parameters for future experiments using this protein. Determining primary through tertiary protein structure was essential for functional and phylogenetic comparisons, and biochemical analysis may help understand observed intra- and intercellular interactions. The first step in biochemical characterization was the purification and analysis of the protein's molecular weight. This was followed by partial determination of primary structure, determination of protein pI and detection of isoforms, analysis of protein stability, and ligand (carbohydrate) specificity.

II.B. Materials and Methods

Animals source and sample collection: Five adult striped bass ranging from 4 to 7 kg were obtained from the Aquaculture Research Center (ARC) located in Fells Point, Baltimore, Maryland, and ARC II, located at the Center of Marine Biotechnology, University of Maryland in Baltimore, Maryland. Animals were anesthetized by adding 0.079 ppt phenoxyethanol to small holding tank containing tank water, and euthanized by severing the spinal cord posterior to the gill slits. Tissues and organs were dissected, immediately frozen in liquid nitrogen, and stored at –80^{o}C until processing.

Protein purification: Galectin was extracted and purified from striped bass muscle, skin and mucus, using an improved protocol as reported by Ahmed, *et al.* (1996). Tissues were homogenized in 1:10 phosphate buffered saline (PBS) containing β-mercaptoethanol (β-ME), 0.1 M lactose, with 1 mM phenylmethylsulfonyl fluoride (PMSF) and 2 mM ethylenediaminetetraacetic acid (EDTA) as protease inhibitors. The homogenate was clarified by centrifugation, and the supernatant was fractionated by ion exchange chromatography as follows: first, the supernatant was absorbed on diethylaminoethyl (DEAE)-Sepharose bymixing with DEAE -Sepharose equilibrated in β-ME, [PBS/10]. After overnight incubation at 4^{o}C, mixture was poured into a sintered glass funnel and the flow through was saved. DEAE-Sepharose was washed with 10 bed volumes of β-ME, [PBS/10] with low vacuum. The bound protein was eluted with 4-bed volumes β-ME, PBS, 0.5 M NaCl. The flow through was subjected to extraction a total of three times. All three high salt eluates were pooled, and used in affinity chromatography.

Affinity chromatography: Affinity column consisting of lactosyl-Sepharose was prepared by cross-linking lactose to Sepharose CL-6B activated by divinyl sulphone (Gabius, 1990), and the column was washed extensively with β-ME/PBS/0.5 M NaCl. The pooled eluate from ion exchange chromatography was absorbed on lactosyl-Sepharose in a 100 mm x120 mm column. The column was washed with β-ME/PBS/0.5 M NaCl, and equilibrated with β-ME/ [PBS/10]. Galectin was eluted with at least 2 bed volumes β-ME/ [PBS/10] /0.1 M lactose. Protein concentrations were measured and homogeneity was analyzed on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), where a single reproducible band represented a sample with little or no contamination by other proteins. Protein was stored absorbed to DEAE-Sepharose in 1:1 β-ME/ [PBS/10]: glycerol at -20.0^{o}C.

Protein Concentration Determination: Protein concentrations were determined on 96-well flat bottom plates with the Bio-Rad Protein Assay following a modification of the manufacturer's protocols, using bovine serum albumin (BSA) as standard. To 100 μl of 5-40 μg/ml protein standard solutions and samples, 100 μl of Coomassie blue dye reagent pre-diluted 2.5 fold with water was added. After 5 min, the reactions were read in a Molecular Devices Plate Reader at 595 nm and the data were analyzed through the Softmax program.

Hemagglutination assay: Erythrocytes from human (Referencells, Immunocor, Norcos, GA), horse, goat, sheep, and rabbit were screened for use in agglutination tests. All erythrocytes except human were fixed in glutaraldehyde by mixing 10:1 whole blood to cold 0.05% glutaraldehyde, PBS for 10 minutes at room temperature. Following incubation, cells were washed in PBS three times and stored in PBS. All

cells except human were treated with pronase by incubating one volume fixed cells with 4 volumes 5 mg/ml pronase for 1 hour at 37^{o}C. Following treatment, cells were washed 3 times with PBS and stored in PBS. Human type O RBC's were chosen for agglutination tests based on reproducibility of agglutination and availability of quality cells. Agglutination tests were carried out in 96 well Terisaki plates (Robbins Scientific, Mountain View, CA) coated with bovine serum albumin (BSA) (Vasta *et al*, 1986). An equal volume of a 0.5% suspension of human type O RBC's was added to 5 μl of two-fold dilutions of MS15 in β-ME, PBS (pH 7.5). Plates are gently vortexed and incubated at room temperature for one hour. Agglutination was read under a microscope (200X) and scored from 0 (no agglutination) to four. The reciprocal of the highest dilution of MS15 showing an agglutination of one was recorded as the titer. The specific activity of MS15 was defined as the titer/milligram of protein/milliliter. Controls were carried out by adding β-ME, PBS buffer instead of galectin.

Relative molecular mass analysis

SDS-PAGE: Analytical polyacrylamide slab gel electrophoresis in the presence of sodium dodecylsulphate (2%) was carried out on 15% (w/v) acrylamide gels under reducing conditions using loading buffer consisting of 4% SDS, 0.1 M Tris pH 8.9, 2 mM EDTA, 0.1% bromophenol blue, 20% glycerol, and 2 mM DTT.

Gel permeation chromatography: Gel permeation chromatography of the purified protein was carried out in a Pharmacia Superose 6 (1 x 30 cm) column equilibrated with β-ME, PBS, 0.25 M NaCl, 0.01 M lactose (pH 7.5) in a high performance liquid chromatography (HPLC) system that consists of a Beckman- 116

pump and a Beckman programmable detector module- 166 (280 nm), at a flow rate of 0.4 ml/min. Gel permeation chromatography of the horseradish peroxidase (HRP)-conjugated galectin was carried out with PBS (no azide), 0.25 M NaCl, 0.01 M lactose (pH 7.5) in the same system and conditions as described above. BSA (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), and ribonuclease A (13.7 kDa) were run as standards.

Western Blot analysis: Samples were separated by SDS-PAGE as described before in duplicate and electrotransferred to polyvinylidene fluoride (PVDF). Blots were blocked for 1 hour at room temperature in 5% BSA, PBS pH 7.4. One blot was probed with rabbit anti-striped bass muscle galectin antiserum, as reported earlier (Ahmed, *et al*., 2004) and the other with pre-immune serum from the same rabbit as a negative control. The anti-striped bass muscle antiserum (Ahmed, unpublished) was prepared in New Zealand white rabbits at Duncroft (Lovetsville, FL) by multiple subcutaneous and intramuscular injections of affinity-purified striped bass muscle galectin (100 μg/ injection), and the antibody titer was determined by enzyme-linked immunosorbant assay (ELISA) as previously described (Vasta *et* al., 1986). Each blot was probed with a 1:1000 serum dilution in antibody incubation buffer (1% BSA, 0.3% Tween-20, PBS pH 7.4) for 2 hours at room temperature. Blots were washed twice in 100 ml PBST (PBS, 0.1% Tween-20, 10 minutes, room temperature) and once in PBS (10 minutes, room temperature) prior to probing for 2 hours at room temperature with a 1:3000 dilution of HRP-conjugated goat anti-rabbit IgG (A6154, Sigma, St. Louis, MO) in antibody incubation buffer. Blots were washed as before and developed with 3,3,5,5 tetramethylbenzidine (TMB, Pierce, Rockford, Illinois) and allowed to develop for 20

minutes. Apparent molecular masses of antiserum-reactive proteins were determined by comparing their migration distances to molecular weight vs. migration distance for molecular weight standards between 14.4 and 94 -kDa (LMW, 17-0446-01, Amersham Pharmacia, Piscataway, New Jersey) run concurrently on the same gel.

Peptide Sequencing and Amino Acid Analysis: The purified galectin was partially sequenced by automated Edman degradation (Hewick, *et al*1981) by Dr. Jan Pohl, at the Winship Cancer Center's Microchemical Facility at Emory University School of Medicine. For this purpose, 50 ⌈γ of affinity-purified 15 kDa lactose-binding protein was subjected to SDS-PAGE in a 15% gel (reducing conditions), and in-gel digested with trypsin in 0.05 M Tris-HCl (pH 8.5), 1 M guanidine. The peptides were separated with a PE-Biosystems 140B/1000S/783A microbore/capillary HPLC system (Foster City, CA, USA), fractionated on an Applied Biosystems Aquapore ODS-300 C-18 silica column, with the flow through manually collected and stored at -20^{o}C prior to further analysis. Fractionated peptides were sequenced on an Applied Biosystems model pulsed-liquid 477A/120A sequencing system (Foster City, CA, USA).

Isoelectric focusing and two-dimensional (2-D) gel electrophoresis: Analytical isoelectric focusing of the purified galectin was carried out on a thin (1 mm) layer 5% polyacrylamide Ampholine PAGplate (Pharmacia, pH range 3-10) in an EC 100I electrophoresis unit (EC Apparatus Corporation). Ten μg of purified galectin in 100mM lactose, PBS pH 7.4 was loaded onto the gel using wicks placed at the halfway point of the gel. The gel was fixed with 4% sulfosalicylic acid, 12.5 %trichloroacetic acid in H_2O, stained with 0.1% Coomassie Brilliant Blue R-250 in 40% ethanol, 10% acetic acid, H_2O, and destained with 40% ethanol, 10% acetic acid, H_2O. The

isoelectric point (pI) was determined from a plot of pIs of markers vs. distance from the cathode of pI standard markers. The pI markers (Sigma Chemical Co.) used were glucose oxidase (pI 4.2), trypsin inhibitor (pI 4.6), β-lactoglobulin A (pI 5.1), and carbonic anhydrase II (pIs 5.4, 5.9). For 2-D electrophoresis, IEF was first performed using the Protean IEF System with immobilized pH gradient (IPG) strips (BioRAD) as follows: 100 μg MS15 was loaded onto rehydrated IPG strip, pH 3-10, and focused for 30,000 V-hours. Strips were equilibrated in dithiothreitol (DTT) equilibration buffer (2% w/v DTT, 6 M urea, 2%SDS, 20% glycerol, 0.05 M Tris-HCl, pH 8.8) followed by iodoacetamide equilibration buffer (2.5% w/v iodoacetamide, 6 M urea, 2%SDS, 20% glycerol, 0.05 M Tris-HCl, pH 8.8) and transfer to a precast gel (Criterion, BioRAD) for second dimension. The separated protein was transferred to a PVDF membrane, and protein as detected by Ponceau Red staining followed by western blot using the previously described protocol.

Stability studies

Thermostability: The temperature stability of the striped bass galectin was determined by incubating 100 μl (3 μg/ml) in β-ME/PBS at various temperatures for 30 min, cooling samples on wet ice and titrating them with human type-O RBC (Vasta and Marchalonis, 1986).

Stability in a Non-Reducing Environment: The striped bass galectin (100 μg) was absorbed on 1 ml lactosyl-Sepharose, and the matrix thoroughly washed with aerated PBS (20 ml/ml matrix). Bound galectin was stored at room temperature. Control matrices contained the same amount of striped bass galectin in β-ME/PBS.

After 8 days, the lactosyl-Sepharose column was eluted with 2 ml of β-ME/PBS/ 0.1 M lactose and the eluate dialyzed with β-ME/PBS in the presence of 2 mg of BSA. The hemagglutinating activity was measured with human type-O RBC.

Carbohydrate Specificity

Alkylation of galectin: The purified striped bass galectin was carboxamido-methylated with iodoacetamide on a solid phase under mild conditions in the presence of excess ligand (Allen *et al.*, 1990), yielding carboxamidomethylated galectin (CAM^a-galectin). This was done by washing a DEAE-Sepharose column (0.5 ml bed volume) containing purified striped bass galectin with PBS [1:10] pH 7.4 (PBS/10) and immediately overlaying the column with 1 ml of 0.1M iodoacetamide-containing 0.1M lactose. After incubation at 4°C for 1 hour in the dark, the column was washed with PBS/10 (no azide) to remove excess reagent and lactose and the CAM^a-galectin was eluted with 0.5 M NaCl/PBS pH 7.4 (no azide).

HRP conjugation: The CAM^a-MS15 was conjugated to HRP through glutaraldehyde coupling as described in Ahmed *et al*. (1994a): to a mixture of CAM^a-MS15 (0.7 mg) and HRP (2.0 mg) in 1.3 ml of 0.5 M NaCl, 0.1 M lactose, PBS pH 7.4 (no azide), 160 µl of 1% glutaraldehyde was added. After overnight incubation at 4°C, the conjugation mixture was diluted 40-fold with cold water and adsorbed onto DEAE-Sepharose (0.5 ml) preequilibrated with PBS/10 (no azide). The column was washed with PBS/10 (no azide) to remove lactose and the conjugate was eluted with 2 ml of l.0 M NaCl, PBS pH 7.4 (no azide) and purified by affinity chromatography on lactosyl-Sepharose as described before. Finally, the conjugate was separated from the unreacted

striped bass galectin by gel permeation chromatography on a Superose 6 column as described above. The purified MS15-HRP conjugate was stored in 1% BSA, 50% glycerol at -20^{o}C.

Solid phase binding-inhibition assay: Binding of striped bass galectin to asialofetuin (ASF) and its inhibition by sugars was determined in 96-wells polystyrene plates (Dynatech Laboratories). First, ASF (0.5 μg/ l00 μl/ well) in 0.1 M Na_2CO_3, 0.02% NaN_3 (pH 9.6) was adsorbed in wells of polystyrene plates and incubated at 37^{o}C for 3 hours. After aspirating the residual ASF solution, fixation was carried out with 2% formaldehyde in PBS at 37^{o}C for 30 minutes. The plates were washed three times with PBS pH 7.4 (no azide), 0.05% Tween 20 (polyoxyethylene (20) sorbitan monolaurate) and incubated with the galectin-HRP conjugate (for binding assays) or with pre-incubated mixture of conjugate and test ligands (for binding-inhibition assays). To determine the optimal pH for striped bass galectin binding, 200 ng/ml galectin-HRP conjugate in water containing 0.1% Tween 20, was mixed with equal volume of various buffers (0.2 M) and 100 μl of this mix was used in triplicate in the binding assay as described above. The buffers used were citrate-phosphate, pH 4.0-6.0; phosphate, pH 6.5-8.0; and carbonate-phosphate, pH 8.5-9.5. The optimal ionic strength for MS15 plate assays was determined by mixing 200 ng/ml with an equal volume of various saline buffers and 100 μl of this mix was used in triplicate in the binding assay as described above. The buffers used were 10 mM phosphate buffer, 0.05% Tween 20, with NaCl at the following concentrations: 100 mM, 200 mM, 300 mM, 500 mM, and 1000 mM.

Pre-incubation of the galectin-HRP conjugate [200 ng/ml in PBS pH 7.4 (no

azide), 0.05% Tween 20] for binding-inhibition assays was carried out by mixing equal volumes conjugate and the test ligand at varying concentrations. After 1 hour at 4^{o}C, the conjugate-ligand mixture (100 μl) was added to the wells in duplicate and the plates were incubated for 1 hour at 4^{o}C. The plates were washed with ice-cold PBS pH 7.4 (no azide), 0.05% Tween 20 and the bound peroxidase activity was assayed with TMB. To quantitate the amount of galectin-HRP conjugate that bound to ASF, at the time the plates were developed; equal volumes containing increasing amounts of galectin-HRP conjugate were added to uncoated wells and developed with TMB under identical conditions (time and temperature) to the binding assay. A standard curve was drawn from the absorbance value of each point.

II.C.Results

Animal source: Although all animals chosen appeared outwardly healthy, upon dissection, some exhibited signs of mycobacteriosis in the form of granuloma formation in the liver. Samples from these were preserved for possible further analysis, but were not used in the extractions for galectin.

Protein purification: Purification a striped bass galectin was performed with a protocol based on Ahmed, *et al*, 1994b, used successfully for galectins with an acidic pI. The flowchart in Figure 2.1 outlines the steps followed. Of the various samples homogenized for extraction, only skin presented any difficulty. Skin samples had to be cut in less than 3 cm x 3 cm pieces, to prevent "clogging" the homogenizer. Following centrifugation, homogenized samples separated into three distinct layers: an upper lipid layer, a pink supernatant and pelleted solids. It was the pinkish supernatant, containing

soluble proteins, that was used for galectin extraction by affinity chromatography. Flow through from the affinity column had no detectable hemagglutination activity, suggesting either galectins had been captured on column or inactivated during extraction. Elution with 0.1 M lactose, PBS/10 pH 7.4 revealed a peak of protein

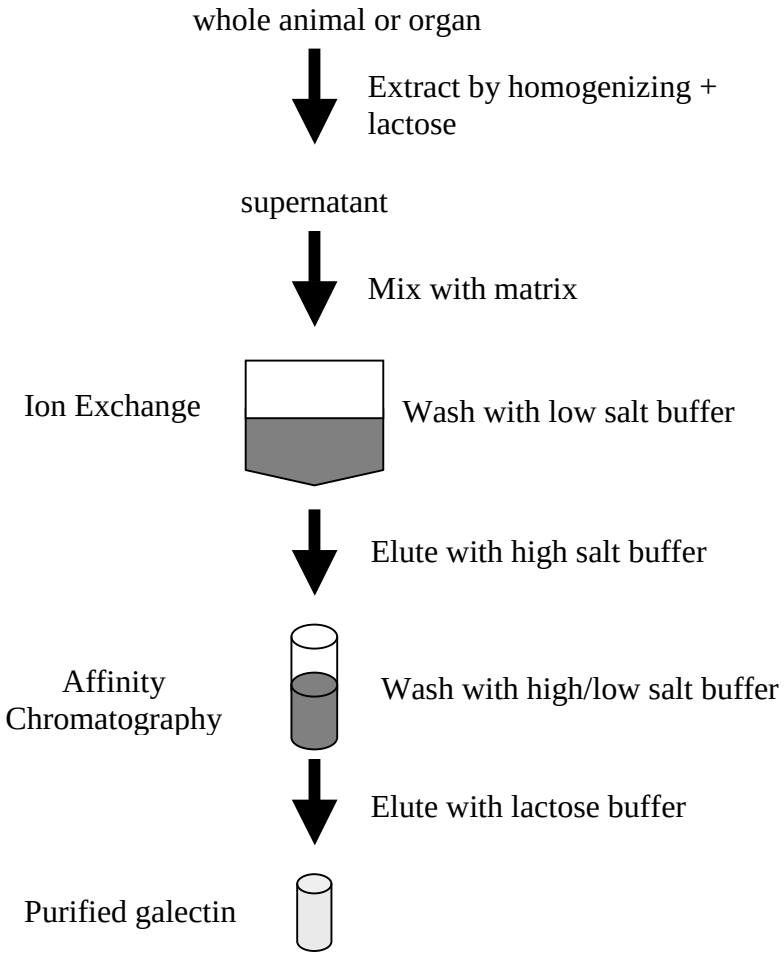


Figure 2.1. Purification strategy for galectins- Based on Ahmed, *et al*, 1994b, purification of MS15 assumes striped bass galectin is a soluble, acidic, lactose-binding protein. This purification scheme allows for buffer exchange while protein is bound to matrix and monitoring of activity and yield after each step. Purification performed at 4^{o}C.

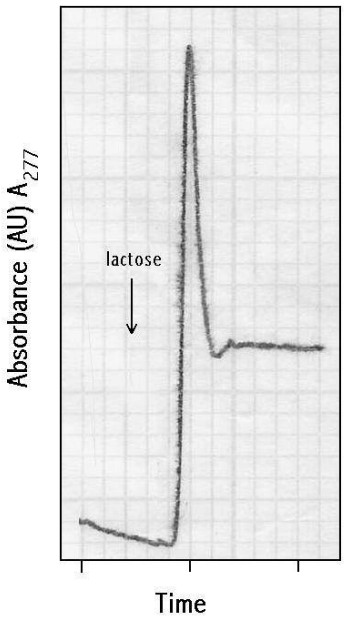


Figure 2.2. Elution profile of MS15 from lactosyl-sepharose. Flowthrough of lactosyl-Sepharose was monitored by absorbance at $Å_{277}$ during equilibration with β-ME/[PBS/10], following addition of β-ME/[PBS/10]/0.1 M lactose. Peak was observed as second bed volume of elution buffer passed through column.

between the first and second bed volumes of eluate (Figure 2.2). Remainder of eluted protein was collected and stored on DEAE-Sepharose at -20^{o}C, with 50% glycerol to prevent freezing of protein sample. SDS-PAGE of samples collected from each stage of purification revealed a progressive reduction in the overall amount of protein and the complexity of the mixture (Figure 2.3). Samples collected at each step of purification were dialyzed to remove lactose, and tested for hemagglutination with human type O blood. The results were used to generate a purification table for both skin and muscle extractions, summarized on Table 2.1. Striped bass galectin was purified 6,000 – 11,000-fold, and the yield of active galectin per gram sample was 77 μg/kg skin, and 15 μg/kg from muscle. The final stage revealed a single band eluting from lactosyl-Sepharose in a competitive ligand-dependent manner. The single band had a relative molecular mass of 15 kDa, as compared with molecular mass standards run in adjacent lanes, and the protein was designated MS15.

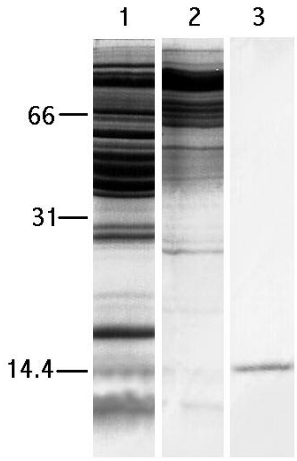


Figure 2.3. SDS-PAGE of protein purification. Protein sample mixed with 2x loading buffer, heated for 1 minute prior to loading. Gel was 15% acrylamide. *Lane 1*, striped bass crude extract; *lane 2*, high salt eluate from DEAE-sepharose (ion exchange); *lane 3*, lactose eluate from lactosyl-sepharose (affinity chromatography).

Sample Muscle	**Protein conc (mg/ml)**	**Titer**	**Vol (ml)**	**Total protein (mg)**	**Specific Activity**	**Total Activity**	**Recovery (%)**	**Puri. fold**
Crude	130.4	8	500	65200	0.061	4000	100	1
High salt 1	3.8	8	370	1406	2.1	2953	74	34.4
High salt 2	3.0	4	400	1200	1.34	1608	40	21.9
High salt 3	2.3	2	450	1035	0.87	900	22	14.3
High salt filtrate	-	0	-	-	0	0	-	-
Lactose elute	.041	16	20	.82	390	320	80	6393
Sample Skin	**Protein conc (mg/ml)**	**Titer**	**Vol (ml)**	**Total protein (mg)**	**Specific Activity**	**Total Activity**	**Recovery (%)**	**Puri. fold**
Crude	12.1	512	1070	12895	42.3	541.5	100	1
High salt 1	5.6	128	200	1120	22.8	25.8	4.76	0.54
High salt 2	2.1	32	200	420	15.2	6.3	1.16	0.35
High salt 3	3.0	8	200	60	2.6	1.8	0.33	0.07
High salt filtrate	-	0	-	-	0	8	-	-
Lactose elute	0.27	128	4	1.08	474	512	94.6	11000

Table 2.1. Purification tables for striped bass muscle and skin galectin- **"**Crude" is homogenized sample in lactose/PBS. The "high salt" steps are the eluate from DEAE-Sepharose, repeated three time with flow through. The "lactose elute" is elute from lactosyl-Sepharose. Recovery of total activity from the crude sample was high (80-95%), and the purification fold was on the order of 10^4.

Transfer of electrophoresed samples to a PVDF membrane was completed in approximately 1 hour. Following development of membrane with TMB, a single band was visualized, at ~15 kDa. No signal was visible on membranes probed with rabbit pre-immune serum. Occasionally, extracts from striped bass muscle revealed a doublet, with the upper band at 15 kDa, and the lower band at ~ 14 kDa. This doublet appeared only in muscle extracts (Figure 2.4) and the lower band was subjected to peptide sequencing by separating the protein using a long format 20% tricine gel.

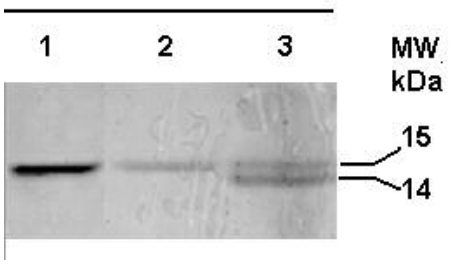


Figure 2.4 . Tricine gel separation of *M. saxatilis* muscle galectin(s). The two proto type galectins of muscle co-purified, and were separated by long format 20% tricine gel for N-terminal sequencing. *Lane 1*- mucus galectin; *Lane 2*-skin galectin; *Lane 3*-muscle galectin

Following electrotransfer to a PVDF membrane and Coomasie staining, the bands were sufficiently separated to be excised and N-terminally sequenced, with results shown in Figure 2.6. Nineteen residues were read, revealing the N-terminus was not blocked. It overlapped and aligned with eight residues determined in peptide sequencing, and aligned (5 positions out of 19) with the N-terminus of human galectin-1. This evidence suggests the 14 kDa galectin band from muscle may be the same protein as the 15 kDa, possessing natural or artifactual modifications, or the 14 kDa galectin is closely related protein, such as the congerins found in conger eel.

The gel permeation elution profile in Figure 2.5, compiled by overlying the molecular mass standards elution profile with the MS15 elution profile, reveals a protein of approximately 30 -kDa. This was interpreted as a non-covalently linked homodimer of MS15. Multiple runs at different dilutions did not reveal a concentration at which there was a dimer: monomer equilibrium shift.

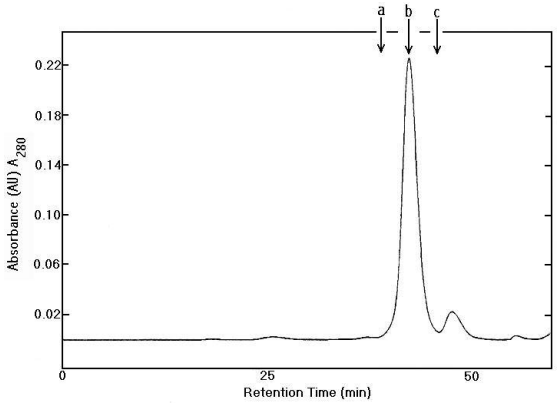


Figure 2.5. Gel permeation chromatography of MS15 with Superose 6 column.
Arrow a, the peak retention time for bovine serum albumin (molecular weight, 66,200); *arrow b*, the MS15 dimer (molecular weight, 30,180); *arrow c*, ribonuclease A (molecular weight, 13,700).

Peptide sequencing: Trypsin cleavage of MS15 prior to sequencing was performed for two reasons. First, many cytosolic proteins of eukaryotes are N-terminally blocked to Edman degradation. Second, the efficiency of Edman degradation only allows at most 25-30 residues to be determined accurately. With MS15 being approximately 136 amino acids in length, 5 or 6 peptides would have to be ideally read to span the whole polypeptide. The peptides isolated and sequenced are shown in Figure 2.6. Of an estimated length of 136 residues, (15 -kDa / 110 Da / amino acid) 86 were revealed in the peptide sequences. Sequencing reads ran between 13 and 24 residues long. The peptides were aligned with the primary structure of human galectin-1, as shown in Figure 2.6. All peptides could be aligned with the human prototype galectin, and the ends of the peptides were at conserved arginines and lysines, which is as expected

considering the specificity of trypsin.

```
Human MACGLVASNLNLKPGECLRVRGEVAPDAKSFVLNLGKDSNNLCLHF
M.U.  ~~~~~~~~~~~~~VGQTMTVVGVAKPDASDFAVNVGPDEK~~~~~~
M.L.  ~~DGLLIKNMSFKVGQTMTVV~~~~~~~~~~~~~~~~~~~~~~~~

Human NPRFNAHGDANTIVCNSKDGGAWGTEQ REAVFPFQPGSVAEVCITF
M.U.  ~~~FNAXGDENVVVXNSYQGGKWEEEH REGGFPFQQGEEFKITIEF
M.L.  ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

Human  DQANLTVKLPDGYEFKFPNRLNLEAINYMAADGDFKIKCVAFD
M.U.   TPTEFLVTLSDGSTIHFPN~~~~~~YSFINFVGDVRIK~~~~
M.L.   ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
```

Figure 2.6. Alignment of sequenced peptides from muscle "doublet" with human galectin-1- Results from peptide digestion and microsequencing (M.U.-muscle, upper band) were aligned with human galectin-1 primary structure. Results from N-terminal sequencing (M.L.-muscle, lower band) were aligned to the first two. X's in sequence were blanks in sequencing reaction, which often occurs with unmodified Cys in Edman degradation. M.L. was not N-terminally blocked. M.U. possessed an N-terminal "D" in place of "FN", possibly by deamidation.

When aligned with human galectin-1, the aligned peptides range from 30% to 59% identical. When aligned with the only fish galectin that had been fully sequenced at that time, electrolectin from *Electrophorus electricus*, the identity was 40% in the N-terminal peptides and 59% through the C-terminal peptide and in the highly conserved carbohydrate-binding site. As previously mentioned, one peptide aligned with no differences with N-terminally sequenced protein from muscle, bringing the total number of residues sequenced to 97.

IEF/2-Dimensional Gel Electrophoresis: Analysis of the purified MS15 by IEF showed some heterodispersion in spite of the apparent subunit size homogeneity

observed by SDS-PAGE. The four major protein species resolved at pI 4.8, 4.9, 5.1, and 5.2 (Figure 2.7*c*). When 2-D electrophoresis was performed on skin and muscle crude extract, the first dimension (pI) revealed proteins dispersed across the entire pH 3 to pH 10 range. The second dimension (MW, SDS-PAGE) revealed proteins ranging in size from 7 -kDa to 200 -kDa. Following western blot analysis of skin extract with anti-MS15, three spots were resolved at ~15 -kDa, and ranging from pI 4.8 to 5.1 (Figure 2.7 *A,C*). Western blot analysis of muscle extract did not resolve as clearly but the protein detected was similar in MW and pI as in the skin extract (Figure 2.7 *B,D*). Galectins in other organisms have resolved as several charged isotypes, but the structural basis for this observation has not been fully understood. Until the entire galectin polypeptide sequence is elucidated, this question will remain unanswered.

Thermal Stability: The stability of native striped bass skin and muscle galectin varied with temperature, as measured by hemagglutination activity (Figure 2.8*A*). From 30^{o}C to 60^{o}C there is a steady decline in activity with no activity observed above 60^{o}C for either skin or muscle galectin. MS15 was inactivated by exposure to an oxidative environment, but could be protected from oxidative inactivation by either the presence of excess ligand, or by alkylation when performed in the presence of ligand (Figure 2.8*B*). Peptide sequencing revealed no cysteines (Figure 2.6), but there were two positions at which sequencing failed to identify any amino acid residues, possibly due to the presence of cysteines. Peptide sequencing could not confirm the presence of cysteines, but the conserved tryptophan of prototype galectins was observed in the most probable peptide sequence alignment.

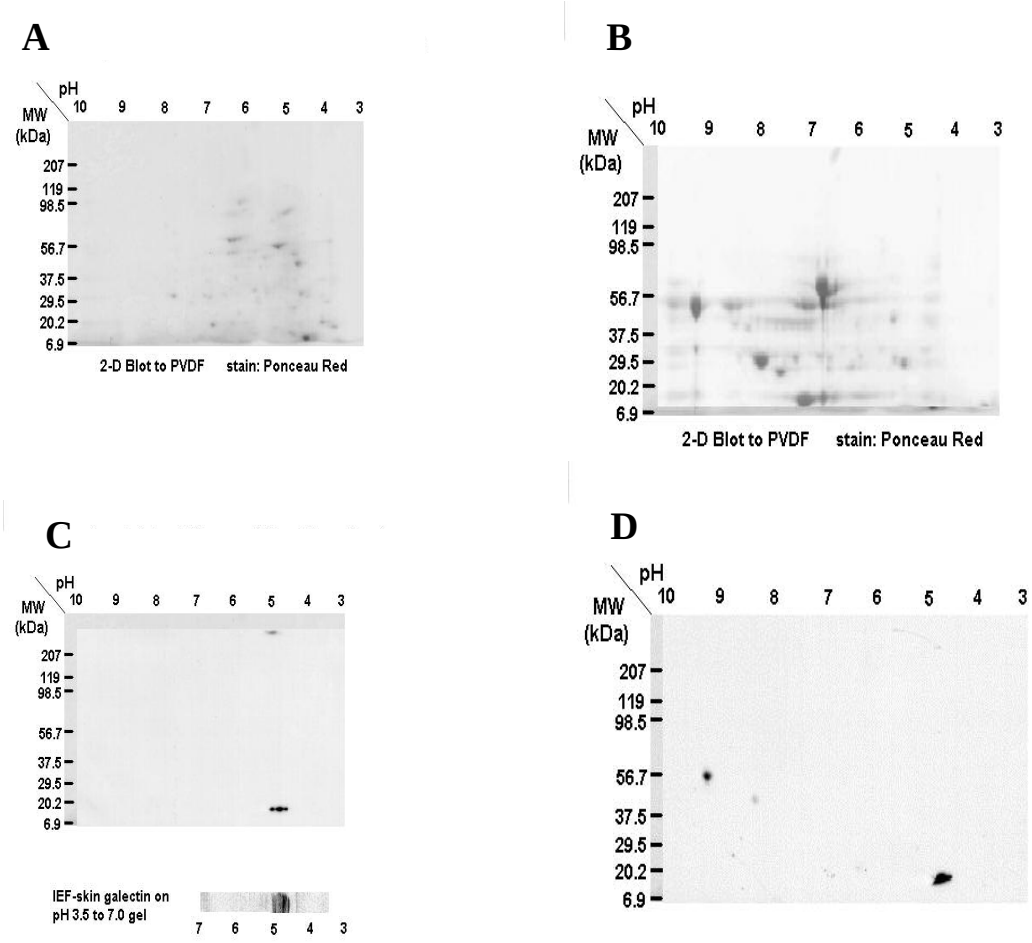
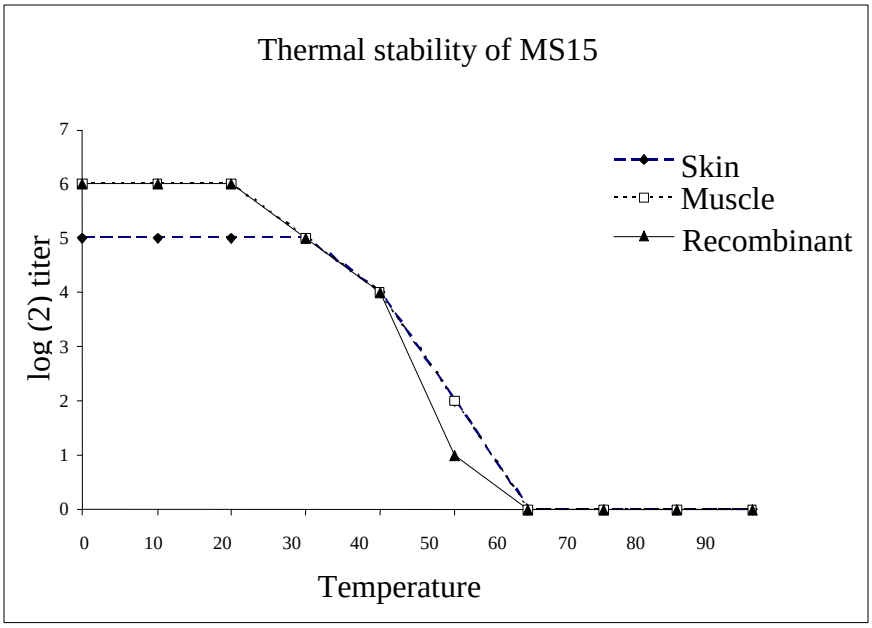


Figure 2.7. IEF and 2-D electrophoresis of MS15: skin and muscle- MS15 purified from striped bass skin (*A, C*) or striped bass muscle (*B, D*), was separated in 2 dimensions based on pI and molecular weight. *A,* Skin extract was separated and stained with Ponceau Red, revealing a wide range of protein sizes and charges. *B,* Muscle extract was separated and stained the same way. *C,* Destained skin extract blot was probed α-striped bass galectin, with α-rabbit:HRP as secondary antibody. Bound Ab was detected with DAB. *D,* Destained muscle extract blot was probed and detected the same way. Below *C* results from IEF, aligned with the pH scale of the 2-D gel, revealing same pattern of charge isotypes in skin extract.

A



B

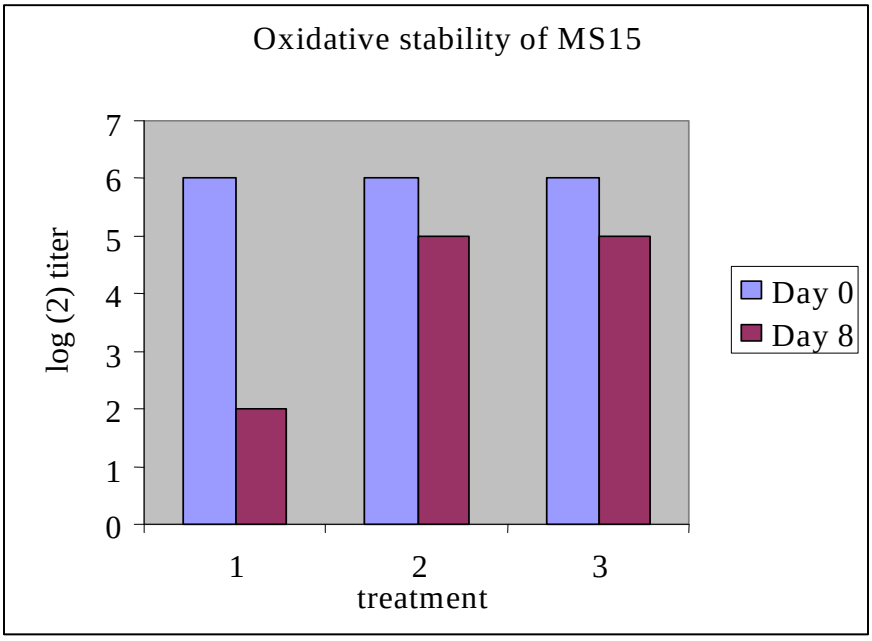


Figure 2.8. Stability studies with MS15. *a*) Thermal stability study was performed on skin MS15, muscle MS15, and recombinant MS15. Temperature range was 0^{o}C to 100^{o}C. *b*) Oxidative stability study was performed with MS15 from skin as outlined in "Material and Methods"

Carbohydrate specificity: The carbohydrate specificity of MS15 was determined using selected saccharides in a solid phase binding-inhibition assay. Preparation for the binding-inhibition studies included the optimization of the assay conditions (Figure 2.9). The optimal pH for binding was pH 8.0, with a test range of pH 4.0 to 9.0 (Figure 2.9 *A*). Optimal ionic strength for binding was 150 mM NaCl, with a test range of 50 mM to 1000 mM NaCl, final concentration (Figure 2.9 *B*). For each saccharide, the molar concentration giving 50% inhibition (I_{50}) was determined from the inhibition curves derived from the assays. These I_{50} values were normalized with respect to lactose standard on each assay plate. Table 2.2 shows the results of binding-inhibition assays, comparing the relative binding of MS15 to the bovine spleen galectin-1 (Ahmed, *et al*., 1996a), the 14.5 kDa galectin from *Bufo arenarum* Hensel (Ahmed, *et al*., 1996b), and Drgal1-L2, a proto type galectin from *Danio rerio* (Ahmed, *et al*., 2004). The poorest inhibitors of agglutination (<< lactose) were the monosaccharides galactose, Gal β-OMe, and Gal-α-OMe. The poorest inhibitors among the disaccharides were Galβ1,3GalNac, Galβ1,6GlcNac and Galα1,6Glc. The only trisaccharide tested, Fucα1,2Galβ1,4Glc, was also a relatively poor inhibitor of agglutination. The medium inhibitors of agglutination (~ lactose) were all disaccharides: Galβ1,4Fru_f, Galβ1,4Glcβ(1-0)Me, and Galβ1,4Man. The best inhibitors of agglutination (>> lactose) among the saccharides tested were the disaccharides Galβ1,3Ara, Galβ1,3GlcNac, Galβ1-3GlcNacβ-OPhNO_2, Galβ1,4Glcβ-OPhNH_2, Galβ1,4GlcNac, MeO-2Galβ1,4Glc and Galβ1S1βGal.

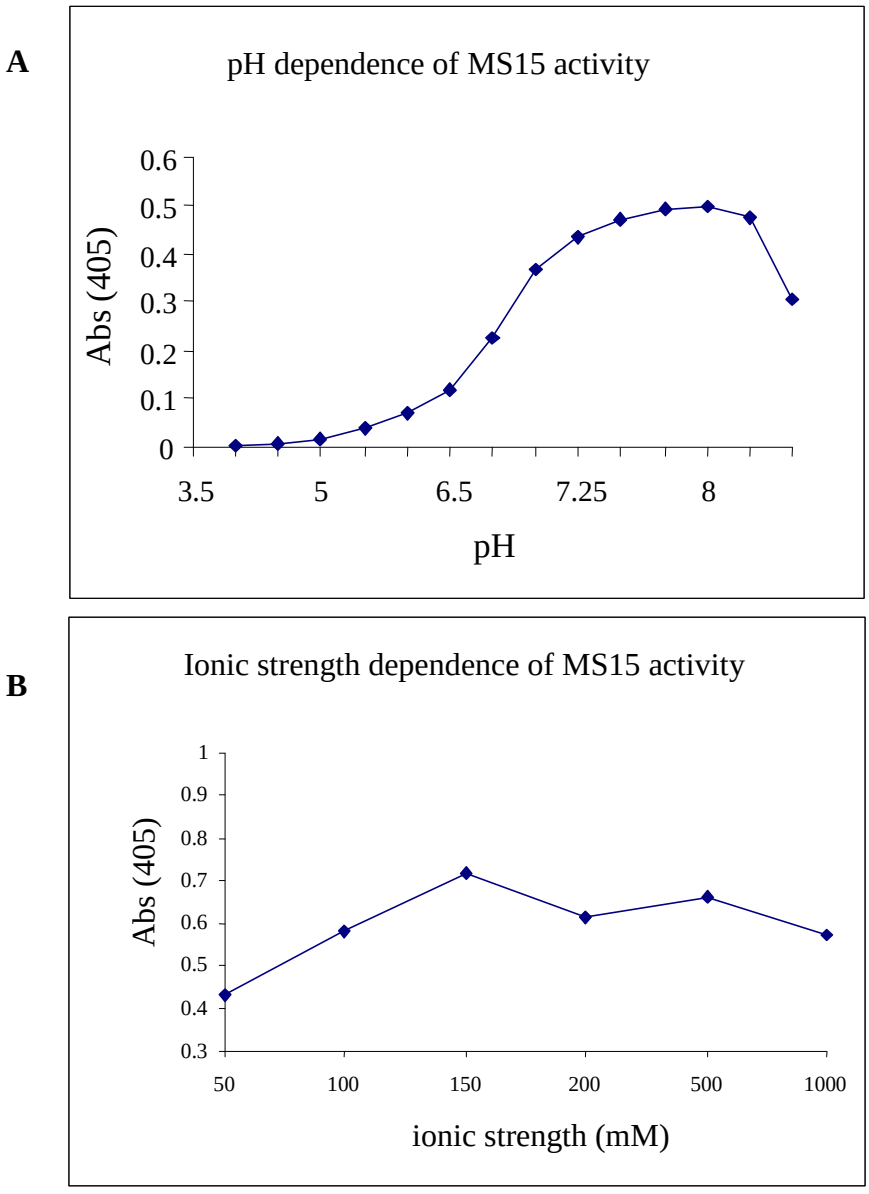


Figure 2.9-Optimization of carbohydrate inhibition assay conditions. *a*) pH-dependent binding of MS15-HRP conjugate to asialofetuin. In *A*, the binding of MS15-HRP conjugate to ASF was carried out at various pH as described under " Materials and Methods." *b*) pH-dependent binding of MS15-HRP conjugate to asialofetuin. In *B*, the binding of MS15-HRP conjugate to ASF was carried out at various salt concentrations as described under "Materials and Methods."

Table 2.2. Relative carbohydrate inhibition of MS15, compared with other proto type galectins: Carbohydrate inhibition assay was used to determine carbohydrate specificity of M15, and to compare these results with galectins from toad (*Bufo arenarum*), domestic cow (*Bos taurus*), and zebrafish (*Danio rerio*).

	Relative inhibitory activity			
Saccharide	*Bufo arenarum*	*Morone saxatilis*	*Bos taurus*	*Danio rerio*
Galβ1,3GlcNac	4.45	2.8	-----	1.4
Galβ1,3GalNac	<0.06	<0.02	<0.02	0.007
Gal	0.005	0.007	0.0079	0.004
Galβ1,3Ara	1.28	3.9	2.56	2.1
Galβ1,4Fru_f	1.0	1.9	0	1.0
Galβ1,4Man	2.5	1.70	2.34	1.5
Galβ1,4Glc	1.0	1.0	1.0	1.0
Galβ1,4GlcNac	3.75	3.0	5.54	7.9
Galβ1,4Glcβ(1-0)Me	2.14	1.8	1.33	2.9
Galβ1,4Glcβ-OPhNH_2 (p)	1.71	2	1.48	1.4
Galβ1-3GlcNacβ-OPhNO_2 (p)	1.78	6.5	4.5	2.3
Galα1,6Glc	0.0075	0.02	0.0053	0.004
Galβ1,6GlcNac	0.83	0.21	0.12	0.03
Galβ1S1βGal	3.9	10.0	9.0	12.2
Galα-OMe	0.03	0.03	0.01	0.006
Galβ-OMe	0.006	0.008	0.0044	0.004
Fucα1,2Galβ1,4Glc	0.17	0.29	0.36	0.35
MeO-2Galβ1,4Glc	2.5	3.0	4.08	2.9

The mammalian, amphibian, and two teleost prototype galectins revealed same basic pattern of specificity relative to lactose. Comparison of relative specificity with other teleosts could be informative with regards to changes in primary structure relating to tertiary structure and binding affinity. *Drgal*-L2 shares high similarity/identity with MS15 and this is reflected in carbohydrate specificity. Relative specificity of congerins, which are more similar to mammalian galectin-2, could provide a useful contrast to MS15 and *Drgal*1-L2 structures. Unfortunately, though congerins have been extensively characterized, only limited carbohydrate specificity has been determined.

II.D. Discussion

The isolation and biochemical characterization of a 15 kDa lectin belonging to the subfamily of galectins and designated MS15, was carried out from selected tissues of the striped bass *M. saxatilis*. Similar lectins had been previously purified from *M. saxatilis* and the hybrid striped bass (*M. saxatilis* x *M. chrysops*), although these were not characterized in detail (Ahmed, Fink, and Vasta, unpublished). The biochemical analysis of MS15 presented here revealed a protein that could be classified within the galectin family. Using an established purification protocol (Ahmed, *et. al*, 1996a) that was developed to isolate acidic lactose binding proteins in a reducing environment, MS15 was purified from most *M. saxatilis* organs or tissues, with the procedure resulting in a single protein band on SDS-PAGE with an apparent molecular weight of 15 kDa. Peptide sequencing performed on the purified MS15 digested with trypsin led to the identification of 85 amino acids, which upon alignment with human galectin-1 (prototype galectin) supported the identification of this protein as a galectin. MS15 agglutinated red blood cells from several mammalian species, including human type-O, which became the RBC of choice for use in hemagglutination assays. Results from relative mobility in SDS-PAGE and size exclusion chromatography suggested that MS15 is a 30 kDa dimer composed by two identical 15 kDa subunits. MS15 is acidic protein, with a similar theoretical and experimental isoelectric point. There is, though, microheterogeneity observed in the native protein subjected to IEF and 2-D gel analysis. Possible causes for this microheterogeneity have been considered, with most being rejected due to improbable biochemistry. The experimental pI's of these different charged species could be accounted for theoretically by a combination of acetylated and

non-acetylated N-termini, and deamidation of asparagine 3. Resistance to direct N-terminal sequencing of MS15 suggests blocking by some form of post-translational modification. N-acetylation is a modification, which blocks sequencing direct sequencing of proteins, and is common to other galectins. N-acetylation is commonly encountered in cytosolic proteins of eukaryotes. Over 50% of the soluble proteins from mammalian cells are acetylated (Brown, 1979). In intracellular proteins (Jolly and Taketa, 1979; Strauss, *et al.*, 1974; Kecskes, *et al.*, 1976), and also in certain secreted proteins such as ovalbumin (Wilson and Dintzis, 1970), the addition of the acetyl group to the amino terminal residue occurs cotranslationally when the polypeptide chain reaches a length of approximately 40-45 residues. However, in secreted or membrane-associated proteins, N-acetylation is a posttranslational event (Woodford and Dixon, 1979). According to tabulations of protein sequences, serine, alanine, and methionine account for the great majority of the N-terminally acetylated residues (Tsunasawa, *et al.*, 1985; Boissel, *et al.*, 1985). These modifications are known as degradation signals. Degradation signals of proteins, called degrons, are protein features that confer metabolic instability (Varshavsky, 1991). The essential component of one degradation signal, termed the N-degron, is a destabilizing N-terminal residue of a protein (Bachmair, *et al.*, 1986). This has led to an N-end rule, whose pathway has been found in all species examined, including the eubacterium *Escherichia coli* (Tobias, 1991), the yeast *Saccharomyces cerevisiae* (Bachmair and Varshavsky, 1989) and mammalian cells (Gonda, *et al.*, 1987; Lévy,, *et al.*, 1996). This enzymatic pathway leads to binding of ubiquitin, and possible protein degradation. A well-characterized modification that creates an N-degron is the cotranslational cleavage of the initiator methionine.

According to N-terminal rule, though, if the second residue has a radius of gyration, also called root mean squared distance, greater than 1.29 Å, cleavage of the initiator methionine is fully inhibited (Boissel, *et al.*, 1988). cDNA sequencing, described in the Chapter III, revealed the N-terminal sequence of MS15 is Met-Phe-Asn-Gly. If acetylated, MS15 must have an acetylated uncleaved initator methionine (iMET), based on the N-end rule. Phenylalanine has a radius of gyration of 1.90 Å, which is significantly greater than 1.29 Å. Therefore, native MS15 should retain its iMET and that Met may or may not be acetylated. A third modification of interest at the N-terminus is deamidation. Striped bass muscle extraction revealed an additional 14 kDa band, which co-purified with MS15. Successful N-terminal sequencing of this protein revealed it a possible cleavage and deamidated product of MS15. Deamidation converts an amide-containing residue into an acid-containing residue (effectively, N->D) with two observable effects. First, the apparent pI of the protein can be lowered, and secondly, the mass of peptides/ proteins will increase by and average of 0.985 Da per deamidation (Wei and Koh, 1978; Rosen *et al*, 1996). Deamidation of Asn to Asp would lead to an increase of only 0.985 Da. The sensitivity of SDS-PAGE and 2-D PAGE would not permit resolution of such a small change.

The deamidation of Asn3 to Asp3 was deduced from N-terminal protein sequencing of what is thought to be a truncated mature MS15. Deamidation can occur non-enzymatically, changing asparagine to isoaspartate or aspartate. The proposed mechanism for deamidation of asparaginyl-glycine is shown in Figure 2.10. This post-translational modification may provide signal for protein degradation, or alter the interactions of proteins. Sequence derived from cDNA (Chapter III) revealed “FNGLL”

as first five residues of mature MS15. Direct sequencing revealed “DGLLI” as first five residues. Since isoaspartate blocks Edman degradation reactions, the “DGLLI” peptide may have been the only sequence recorded. The characteristics of a potential deamidation site are solvent accessibility and local conformational flexibility, with a preference for (N + 1) being Asn-Gly. The N-terminus of MS15 appears to meet all of these criteria. Deamidation may have occurred while protein was being extracted, but the conditions that can accelerate the rate of reactivity (slightly alkaline buffer, <pH ~ 8, and/or elevated temperature (i.e. 37^{o}C) was well outside the parameters of the protocol used.

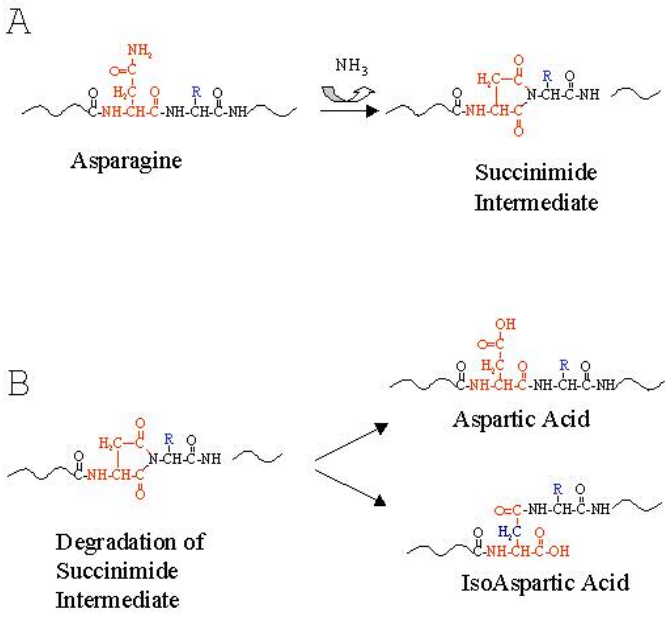


Figure 2.10. Deamidation of N-terminal asparagines in MS15- Step 1 The peptide bond nitrogen (reactive anion) of the N + 1 amino acid attacks the carbonyl carbon of the asparagine or aspartate side chain forming a five-membered ring structure referred to as a succinimide or cyclic imide. Step 2The succinimide is rapidly hydrolyzed at either the alpha or beta carbonyl group to yield iso-aspartate (beta-aspartate) and aspartate in a ratio of approximately 3:1. From “Deamidation in Proteins and Peptides” Glen Teshima, 2000

Deamidation may be influenced by the degree to which the protein is dimerized. The galectin as a monomer would have greater exposure of the N-terminus. In addition, the net charge of the protein would be little changed, and the molecular weight would only increase by one Dalton. The loss of the hydrophobic phenylalanine and the change in charge of asparagine to aspartate (negative), though, could have important effects in the dimerization of MS15.

Biochemical characterization of *M. saxatilis* tissue extracts suggested that in addition to a proto type galectin, other lactose-binding proteins, possibly other galectins are present (Figure 2.11). Antiserum produced in rabbits against MS15 from *M. saxatilis* muscle specifically bound MS15 in *M. saxatilis* whole body extract, and enabled the tissue/organ-specific detection of MS15 in brain, eyes, liver, spleen, stomach, intestines, heart, gills, skin, mucus, and gonads.

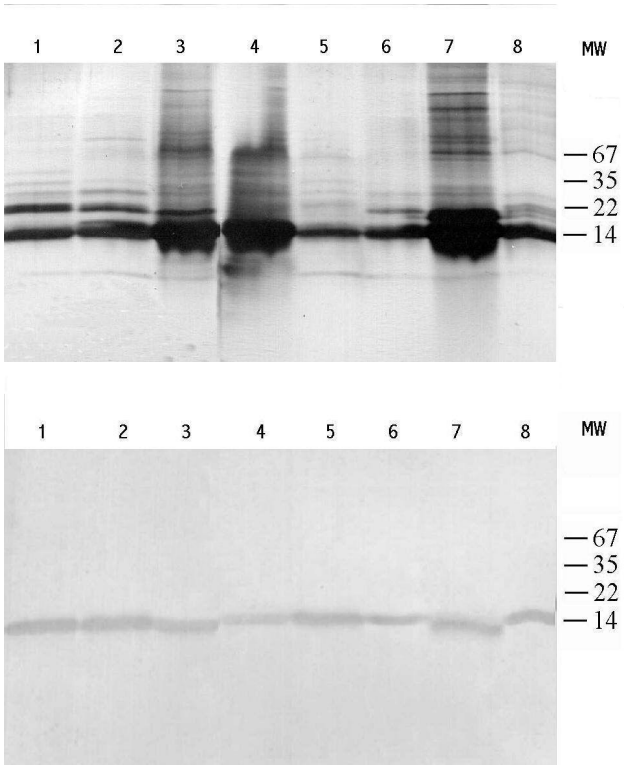


Figure 2.11. Affinity purification and Western blot of MS-15 from various tissues. *Lane1*, gill; *lane 2*, heart; *lane 3*,intestine; *lane 4*,muscle; *lane 5*, ovary; *lane6*, skin; *lane 7*, spleen; *lane 8*, stomach. Blot was performed using rabbit anti-striped bass galectin polyclonal serum, and goat anti-rabbit Ig-HRP. MW= kDa

Furthermore, the antiserum cross-reacted with proto type galectins from bovine (*Bos taurus*), toad (*Bufo arenarum*), zebrafish (*Danio rerio*), and a tunicate (*Clavelina picta*). When galectin(s) were purified directly by affinity chromatography, without prior ion exchange chromatography to select for acidic proteins, bands of 33 kDa, 17 kDa, and 15 kDa were detected by Coomassie staining (Figure 2.12). Because tandem repeat and chimera galectins have already been identified in zebrafish (Ahmed, *et al*., 2004) and possibly in the pufferfish, due to its molecular weight, binding to lactose, and being a neutral→basic protein, the 33 kDa was tentatively identified as one of these types of galectins. N-terminal sequencing, however, determined it to be the fucose-binding protein FBP32, identified and characterized by Odom and Vasta, 2004. The 17 kDa component was neutral→basic, bound lactose, and was present in most adult tissues, as revealed in western blots. No further work was done to characterize this protein. Proto type galectins of approximately 17 kDa, are present in several mammalian species, and include human galectin-10 (Ackerman, *et al.*, 1993) and rat galectin-5 (Gitt, *et al.*, 1995).

The thermal stability of MS15 revealed that the activity of MS15 does not decrease in the range of temperatures that striped bass thrive. Striped bass eggs and larvae cannot survive outside the range of 10^{o}C and 26^{o}C (Doroshev 1970), and juveniles experience zero growth outside the range of 10^{o}C and 33^{o}C (Cox and Coutant, 1981). There is no reported lethal temperature for striped bass, but there is a downward shift in optimal temperature as the fish age (Coutant 1985). Within these temperature ranges, MS15 retains almost all its activity. The thermostability profile of *Bufo arenarum* galectin (Ahmed, *et al.*, 1996b) has a similar pattern, but the activity of toad

galectin does not reach zero till ~84^{o}C. The thermostability of the congerins (I and II) is much greater, remaining fully active until 50^{o}C and 45^{o}C, respectively, and having total loss of activity at 70^{o}C and 60^{o}C, respectively. Structural comparisons between *B. marinus*, *M. saxatilis*, and *C. myriaster* galectins may help explain these differences.

Oxidative instability has been attributed to the presence of cysteines (creating disulphide bonds) or tryptophan in the binding site (unoccupied by ligand when oxidized).The mammalian galectins possess six conserved cysteines, but drgal1-L2 (*Danio rerio*) possesses 3 cysteines and congerin I and II and electrolectin (*Electrophorus electricus*), possess no cysteines.

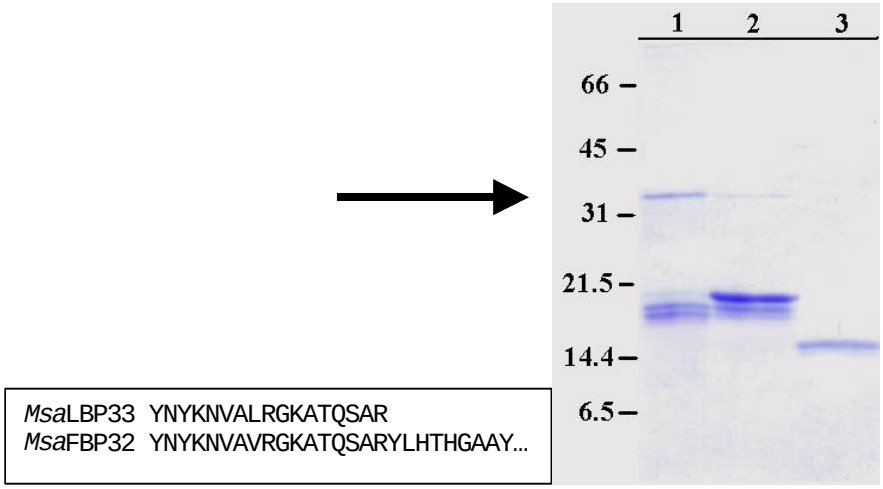


Figure 2.12. N-terminus alignment of ~33 kDa lactose-binding protein from *M. saxatilis*: ~33 kDa protein was separated by SDS-PAGE, transferred to a PVDF membrane, and N-terminally sequenced without prior digestion on a Beckman LG3000 Sequencer. Results were 17 amino acids, which had 100% identity to N-terminus of *Morone saxatilis* FBP32, a fucose-binding lectin characterized by Odom, *et al.*. *Lane 1*-lactose elute from lactosyl-Sepharose, with DEAE-Sepharose + EDTA flow through as starting material. *Lane* 2-lactose elute from lactosyl-Sepharose, with DEAE-Sepharose + Ca^{+2} flow through as starting material. *Lane* 3- lactose elute from lactosyl-Sepharose, with DEAE-Sepharose high salt elute as starting material.

The relative carbohydrate specificity of MS15 is similar to proto type galectins in other Classes of jawed vertebrates namely mammalian galectin-1 (*Bos taurus*), amphibian prototype galectin (*Bufo arenarum*), and teleost prototype galectin (*Danio rerio*). Though other fish galectins have been identified and characterized, these characterizations did not include carbohydrate specificity with sufficient range of compounds that could be contrasted with *M. saxatilis* carbohydrate specificity results. Based on similarities in carbohydrate-binding patterns, and other biochemical properties, the carbohydrate recognition domain (CRD) of MS15 is predicted to be more similar to mammalian galectin-1 than to other mammalian proto type galectins (-2, -5, -7, -10, -13, -14, -15), chimera type galectin-3, or the mucus galectins of conger eels. The relative inhibitory efficiencies of four key oligosaccharide structures in the order *N*-acetyllactosamine> lactose> human blood group A-tetrasaccharide> Galβ1,3GalNAc have been suggested as a way to group galectins as 'Type I' (conserved) or 'Type II' (variable) in reference to the primary structure of the CRD (Ahmed and Vasta, 1994). MS15 was not tested with human blood group A-tetrasaccharide, but it was tested with blood group A-trisaccharide [(Fucα1,2)Galβ1,4Glc], and human blood type A was agglutinated by MS15 (data not shown). The effectiveness of *N*-acetyllactosamine was 3-fold higher than lactose, with A-trisaccharide 3-fold less effective than lactose, and the effect of Galβ1,3GalNAc negligible. Thus, MS15 can be grouped with the galectins that have the 'Type I' CRD.

Mammal, toad and fish galectins exhibited similar specificity profiles, with slight variation in the actual inhibitory concentrations. MS15 shares this pattern, further supporting its inclusion in the galectin family. Comparison of relative specificity with

other teleosts could be informative with regards to changes in primary structure relating to tertiary structure and binding affinity. *Drgal*-L2 shares high similarity/identity with MS15 and this is reflected in carbohydrate specificity. The relative specificity of congerins, which are more similar to mammalian galectin-2, could provide a useful contrast to MS15 and *Drgal*1-L2 structures. Unfortunately, though congerins have been extensively characterized, their carbohydrate specificity has been determined only to a limited extent. It will be interesting to see if the results from the determination of the complete MS15 primary structure support this conclusion.

When characterizing galectins, it may be difficult to determine whether orthologous or paralogous genes are being compared. The hypothesized gene duplication events that have occurred in teleosts is one such complication. Extending the similarity of specificity from mammalian galectin-1 and MS15 to possible functions within these organisms is a big leap. Galectins appear to vary their function from tissue to tissue, from cell to cell and from time point to time point. This is probably due to the temporal and spatial variations in galectin ligand expression. Of the posited ligands for prototype galectins, the two major ligands appear to be poly-N- acetyllactosamine-enriched glycoconjugates found ubiquitously expressed by many cells, and poly-N-acetyllactosamine extensions on mucin-like O-glycans (Wilkins, *et al.*, 1996), both of great interest. Besides the canonical residues of the binding site, it has been proposed that there is an extended binding site, which allows for finer specificity and greater affinity for oligosaccharides longer than the disaccharides Galβ1,4Glc or Galβ1,4GlcNac (Seetharaman, *et al.*, 1998). There are differences seen between human galectin-3 structure and bovine galectin-1 structure that, coupled with carbohydrate

specificity studies, suggest an extended binding site. Substitutions of amino acids that align in primary structure and in tertiary structure (human galectin-3, 1A3K, Seetharaman, et al., 1998; bovine galectin-1, ISLT, Liao, et al., 1994) favor carbohydrate moieties attached to the 3-OH of galactose for galectin-3. MS15 shares substitutions with galectin-3 at the sites attributed to an extended binding site. Previous analysis of carbohydrate specificity of striped bass muscle galectin, which is now known as MS15, revealed very weak binding for 3-OH substituted galactosides. This suggests that though the substitutions in primary structure exist, MS15 maintains a fine specificity more similar to mammalian galectin-1 than galectin-3.

II.E. SUMMARY

A 15 kDa β-galactoside binding protein was purified from striped bass, and subsequent biochemical characterization supports the identification of this protein, designated MS15, as a proto type galectin. It can be purified from almost any tissue or organ, but with variable yield. Its subunit mass is 15 kDa, and its native mass is 30 kDa, suggesting a tertiary structure consisting of a non-covalently linked homodimer. Antibodies to MS15 cross-reacted with proto type galectins ~~of~~ from other vertebrate and protochordate species. Partial peptide sequences aligned well with other proto type galectins from teleost, amphibians, birds, and mammals. Carbohydrate specificity was similar to that of bovine galectin-1, toad 14.5 kDa galectin, and a zebrafish proto type galectin. Though it appears to be a proto type galectin, final classification must wait until entire primary structure is determined.

CHAPTER III: CHARACTERIZATION OF CDNA AND GENE ENCODING THE 15 KDA GALECTIN OF THE STRIPED BASS (*MORONE SAXATILIS*)

III.A. Introduction

MS15 is biochemically related to animal galectins, in particular, the prototype galectins. Determining the molecular biology of this protein-its cDNA, gene organization, control of gene expression, redundancy, and phylogeny – are essential for confirming that this protein is a member of the galectin family, and to place this protein in a functional as well as an evolutionary context. Sequences for proto type galectins have been determined in insects, nematodes, sponges, tunicates, fishes, amphibians, birds, and mammals. With the rapid pace of genome projects, entire genes are available for analysis, and putative galectins are being identified in these databases at an astounding rate. EST databases also allow for cDNA matched with a *bona fide* protein to be compared to cell-specific transcripts from various species. With these data coupled to rapid computer-based analyses, sequence data from MS15 and its corresponding gene can be compared to other galectins in a variety of ways. To begin this analysis, the strategy outlined in Figure 3.1 was followed. To start, peptide sequence was used to identify and amplify the cDNA and entire gene coding for MS15. This was accomplished by designing primers based on alignment of partial peptide sequences with known galectins, and using various PCR protocols and DNA sequencing to determine the coding sequence for MS15. The complete coding sequence was aligned with known galectin genes, and primers were designed to determine upstream, downstream, and introns of the gene coding for MS15. The translation of the

coding region for MS15 allowed for primary structure analysis, including pI and molecular weight analysis, prediction of post-translational modifications, sequence alignment and phylogenic analysis, and homology modeling of the protein to galectins with crystallographic structures. Complete gene sequence was used to analyze conserved transcriptional control elements, conserved intron/exon boundaries, and conserved galectin gene organization. Finally, sequence data allowed for the creation of a functional recombinant MS15, using directional ligation independent cloning technology in bacteria. This recombinant MS15 allowed for further biochemical analysis without sacrificing any more animals.

III.B. Materials and Methods

Isolation and Characterization of cDNA Encoding MS-15: Elucidating the nucleic acid sequence for striped bass galectin was begun by acquiring fresh sample material (spleen, muscle, and skin) from fresh-killed fish and immediately freezing samples in liquid nitrogen. Isolation and quantitation of total RNA from the tissue was performed using an adapted protocol from Sambrook, *et al.*, 1989, as follows: 5.0 mL guanidinium thiocyanate (GTC)/ buffer/β-ME was mixed with 500 mg homogenized tissue (mortar and pestle, tissue in liquid nitrogen), and separated into 500 µl aliquots. To each was added 50 µl 3 M NaOAc pH 5.2, 500 µl phenol, and 100 µl $CHCl_3$. This was chilled, centrifuged, and the aqueous layer was removed. RNA was precipitated with 1 volume isopropanol (1:1 final) at -80.0^{o}C for 30 minutes. RNA was redissolved in 500 µl Tris/EDTA, and the phenol/ chloroform extraction repeated. Lastly, RNA was precipitated overnight in ethanol at -80.0^{o}C. RNA was checked for quality and quantity

by Shimadzu UV-VIS spectrophotometer by 260 nm: 280 nm, and by electrophoretic analysis of RNA using a 1% agarose/ formaldehyde gel (Sambrook *et al*., 1989).

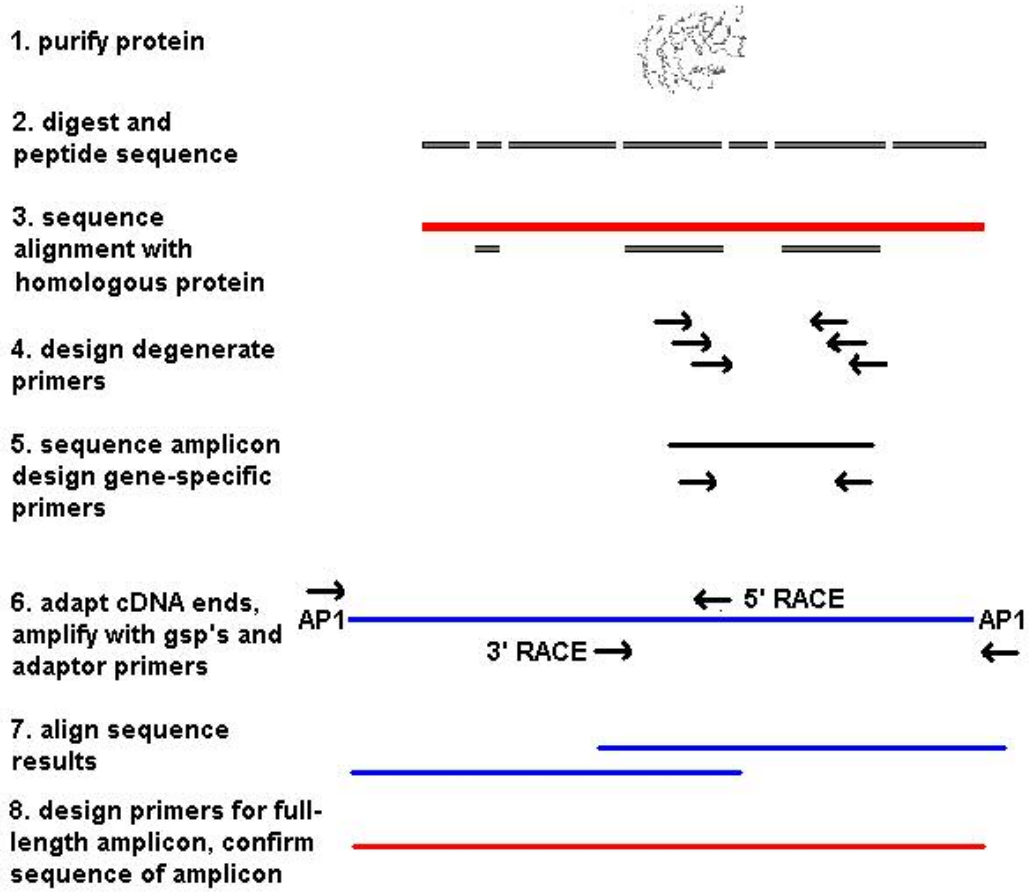


Figure 3.1. Strategy for determining sequence of full-length cDNA of *Msgal1*- Protein was purified based on properties of proto-type galectin. Protein was digested with trypsin, and resulting peptides sequenced. Peptides were aligned with prototype galectin of known sequence, and degenerate primers were created to amplify most conserved region. Sequencing results from degenerate primer amplicon was used to design gene-specific primer's (gsp's). 5' end and 3' end of cDNA were separately amplified and sequenced and the results were compiled into the complete coding region for *M. saxatilis* galectin.

Preparation of *E. coli* competent cells-A single colony of strain DH5α was grown in 10 ml LB broth at 37°C with shaking. 500 ml LB broth medium was inoculated with 5 ml of the overnight culture, and grown at 37°C with shaking (250 rpm) to early log phase (0.20 OD_{600} , 3 hours). The culture was centrifuged at 5°C at 5000 rpm for 10 minutes and the pellet was resuspended in 50 ml ice-cold 100 mM $CaCl_2$ solution, placed on ice for 30 min, and centrifuged again at 5000 rpm for 10 min. This pellet was again resuspended in 5 ml ice-cold 100 mM $CaCl_2$ with 10% of glycerol. 200 µl of the cells was transferred into microcentrifuge tubes and frozen at -80°C until later use.

Primer design: Primers were designed from the peptide alignment described in II.B.6. Degenerate primers were generated using Primer3 CyberGene AB Primer design utility software at www.cybergene.se/primer.html. One forward primer and three reverse primers were generated based on the peptide alignment with human galectin-1: forward primer (peptide 1); reverse primer 1 (peptide 2); reverse primer 2 (peptide 3); and reverse primer 3 (peptide 4).

Degenerate PCR for galectin cDNA - Using a PCR protocol known as "touchdown PCR" (Table 3.1), degenerate primers designed above were used to amplify cDNA coding for galectin, in the presence of both positive (universal actin primers) and negative (no template) PCR controls. PCR products were purified by separating PCR products in a 1% agarose gel prepared in 89 mM Tris, 89 mM boric acid, 2 mM EDTA (TBE) buffer, coring major bands, and extracting the DNA from the gel using QIAquick Gel Extraction Kit (QIAGEN, Valencia, CA). Briefly, the gel slice was dissolved in 3 volumes of chaotropic agent at 50°C for 10 minutes, applying the

solution to a spin-column and spinning for 1 minute (the DNA remains in the column), washing the column by passing 70% ethanol through (the DNA remains in the column, salt and impurities are washed out), and finally eluting the DNA in a small volume (30ml) of DNase-free water by spinning to collect flow through.

Table 3.1. Thermocyling programs used for striped bass DNA analysis

touchdown PCR	sequencing	RACE	standard
94°C for 1 minute 92°C 20 seconds 70°C for 20 seconds minus 0.5°C per cycle 72°C for 30 seconds go to step 2 for 19 cycles 92°C for 20 seconds 65°C 30 seconds 72°C for 30 seconds go to step 6 for 14 more cycles 4°C hold	96°C for 30 seconds rapid ramp to 50°C 50°C for 10 seconds rapid ramp to 60°C 60°C for 4 minutes go to step 2 for 24 more cycles rapid ramp to 4°C hold	94°C for 1 minute 94°C for 30 seconds 68°C for 30 seconds go to step 2 for 14 cycles 72°C for 10 minutes 4°C hold	94°C for 2 minute 94°C for 20 seconds (variable)°C for 20 seconds 70°C for 30 seconds go to step 2 for 24 cycles 70°C for 10 minutes 4°C hold
Suppression PCR			
5 cycles: 94°C 25 sec 72°C 4 min • 18–22 cycles: 94°C 25 sec 67°C 4 min • 67°C for an additional 4 min after the final cycle.			

Utilizing the fact that Taq DNA Polymerase preferentially adds a single 3´ adenine to double-stranded DNA fragments by a non-template-dependent extension reaction, purified PCR products were ligated to pGEM-T vector plasmid (Promega), and constructs were used to transform competent DH5α *E.coli* (Sambrook and Russell, 1989), as follows: *E. coli* DH5α was transformed by thawing one vial (about 200 µl) of

competent cells on ice. DNA was added into the vial and gently mixed with the cells by stirring with pipette tip. The cells were allowed to stay on ice for 30 minutes and heat-shocked at 42^{o}C for 2 minutes in a water bath, followed by chilling on ice for another 2 minutes. 1 ml of fresh SOC broth (0.5% Yeast extract, 2.0% tryptone, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$) was added and the cells were incubated at 37^{o}C with shaking for 1 hour. 50 µl of the cells were plated on a LB agar plate with appropriate antibiotic(s) for selection. For vectors that contains the DNA sequence *lac*Z', coding for the amino terminal portion of β-galactosidase, 25 µl isopropyl-beta-D-thiogalactopyranoside (IPTG) (25 mg/ml) and 50 µl 5-bromo-4-chloro-3-indolyl-b-D-galactoside (X-gal) (25 mg/ml) were spread on the plate 1 hour prior to the plating of the transformants. Clones harboring plasmid with insert form white colonies while those harboring plasmid without insert form blue colonies. After sufficient growth in the presence of selective antibiotic (ampicillin), colonies were selected for 5 ml batch culture grown overnight at 37^{o}C with shaking. Plasmids were extracted from this culture and electrophoretically analyzed for insert size. Plasmids with inserts were used for sequencing the inserts utilizing SP6 and T7 promoter sites for bi-directional sequencing (Table 3.2.), using the "sequencing" protocol in Table 3.1. Sequencing was performed at the Center of Marine Biotechnology's BioAnalytical Services Laboratory (BASLab), using an ABI 377 DNA sequencer with PE Biosystems 310 capillary sequencer. Sequence output file was analyzed using CHROMAS v. 2.24 software.

Rapid Amplification of cDNA Ends (RACE): Following the manufacturer's protocol, Marathon cDNA Amplification Kit (Clontech) was used with gene-specific primers to amplify complete cDNA of striped bass galectin. 3'- and 5'- gene-specific

primers were generated using the previously described Primer3 utility. For 3'- RACE, forward and reverse primers (Table 3.2) were used. For 5'- RACE, forward and reverse primers (Table 3.2) were used. Starting with striped bass poly A^+ RNA, first and second strand cDNA synthesis was performed and Marathon cDNA Adapter was blunt-end ligated to double stranded (ds) cDNA. From this library of ds-cDNA with adapters, PCR were performed, using designed primers and adaptor primers. Products were characterized by agarose gel electrophoresis, cloning of amplicons, and DNA sequencing.

Amplification and sequencing of full-length cDNA of galectin: Gene specific primers were generated (Genosys) and amplify the entire coding region of MS-15 from striped bass cDNA library, based on results of RACE amplifications and analysis. Full-length cDNA forward primer and reverse primer (Table 3.2) were design to straddle the start codon and stop codons, respectively. Following amplification, the single amplicon was cloned into pGEM-T vector and sequenced as previously described.

Analysis of complete cDNA coding for MS15: The gene coding for MS15 (hereafter *Msgal1*) was analyzed *in silico*, and predicted values were compared to the experimentally derived values. Calculations were made with and without N-terminal methionine, and with and without N-terminal cleavage and deamidation. Molecular weight, pI, Grand average of hydropathicity (GRAVY), and estimated half-life were calculated using Protparam tool at The ExPASy (Expert Protein Analysis System) proteomics server of the Swiss Institute of Bioinformatics. Other tools used on this server were NetPhos, for prediction of serine, threonine and tyrosine phosphorylation sites in eukaryotic proteins; NetOGlyc, for prediction of O-GalNAc (mucin type)

glycosylation sites; NetNGlyc, for prediction of N-glycosylation sites; and Signal P, for prediction of signal peptide cleavage sites. For theoretical support of homology modeling results, full length MS15 (translation) and bovine galectin-1 (model template) was submitted to Protscale (Expasy server) for Kyte-Doolittle hydropathy mapping and Chou-Fasman beta sheet prediction.

Homology modeling of MS15: Secondary and tertiary structure was modeled using the Swiss-MODEL Server (Schwede T, Kopp J, Guex N, and Peitsch MC (2003)). Primary structure of MS15 was submitted via Internet to http://www.expasy.org/swissmod/SWISS-MODEL.html. First, BLASTP2 (Altschul and Lipman, 1990) was used to find all similarities of target sequence with sequences of known structure in the ExNRL-3D sequence database, reflecting the protein sequences of ExPDB. The residues with structural modifications (HYP, PCA, SEP, MSE etc.) are replaced by their genetic precursors (i.e. PRO, GLU, SER, MET etc.). Next, SIM (Huang, *et al*., 1990) selected all templates with sequence identities above 25% and projected model size larger than 20 residues. Furthermore, this step detected domains, which can be modeled based on unrelated templates. Next, Swiss-Model used ProModII to generate a model by alignment, framework, lacking loops, backbone correction. The reliability of the model is determined by the degree of sequence identity and alignment. Gromos96 (GROningen MOlecular Simulation) generates energy minimization of the models. The results were sent back to the submitter via e-mail .

Table 3.2. Primers for *Msgal*1

Target	FORWARD (5'>3')	REVERSE (5'>3')
full length	GCACATATGTTTAATGGTTTGCTCATA	GGTGGATCCTTATTTGATCTCAAGG
Upstream	GTAATACGACTCACTATAGGGC (AP1)	GCAAACCATTAAACATGATTGCAGATG
Intron I	TCTCACTTCTCCTCAGCTGTACTTGAC	ATGGTCTGCCCGACCTTGAAGGAC
Intron II	TTCA AGGTCGGGCAGACCATGACC	GACCACCACATTCTCG TCTCC
Intron III	GAGGCTTTCCTTTCCAACAGGG	ATCCCCAACAAAGTTGATGAAGGAGTAC
Downstream	GAGGCTTTCCTTTCCAACAGGG	GTAATACGACTCACTATAGGGC (AP1)
cDNA for LIC	GACGACGACAAGATGTTTAATGG TTGC TC	GAGGAGAAGCCCGGTTCCTTAT TTGATC TC
3' RACE-1 -2	ACCAGACGCCTCGCGGCACTCCC TGGTGTGAGGAGCACCGTGAGGGAGG	
5' RACE–1 -2	ATCCCCAACAAAGTTGATGAAGGAGTAC AAGTTGCAGGTTTATTGATCTCA	

Isolation and Characterization of *Msgal1*: High molecular weight genomic DNA was extracted from striped bass blood (RBC's of striped bass are nucleated) using the GenomicPrep Kit (Amersham Biosciences, Piscataway, New Jersey). Samples were collected from striped bass by tail venipuncture. Blood was collected in EDTA to prevent clotting and reduce degradation, and placed on wet ice until DNA extraction. Following manufacture's protocol for extraction from nucleated blood cells, 40 µl of striped bass blood was added to 6 mL cell lysis solution. After 1-hour incubation at room temperature, sample was RNAse treated. Protein was precipitated using kit's Precipitation Solution, and the supernatant was transferred to a 15 mL tube containing 6 mL 100% isopropanol. Sample was mixed gently by inverting until DNA began to precipitate. Precipitate was pelleted by centrifugation, and pellet was washed twice with 70% ethanol. Wash was decanted, and pellet was allowed to dry at room temperature. DNA was rehydrated using 500 µl DNA Hydration Solution (Amersham Biosciences), and stored at 4^{o}C.

Southern blot with genomic DNA: Genomic DNA was digested with *Bam*HI, *Xba*I *and Ssp*I by incubating 5 ug DNA with 1 µl enzyme for 3 hours at 37^{o}C. Enzymes were chosen following restriction mapping of complete *Msgal1* cDNA sequence. DNA digests were gel electrophoresed overnight, (25 V, constant voltage) and transferred to a Nytran SuPerCharge Nylon membrane (Schleicher &Schuell, Keene, New Hampshire) using the TurboBlotter System (Schleicher &Schuell) and following manufacturer's protocol. DNA was covalently cross-linked to damp membrane using a UV Stratalinker 2400 (Stratagene, La Jolla, California) by exposing blot to total dose of 120 mJ/cm^2. Detection was performed using DIG-labeled probe described previously, and reagents from DIG High Prime DNA Labeling and Detection Starter Kit II (Roche Applied Science, Indianapolis, Indiana). Southern blot was performed using protocols from DIG High Prime DNA Labeling and Detection Starter Kit II (Roche Applied Science). Genomic DNA was prepared, digested with restriction enzyme(s) as described above, and electrophoresed onto an agarose gel for DNA fragment separation. DNA probes were labeled with the non-radioactive digoxigenin (DIG)-based Genius kit (Roche Applied Science) following the instructions provided by the vendor. After electrophoresis, the gel was treated with 0.25 M HCl with shaking for no longer than 10 min (depurination), denatured by alkaline solution (0.5 N NaOH, 1.5 *M* NaCl) for 1 hour and neutralized with neutralization solution (1.0 *M* Tris-HCl, pH 8.0, 1.5 M NaCl) for 1 hour. The DNA was transferred onto nylon membrane (MSI) in 3M sodium chloride, 0.3M sodium citrate, pH 7.0 (20X SSC) by capillary action, overnight. The fragmented DNA-bearing

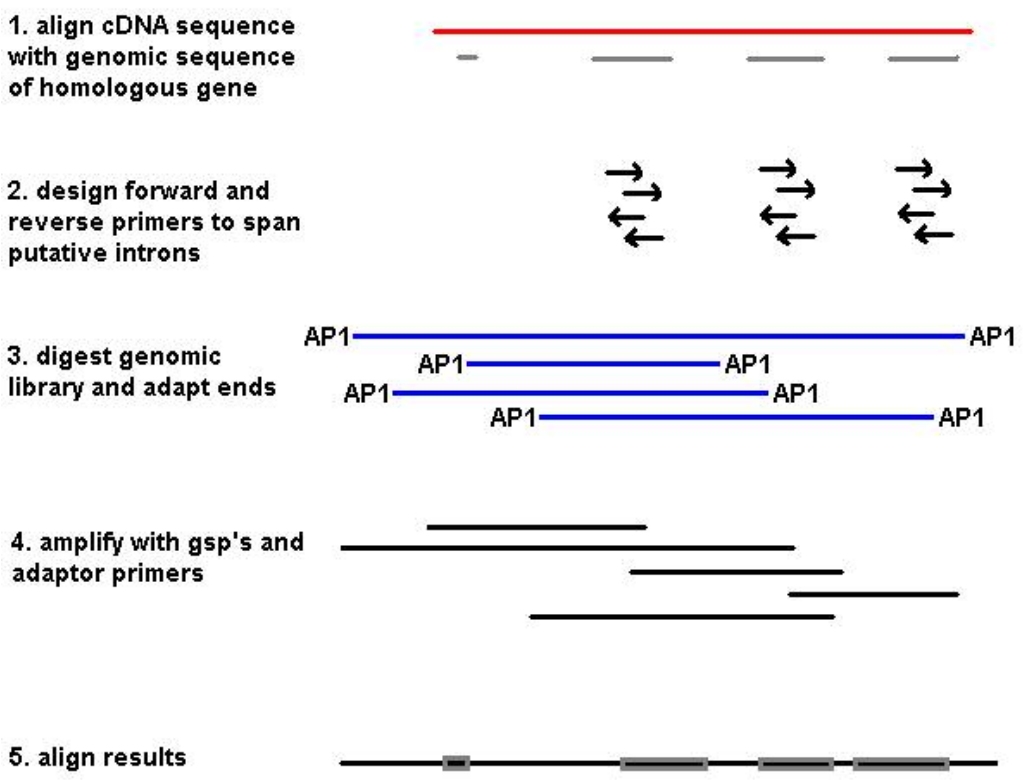


Figure 3.2. Strategy for determining gene organization of *Msgal1*- The results from the complete cDNA sequence of *M. saxatilis* 15-kDa galectin were aligned with the genomic sequence of human 14.5 ka galectin. *M. saxatilis* genomic DNA was restriction digested and blunt-end ligated with an AP1 adaptor. Gene-specific primers were designed and used with AP1-specific primers to amplify regions between putative exons and upstream and downstream of gene. Following amplification, amplicons were sequenced by cloning into pGEM-T and determining insert sequence, or by direct sequencing of amplicons. Results of sequencing were aligned with each other to yield complete gene structure.

nylon membranes were treated with UV crosslinker to fix the DNAs on the membrane. The membranes were prehybridized with the prehybridization solution, containing 5X SSC, 0.1% of N-lauroylsarcosine, 0.02% of SDS, 2% of blocking reagent, and 50% of formamide (Roche) for 2 hours and hybridized with the DNA probe overnight. Both prehybridization and hybridization were performed at 42 °C in a water bath with

shaking under high stringency conditions. Before detection, the membranes were washed with 2X SSC (containing 0.1% SDS) twice and 0.5X SSC (containing 0.1% SDS) twice at room temperature, were blocked with buffer 2 (0.15 M NaCl, 0.1 M Tris-HCl, pH 7.5, 2% blocking reagent) for 0.5 hr, and incubated with anti-DIG-alkaline phosphatase (1:5,000-1:10,000, v/v, in buffer 2) for 0.5 hr. The membrane was washed twice with buffer 1 (0.15M NaCl, 0.1 M Tris-HCl, pH 7.5). NBT (1:1000, v/v, in buffer 3: 0.1 M Tris-HCl, pH 9.5, 0.1 M NaCl, 0.05 M $MgCl_2$) was used as the substrate for alkaline phosphatase. Development time was approximately 20 minutes at room temperature.

Gene Primer Design: Using the sequence information from the completed full-length *Msgal1* cDNA aligned with the known gene organization of galectin-1 in mouse and humans, primers were designed to bracket the predicted introns, and to amplify 5'-upstream and 3'- downstream. Primers are listed in Table 3.2. Following amplification, the products were either directly sequenced, or sequenced after cloning into pGEM-T vector.

Amplification of 5' upstream region, 3' downstream region, and introns of *Msgal1*: Clontech's "Gene Walker" DNA Walking Kit was used to amplify and sequence unknown regions of *Msgal1*. Starting with *M. saxatilis* genomic DNA previously extracted, five genomic libraries were created, each one the result of digestion with a different restriction enzyme. The restriction enzymes used were *Pvu*II, *Sca*I, *Eco*RV, *Stu*I, and *Dra*I, selected based on results restriction mapping. Each of these libraries was ligated with the Genome Walker Adapter using T4 DNA ligase in a blunt-end ligation reaction. With gene specific primer (GSP, Table 3.2) and adapter-

specific primer (AP-1), suppression PCR (Table 3.1) was used to amplify the intervening region. When products consisted of "smear" nested adapter primer (AP-2) and GSP were used in amplification reaction. Following sequencing (BASLab, Center of Marine Biotechnology), which averaged 400 to 600 bp, new gene specific primers were designed 100 bp from end of previous sequencing results, and sequencing was continued, thus "walking" up and down restricted genomic DNA.

Analysis of *Msgal1* transcribed and non-transcribed regions: Analysis of *Msgal1* was performed using Transcription Element Search Software (TESS) at http://www.cbil.upenn.edu/tess, BLAST searches at the Fugu Genomics Project at http://fugu.hgmp.mrc.ac.uk/Analysis , CLUSTALW: Multiple Sequence Alignment at http://clustalw.genome.jp,

Preparation of expression vector/insert: Novagen's pET30 Ek/LIC System was used to create expression construct, following the manufacturer's protocols. First, primers were designed that are specific for the 5'-end (including start codon) and the 3'-end (including stop codon) of *Msgal1*, to incorporate Ligation Independent Cloning site specific-sequence. The forward primer (sblicf1) the reverse primer (sblicr1) is shown on Table 3.2. Following PCR ("standard", Table 3.1) the full-length amplicon was purified as previously described, and annealed to the pET30 Ek/LIC vector using manufacturer's protocol. After transformation and growth, recombinant plasmid was extracted and purified by Wizard Mini-prep Kit (Promega). Purified plasmid was used as a template, and two nested insert-specific primers EX2F1 and EX4R1 were used to confirm insert, while vector-specific primers T7 and T7 terminus were used to confirm insert size. Plasmid preps that were positive for galectin insert and possessing

predicted size insert were introduced into *E. coli* BL21 (DE3) cells by transformation as described above.

Characterization of expression construct: DH5α containing recombinant plasmids were grown on LB agar with 50 ug/mL kanamycin overnight at 37^{o}C. Colonies were picked and applied to both labeled master plates and to 50 µl dH_2O in microcentrifuge tubes. Bacteria samples in water were boiled at 100^{o}C for 10 minutes, and centrifuged. Supernatant was used as template for PCR, using plasmid-specific primers flanking insert site (T7 and T7 terminus), and nested primers for galectin. Colonies positive for insert of the predicted size were found, corresponding colonies on master plate were used to prepare sufficient plasmid for sequencing to confirm presence, orientation, and reading frame of construct. The DNA sequence of the product was confirmed by automated DNA sequencing as previously described (BASLAb, Center of Marine Biotechnology).

Expression of recombinant galectin: The recombinant MS15 (rMS15) was expressed in *E. coli* BL21 (DE3) by induction of an actively growing culture with 1 mM IPTG at an O.D. A_{600} of 0.6. For negative control, a duplicate culture was grown, but not induced with IPTG. Cells from 1 L of culture were harvested after an additional six hours of growth by centrifugation and resuspended in 10 mL β-ME/PBS pH 7.4. Rapid freeze-thaw using dry ice/ethanol bath was used to lyse cells, and sample was sonicated briefly to shear DNA, and cleared by centrifugation at 15,000 rpm, 20 min, and 4°C in a Sorvall SS-34 rotor. The cleared lysates were mixed with 5 mL (bed volume) lactosyl-sepharose pre-equilibrated in β-ME/PBS and mixed gently at 4^{o}C for 2 hours. Each batch of slurry was loaded into a separate column and washed once with

50 ml β-ME/PBS and twice with 50 ml each β-ME/ [PBS/10]. The bound protein was eluted with β-ME/[PBS/10]/0.1 M lactose. To cleave His-tag from protein, rMS15 was loaded on 10 mL DEAE-sepharose and washed with 100 mL β-ME/[PBS/10] to remove the lactose, and eluted with 20 mL β-ME/PBS/0.5 M NaCl. The eluted protein was mixed with 5 mL pre-equilibrated lactosyl-Sepharose, and incubated for one hour. Enterokinase (rEK, Invitrogen) was added incubated with slurry, with mixing. Fractions at different time points were removed to monitor cleavage. At 16 hours, the slurry was loaded in a column and the matrix was washed with 50 mL β-ME/PBS followed by 50 mL β-ME/ [PBS/10]. The bound protein was eluted with 10 mL β-ME/[PBS/10]/0.1 lactose and stored on DEAE-sepharose in 1:1 β-ME/[PBS/10]/0.1 M lactose: glycerol at -20^{o}C. In later purifications, cleavage with rEK began immediately following first step of purification, during initial affinity chromatography step.

Characterization of expressed galectin: Characterization of rMS-15 began with SDS-PAGE to monitor purification steps and cleavage of His-Tag with rEK. Purification steps were characterized with 15% SDS-PAGE gels stained with Coomassie-R250, and used to locate the purified protein in the unstained portion of each gel. Protein was further characterized by western blot and thermal stability.

Sources for sequence data sources for galectins used in analysis: Accession numbers for galectin sequences used for analysis of *Msgal1* and MS15 are human galectin-1, BC020675; chicken 16, NM206905; lizard galectin, P82447; toad galectin, P56217; flounder galectin, AF220550; electric eel galectin, P08520; nematode 16, NM064814; congerin I and II, AB010276 and AB010277; Japanese eel, BAC67210;

catfish, CF261531; killifish, CN983392; Haplochromis (cichlid, BJ702281; Atlantic salmon, CK880684 and zebrafish , AW174841, AY421704, and BM182089. Sequences for *Takifugu* and *Tetraodon* were retrieved from International Fugu Genome Consortium, www.fugu-sg.org/project/info. Tilapia and Atlantic salmon sequences were from The Institute for Genome Research, (TIGR) www.tigr.org.

III.C. Results

Isolation and Characterization of cDNA encoding MS15: cDNA solution libraries were created from spleen, skin, and muscle RNA. Primers for actin used to confirm quality of libraries. When degenerate primers for *Msgal1* were used, one set revealed a single band at ~190 bp. Upon sequencing, a 183 bp insert translated to a 61 amino acid peptide that included sequence from the peptides used to design primers and an intervening 47 amino acids. The nucleic acid sequence and its translated amino acid sequence aligned with the highly conserved exon III region and protein (respectively) of prototype galectins from mammal, bird, and fish. It is this nucleic acid sequence that was used for RACE primer design (Table 3.2).

RACE: The primers originally designed for RACE gave mixed results. The primers designed as nested primers gave better and more consistent results (Figure 3.3). These nested primers eventually were used to amplify 5’ and 3’ cDNA ends. 5’ RACE revealed a 66 bp 5’-UTR, in which the first ATG codon aligns with the first (start) methionine of MS15. 3’-RACE revealed a long 500 bp 3’UTR, that ended with a predicted polyadenylation site, and at least 16 adenines. The 150 bp overlap of 5’-RACE and 3’-RACE revealed no mismatches, so the entire cDNA for MS15 was 992

bp long, with an open reading frame (ORF) 405 bp in the coding region. 405 bp translates to 135 aa with molecular weight of 14,983 Daltons, which is close to the predicted size of MS15 based on protein analysis. The continuity of 5'- and 3'- RACE was confirmed by sequencing the amplicon generated with the primers

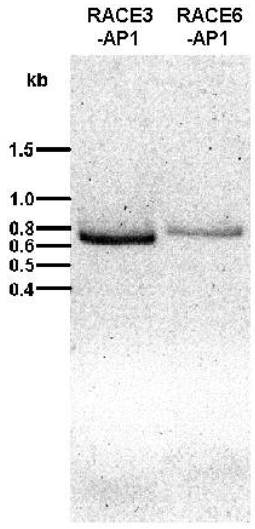


Figure 3.3. Amplicons from 3'-RACE reactions- RACE6 and AP1 primers were used to amplify predicted 3' end of striped bass galectin. Template was AP1-adapted striped bass muscle cDNA library. RACE3, a downstream (nested) primer, amplifies product at predicted size (~45 bp < RACE6-AP1)

sbfullf1 and sbfullr1. PCR with sbfullf1->sbfullr1 primers yielded a single amplicon (Figure 3.4) The 426 bp sequence that coded for all the peptide sequences from protein analysis, and was 100% match to the aligned RACE products described in III.C. Translation of full-length cDNA revealed 135 amino acid protein that aligned with previously described prototype galectins of animals (Figure 3.5).

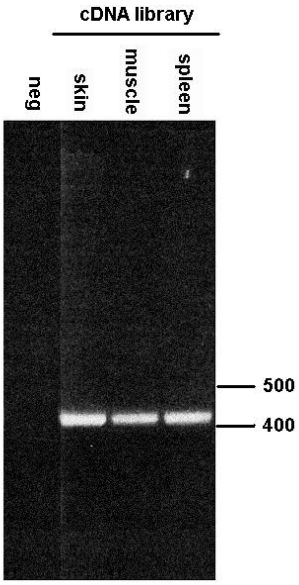


Figure 3.4. Full-length coding region amplified from *M. saxatilis* cDNA libraries. cDNA libraries were created using oligo-dT primers, with RNA from skin, muscle, and spleen used as template. Amplicons were ~425 bp, with 423 bp predicted length. Sequencing of amplicons confirmed alignment from RACE amplification. Translation yielded 134 aa protein that contained all residues from peptide sequence.

	predicted	**experimental**
MW (no N-ter Met)	14,931.9	15,000 Denatured 30,000 native
pI	5.15	4.7, 4.9, 5.1

Table 3.3. Theoretically Predicted vs experimental values for pI and sub-unit molecular weight- Using full-length cDNA translation, MW and pI were theoretically predicted using the ProtParam tool at kr.expasy.org. Experimental values were obtained from SDS-PAGE, gel permeation chromatography, IEF, and 2-D electrophoresis.

```
                                                          *   *   * *
SBGAL 15      MFNGLLIKNMSFKVGQTMTVVGVAKPDASDFAVNVGPDEKDITMHINARFNACGDENVVV
H L-14-I      MAC..VAS.LNL.P.ECLR.R.EVA...KS.VL.L.K.SNNLCL.F.P....H..A.TI.
C-16          MEQ..VVTQLDVQP.ECVK.K.KILS..KG.S....K.SSTLML.F.P..DCH..V.T..
LG-14         --P.ITXTSLHVAP.ARLA.K.DIPAG.KSWVI.L.KG.N..ML.F....D.H..IRTI.
BUFO-I        ASA.VAVT.LNL.P.HCVEIK.SIP..CKG.A..L.E.ASNFLL.F....DLH..V.KI.
PARA          MMKNMM.............II..P....TN..L.I..TDQ..V....P....H....A..
Electro       SM..VVDER....A..NL..K..PSI.STN..I...NSAE.LAL...P..D.H..QQA..
Nema 16    ...PQTPVN.PVQG.SN.ARLRLVLLPTSAD.R.HI.LRTPD-..VL.F....-----..GA..
Sponge I   ...V.GD.KLTVP.LT....IE.LY.NP.TGA-LSI.LVTADD.VAL.F.P.Y--SSTGGYL.

               *      *  * *
SBGAL 15      CNSYQGGKWCEEHREGGFPFQQGEEFK---ITIEFTPTEFLVTLSDGSTIHFPNRM--GA
H L-14-1      ...KD..A.GT.Q..AV....P.SVAE---VC.T.DQANLT.K.P..YEFK....L--NL
C-16          ...KED.T.G..D..AD......DKVE---.C.S.DAAEVK.KVPEVEFE ....L--.M
LG-14         ------------------------------------------------------------
BUFO-1        ...KEADA.GS.Q..EV......A.VM---VCF.YQTQKIIIKF.S.DQFS..V.K—-VL
PARA          ....I.RQ....L........L.....---.V.....Q..........I......I--..
Electro       V..F...N.GT.Q.......K...D..---.Q.T.NSE..RII.P...E.....N-----
Nema 16       N..TS..G.QS.D.HAN-....NKIYT---LEFVSNGGIISIFVNGAHFAD.VE.T-—PS
Sponge I      L.TLLN.N.QT.VHPT....PANNVKTRVLVS.TVQEKD..LQVNGIDITT.SY.PGLSY

                                                        % Identity
SBGAL 15      EKYSFINFVGDVRIKSLEIK                          --
H L-14-1      .AINYMAAD..FK..CVAFD                          41
C-16          YLAVE..FKV.AIKFS                              39
LG-14         ----------------                              31*
BUFO-1        PSIPFLSLE.LQF-..ITTE                          36
PARA          .....MS.E.EA..R.F...                          74
Electro       ---RYMH.E.EA..Y.I...                          52
Nema 16       HGVHL.EIE.GVHVHSAHVSH                         24
Sponge I      D.VRHITCK.LEHAVL...                           22
```

Figure 3.5. Complete amino acid sequence from MS-15 . Amino acid sequences of other galectins are presented for comparison. These include human galectin-1 (H L-14-I), chicken 16 kilodalton galectin (C-16), partial sequence for *Podarcis hispanica* lizard galectin (LG-14), *Bufo arenarum* toad galectin (Bufo-1), *Paralichthys olivaceus* flounder galectin (Para), *Electrophorus electricus* electrolectin (Electro). Identical residues to MS-15 are shown by dots. Asterisk by % identity for LG-14 because calculation is based on partial sequence.

Table 3.4. Percent identity and similarity to striped bass MS15- The full-length coding sequence for MS15 (405 bp) was used for comparison. Some sequences below are "predicted' proto type galectins from genome sequencing projects.

Genus/ species	*T. r.*	*T. n.*	*M s.*	*P. o.*	*D. r.* L2	*D. r.* L1	*E. e.*	*O.m.*	*S. s.*	*D. r.* L3	*G. a.*	*O. l.*	*C. m* I	*C. m* II	*A. j.*
Takifugu rubripes	100	76 86	73 82	68 82	50 71	47 65	47 64	42 60	40 60	44 60	44 64	33 44	28 50	30 52	29 50
Tetraodon nigroviridis	-- --	100	72 85	65 82	54 75	51 71	48 67	43 62	42 62	44 63	45 63	30 44	29 54	35 58	30 53
Morone saxatilis	-- --	-- --	100	74 86	58 77	55 74	51 68	44 61	42 61	46 63	43 60	31 42	32 51	36 58	30 53
Paralichthys olivaceus	-- --	-- --	-- --	100	61 77	57 76	54 69	45 63	42 64	45 60	43 60	31 42	28 49	32 55	27 53
***Danio rerio* L2**	-- --	-- --	-- --	-- --	100	76 87	60 75	45 66	42 65	48 66	47 66	32 45	30 52	34 54	33 54
***Danio rerio* L1**	-- --	-- --	-- --	-- --	-- --	100	58 73	43 65	42 62	44 62	45 62	29 42	27 51	33 56	32 54
E. electricus	-- --	-- --	-- --	-- --	-- --	-- --	100	40 60	39 60	43 62	45 63	30 42	29 51	33 55	28 49
O. mykiss	-- --	-- --	-- --	-- --	-- --	-- --	-- --	100	93 97	59 69	56 72	42 49	25 47	30 50	28 49
Salmo salar	-- --	-- --	-- --	-- --	-- --	-- --	-- --	-- --	100	60 70	54 72	42 50	25 47	31 50	29 47
***Danio rerio* L3**	-- --	-- --	-- --	-- --	-- --	-- --	-- --	-- --	-- --	100	50 70	39 47	28 54	36 55	30 52
Gasterosteus aculeatus	-- --	-- --	-- --	-- --	-- --	-- --	-- --	-- --	-- --	-- --	100	48 58	29 50	30 53	31 50
Oryzias latipes	-- --	-- --	-- --	-- --	-- --	-- --	-- --	-- --	-- --	-- --	-- --	100	23 39	23 39	23 35
***Conger myriaster* I**	-- --	-- --	-- --	-- --	-- --	-- --	-- --	-- --	-- --	-- --	-- --	-- --	100	45 71	29 52
***Conger myriaster* II**	-- --	-- --	-- --	-- --	-- --	-- --	-- --	-- --	-- --	-- --	-- --	-- --	-- --	100	38 55
Anguilla japonica	-- --	-- --	-- --	-- --	-- --	-- --	-- --	-- --	-- --	-- --	-- --	-- --	-- --	-- --	100

Analysis of *Msgal1* cDNA: Table shows the results of calculations for molecular weight, pI, grand average of hydropathicity (GRAVY), and estimated half-life with and without N-terminal methionine, and with and without N-terminal cleavage and deamidation. Signal P detected no signal peptide cleavage sites (Figure 3.6). Predictions for phosphorylation revealed by NetPhos were four sites in the C-terminus of the protein. All four sites had confidence scores over 0.800, on a scale from 0.000 to 1.000 (Figure 3.7). NetOGlyc and NetNGlyc, for prediction of O-GalNAc (mucin type) and N-glycosylation sites, detected no sites with sufficiently high levels of confidence (Figure 3.8, 3.9). This, combined with no signal sequence, and no differences between calculated and experimental molecular weight, supports the idea that this galectin is not glycosylated.

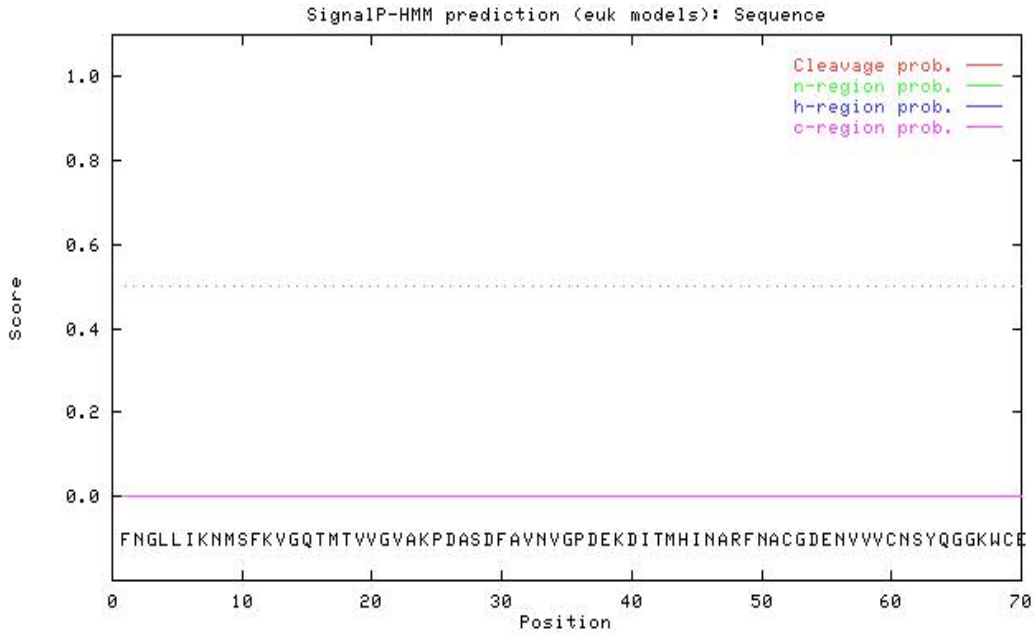
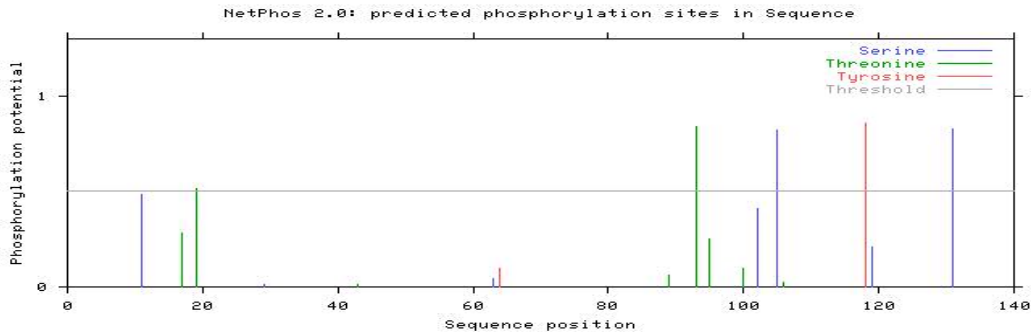


Figure 3.6. Analysis for signal sequence, using SignalP 3.0 Server at Expasy.org: The prediction was that MS15 is a non-secretory protein, with a probability of having a signal peptide at 0.002.



Serine predictions

Name	Pos	Context	Score	Pred
Sequence	11	IKNMSFKVG	0.487	.
Sequence	29	KPDASDFAV	0.015	.
Sequence	63	VVCNSYQGG	0.044	.
Sequence	102	LVTLSDGST	0.408	.
Sequence	105	LSDGSTIHF	0.823	*S*
Sequence	119	AEKYSFINF	0.206	.
Sequence	131	VRIKSLEIK	0.827	*S*

Threonine predictions

Name	Pos	Context	Score	Pred
Sequence	17	KVGQTMTVV	0.280	.
Sequence	19	GQTMTVVGV	0.514	*T*
Sequence	43	EKDITMHIN	0.012	.
Sequence	89	EFKITIEFT	0.064	.
Sequence	93	TIEFTPTEF	0.841	*T*
Sequence	95	EFTPTEFLV	0.253	.
Sequence	100	EFLVTLSDG	0.100	.
Sequence	106	SDGSTIHFP	0.026	.

Tyrosine predictions

Name	Pos	Context	Score	Pred
Sequence	64	VCNSYQGGK	0.101	.
Sequence	118	GAEKYSFIN	0.859	*Y*

Figure 3.7. Prediction of MS15 phosphorylation- Using the Net-Phos program at the EXPASY server, potential sites of phosphorylation were identified in the primary structure of MS15. Scores over 0.500 represented strong potential for phosphorylation.

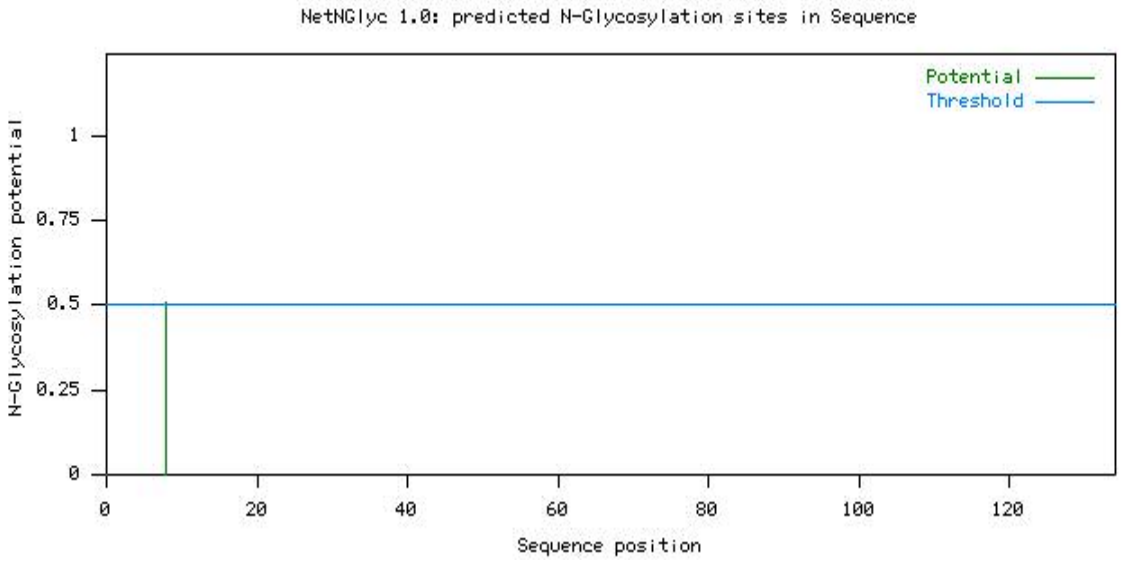


Figure 3.8. Predicted N-glycosylation sites in MS15- Site of N-glycosylation is predicted at position 8 (NMSF) of MS15, though if secreted in non-classical manner, MS15 would not be exposed to N-glycosylation machinery. There are no predicted O-glycosylation sites (NetOGlyc 3.1 Server and YingOYang 1.2 Prediction Server, expasy.org)

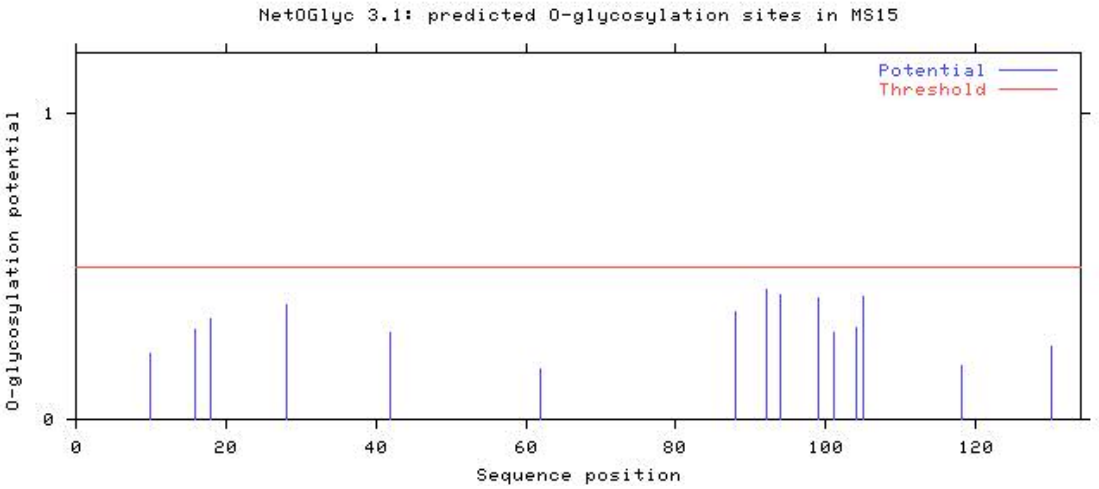


Figure 3.9. Predicted O-glycosylation sites in MS15- Graphical output of scoring O-glycosylation shows many potential sites, but both general prediction scores and isolated site scores are well below 0.500 confidence level. There are no predicted O-glycosylation sites in MS15, (NetOGlyc 3.1 Server and YingOYang 1.2 Prediction Server, expasy.org).

Additional evidence is found in the peptide sequencing of this gene product. Residues modified by glycosylation and phosphorylation will give a “blank” cycle during sequencing. All predicted sites of glycosylation and phosphorylation were within the sequenced peptides, except for serine 130 near the C-terminus. Therefore, they could not have been modified. Comparisons were made between bovine galectin-1 and MS15 to see if there was theoretical support for results in homology modeling. Hydropathy plots (Figure 3.10) show how similar the hydrophobic and

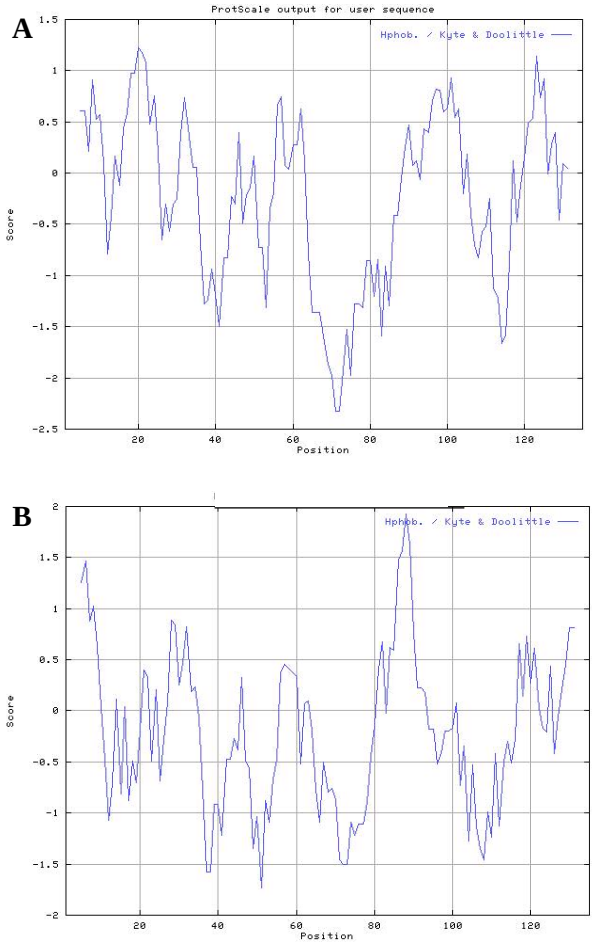


Figure 3.10. Hydrophobicity plot for MS15 vs. bovine galectin-1: Bovine galectin-1 (*A*) and MS15 (*B*) were submitted to Protparam at the Expasy server for hydrophobicity analysis of their primary structure, using the Kyte& Doolittle algorithm. Position 50-80 approximates the amino acids involved in the carbohydrate-binding cleft. Both proteins are similar in this region. MS15 has regions of greater hydrophobicity (N- and C-termini, F3 strand) and less hydrophobicity (F2 and F4 strands). Interestingly, these strands are on the opposite side of the binding site, and have been implicated in non-carbohydrate dependent activities.

hydrophilic regions are for both proteins, even with the difference in primary structure. beta sheet prediction results (Figure 3.11) show a very similar pattern. The crystal

structure of bovine galectin-1 (Liao, *et al.*, 1994) confirmed the secondary structure predictions of the Chou-Fasman equations, at least for that protein. These two predictors of structure, coupled with what is known about the structure of bovine galectin-1, gives support to the use of this mammalian galectin as a template for homology modeling.

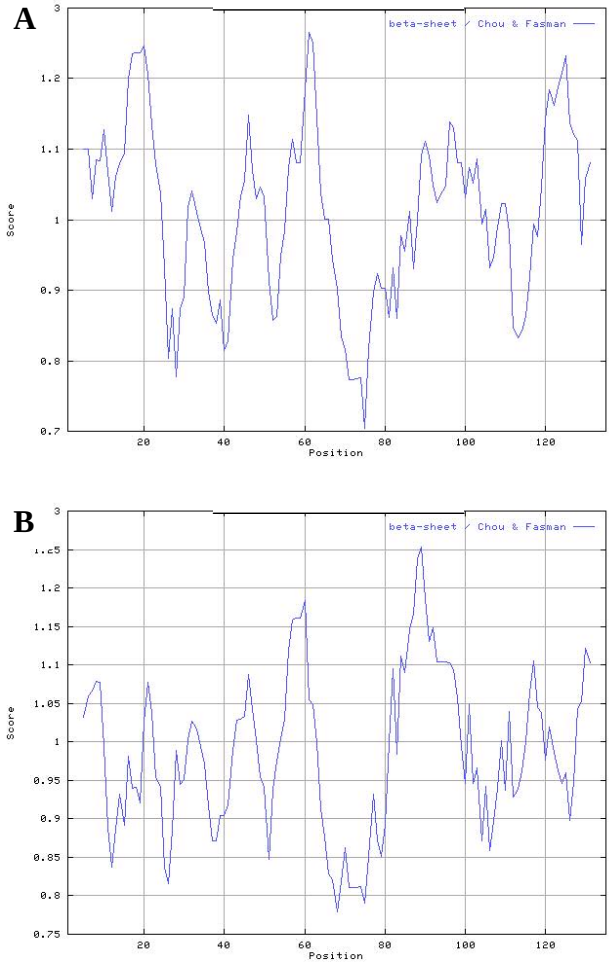


Figure 3.11. Prediction of beta sheet plot for MS15 vs. bovine galectin-1- Bovine galectin-1 (*A*) and MS15 (*B*) were submitted to Protparam at the Expasy server for beta sheet prediction based on primary structure, using the Chou & Fasman algorithm. Though there are regions of stronger prediction for beta sheets in one protein or the other, both appear to have the same number and location of predicted sheets.

Homology modeling of MS15: Using previously describe galectins that have had their structure solved by diffraction, a model for MS15 was obtained from the SWISS-Model server. Probabilities of modeling accuracy were considered (Table 3.5), and bovine galectin-1, congerin II, and chicken 16 galectin were used because of

similarity of primary structure, and because these represent mammal, bird, and fish galectins. Bovine galectin-1 generated the model that was most correct and accurate (Figure 3.14, 3.15). The theoretical model of MS15 was analyzed for correctness (bond angles/lengths, non-covalent contacts, surface hydrophobic residues) and accuracy (how well it fits the template), and the model was examined for how well it supported the biochemical data collected to date.

Table 3.5. Probabilities of SWISS-MODEL accuracy for target-template identity classes

Percent sequence identity[a]	Total number of models[b]	Percent[c] models with rmsd lower than 1 Å	Percent models with rmsd lower than 2 Å	Percent models with rmsd lower than 3 Å	Percent models with rmsd lower than 4 Å	Percent models with rmsd lower than 5 Å	Percent models with rmsd higher than 5 Å
25-29	125	0	10	30	46	67	33
30-39	222	0	18	45	66	77	23
40-49	156	9	44	63	78	91	9
50-59	155	18	55	79	86	91	9
60-69	145	38	72	85	91	92	8
70-79	137	42	71	82	85	88	12
80-89	173	45	79	86	94	95	5
90-95	88	59	78	83	86	91	9

a: Range of sequence identity between target and template sequence.
b: Total number of models in any given class of sequence identity. The table summarises 1201 model – control structure pairs.
c: Probability in percent that a model, sharing X% sequence identity with its template, deviates by 1 Å or less from the corresponding experimental control structure. The following columns provide these probabilities for other rms deviations. Table courtesy of "swissmodel.expasy.org"

Isolation and characterization of genomic DNA containing *Msgal1*:

Southern blot with genomic DNA: Southern blot analysis of striped bass genomic DNA following restriction digest reveals a single copy gene detected by a 200 bp probe directed at Exon III of *Msgal1*, which encodes the conserved residues of the carbohydrate binding site of proto type galectins (Figure 3.12). The stringency chosen for this blot should have detected other galectin genes that were within 40% identity.

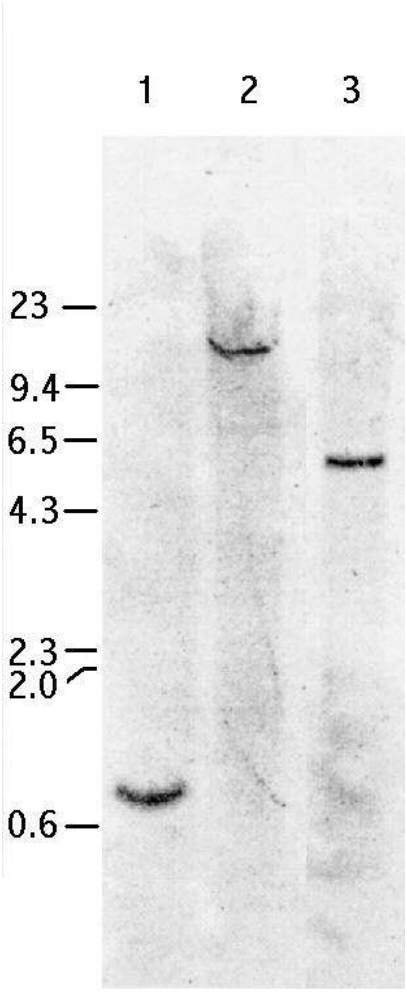


Figure 3.12. Genomic Southern blot of *Msgal1*. *M. saxatilis* genomic DNA was cut as follows: *lane 1*, BamHI cut; *lane 2*, XbaI cut; *lane 3*, SspI cut. The probe used is 190 bp *M. saxatilis* galectin exon III PCR product . Migration of DNA size markers is noted.

Amplification of 5' upstream region, 3' downstream region, and introns of *Msgal1*: The gene organization of MS15 is similar to that elucidated in human, mouse, and chicken prototype galectins (Figure 3). There are 4 exons and three introns, all with canonical exon/intron boundaries of (GT/AC). The sizes of the exons are 9 bp, 81 bp, 171 bp, and 147 bp for exons I-IV, respectively. The sizes of the introns are 2000 bp, 871 bp, and 425 bp for introns I-III, respectively. Not shown is the genomic amplicon from start of exon I to predicted stop codon in

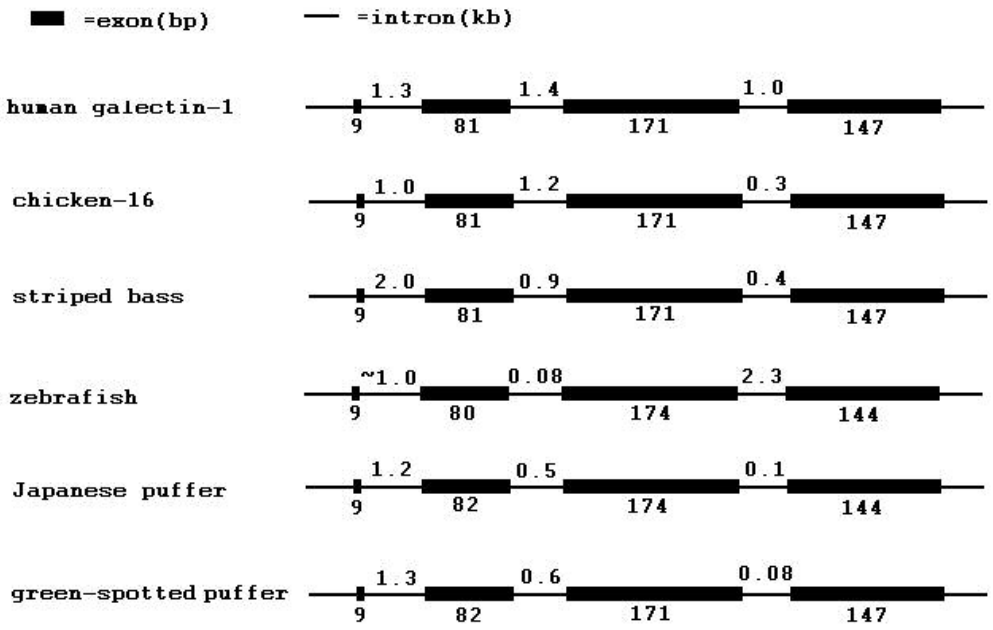


Figure 3.13. Genomic organization of *Msgal1* compared to other prototype galectins- 1600 bp upstream sequence not included for *M. saxatilis*, to simplify comparison. Human and chicken galectins were sequenced as known genes. Zebrafish was sequenced as a known gene, confirmed in the zebrafish genome project. The two pufferfish galectins were retrieved from recent genomic databases for these organisms. Exons and introns are not to scale.

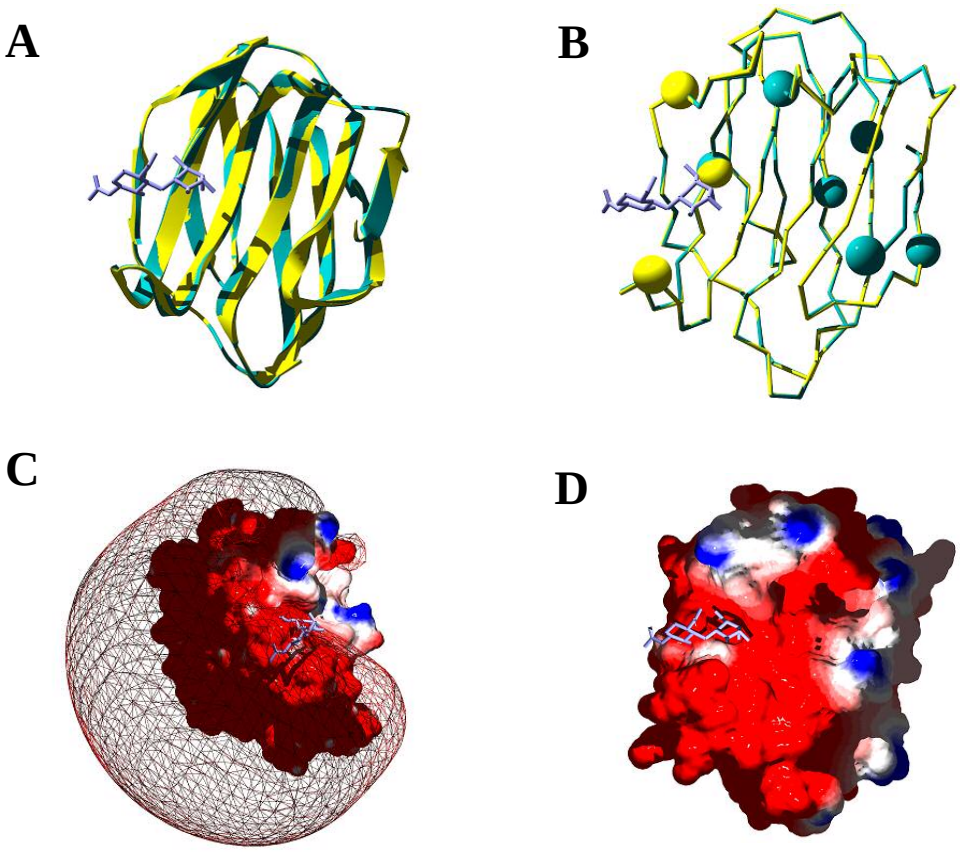


Figure 3.14. Analysis of protein surface, charge and cysteines conservation with homology model- 15-kDa galectin modeled to *Bos taurus* galectin-1. *A. B.taurus* galectin-1 was crystallized with N-acetyllactosomine. *B. taurus* galectin is green, *M. saxatilis* is yellow. Side chains relevant to carbohydrate binding and conserved throughout galectins are seen in binding cleft. ***B.*** Relative positions of 6 conserved cysteines in mammalian galectin (*green*) vs. 3 cysteines in MS15 (*yellow*) C. Electrostatic potential map of MS15, forming a characteristic anisotropic electric field protruding into the surrounding solvent. Molecular view is rotated 90^{o} around x-axis to see "back" of molecule. *D.* Electrostatic potential map of MS15 computed at molecular surface and viewed into the carbohydrate-binding site. Blue and red indicate positive and negative electrostatic potentials respectively. The bound LacNAc moiety is shown in stick representation in *violet.*

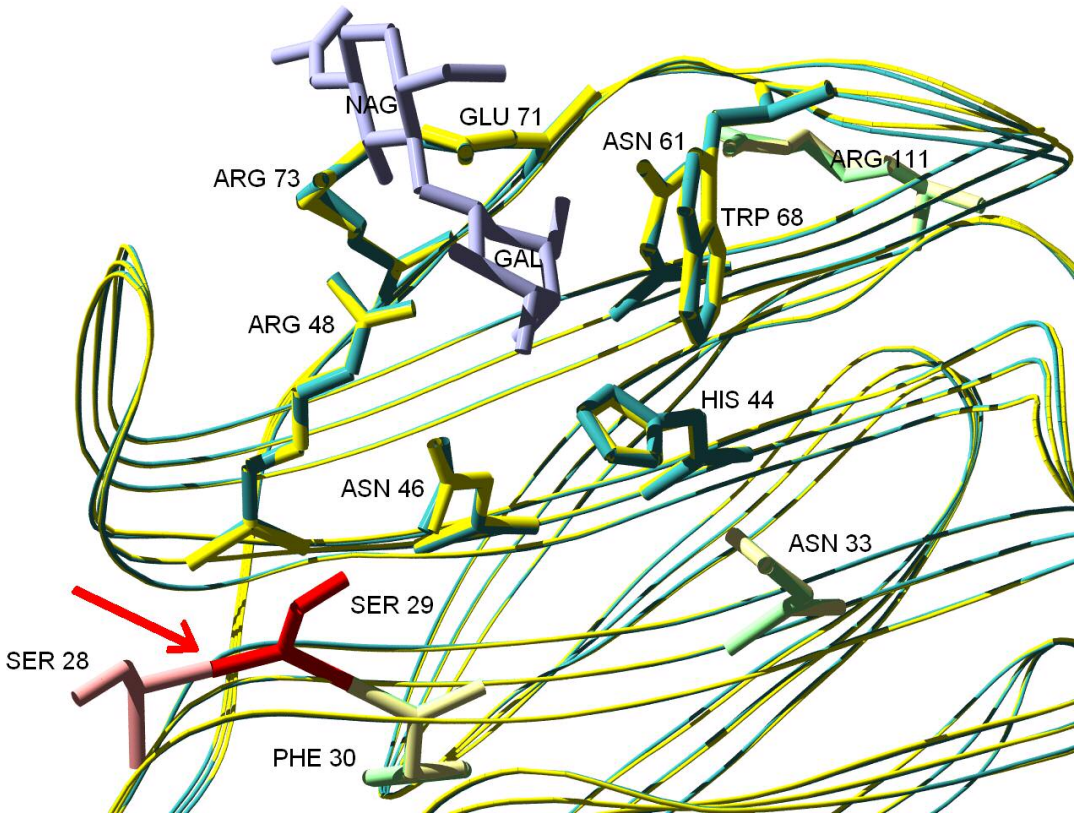


Figure 3.15. Canonical galectin-1 side chains, involved in ligand interactions and CRD architecture. Red arrow points to only difference between MS-15 and bovine galectin-1 conserved residues. MS-15 possesses Asp at position 29, similar to mammalian galectin-3. GAL and NAG label the galactose and *N*-acetylglucosamine moieties of the bound LacNAc from the bovine galectin-1 resolved structure.

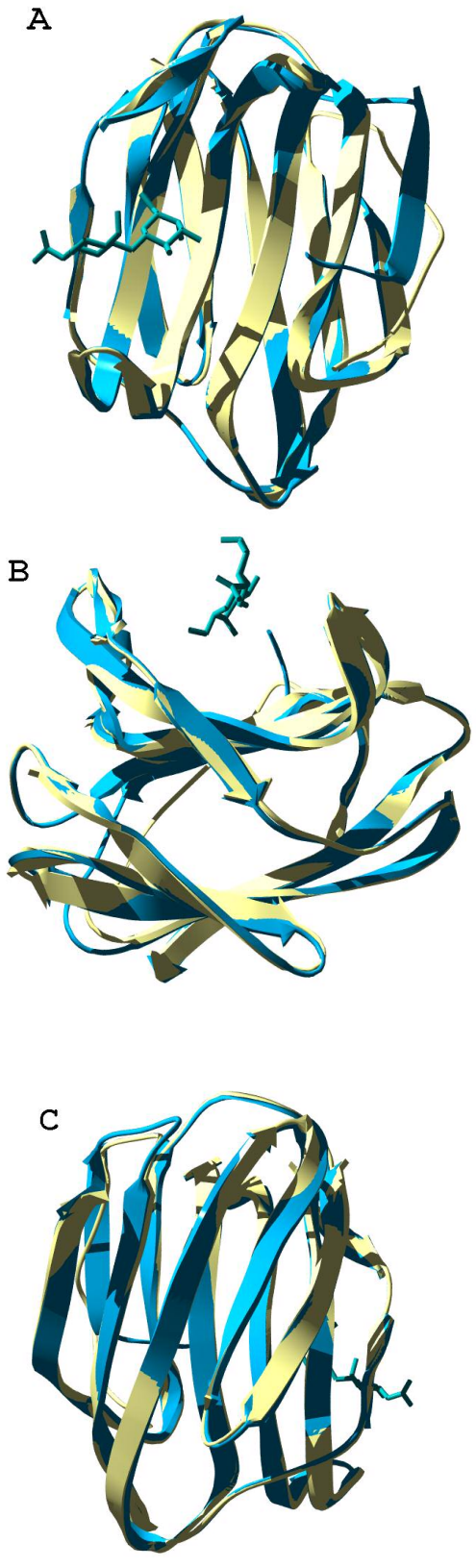


Figure 3.16. Homology models of *M. saxatilis* 15 kDa galectin using *Bos taurus* galectin-1- Following elucidation of MS15 primary structure, sequence was submitted to SWISS-Model with bovinegalectin-1 structure as a template. MS15 is shown in yellow, bovine galectin-1 in blue, and N-acetyllactosamine in green. .*A*, model shows N-and C-termini (dimer interface) to the right, and the binding cleft running left to right across subunit. *B*, looking down binding cleft, with N- and C-termini at far end. Note beta sheet folding in the structure, and loops at binding site extend to create pocket. *C*, 180^{o} rotation from *A*, the three loops seen at top of "back" of some galectins are being implicated in non-carbohydrate binding activities. The four beta strands forming this sheet exhibit the greatest differences in primary structure, charge, and hydrophobicity, between mammalian galectin-1 and MS15.

Exon IV, which yielded a product of about 3,700 bp, which matched the predicted gene size. This amplicon also yielded products of predicted sizes with nested primers.

Analysis performed on upstream regions of *Msgal1* by automated searches for transcription factors (TFAC) identified numerous potential control elements. The same search performed on the introns suggested the presence of control elements in Intron I regions. The sequence downstream of the predicted stop codon, both transcribed (500 bp) and non-transcribed (500bp genomic beyond poly A signal) revealed a predicted polyadenylation signal and a weak T/GT-rich region.

Expression and characterization of rMS15: Sequencing of the pET30: MS15 construct showed that *Msgal1* was in frame with the start codon of the vector, and that the stop codon of *Msgal1* was present and in frame. Active rMS15 was expressed in *E. coli* using an IPTG-induced construct. No protein was recovered in the supernatant of the culture broth, the periplasm of the bacteria, or in inclusion bodies, or in the control culture (no induction). The need of MS15 for a reducing environment appears to be met in the reducing interior of the bacteria. Following induction and lyses, active MS15 was purified in one step by affinity chromatography with lactosyl-Sepharose (Figure 3.17). Cleavage of protein tags introduced by the vector was accomplished by incubating lactosyl-Sepharose bound rMS15 with rEK for 16 hours, washing to remove enzyme and cleaved tags, and eluting rMS15 with lactose. SDS-PAGE revealed a highly expressed protein in the cytoplasm of *E. coli* transformed with the pET30: MS15 construct and induced with IPTG. This protein was about 19.5 kDa, which matched the predicted size of MS15 with an N-terminal S- and His-tag.

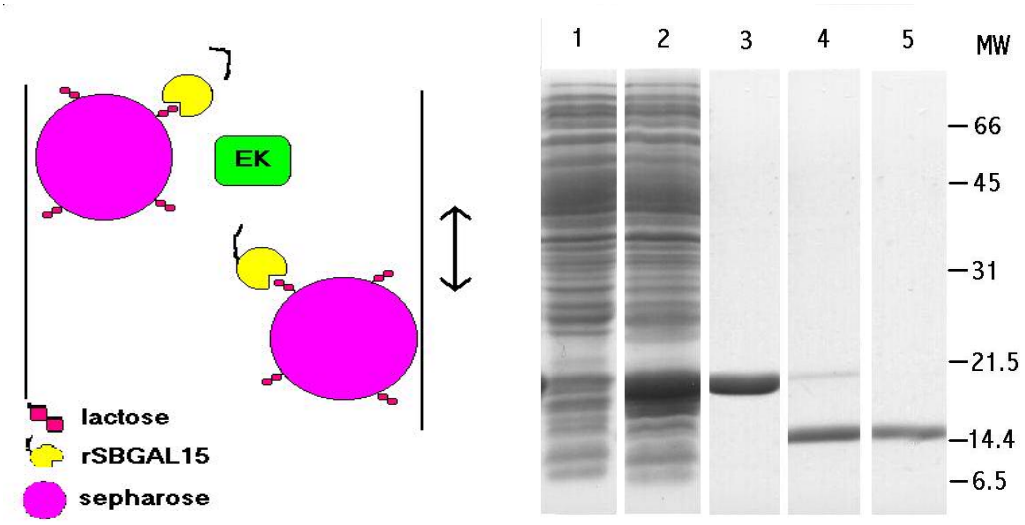


Figure 3.17. Enterokinase treatment of rMS15-Supernatant of lysed bacterial culture (IPTG-induced) containing recombinant galectin was mixed with lactosyl-Sepharose, washed, and enterokinase was added to bound protein: matrix. Cleavage was monitored at various time points by SDS-PAGE, until no 19 kDa protein was detected. Bound protein was washed to remove enzyme and unbound cleavage products, and eluted with PBS/lactose.

Following cleavage with enterokinase, rMS15 migrated the same as native MS15 in a 15 % SDS-PAGE gel.

Western blot with anti-striped bass muscle galectin detected rMS15 and native MS15 suggest maintenance of epitopes (primary through tertiary structure) during production of recombinant. Thermostability of rMS15 was the same as MS15 from both striped bass muscle and striped bass skin. This also supports the conclusion that this recombinant protein folded properly following expression in bacteria.

III.D. DISCUSSION

Results of degenerate PCR and RACE using material from striped bass revealed a complete coding region for a prototype galectin. Sequence similarity between the translated nucleotide sequence and known prototype galectins strongly support the conclusions founded on the biochemical characterization of MS15. Sequences for fish prototype galectins have been determined in electric eel, *Electrophorus electricus* (Paroutaud *et al.,* 1987); Japanese eel *Anguilla japonica* (Tasumi, *et al.*, 2004); Conger eel *Conger myriaster* (Ogawa and Ishii, 1999; Muramoto and Kamiya, 1992); bastard flounder *Paralichthys olivaceus* (Lee, *et al.*, unpublished); zebrafish *Danio rerio* (Ahmed, *et al*., 2004); cichlid *Haplochromis* sp. (Watanabe, *et al.*, unpublished) and can be found in the databases of various genome sequencing projects, such as catfish *Ictalurus punctatus*, medaka *Oryzias latipes* and rainbow trout *Oncorrhynchus mykiss* (The Institute for Genome Research, www.tigr.org); Japanese pufferfish *Takifugu rubripes* and the green spotted pufferfish *Tetraodon nigroviridis,* (International Fugu Genome Consortium, www.fugu-sg.org/project/info; Center for Genome Research, genome.jgi-psf.org/fugu), zebrafish *Danio rerio* (Sanger Institute, Strausberg); and the stickleback *Gasterosteus aculeatus* (Stanford Genome Evolution Center). All of these were used in the analysis of MS15.

Primary structure for galectins have been determined in species other than fish and mammals, including chicken C16 (Sakakura, *et al*., 1990), chicken galectin-3 (Nurminskaya and Linsenmayer, unpublished), toad galectin (Ahmed, *et al.,*, 1996b) frog Xgalectins (Marshal, *et al*., 1992; Shoji, *et al.*, 2003), tunicate galectin (Oleary, 2003); nematode galectins (Hirabayashi *et al*., 1992, 1996; Klion and Donelson, 1994),

marine sponge galectins (Pfeifer *et al.*, 1993), and fungus galectins (Cooper *et al.*, 1997). The cDNA sequence (Figure 3.5) and genomic organization (Figure 3.13) of striped bass 15 -kDa galectin, called *Msgal1*, is similar to the proto type galectins mentioned above. The percent identity/ similarity is quite high for some other teleosts (bastard flounder, 74%/86%; pufferfish, 73%/82%). The resulting translation showed no coding for a leader sequence, and there was no evidence for alternative splicing events. The amino acid translation aligns with all the peptides previously sequenced from trypsin digested MS15, and with the results of N-terminal sequencing of the truncated MS15 from striped bass muscle. The only conflicting data was seen in the peptide that began with WEE, which translated from repeated nucleic acid sequencing as W*C*E. The coding region for these amino acids has been bidirectionally sequenced several times, and always the results code for WCE. Comparison with other prototype galectins from teleosts *P. olivaceus, F. rupribes,* and *D. rerio* showed all share WCE, and *T. nigroviridis* has WCG. In teleosts, to date, only congerin I from *C. myriaster* has a glutamic acid following this tryptophan (WET). Technically, Cys must be modified prior to sequencing, and modified Cys may co-elute with other residues, giving an inaccurate molecular weight during sequencing. Considering these points, WCE is being accepted as the true native primary structure. The other unusual result was found, coincidently, in another cysteines. Mammalian galectin-1 has a histidine at position 52, and many proto type galectins in other organisms share this conserved amino acid. This includes most of the teleost galectins. Substitution or deletion of this His has been attributed to variations in fine carbohydrate specificity (Ahmed, *et al.*, 1994, 1996a). MS15 has a cysteine substituting the histidine at this position. From a DNA point of

view (mutation), this represents a major change- CAC/T to TGC/T. From a protein point of view (functional), cysteines and histidines are frequently found in active and metal-binding sites, often substituting for each other (Betts and Russell, 2003). Mutational studies involving cysteine and histidine have shown little change in activity of proteins (Golinelli, *et al.*, 1999; Núria, *et al.*, 1999). The difference lies in cysteines present in intra- vs. extracellular proteins. Intracellularly cysteines behave as a small, neutral residue. Extracellularly, cysteines are frequently involved with disulphide bonds. Regarding galectins, instability without bound ligand in an oxidizing environment has been attributed to intramolecular disulphide bonds (Tracey, *et al.*, 1992) and tryptophan oxidation (Levi and Teichburg, 1981). With the overall relative specificity of MS15 being quite similar to mammalian galectin-1 (Chapter II), perhaps cysteine 52 represents a modification to control extracellular activity rather than specificity.

In part to address these differences, homology modeling was employed. Homology modeling provided a 3-D structure that, due to conservation of the protein, permitted high confidence in the model. There are two criteria to determine quality of a "theoretical" protein model. First, the *correctness* of a model is essentially dictated by the quality of the sequence alignment used to guide the modeling process. Second, the *accuracy* of a model is essentially limited by the deviation of the used template structure(s), which deviate 0.5 to 1.5 Å from different structures of the same protein. Once modeled, residues that are thought to contribute to an extended binding site in galectin-3 are similar and/or identical to those of MS15. For modeling MS15 on bovine galectin-1, the software performed the sequence alignment, and MS15 was threaded

onto the template. Following energy minimization

It has been proposed that the entire CRD of galectins are involved establishing the carbohydrate binding site (Abbott and Feizi, 1991). More recent analysis, using mutagenesis, suggests that loss of function due to substitutions or deletions of N- and/or C-termini (Cho and Cummings, 1996) is due to weaker binding by monomer vs. dimer (avidity), and not due to altered conformation of the carbohydrate-binding site. Therefore, conservation of primary structure of binding site may relate to conserved function, while divergence in the N- and C-termini may represent less evolutionary pressure in these regions of the protein. What are obvious in the N- and C-termini are the highly conserved phenylalanines and glycines that contribute to the beta-sheet and beta–turns of galectins. An exception to this is seen in Conger eel galectins congerin I and II, where the exons coding for the N- and C-termini appear to have diverged rapidly under positive pressure to create an extended binding site and strand swapping potential at the dimer interface, setting it apart from other fish galectins (Shirai, *et al*., 1999). With this in mind, comparisons were made between translations of *Msgal1*exon III, which codes for the carbohydrate binding site, and complete primary structure of prototype galectins. Using BLAST to search through the *Fugu* genomic database, nine predicted proteins were retrieved, with six being ~130 amino acids and three being ~300 amino acids. Of the six ~130 aa proteins from *Fugu*, percent identities for translated exon III and complete coding sequences range from 33/27% to 82/77%, respectively. Of the three *Danio* proto type galectins identified to date, percent identities are 48/63%, 56/56%, and 54/60%, respectively. With the *Fugu* sequences, percent identity of exon III's correlated with percent identities of the complete coding

region. This was not the case with *Danio*, where an inverse relationship was noted. It has been suggested that with multiple prototype galectins present, some genes may evolve at an accelerated rate due to removal of functional constraints (Ahmed, *et al.*, 1996). With only one prototype galectin known to date in striped bass, comparisons of rates of gene mutations is impossible. The genomic sequencing of *Takifugu*, *Tetraodon*, *Danio*, and other fish, coupled with the presence of multiple galectin genes in each organism, will be more useful as models for this type of analysis.

Though some other species appear to have alternatively spliced transcript variants (Wada and Kanwar, 1997; Moisan, *et al.*, 2002), and others possess paralogous galectins, no other galectin sequences were revealed in the selected cDNA libraries, in the southern blot analysis, or in the genomic sequencing results. This does not mean they do not exist, but merely that paralogous prototype galectins in striped bass may be expressed in a spatially and/ or temporally controlled manner, and have not been revealed under the conditions used to date.

Table 3.6. Conservation of start, intron/exon boundaries, and stop site in *Msgal1*

	start	exon/intron	intron/exon	lariat	stop
consensus	C/TATGG/A	(C/A)AG*GT(G/A)AGT	C/T-AG*G	YNYYR**A**CY$_9$N AG*	TA/GA/G
Start	C**ATG**TT	----------	----------	----------	--------
intron I	---------	AAT*GTAAGT	TTCTCCACAG*G	CGCT**A**CCCCCTCTC CTN$_{14}$AG*	--------
intron II	---------	AGA*GTAAAT	TGTTCCGCAG*T	CAATA**A**CGTGCTGT TTCN$_{28}$AG*	--------
intron III	---------	AAG*GTGAGA	TACAATCAAG*A	TGCAT**A**TTCTTCCC CTN$_{35}$ AG*	--------
stop	---------	----------	----------	----------	AA**TAA**ACC

The majority of eukaryotic mRNAs are polyadenylated. There are three cis-acting elements that involved in this process: the polyadenylation signal, with a the consensus

sequence of AATAAA, which lies 6-30 bp upstream of the polyadenylation site (Fitzgerald and Shenk, 1981; Proudfoot and Brownlee, 1976); the poly A+ addition site, which is usually occupied by an adenosine and often follows a cytosine (Moore *et al.*, 1986; Sheets *et al.*, 1987); and a downstream U or GU-rich region (Fitzgerald and Shenk, 1981; Gil and Proudfoot, 1984; Sadofsky *et al.*, 1985) . The 5'-UTR of eukaryotes possesses a short sequence called Kozak sequence (GCC) GCCRCC*ATG*G (Table 3.7), where R is a purine and which includes the ATG start site (Kozak, 1987). Analysis of *Msgal1* reveals a 43 bp long 5'-UTR, with (T<u>C</u>T)<u>GC</u>A<u>A</u>T<u>C</u>*<u>ATG</u>*T) present in place of the Kozak sequence. The underlined bases match the consensus sequence. The single most important residue in this sequence is the A at -3 (Kozak, 1986), and this is found in the *Msgal1* 5'-UTR. A BLAST search with UTR revealed hits with up to 28 bp at 100%, but these were from partially annotated BAC clones and genomic fragments, and no UTR's of galectins. On the other end, the 3'-UTR, from the end of the stop codon to the poly A+ tail, is 502 bp. The poly A+ site of *Msgal1* has a weak similarity to the "canonical" site of vertebrates. The polyadenylation signal is ATTAAA vs., AATAAA, but ATTAAA has been found to be only slightly less efficient as a signal. Approximately 30 bp downstream of the polyadenylation signal there is an increase in G and T, but no strong GT dinucleotide pattern. In the 3' UTR there are extended stretches that match well with *Paralychthys* galectin 3' UTR, but no other galectin analyzed to date. Phylogenetic analysis of MS15 was performed using ClustalW software, and included representative prototype galectins from mammals, birds, fish, and tunicates. Analysis was also done with MS15 vs. all available fish galectin

Table 3.7. Conservation of Kozak and polyadenylation sites- the consensus sequence for translation initiation is GCCACCATGG, where the A in the bold ATG start codon is coordinate 1 and the A at position –3 (italicized) could also be a G (Kozak, 1987a). Functional studies on preproinsulin and alpha-globin translation in cells indicated that a purine (usually A) in position -3 is crucial for efficient initiation of translation, and in its absence, a G at position +4 is essential (Kozak, 1989).

galectin cDNA sequence	Kozak	poly A site
consensus	CCA*G*CC**ATG**(G)	AATAAA
Msgal1 (fish)	GCA*A*TC**ATG**TTT	ATTAAA
Lgals1 (human)	TCA*A*TC**ATG**GCT	AATAAA
Lgals1 (mouse)	TCA*A*TC**ATG**GCC	AATAAA
Lgals1 (cow)	CCA*A*TC**ATG**GCT	AATAAA
C-16 (chicken)	GGC*A*TC**ATG**GAG	AATAAA
Pogal1 (fish)	GCA*A*AA**ATG**ATG	ATTAAA
AJL-1 (fish)	GCC*A*AG**ATG**GAT	AATAAA
pCon I (fish)	GCC*A*AG**ATG**AGT	AATAAA
pCon II (fish)	GCC*A*AG**ATG**AGT	AATAAA
Trgal1 (fish)	CCA*T*AG**ATG**ATT	ATTAAA
Trgal2 (fish)	******ATG**ACC	ATTAAA

sequences. The sequences used for comparison are from genome sequences and cDNA sequences found deposited in GenBank and at genome databases of TIGR and HGMP Resource Centre (Fugu Genomics Group). The phylogenetic trees were generated using the ClustalW program. The program uses the NJ (Neighbor Joining) method of Saitou and Nei (1987). First, distances are calculated (percent divergence) between all pairs of sequence from a multiple alignment; the NJ method is applied to the distance matrix. The phylogenetic tree (Figure 3.18) shows that although there is a close relationship among all of the galectins, the teleost galectins are removed from the tight group formed by mammals, birds, and amphibians, and MS15 clusters with teleosts. Specifically, MS15 cluster closely with bastard flounder, green-spotted pufferfish, and

the Japanese pufferfish. Slightly more distant is electric eel and two of three zebrafish prototype galectins. The galectins of salmonids, stickleback fish, and the anguillids are as distant as the mammalian and bird proto type galectins. Phylogenetic analyses of prototype galectins of teleosts match current taxonomy to the level of order in most of the species analyzed. The Order Anguilliformes group together, as do the Superorder Percomorpha and the Order Salmoniformes. The exceptions are the galectins of medaka, stickleback (both Percomorpha) and Drgal1-L3 from zebrafish (Cypriniformes) groups with the Order Salmoniformes. With the proposed genomic duplication events in teleosts, it is possible that these species possessed multiple prototype galectins, and it is the products of extant paralogous galectin genes that are being analyzed, rather than orthologues. Evolutionary relatedness must be confirmed either by experimental evidence for evolutionary history or experimental establishment of similar function.

Southern blot analysis with several restriction enzymes and a probe that directed to the highly conserved exon III of proto type galectins revealed only one copy of the *Msgal1*. The formula for determining the Tm of the probe fragment is:
Tm = 69.3°C + 0.41[%(G+C)] - 650/l, where l = the length (in nucleotides) of the probe. Therefore, Tm for the Exon III probe is 69.3 +0.41 (50)-650/186=69.3+20.5-3.5=86.3. High stringency conditions would therefore consist of hybridization temperatures above 74.5^{o}C. Though many factors contribute to hybridization, probe: target identity as low as 40% should theoretically hybridize. This degree of identity is well within the range of paralogous galectin genes in other organisms. Therefore, this

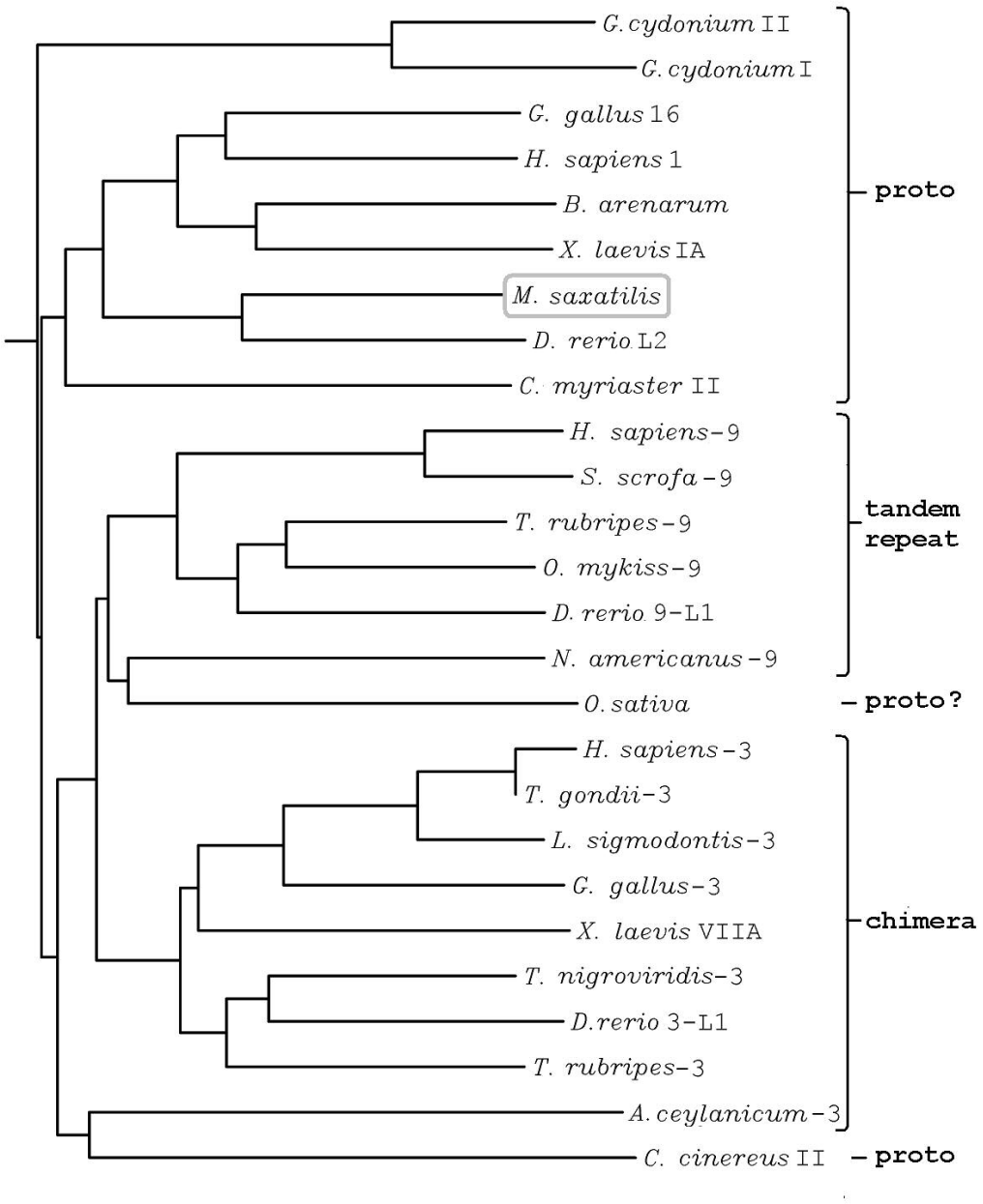


Figure 3.18. Phylogenetic Analysis: MS15 vs. proto, chimera and tandem repeat galectins- ClustalW (1.81) multiple sequence alignment tool was used to generate an amino acid alignment and Neighbor Joining method to generate a rooted phylogeny tree. 135 amino acids were used from MS15. *M. saxatilis* is outlined in gray. Proto type, chimera, and tandem repeat fall into distinct groups, with MS15 grouping with other proto type galectins. Bootstrap values are not provided by ClustalW server.

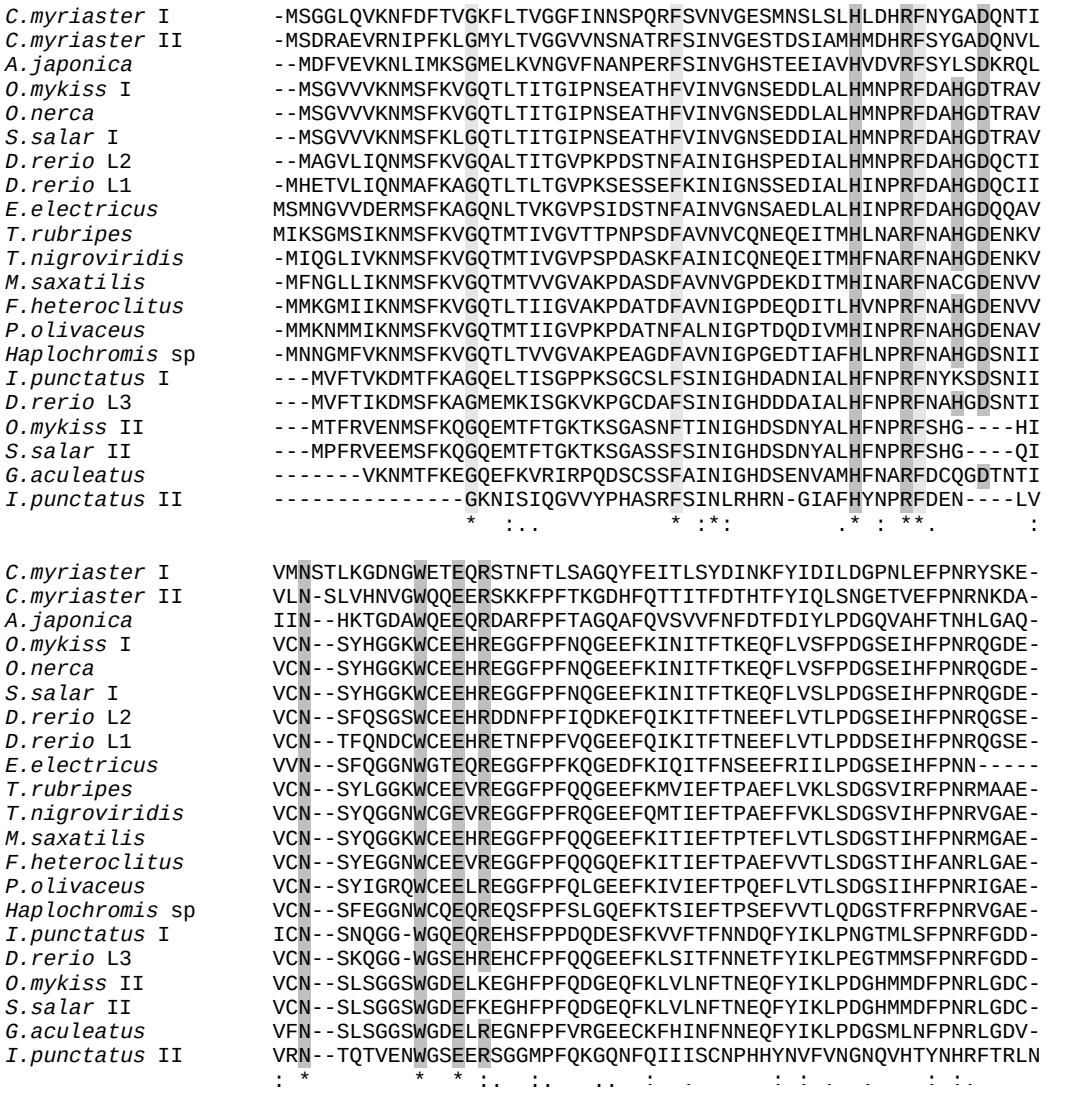


Figure 3.19. Phylogenetic Analysis: MS15 vs. fish prototype galectins - CLUSTAL W (1.81) multiple sequence alignment tool was used to generate an amino acid alignment using sequences from both published and unpublished sources. Dark gray bars mark the conserved residues involved in carbohydrate specificity most similar to mammalian galectin-1. The light gray is conserved residues involved in tertiary structure. Note residues involved in protein folding are more conserved than those involve with carbohydrate binding. See Fig. # for phylogenetic tree based on these data.

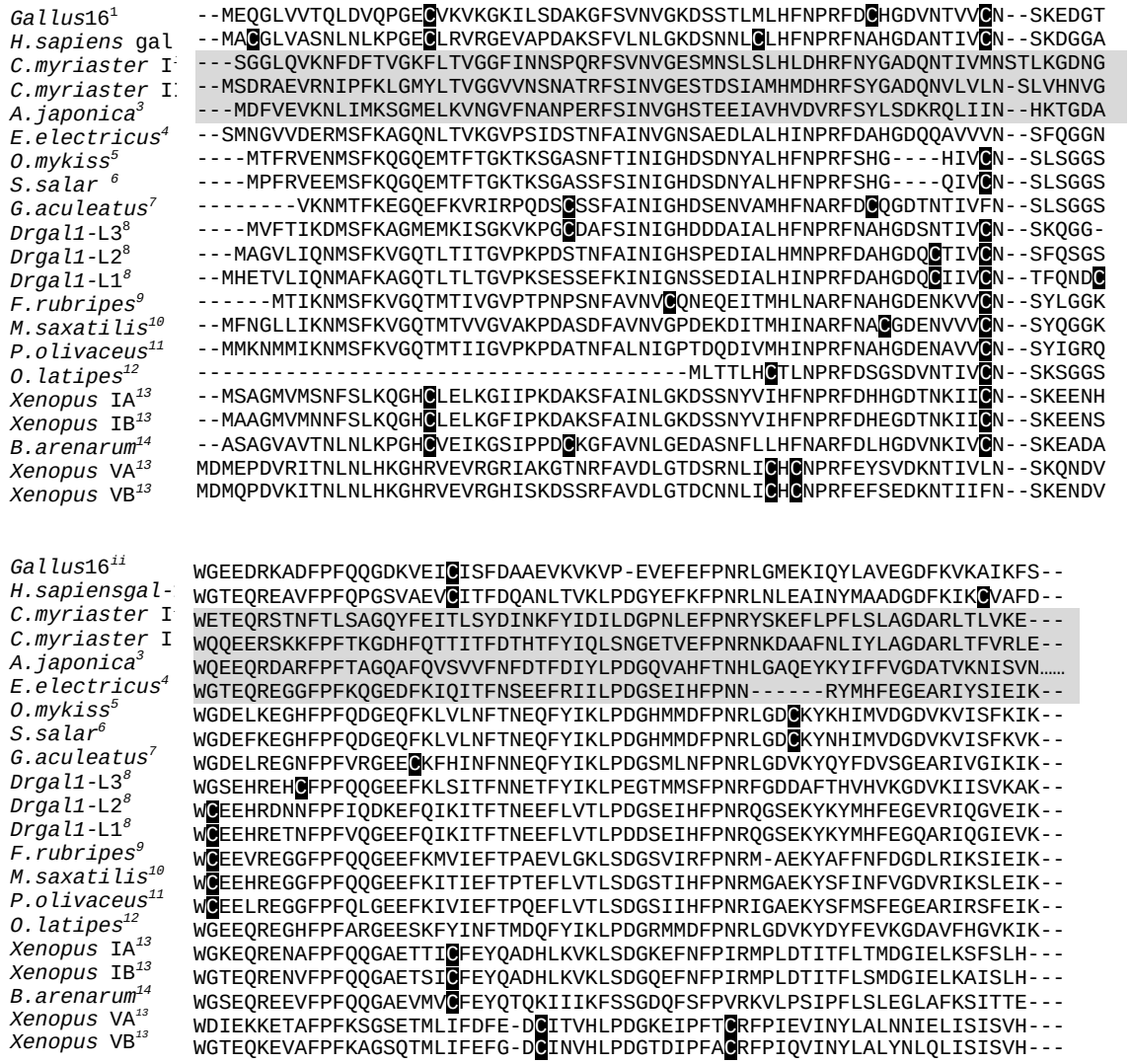


Figure 3.20. Trends in cysteines of prototype galectins: Cysteines are conserved in phylogenetically close species, but cysteine in highly conserved carbohydrate-binding site appears across many classes (mammals, birds, amphibians, fishes) Cysteines are in black. Grey bars highlight species with no cysteines. Species common names are given prior to Chapter I.

Source:

[1] skin,[1] placenta,skin,spleen, [1] skin, mucus, [4] electric organ, [5] genomic DNA, [6] genomic DNA, [7] genomic DNA, [8] whole body cDNA, genomic DNA, [9] genomic DNA, [10] skin, muscle, spleen, [11] muscle cDNA, [12] genomic DNA, [13] kidney cDNA, [14] ovary

level of stringency should detect both multiple copies and paralogous genes in the striped bass genome.

All genes for prototype galectins determined to date (mammalian galectin-1 and -2, chicken 14 kDa galectin), the galectin domain of the chimera-type galectin (mouse galectin-3), and gene sequence derived from genome project databases (*Takifugu, Tetraodon, Danio*) possess three conserved introns. The coding region of these galectins consists of four exons, the sizes being: exon I (6-9 bp), exon II (80-83 bp), exon III (160-172 bp), and exon IV (144-150 bp). Exon III encodes the largest, most conserved region, and includes the amino acids considered being important in forming hydrogen bonds or in van der Waals interaction with sugar ligand. This familial gene organization was used to determine the organization of striped bass proto type galectin. The coding region of MS15 was aligned with the human galectin-1 gene to help determine possible exon/ introns boundaries, and primers were designed to amplify the intervening regions (putative introns).

Sequencing results from the "introns" and "exons" were aligned and merged to create a single contiguous sequence. This construct revealed a gene that when aligned with the cDNA sequence of MS15 derived from mRNA, has four exons and three introns, with exon/introns boundaries following the GT/AG rule. The genomic sequence matches the cDNA sequence perfectly. The intron-exon organization of the MS15 galectin gene is identical to that of previously described galectin genes. The complete gene is organized the same as chicken-14 (Ohyama and Kasai, 1988), human galectin-1 (Gitt and Barondes, 1991), mouse galectin-1 (Chiariotti, *et al*, 1991), and *C.*

picta 14-kDa galectin (Oleary, 2003). The major difference between organisms is the intron sizes (Figure 3.13). This leads to the speculation that some control elements (enhancers) for expression may be located in the introns. An enhancer can activate a promoter when it is positioned in either orientation relative to the promoter, and when positioned upstream or downstream of the transcribed region, or within an intervening sequence (intron), which is eventually removed from the RNA by splicing. With this in mind, analysis of the 1800 bp upstream non-coding region of *Msgal1* was performed. Both the 5'-upstream region and Intron I possesses numerous potential transcription factor binding sites. Bias may lead one to focus on some more than others, due to their prevalence in cell types relevant to hypothesized role of protein (immune effector cells, for instance), and their relevance during different stages of development. A comparison with other proto type galectin sequences may suggest conserved factors, with the caveat that mammalian galectins have diversified in numbers, and possibly function. What are less common are pairs, or modules, of transcription factors in a particular orientation/spacing, such that they can interact with each other and the transcriptional machinery. Several of these were identified in *Msgal1*. In the 5' upstream region, there were three modules with strong identity in their core sequence. Intron I had 5 modules, most notable being two modules identical to the 5'-region. Intron II and III each had one, and the downstream 3'- region had 7. Modules containing c-EBP binding sites were in every region except Intron III. The NFAT/ Ap1 module is identified with regulation of IL-4 expression specifically, and generally involved in upregulating numerous cytokines and present in immune effector cells. Interestingly, no modules were identified in the exons of this gene. Functional characterization of galectin-3

revealed that it has genomic fragments encompassing -836 to +141 nt (relative to start of exon I) that have significant promoter activity when linked to the luciferase reporter gene (Kadrofske *et al*., 1998). Interestingly, human galectin-3 is bicistronic, coding for galectin-3 and for *galig*, a protein from a different reading frame (Guittaut *et al.*, 2001). Analysis of methylation patterns in mammalian galectin-1 revealed that density of methylation in the promoter region determined whether the gene was expressed (Salvatore *et al.*, 1998), and that these CpG-rich islands were located both upstream (to –116 nt) and downstream (to +53) of transcription initiation. In these studies, hypomethylation led to increased expression, supporting the idea that methylation sensitive transcription factors were involved with expression. SP1 sites have also been implicated in basal transcription of galectin-1 in mammals, but SP1 bound by antibody did not abolish basal transcription (Kondoh *et al.*, 2003) No functional characterization has been performed in fish.

Submission of the 1775 bp upstream of the start site for BLAST analysis revealed no significant similarity or identity to known non-coding regions or proteins, though similar range of galectin upstream sequence has been deposited from human, mouse, zebrafish, and pufferfish genomic sequencing projects. BLAST analysis of the large 2000+ bp Intron I reveal no significant regions of identity. There are some stretches of up to 25 bp with 100% identities, but never more that one stretch per nucleic acid sequence, and none of the stretches occur in known galectin gene sequence. *In silico* prediction of transcription factor binding sites in Intron I of *Msgal*1 reveals many putative sites that may be relevant to the hypothesized function(s) of MS15. Using TRANSFAC v4.0, each sequence has a Log-likelihood score (La)

calculated, and from that other scores are calculated (La / Length, La / L_M, where L_M is the maximum La possible for the site model). Among the best scores are the following factors: NFκB, a key regulator of genes involved in responses to infection, inflammation, stress (Baeuerle and Henkel, 1994); MEF-2 , which involved in myogenesis and induces MyoD (Kaushal *et al.*, 1994); C/EBPbeta, involved in inflammation and haematopoiesis, master regulator of the acute-phase response, induced by IL-1, IL-6, LPS (Akira *et al.*, 1990); HNF-4α1, a transcriptional activator (Malik and Karathanasis, 1996); E12, an activator, that may be involved in tissue-specific gene regulation of muscle, lymphoid, or neural cells (Peverali *et al.*, 1994); and AP-1, which is down-modulated by glucocorticoids through direct interaction with glucocorticoid receptor (Jonat *et al.*, 1990). Confirming that these factors/ sites are functional involves two approaches: 1) using the upstream region and Intron I to drive expression of a reporter gene, and 2) providing evidence that the factor in question is in the cells/ tissues in question, though competitive binding and microarray assays.

Recombinant MS15 was created to provide sufficient material for biochemical analysis, to simplify and improve purification, and to eliminate potential isoforms that may co- purify with MS15. Once rMS15 was found to be active, single step purification could be performed using affinity chromatography. The original recombinant design included an N-terminal His-Tag, to facilitate purification, but this proved unnecessary, leading to incorporating the tag cleavage step with the affinity purification step. The recombinant galectin had an equivalent mobility in SDS-PAGE, which closely approximated the predicted molecular weight of MS15. This suggested that rMS15 and native MS15 experienced similar (or no) post-translational modifications.

Thermostability of rMS15 was the same as native MS15, as well as the relative carbohydrate specificity for all sugars tested with rMS15. The yield of active rMS15 was substantial- 50-60 mg/L. There was some loss while cleaving tag from recombinant, though. Since active galectin could be expressed well in this system, consideration was given to a construct with out a His tag, but was thought unnecessary because yield was sufficient. This system would also work well for expressing MS15 mutants, such as N- and C-termini alterations to investigate monomer only effects in fish.

Summary: The determination of the full-length coding sequence for MS15 confirms that this protein is a proto type galectin, and of the 14-15 mammalian galectins identified to date, MS15 is most similar to mammalian galectin-1. Comparison of MS15 to galectin primary structures from fly, sponge worm, fish, amphibian, reptile, bird and mammal shows that MS15 is most similar to other fish proto type galectins, with relationship to other animal galectins being relative to evolutionary distance. The gene coding for MS15, *Msgal*1, is single copy per genome, and has the same intron/exon organization as mammal, bird, and fish galectins. *Msgal*1 possesses canonical signals for start, intron/exon boundaries, stop, and polyadenylation, but control elements vary from those of other galectins. Homology models of the protein, based on bovine galectin-1 (template), shows conserved residues of the carbohydrate binding site aligning well crystallographic data. No residues created conflicts with the template, and the predicted electrostatic potential revealed positive charge in the binding cleft, and mixed charges at the predicted dimer interface. This conforms to the hypothesized model of how MS15 subunits interact with each other and

the environment. Lastly, a recombinant MS15 was expressed in bacteria with high yield, and subsequent biochemical characterization confirmed that it functioned the same as native MS15, providing confidence in its use in future experiments.

CHAPTER IV: LOCALIZATION OF MS15 IN *M. SAXATILIS*

IV.A. Introduction

Understanding the function(s) of a galectin in an animal involves not only knowing its biochemical properties and genetic control of its expression, but also what cells, tissues, and organs it is expressed, and at what stage of the organism's development the galectin is expressed. The complex expression patterns of galectins can be correlated with the rapid and dynamic redistribution of carbohydrates during embryogenesis (Thorpe *et al*., 1987, Colnot *et al.*, 1997). However, there is also a non-uniform distribution of different galectins in adult animals. The repertoire of proto type galectins in a mammal may share similar carbohydrate specificities, but when and where they are expressed in part determines their function. Comparing location and proposed function of various animal proto type galectins can show this. Galectin-1 has been localized in mouse muscle tissues (Poirier and Robertson 1993; Cooper *et al.*1991; Wasano *et al.*1990), in neurons that interact with peripheral tissue (Regan *et al.*. 1986; Hynes *et al.*1990), and in cultured fibroblasts (Roff and Wang 1983). Mucins of the gastrointestinal tract have been shown to bind galectin-1, but though externalized into extracellular matrix (ECM) (Barondes *et al.*, 1994), galectin-1 is confined to the subepithelial connective tissue of the gastrointestinal tract and therefore appears not to directly interact with lumenal mucin or epithelial cell surface glycocalyses (ECSG) (Wasano *et al*., 1997). Galectin-1 has been localized to smooth muscle layer of large vessels such as arteries and veins, where it is able to modulate SMC attachment, spreading and migration via interactions with ECM proteins and integrin (Moiseeva *et*

al., 1999). CG14 is found in intestine (Beyer and Barondes, 1982), and CG16 is found in adult chicken retina and intestine (Maldonado *et al.*1999). Though expression is high in the mouse mesodermal cell types (most organs), it was undetectable in endodermal (epithelial) cells (Colnot *et al.*, 1997). Immunohistochemical studies in adult mammals showed that galectin-1 is expressed profusely at sites of immune privilege (Maldonado *et al.*, 1999; Phillips *et al.*, 1996; Wollina *et al.*, 1999). Galectin-5 is found in erythrocytes, with a possible role in erythroblast maturation, and localization in other tissues is attributed to presence of blood (Gitt *et al*., 1995). Galectin-7 is primarily distributed in stratified epithelial cells of the epidermis (Magnaldo *et al*., 1995; Madsen *et al*., 1995; Sato *et al.*, 2002). This complex expression pattern observed in animals with multiple prototype galectins can hinder determining a function for a particular galectin. It is possible that in animals with a simpler galectin repertoire, narrowing the function(s) for galectin may be simplified. As previously mentioned, galectins appear to associate with the cell surface of the cell that produced them, or remain in the ECM surrounding that cell (Barondes *et al*,.1994). Therefore, cells and tissues that provided the source RNA and DNA for molecular biology should have detectable levels of expressed galectin by immunocytochemistry, as well as the extracellular spaces immediately surrounding the cells and tissues. This is the justification for investigating tissues and organs throughout the striped bass adult. This concept cannot be extended to mucosal surfaces, though, where galectins can migrate with flowing mucus. This is important when considering MS15 in fish skin mucus. With regards to fish, galectins of Conger eel, *Conger myriaster* have been localized to the club cells of the skin, and in the mucosal epithelial lining preceding the stomach and in the gills (Nakamura *et al*.,

2001). Galectin has been found in the skin mucus of Japanese eel, *Anguilla japonica* (Tasumi *et al.*, 2002). MS15 has been purified from mucus and skin of striped bass, *M. saxatilis*, but the source of galectin in striped bass mucus is unclear. It is assumed that galectins are released to the surface of the animal from epidermal glands. Another study revealed one of three prototype galectins in zebrafish *Danio rerio* localizing to the developing notochord (Ahmed *et al.*, 2004). MS15 has been purified from almost every tissue and organ investigated, but only in juvenile and adult fish. This will be the first investigation into the localization of galectin in striped bass using immunocytochemical methods. Understanding where MS15 is located may help determine why it is present, and perhaps will help understand some of the observations in other fish.

In this chapter, conventional histochemical stains help identify cell and tissue types, including circulatory cells, as well as extracellular components. With an understanding of the structure and possible constituents of striped bass tissues and organs, localization of MS15 in adult striped bass is determined using immunocytochemistry and electron microscopy using the immunogold technique. The relationship of galectin location to putative function can be explored.

IV.B. Methods

Preparation and Characterization of Morphology of Circulatory Blood Cells: Blood was collected from euthanized striped bass from the caudal fin vein, and used to create blood smears to help identify cells in immunohistochemistry. Fifty µl of whole blood was placed on slide, smeared by dragging one slide over the other, and left to air dry. Replicate slides were subjected to staining with Diff-quick (Dade

Diagnostics, Aguada, Puerto Rico).

Preparation of Samples for Histological Staining and Immunocyto-chemistry: Adult and juvenile striped bass were euthanized by overdose with phenoxyethanol, 0.079 parts per thousand (ppt), and dissected immediately. Samples of skin, lips, tongue, buccal cavity, gills, esophagus, stomach, pyloric caeca, intestine, muscle, liver, spleen, testis, were fixed by placing in neutral buffered formalin (NBF) (100.0 ml formalin, 6.5 g sodium phosphate dibasic (anhydrous) / 4.0 g sodium phosphate monobasic / 900 ml distilled water; from "A Manual of Histotechniques" 3rd ed. Ann Preece, 1972) for up to 12 hours. For mounting of samples, Superfrost slides (Fisherbrand) were immersed in a freshly prepared 2% solution for 3-aminopropyltriethoxysilane (APES) in dry acetone for 5 minutes, washed briefly in distilled water twice. The slides were dried overnight at room temperature and stored at room temperature till use.

The fixed tissues were placed in labeled polypropylene Histoprep tissue capsules (Fisherbrand), and processed using a Leica TP1010 Tissue Processor with each step set to one hour; fixed samples were dehydrated through an ethanol series (50% to 100%) and passed through two xylene baths. Finally, tissue was infiltrated with paraffin in the final two baths. These samples were embedded in paraffin using a Leica EG 1160 Tissue Histoembedder, with 24 mm X 24 mm X 5 mm embedding rings (VWR) and disposable polypropylene base molds (VWR). Embedded tissue was sectioned using a Microm HM 340, with Leica 819 low profile microtome blades, at 12^{o} and 4 microns. Tissue sections were placed in a 40^{o}C water bath to flatten and orient the sections, and mounted on previously described APES slides.

Histological staining of striped bass mucus, tissues and organs: Sections were stained by three methods. Hematoxylin and eosin (H&E) were used as differential stains to determine quality of section preparation, and to identify cell types. Periodic acid-Schiff (PAS) staining was performed to detect glycogen, glycoproteins (such as mucus), and basement membranes. Since PAS stains predominantly neutral mucins, Alcian Blue (AB) staining was also done, to detect sulfated and carboxylated acid mucopolysaccharides and sulfated and carboxylated sialomucins (glycoproteins). All three techniques began by rehydrating the section to water through an ethanol series (100%, 95%, 75%, 50 %, water). For H&E, sections were rinsed in deionized water and submerged in hematoxylin for 3 minutes. The sections were washed in deionized water, followed by 'Bluing' sections in Scott's tap water. Sections were decolorized by quickly dipping sections in 1% hydrochloric acid in ethanol (acid alcohol) 4 to 8 times. Following another wash in tap water, sections were submerged in eosin for 1 minute, and rapidly dehydrated in 95% ethanol (5 minutes) and 100% ethanol (5 minutes), the cleared in xylene (5 minutes). Mounting sections was done with Permount and glass coverslips. For PAS staining, rehydrated sections were treated with 1% periodic acid for 5 minutes, and washed in tap water for five minutes. Sections were treated with Schiff's reagent for 30 minutes. To counter stain (nuclei), sections were submerged in hematoxylin for 3 minutes, destained with acid alcohol, and blued in Scott's tap water. The sections were dehydrated and mounted as described previously. For AB staining, rehydrated sections were submerged in AB solution, pH 2.5 (1g AB in 100ml with 3% acetic acid) for 5 minutes, and washed well in water. Hematoxylin was used to counterstain nuclei of cells, as previously described. Crude mucus was scraped from

live striped bass using a plastic spatula. This mucus was homogenized by pipetting up and down several times, and a drop was placed on an APES treated slide and smeared using another slide. Smears were allowed to air-dry. The dried mucus smears were fixed in NBF for five minutes, and stained with H&E and AB/PAS.

Immunocytochemistry of striped bass mucus, tissues and organs: Serial sections at 4 µm thickness mounted on APES coated slides were incubated with methanol containing 0.3% H_2O_2 for 30 minutes at room temperature to inactivate endogenous peroxidase and washed twice in PBS. The sections were blocked with 2% (w/v) BSA for 1 hour at room temperature. Endogenous biotin and avidin activity were blocked using the Avidin/Biotin Blocking Kit (Vector Laboratories), by incubating sections with Avidin D solution for 15 minutes, rinsing briefly with PBS, followed by 15 minute incubation with the biotin solution. For probing with primary antibody, the sections were incubated with protein A-purified IgG from MS15 polyclonal antiserum (from Dr. Hafiz Ahmed) at 1:1,000 dilution for 1 hour at room temperature and treated with biotin-conjugated goat anti-rabbit IgG (VECTASTAIN® Elite ABC Kits, Vector Laboratories) for 30 minutes at room temperature. Negative controls were purified polyclonal IgG from rabbit pre-immune serum and blocking buffer only (no primary antibody). The bound antibody:enzyme was visualized using Stable DAB (3, 3'-diaminobenzidine tetrahydrochloride, 0.0045% H_2O_2 in 50 mM Tris-HCl pH 7.5) (Invitrogen). To elucidate source of galectin in mucus, crude mucus was spread on glass slides and allowed to dry. Slides were hydrogen peroxide treated, avidin and biotin blocked, and probed with anti-MS15, as described previously. Bound antibody:enzyme was detected with Stable DAB, with 3-5 minutes reaction time, and

reaction was stopped by washing in tap water. Controls include non-immune serum and no primary antibody.

IV.C. Results

Identification of Peripheral Blood Cells: Stained blood smears provided a basis for cell identification in tissue and organ sections. Morphology of the cells was important for identification, but differential staining with the modified Giemsa stain allowed for differentiation based on nuclear and cytoplasmic staining (Figure 4.1). The nomenclature of the leucocytes is based on the morphological criteria described in several previous papers (Weinreb, 1963; Ferguson, 1976; Ellis, 1976, 1977; Cannon et al., 1980; Savage, 1983). Red blood cells, which are nucleated in fish, stained pale blue in the cytoplasm and dark blue in their nuclei. Immature RBC's stained the same way, but was up to 50% larger than mature RBC's. The thrombocyte (Figure 4.1 *B*.) is a small spindle-shaped cell with a large nucleus, which occupied most of the cell. The nucleus contained dense chromatin and was often indented. The lymphocyte (Figure 4.1 *C*.) was a small round cell and sometimes possessed pseudopodia on its surface. The round to slightly irregular nucleus contained heavily clumped chromatin and was surrounded by a small area of cytoplasm. The heterophil (Figure 4.1 *D*), equivalent to the mammalian neutrophil, is large and sometimes irregular in outline. The nucleus is ovoid to horseshoe-shaped and often more varied in shape than that of the monocyte.

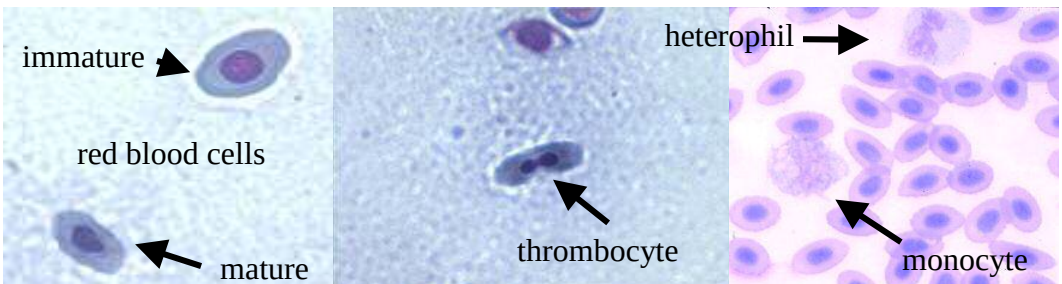


Figure 4. 1. Peripheral blood cells of striped bass: Blood smears were stained with a modified Giemsa stain, and results were compared to blood cells of other teleosts.

Chromatin was moderately condensed on the nuclear membrane and clumped inside the nucleus. The monocyte (Figure 4.1 *E*.) is a large cell with cytoplasmic pseudopodia varying in number and size. The nucleus was ovoid or kidney-shaped and often positioned off-center due to the abundant cytoplasmic organelles. Condensed chromatin is less prominent.

Histochemistry of striped bass mucus, tissues and organs: Sectioning was successful to 4-5 μm, with tissues from all samples losing integrity when cut thinner than 4 μm. Sections over 7 μm gave indistinct morphology, due to overlapping subcellular structures. In addition, the quality of the sections was often dependent on the consistency of the starting tissue, i.e., collagen filled skin with a layer of mineralized scales was more difficult to section than spleen or brain. Many cell and tissue types could be identified by hematoxylin, which stained nuclei pale blue, and

eosin staining, which stained cytoplasm pink. PAS, which stains basement membranes, mucosubstances secreted from the epithelia of various organs, and glycogen present in skin, liver, and parathyroid glands and skeletal and cardiac muscle, was used to highlight these structures and substances with a deep magenta. AB staining was performed with PAS, permitting the differentiation of acidic mucins (blue) from neutral ones (pink). DAB staining resulted in light to dark brown coloration in sections that contained MS15.

Hematoxylin and eosin staining: Results of H&E staining were consistent with results of other animals. Nuclei stained a deep blue, and cytoplasm stained various shades of pink. The nuclear stain was the most useful, helping to differentiate tissue layers and overall quality of sample preparation and processing.

Periodic acid-Schiff and hematoxylin staining: PAS/H staining revealed the relationship of cell and tissue types to some of the putative endogenous ligands of MS15. The basal lamina (BL) is a distinct layer dividing the epidermis and the dermis, consisting of glycan-rich molecules such as laminin, collagens, fibronectin, vitronectin, and glycoaminoglycans and stains strongly with PAS. The loose connective tissue between the skin and the muscle, containing many of the same proteoglycans as the BL, also stains strongly. Mucins present in goblet and mucous cells throughout the body stain strongly with PAS (Figure 4.7). In the esophagus, stomach, pyloric caeca, and intestine the ECM bordering the lamina propria stains strongly with PAS. Lastly, blood vessel walls stained strongly with PAS. All of these PAS positive substances/structures are known to stain in other animals. The location of these PAS positive areas will be important to colocalizing MS15 to putative endogenous ligands.

Alcian Blue/periodic acid-Schiff staining: With AB/ PAS, goblet cells stained either blue or deep pink, respectively. AB is used to stain a section first, because PAS will competitively stain the same structures, but not vice versa. AB positive areas were the goblet cells throughout the body (Figure 4.2), and in cell layer surrounding the deep crypt lumen of esophagus (Figure 4.2.B) and stomach (Figure 4.2.C). Some of the AB positive cells also have hematoxylin positive cytoplasm, which can be found in the acidophilic parietal cells of the stomach (Figure 4.2.C). The PAS positive sub-dermal connective tissue, basal lamina, and lamina propria exhibit no AB staining (Figure 4.2).

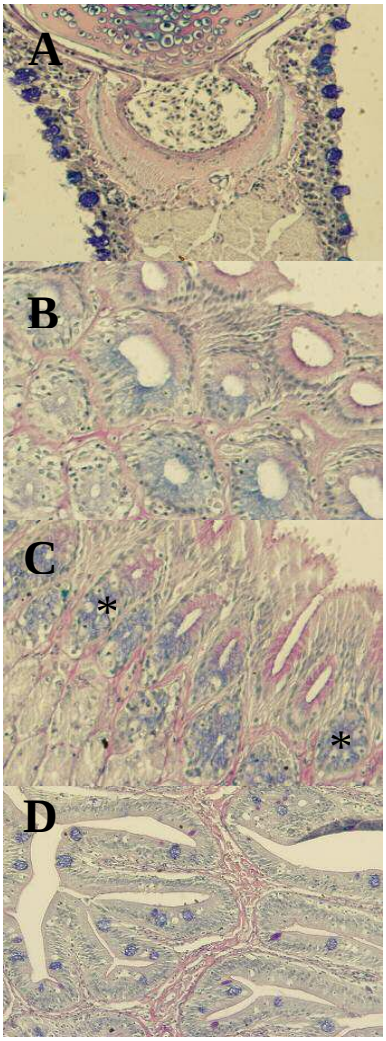


Figure 4.2. Alcian Blue/ PAS staining of mucins: *A*) Mucous glands of gills contain mostly acid mucins. *B*) Esophagus reveals complexity of developing lumenal spaces, with acid mucins present in deepest portion of lumen. *C*) Stomach resembles esophagus in organization, with parietal cells discernable (*stars) D*) Lamina propria contains loose connective tissue (collagens); gobet cells of intestine contain acid mucins similar to gills.

Detection in External Surfaces of Striped bass: Detection of MS15 in the

external surfaces, i.e., the skin, lips, tongue, buccal cavity, mucus and the gills was complicated by the presence of mineralized scales covering the surface of the skin. Focusing on the skin of the forehead of striped bass solved this problem. Here, the scales are reduced or even absent. The thickness of the epidermal layer is greater, though. The lips, tongue, buccal cavity and gills were simpler to dissect and process, but conversely were more complex, with sensory cells (taste buds), mucous cells and neuromasts. Analysis of cell debris in mucus revealed MS15(+) cells, but little staining in the background mucus (Figure 4.3)

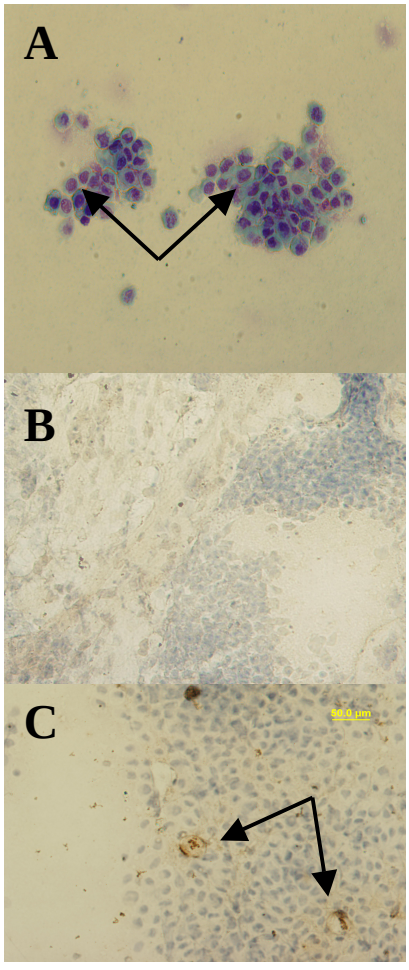


Figure 4.3. Striped bass mucus has MS15(+) cells: *a*) HE stain of cellular debris from striped bass mucus scraping. *Arrow*-basophilic cells (possibly monocytes or macrophage) *b*) Detection using purified Ig from rabbit pre-immune serum. Little background is detected. *c*) Detection with purified anti-MS15 Ig reveals galectin (+) cells in mucus (*arrows)*. These cells have large nuclei, and are about 1.5X larger than other cells present (epithelial cells)

Detection in Mouth, Pharynx and Alimentary Canal: Most epithelial

layers revealed epidermis of various numbers of cell layers, with squamous epidermal cells on the surface and cuboidal epidermal cells proximal to the fusiform layer (Figure 4.6). Samples from the alimentary canal included esophagus, stomach, pyloric caeca, and proximal intestine. MS15 colocalized with PAS staining in almost every sample studied, except in the goblet cells, where there was no MS15 detected (Figure 4.6). Rather, MS15 was detected in two strongly staining, distinct cell types. The first type's location and morphology suggest leukocytes (monocyte/ tissue macrophage, Figure 4.6). The second type is a cell type found only in fish and birds, and is called a rodlet cell (Figure 4.4). There is tissue-specific staining also, namely the smooth muscle surrounding arteries and veins (Figure 4.7). Where MS15 is not detected is in the epithelial layer of any given section, which is similar to the localization of galectin-1 in mammals. There is signal from some goblet cells of the skin, but this signal is of equal intensity in the pre-immune and the "no primary Ab" sections. With peroxidase activity blocked with H_2O_2 treatment, and avidin and biotin blocked, the source of this signal remains a question.

Detection in other organs: The muscle, liver, spleen, testes, and brain were investigated for endogenous MS15. Muscle was positive for MS15 throughout its fascia (epi-, peri- and endomysium), and lightly distributed in the muscle fibers. It is of interest that the first galectin identified from an animal was electrolectin, from the electric organ of *E. electricus*, which is a modified fish muscle. In the liver, the only distinct staining was in the blood vessel walls (Figure 4.7) In the spleen, there were numerous cells staining positive for MS15. These were distributed throughout

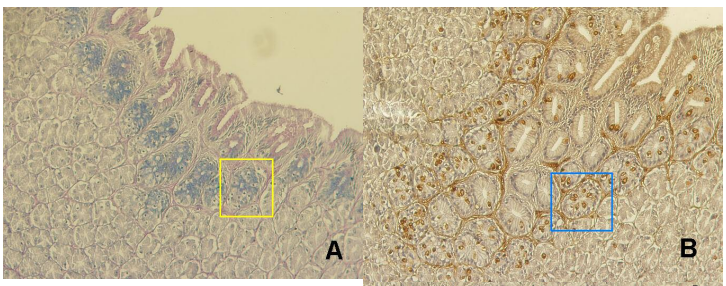
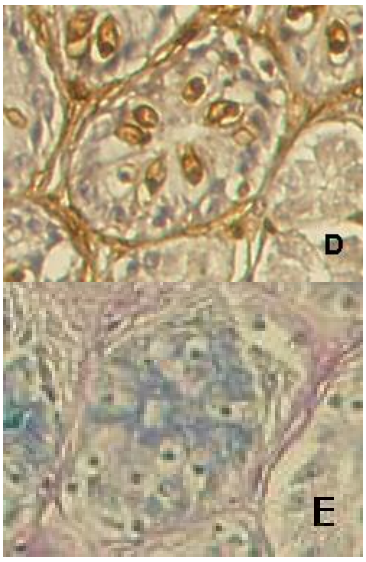


Figure 4. 4. Detection of striped bass rodlet cells with anti-MS15 in striped bass -
4 um section of striped bass corpus ventriculi, (**A)**, stained with alcian blue and PAS, reveals distinct areas of neutral glycoproteins (*pink*) and sulpho- and/or sialomucins (*blue*). DAB+ tissues (**B**) in the presence of anti-MS15 are found in the connective tissue as well as strongly staining rodlet cells. No DAB is visible in pre-immune control (**C**). Close-up of blue box in **B** (**D**) reveals staining of rodlet cells and surrounding connective tissue. Close-up of yellow box in **A** (**E**), reveals AB+ cells and PAS+ connective tissue.



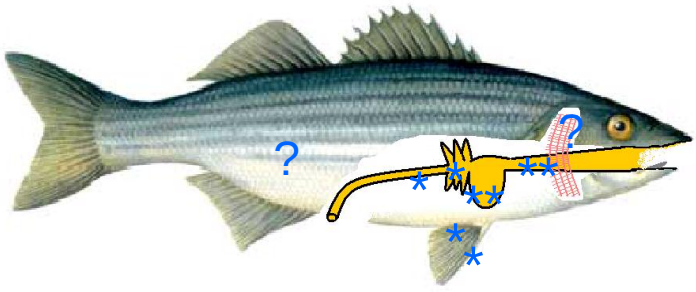


Figure 4.5. Distribution of rodlet cells: Rodlet cells were identified in the esophagus, stomach, pyloric caeca, and proximal intestine of striped bass. None were clearly identified (morphology) in the skin, and gills, *= MS15+ rodlet cells
? = not observed, though in literature

the spleen. The staining appears cell-specific, rather than ECM-specific. One source of

background signal in the spleen was the melanomacrophage centers, which are seen as brownish-blackish collections of cells. These are seen in PAS stained, and in the preimmune serum controls. This background is independent of the MS15 staining in other areas of the spleen. MS15 detection in the testes gave positive signals in the tunica albuginea surrounding the testis (Figure 4.7), and in the connective tissue creating the sperm ducts, but no signal is seen in sperm at any stage of development. The brain revealed what appeared to be a large MS15-positive structure, which turned out to be a transverse section of a blood vessel running between the medulla oblongata and the tegmentum. There were three areas that were positive for MS15, but the cell types in these areas are unknown (Figure 4.7). The source of MS15 would probably be cells localized in the same areas.

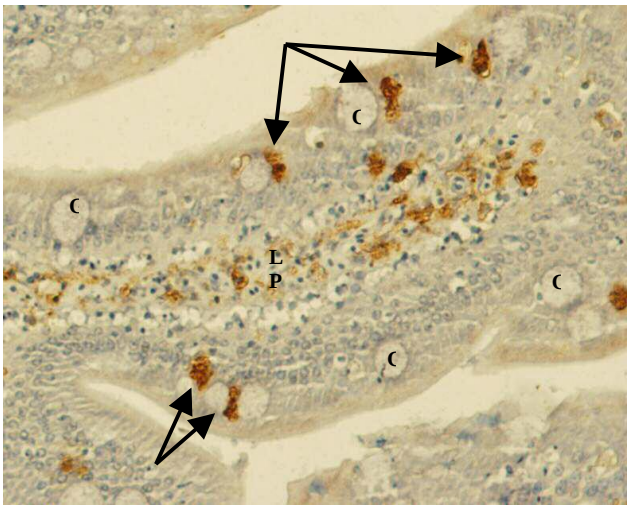


Figure 4.6. MS15 (+) macrophage in the pyloric caeca: Cross section of pyloric caecum reveals goblet cells(G), lamina propria (LP) **,** and resident tissue macrophage (*arrows*). Note no MS15(+) mucus or epithelial cells.

Figure 4.7. Detection of MS15 in striped bass: Various tissues and organs reveal distribution of MS15 in striped bass. Note that not all PAS(+) tissues are MS15(+). Most MS15 detected in loose connective tissue (dermis, lamina propria), rodlet cells of alimentary canal, fibroblast of skin, and peripheral leukocytes. (Con next page)

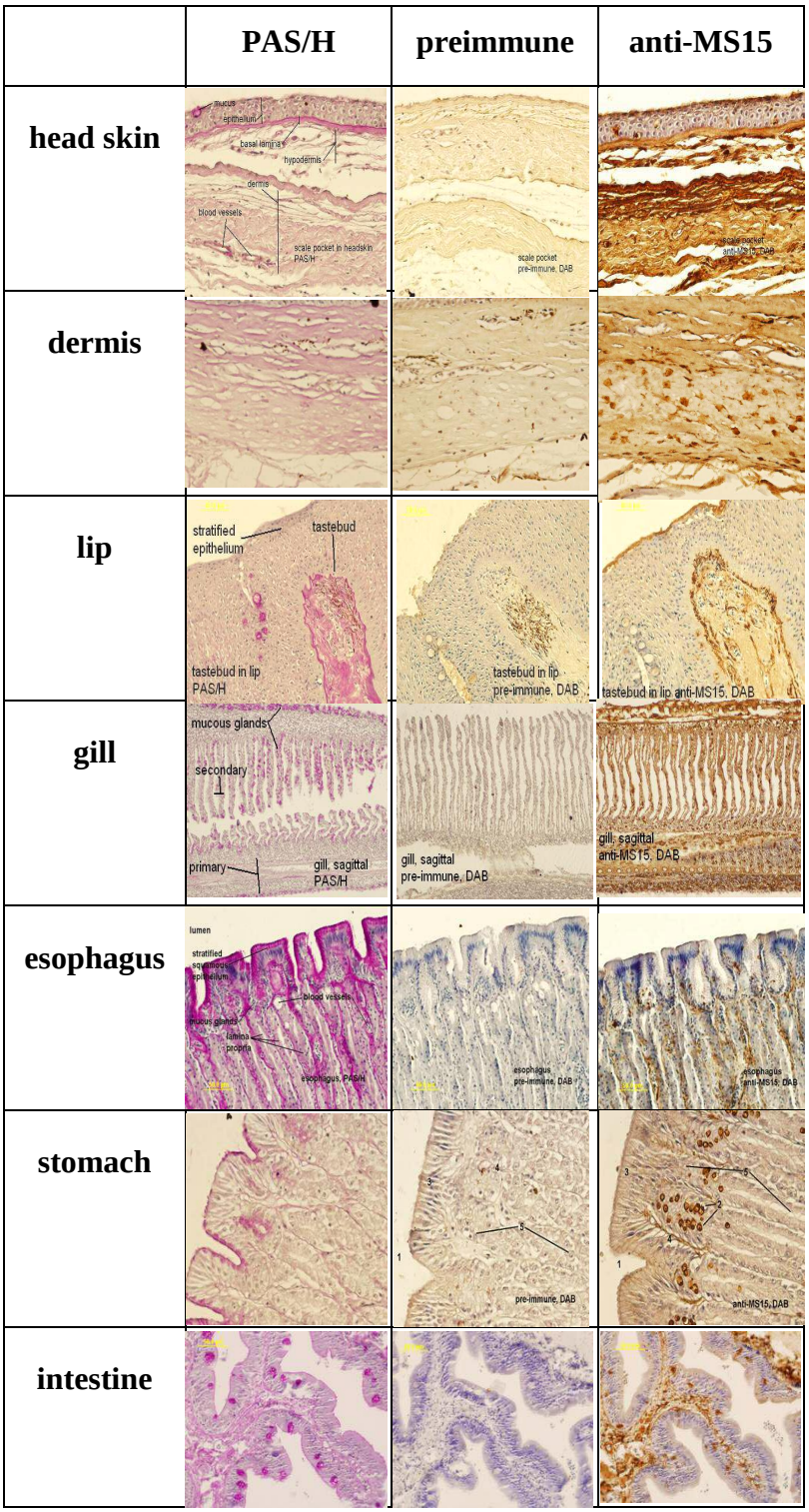
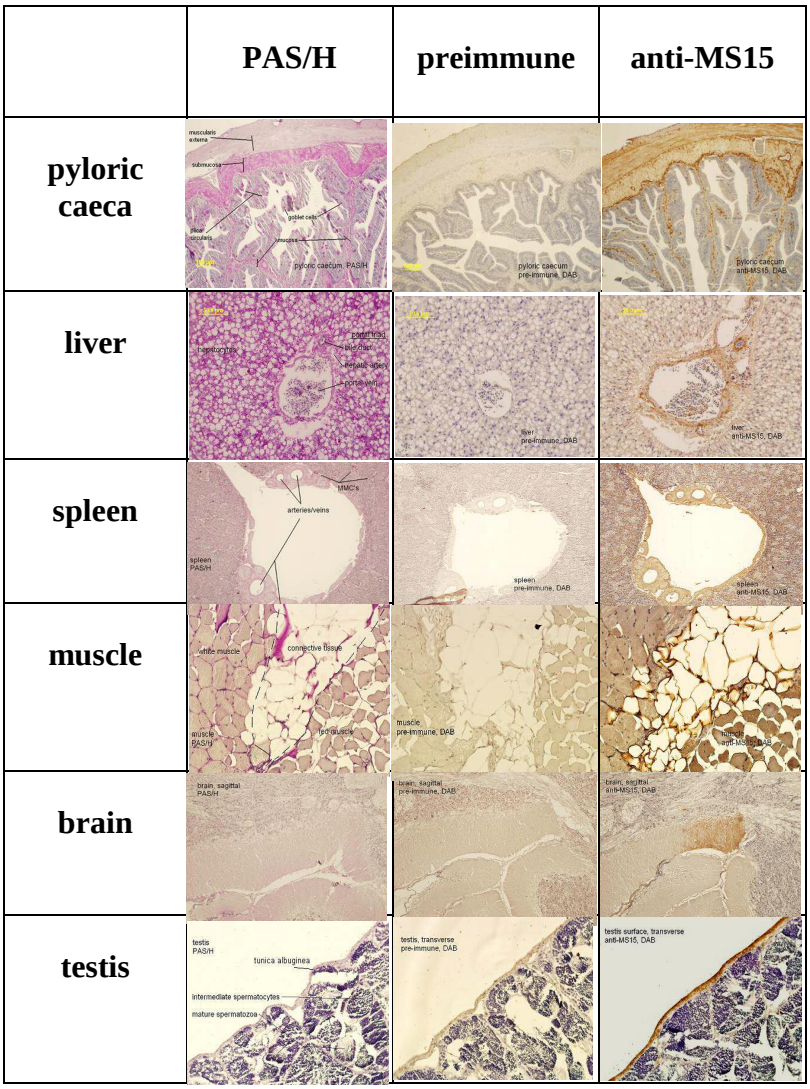


Figure 4.7. (con) Detection of MS15 in striped bass: Various tissues and organs reveal distribution of MS15 in striped bass. Note that not all PAS(+) tissues are MS15(+). Most MS15 detected in loose connective tissue (dermis, lamina propria), rodlet cells of alimentary canal, fibroblast of skin, and peripheral leukocytes.



IV.D. Discussion

Approximately 450 million years of evolutionary divergence separate mammals and bony fish (teleosts, including striped bass), providing considerable time for divergence in the molecular and cellular features of myeloid lineages, not only between different species of teleosts themselves, but also between teleosts and mammals (Rowley *et al*., 1988). Like mammals, teleosts possess red blood cells, several types of granulocytes and a separate macrophage lineage. Instead of platelets, though, teleosts have nucleated cells called thrombocytes. Descriptive studies are available for a wide range of fish species (reviewed in Rowley et al., 1988 and Secombes, 1996), and indicate that even between major teleost groups, for example, salmonids and cyprinids (zebrafish), there are significant morphological differences (Bielek, 1981). Caution must be exercised in inferring the function or identity of a cell merely from its appearance. Comparison of cells between different species must be based on functional and molecular studies as well as morphology. The evidence reviewed here indicates that at least morphologically, striped bass possess blood cells with features in common with other teleost, though functional comparisons have not been reported. Though each cell type exhibits a range of sizes, the staining of their respective subcellular components is not altered. Another factor in describing the peripheral blood cells of striped bass are the relative amounts of each cell type, which is not presented here, but has been found to change as the fish ages (Hrubec *et al.*, 2001).

Erythrocytes are the most abundant cell type found in the peripheral blood. They are oval in shape and have round to oval, centrally located nuclei, with densely packed chromatin. In the adult striped bass, they average 11 μm X 7 μm. The function

of red blood cells is to transport oxygen to and carbon dioxide from the tissues (Satchell, 1971). Their consistent appearance in sections containing blood vessels allowed for a constant scale between various tissue sections, helping to make comparisons between other peripheral blood cells. Immature erythrocytes, called reticulocytes, are also found in the peripheral blood, and are slightly larger than mature red blood cells. The nuclei of reticulocytes are more irregular and have less-condensed chromatin than mature red blood cells.

The striped bass thrombocytes are the second most common cells in the peripheral blood. They are oval in shape, and sometimes spiked at one end, and their nuclei are oval or dumbbell-shaped and centrally located. The heterochromatin stains dark blue/ purple, and their cytoplasm hardly stains at all, appearing light gray. Thrombocytes are small, about 4 μm to 7 μm in diameter, and can appear similar to striped bass lymphocytes (described below). Their primary function is clotting, but they release inflammatory mediators at wound sites, and there are some reports of teleost thrombocytes having phagocytic activity.

In teleost, three types of granulocytes- heterophils, eosinophils, and basophils, have been reported (Ellis, 1977; Rowley *et al*, 1988; Hine, 1992; Ainsworth, 1992). In striped bass, heterophils, called neutrophils in mammals, are the largest leukocytes in the peripheral blood. They are round, and range from 7 μm to 18 μm in diameter. Heterophils often contain small blue-gray cytoplasmic granules and have indented or multi-lobed (2-3) nuclei. The function(s) of heterophils have yet to be established, but heterophils stain strongly for myeloperoxidase, and this is certainly related to the known efficient bactericidal system (H_2O_2- myeloperoxidase -halide system) used as a

defensive mechanism similar to that one observed in mammals (Badwey and Karnovsky, 1981). No basophils or eosinophils were positively identified in this work. There are no conclusive data in the literature about the possible functions of eosinophils and basophils in fish blood. In some fish, eosinophils appear to be phagocytotic (Hine and Wane, 1989; Suzuki, 1986) and accumulate in parasitic infections and in inflammation (Lester and Desser, 1975). It is in eosinophilic granulocytes that the antimicrobial peptide piscidin was localized in hybrid striped bass, *M. saxatilis* X *M. chrysops* (Silphaduang and Noga, 2001). During studies on the distribution of mast cells/eosinophilic granule cells (Reite, 2001) in species representing 5-12 genera from each of the teleost families Salmonidae, Cyprinidae, Gadidae, Labridae and Pleuronectidae, a characteristic distribution pattern common to species of the same genus and great similarities also between the different genera of a family were found. Furthermore, the studies showed that persistent inflammatory reactions, e.g. those caused by helminths in tissues of the viscera, induced local recruitment of mast cells/eosinophilic granule cells, except in gadids, where this cell type was not found in any tissue. In all families, rodlet cells (discussed below) were recruited when cestodes or trematodes affected epithelial tissues.

There is enormous variation within the teleosts in both relative abundance and staining reaction of the granulocytes. For example, in the carp, *Cyprinus carpio*, all three types of granulocytes are found in the blood (Rowley, 1988). Among them, the heterophils and basophils are the least numerous. In salmonids, heterophilic granulocytes predominate with eosinophils and basophils either absent or present in low numbers (Rowley, 1998). In plaice, *Pleuronectes platessa* (Ellis, 1976) and the eel

(Kusuda and Ikeda, 1987), only one type of granulocyte has been reported. In the gilthead sea bream, *Sparus auratus* (Meseguer *et al*. 1994) acid phosphatase activity is evaluated as a cytochemical marker to differentiate the eosinophils from the heterophils. Eosinophils of the loach, *Misgurunus anquillicaudus*, have a unique feature, the presence of one large eosinophilic granule (Ishizeki *et al*, 1984).

The spherical lymphocytes ranged from 5 μm to 9 μm in diameter, with little basophilic cytoplasm and frequently showed cytoplasmic blebs, and some eosinophilic granules. The nucleus was spherical, dark blue and filled most of the cell.

In this work, no differentiation was made between basophilic phagocytes into categories of "monocyte" and "macrophage", because it is generally understood that “monocytes” are circulating macrophage, and “macrophage” are tissue resident cells. Monocytes in adult striped bass averaged 8.5 μm in diameter, have large irregularly shaped nuclei, and a highly vacuolated cytoplasm. Their nucleus is large and eosinophilic. These cells are negative for peroxidase activity. This is an advantage when using DAB detection, for although the hydrogen peroxide blocking of peroxidase activity appears to be complete (based on controls), no endogenous peroxidase is present to complicate interpretation of results. Functionally, the macrophage is a resident phagocytic cell, present before, during and after inflammation.

Morphological identification of monocytes may be helped by the fact that these cells are very similar in appearance in some species of fish. For example, the ultrastructure of the medaka monocyte is similar to that of plaice (Ferguson, 1976) and channel catfish (Cannon *et al*., 1980). The last cell type is the mast cell equivalent- the eosinophilic granular cells. These were not clearly identified in this work.

MS15 was found to be ubiquitous throughout samples of adult striped bass. All loose and irregular dense connective tissue contained MS15, as observed in the gills, spleen, intestine, stomach, esophagus, muscle and skin of striped bass. MS15 colocalized with PAS staining in these tissues, which suggests the connective tissue possesses endogenous ligand(s) for MS15. The most probable ligands are laminin and cell-surface integrin receptors, based on carbohydrate specificity and results for other proto type galectins in other animals (Gu *et al.*, 1994; Akimoto *et al.*, 1995; Seyrek *et al.*, 2000; Hielmann *et al.*, 2000; Uehara *et al.*, 2001; Ahmed *et al.*, 2004). Another common site for MS15 was vascular smooth muscle of the arteries, veins, and throughout the digestive tract. This was observed in the brain, liver, spleen, intestine, stomach, esophagus, muscle and skin of striped bass. This is similar to what is seen with human galectin-1 in vascular smooth muscle (Lotan *et al,*. 1994; Moiseeva *et al.*, 2002). In the digestive tract, there was one cell type, the rodlet cells, which produced both nuclear and cell surface signal for MS15. Though the alimentary canal in nearly indistinguishable at the microscopic level from other vertebrates, rodlet cells are an enigmatic cell type detected in fish and birds, and not yet in other vertebrates. There has been debate over the last 30 years as to their origin and function; it is now generally believed that they are involved in immune functions, either by releasing antibiotics into lumenal spaces, or by playing a role in inflammation. If rodlet cells secrete an antibiotic substance, perhaps their secretion into a wide spectrum of tissue compartments has been selected for in ancestral species with a particular life style, for instance, one that exposed them to large numbers and varieties of parasites.

EPITHELIAL ONLY	EPI- & MESOTHELIAL	EPI-, MESO- & ENDOTHELIAL
Percidae Yellow perch, *Perca flavescens* Walleye, *Stizostedion vitreum* Darter, *Etheostoma nigrum*	*Centrarchidae* Smallmouth bass, *Micropterus dolomieu* Rock bass, *Ambloplites rupestris* Bluegill, *Lepomis macrochirus*	***Cyprinidae*** Fathead minnow, *Pimephales promelas* Golden shiner, *Notemigonus crysoleucas* Redfin shiner, *Notropsis umbratitis*
Gadidae Burbot, *Lota lota* Cod, *Gadus morhua* [(1)]	***Umbridae*** Mud minnow, *Umbra limi*	***Catastomidae*** White Sucker, *Catastomus commersoni*
		Poeciliidae Swordtail, *Xiphopherus helleri*

from *Morrison & Odense, J. Fish Res. Bd. Can. 35:101-116 (1978)*

Table 4.1. Rodlet cell distribution vs. phylogeny: The data in the table demonstrate that closely related species are in the same rodlet cell distribution category, and that, as illustrated in category 3, this grouping may include related families *(Cyprinidae, Catastomidae, Poeciliidae).* The reason for the different distribution of rodlet cells in individual species is not understood.

Conversely, perhaps other host defenses such as leukocytes, complement, and antibody production take the place of rodlet cell functions in certain tissues of many species, particularly in the cardiovascular system. Rodlet cells are a common histological finding in almost all freshwater, brackish, and marine species of fish. However, their exact origin and function are presently unknown. Though described in at least 114 species of fish, they have not been described in *M. saxatilis,* and never has galectin been localized to rodlet cells. There is a distinct signal for MS15 in the ECM immediately surrounding the rodlet cells, and this signal disappears a short distance

from the MS15+ rodlet cells. This supports the conclusions of Barondes, *et al.*1994, where galectin stay in and around the cells that produce it.

In the testes, MS15 localized to the connective tissue that created the sperm ducts, and the membrane surrounding the testis, but none was observed in the spermatozoa. This contrasts sharply with the presence of galectin-1 in and on the spermatozoa rat in rat testes (Dentin, *et al.* 2003).

In the blood vessels, several morphotypes of cells are MS15+. These findings are what motivated the cataloging of different peripheral blood cells. The MS15+ cells are spherical, 1.5X to 2X the size of mature erythrocyte, and some have a discernable nucleus through the intense DAB staining. The only circulatory cells that match this description are the heterophils and monocytes (Figure 4.1, 4.9).

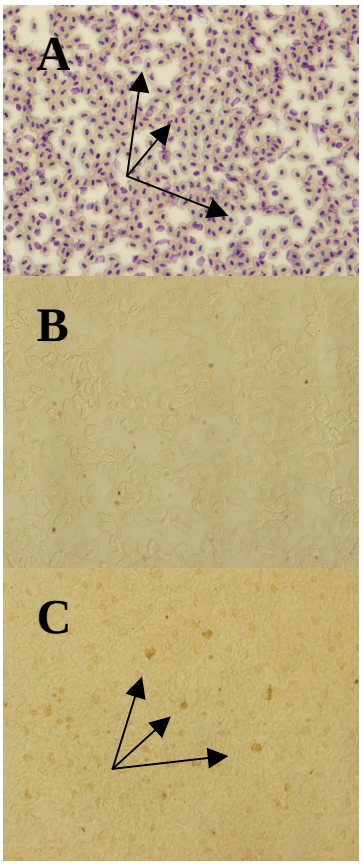


Figure 4.8. Striped bass blood has MS15(+) cells: *a*) Striped bass blood smears reveal red blood cells and leukocytes in Geimsa stain. *b*) Detection using purified Ig from rabbit pre-immune serum. *c*) Detection with purified anti-MS15 Ig reveals galectin (+) cells. These cells have large nuclei, and are probably heterophils or monocytes found in circulation.

They appear in different tissues in cross sections of blood vessels, and they are numerous in the gills, which are a vascular tissue. In gills, spleen, lips, tongue, skin, stomach, pyloric caeca, and intestine, MS15+ cells about the size and shape of the heterophils and monocytes are observed in the loose connective tissue, appearing to squeeze their way though the different cell layers. The only cell type that matches this description is the tissue resident macrophage. In mammals, galectin-1 has been localized to activated macrophage, and has a proposed role in signaling T-cell death (Rabinovich *et al.*, 1999) and promotes phagocytosis of activated neutrophils by activated macrophage. (Dias-Baruffi *et al.*, 2003).

In the dermis, a lightly staining cell morphologically and spatially matches the description of dermal fibroblasts. In humans, galectin-1 has been localized to the nucleus and cytoplasm of skin (dermal) fibroblasts (Akimoto *et al.*, 1995). Recently, mouse dermal fibroblasts, when placed in muscle and exposed to galectin-1, differentiated into myoblast and created new myofibrils (Goldring *et al.*, 2002). These cell types and tissue will be closely observed for changes in dermal bacterial challenge or wounding experiments. Galectin purified from the mucus of striped bass, *M. saxatilis*, though, does not appear to come from goblet or club cells in the skin. It has been localized to cells that appear to be leukocytes, most probably macrophage, because heterophils are thought to migrate into areas of inflammation, and the sections are of healthy tissue.

In the brain, there were areas of distinct MS15+ cells and tissue. The first was dismissed as a fortuitous sagittal sectioning a blood vessel, but within the blood vessel are red blood cells and several MS15+ cells morphologically resembling monocytes.

Two other areas that were MS15+ reveal several MS15+ cells surrounded by diffuse staining. It cannot be determined if these cells are brain cells or if they are migratory leukocytes. In some fish, the macrophage and lymphocytes outnumber the oligodedroglia in the spinal cord and in parts of the brain (Dowding and Scholes, 1993), but this appears to not be the case with striped bass. The other major area is a grouping of nerve bundle traveling laterally across the striped bass brain. These were first thought to be Purkinje cells based on their shape and location, but they are too large to be single cells. The identity of these nerves is not known, and they were the only ones to stain in the striped bass brain. In the mammalian nervous system, galectin-1 has been localized in neuroglial cells and astrocytomas (Camby *et al.*, 2001), Schwann cells (Horie *et al.*, 2004), and primary sensory neurons and motoneurons in the spinal cord and brain stem (Hynes *et al.*, 1990). Teleost possess these cell types also, but no staining in the brain could be directly attributed to these types of cells.

Finally, the overall staining pattern skin of striped bass was little in the mucus, none in the mucus producing cells or other epidermal tissue, and strong staining in the dermis/hypodermis. The detection of galectin in mucus is difficult with paraffin-embedded tissues, since this technique does not consistently preserve the soluble surface mucus. Mucus present in goblet or mucous cells is well preserved, and no galectin is seen anywhere on the skin, a result repeated in mucus-producing cells through out the animal. The epithelial cells, ranging from 3 or 4 cells to 20+ cells thick, are MS15 negative, but individual cells in the epidermis that are morphologically similar to tissue resident macrophage stain MS15+. Loose connective tissue of the dermis and hypodermis stains strongly for MS15, as do individual cells that are

morphologically similar to either macrophages or fibroblasts. Smooth vascular tissue of the arteries and veins stains positive for MS15, but the erythrocytes contained within do not. This staining pattern is most similar to what is observed in mouse when looking at galectin-1 distribution (Colnot *et al.*, 1997). Mammals possess a proto-type galectin, galectin-7, that localizes to the epithelial cells of the skin, in particular, keratinocytes (Magnaldo *et al.*, 1995). Though teleosts produce epidermal keratin, they do not keratinize their epidermis as do reptiles, birds and mammals. Perhaps the differences in galectin localization and skin cell function(s) in mammal and fish skin may be related. The structural sequences for skin mucus lectins in animal groups other than fish are limited and have been reported in two species, the land slug *Incilaria fruhstorferi* (Yuasa *et al.*,1998) and the African clawed frog *Xenopus laevis* (Marschal *et al.*, 1992). Though lectin activities have been found in the mucus of several fish species, the only structural sequences known are for conger eel mucus galectins (Muramoto and Kamiya, 1992; Muramoto *et al.*, 1999) and Japanese eel mucus galectin (Tasumi *et al.*, 2004). From conger eel mucus and skin, two galectins called congerin I and congerin II have been localized (Nakamura *et al.*, 2001) to the skin and mucus, but not observed in any other tissues. It was demonstrated that Japanese eel mucus galectin, called AJL-1, was synthesized in the skin, and secreted into mucus. AJL-1 gene expression was found in skin, but not in other tissues, i.e., gill, intestine, spleen, kidney and liver, by Northern blot analysis (Tasumi *et al.*, 2004). This is in strong contrast to MS15 in striped bass, localized in tissues throughout the body except for the epidermis and mucus. The question remains as to the source of MS15 purified from mucus. One possibility is that MS15 is from cells present in the mucus, either from sloughing off or from leukocytes

present on surface of skin. Striped bass skin mucus does not stain for MS15, but individual cells in mucus debris do stain MS15+. What type of cells these are has not been determined, but it does suggest that active MS15 can be in the mucus without having been produced by mucus-producing cells, which is different from the two eel species. Scaleless fish have been shown to possess different populations of mucous cells, containing different types of mucus (Saxena and Kulshrestha, 1981). There is a distinct difference between striped bass skin and eel skin, and that is the presence of scales in striped bass vs. no scales in eels. As with the differences in teleost skin and mammals, perhaps differences in fish mucus galectin localization are due to structural and functional requirements of scales vs. no scales.

IV.E. SUMMARY

Striped bass cells and tissues reacted to conventional cytological stains (H&E, PAS, AB, and Giemsa) the same as other animals. With these tools, the tissue and organ organization of striped bass was investigated, and major structures and cell types were identified. Among these were the peripheral blood cells, the goblet and mucous cells of the skin and alimentary canal, smooth vascular tissue, basal lamina and lamina propria, epidermal and dermal components, spleen, liver, brain, gills, and components of the alimentary canal. Stain used to detect anti-MS15 bound to endogenous MS15 revealed a distribution pattern most similar to mammalian galectin-1, which is ubiquitous in the dermis and loose connective tissues, smooth vascular tissue, muscle, fibroblasts, myeloid cells (tissue resident macrophage, monocytes, and heterophils, but not observed in the epidermis, mucus-producing cells, serum or red blood cells, or liver.

This contrasts sharply with galectin in eel skin and mucus, which is found only in mucus-producing cells in the skin and epithelial layer of the esophagus to the stomach, but not in the connective tissues or myeloid cells. The source of striped bass skin mucus galectin (MS15) appears to be from cells present in the mucus.

CHAPTER V: INTERACTIONS OF 15 -KDA GALECTIN WITH MUCUS AND BACTERIA ON THE STRIPED BASS (*MORONE SAXATILIS)*

V.A. Introduction

A recurring problem in aquaculture and in wild populations of striped bass is disease (Baya *et al.*, 1992; Toranzo *et al*., 1983; Snieszko, 1964; Eldar *et al*., 1995). Three major types of stresses that effect fish health in the Chesapeake Bay and in aquaculture are chemical stress, biological stress, and physical stress. Chemical stressors, such as low dissolved oxygen (Wannamaker and Rice, 2000), improper pH, pollution, diet composition, and nitrogenous and other metabolic wastes (Francis-Floyd, 2002) may lead to changes in growth, disease resistance, and behavior (Barton, 2002) Stress and injury initially trigger an alarm reaction (fight or flight response), which results in a series of changes within the fish. A blood sugar increase occurs in response to hormone secretion from the adrenal gland as liver glycogen is metabolized (Nolan *et al.*, 2003; Vijayan *et al*., 2003). This produces a burst of energy, which prepares the animal for an emergency. In addition, hormones released from the adrenal gland suppress the inflammatory response, a defense used by fish against invading disease organisms. Water balance in the fish (osmoregulation) is disrupted due to changes in the metabolism of minerals (Wendelaar Bonga *et al.* 1997). Under these circumstances, freshwater fish absorb excessive amounts of water from the environment (over-hydrate); salt-water fish lose water to the environment (dehydrate), this disruption increases energy requirements for osmoregulation. Respiration increases, blood pressure increases, and reserve red blood cells are released into the

blood stream. Biological stressors, such as crowding and presence of other species of fish leads to aggression, territoriality, and lateral swimming space requirements which all induce changes in hormone levels, such as cortisol, that if maintained over a period of time, having adverse effects on fish. Pathogenic and nonpathogenic microorganisms, and internal and external parasites can survive and multiply more easily on fish that have their innate immune system weakened (compromised skin/gill integrity, inhibited inflammation response).

Physical stressors, such as temperature, light, sounds, and dissolved gases, influence to immune system of the fish. Though low temperature has been shown to negatively affect specific immune responses in teleosts (Avtalion, 1969; Avtalion *et al.*,1970), nonspecific defenses in teleost fish tend to offset specific immune suppression at low environmental temperatures until the specific immune system adapts (Yano *et al.*, 1984; Hayman *et al.*, 1992; Collazos *et al*., 1994). Although the species studied comprise a small and non-representative sample of the over 29,000 known teleost species, there are many indications that the stress response is variable and flexible in fish, in line with the great diversity of adaptations that enable these animals to live in a large variety of aquatic habitats.

The innate immune system includes mechanical barriers to infection. The surface of the striped bass is covered with a mucus coating- from the exterior skin through the mouth and alimentary canal. This layer has evolved to allow gas and nutrient exchange, improve locomotion, and relevant to immunity, provide a matrix to surround the animal with antibacterial peptides, antibodies, toxins, and lectins. Bacteria colonize and invade fish through the mucus of the skin, mouth, gills, and

alimentary canal, having co-evolved to evade fish defenses and adhere to the mucus and /or epithelial layers. Two ways that bacteria adhere are with proteins specific for host glycolipids and glycoproteins, and through surface charge or hydrophobicity (Absolom, 1988). Utilizing specific proteins, bacteria adhere to mucus and epithelial cells using pili (or fimbriae), tipped with adhesins that recognize receptors on the host cell. Bacterial surface hydrophobicity (surface tension) plays a role in determining the extent of adhesion to host cells and phagocytic ingestion. Bacterial surface hydrophobicity can be altered significantly through exposure to sub-inhibitory concentrations of antibiotics, surfactants, lectins, etc. Lectins, such as galectins, present in the mucus and specific for bacterial surface glycans (Shiomi *et al*., 1990; Tasumi *et al.*, 2004) may have role in opsonization, but also in altering the bacteria's ability to adhere and invade. Finally, interactions between galectins and mucins may stabilize the mucus matrix, or bind bacteria to mucus until mucus is washed away.

Galectin has been isolated from striped bass skin and mucus (Chapter II). Galectins are proteins that may have many roles, including defense in mucus. Fish mucus galectins have been shown to agglutinate a marine pathogenic bacterium, including *Vibrio anguillarum* (Shiomi *et al.,* 1990) and *Streptococcus difficile* (Tasumi *et al.*, 2004). Galectin may recognize moieties on the surface of bacteria (agglutination) and may recognize N- and O-linked glycans on mucins and epithelial cells (Lindstedt *et al.*,1993; Sparrow *et al.*, 1987). There is evidence that galectins externalized by the GI tract epithelia into the lumen may participate in crosslinking mucin to mucin and mucin to epithelial cell surface glycocalyses (ECSG) (Wasano and Hirakawa, 1997). Such crosslinking may efficiently prevent the loss of mucin into the lumen and protect the

epithelial surface from the attack of lumenal acid, digestive enzymes, and/or microorganisms. The presence of such a system has been documented in chicken intestine (Barondes, 1984; Beyer and Barondes, 1980). Chicken intestine goblet cells secrete a galectin called CL-16 into the intestinal lumen, along with mucin. This protein has high affinity for both intestinal mucin and ECSG and is thought to play a role in crosslinking these two glycoconjugates.

Interactions with mucus and/or bacteria may be part of the function(s) of MS15 in striped bass. To address the possible interactions of MS15 with mucus and bacteria, striped bass mucus was collected from the animal's skin, tested for containing detectable levels of MS15, and tested for interactions with MS15. A variety of bacterial strains (pathogens vs. non-pathogens, Gram (-) vs. Gram (+)) were tested for interactions with MS15 by agglutination assays. Two bacterial strains were chosen to be used in bacterial challenges (injection) in skin and muscle of striped bass, followed by immunocytochemistry of challenged areas.

V.B. Materials

Mucus Collection and Processing: The mucus was collected from anesthetized healthy adult striped bass, by carefully scraping from head to tail the body mucus of the fish. Crude mucus was centrifuged in a Beckman J2-MC 20 centrifuge (JA 20 rotor, 17,000 revolutions/minute, 4°C, and 45 minutes) and the three visible fractions generated from multiple centrifugations were separated and corresponding layers were pooled.

Enzymatic treatment of MS15: Each pooled fraction of mucus was treated

separately with O-Glycosidase (O-Glycopeptide endo-D-galactosyl-N-acetyl-α-galactosamino hydrolase, Roche), N-glycosidase F (peptide-N4-(acetyl-β-glucosaminyl) asparagine amidase), or Endoglycosidase H, and with all three enzymes combined. O-Glycosidase treatment was as follows: 100 µL of mucus sample was added to 0.05 M sodium phosphate buffer (pH 6.0) containing 15 milliunits/ml O-glycosidase at 37°C for 4 hours. N-Glycosidase F treatment was as follows: 100 µL of mucus sample was added to 0.05 M sodium phosphate buffer (pH 6.0) containing 15 milliunits/ml N-Glycosidase F at 37°C for 4 hours. Endoglycosidase H treatment was as follows: 100 µL of mucus sample was added to 0.05 M sodium phosphate buffer (pH 6.0) containing 10 milliunits/ml enzyme at 37^{o}C for 4 hours.

Mucus blots: Mucus was transferred to PVDF using the Bio-Dot Microfiltration Apparatus (Biorad), in which clarified mucus was blotted by vacuum in replicate wells at 100 µl/well, with controls present in each column. Blotted membrane was cut into replicate lanes for different detection methods. Mucus was analyzed in three ways: by PAS staining to detect carbohydrates, Coomassie Blue to detect total protein and rMS15 to detect endogenous ligand. PAS was performed by washing membranes in three changes of water (1 ml/cm^2) and transferred to freshly prepared solution of 1% (v/v) periodic acid in 3% (v/v) acetic acid (1 ml/cm^2) for 30 minutes at room temperature. Membranes were rinsed twice in deionized water. Membranes were transferred to Schiff's reagent (Sigma) for 30 minutes (0.5 mL/cm^2). The membranes were washed three times for 2 minutes each in deionized water and air-dried. Total amount of protein was detected by transferring membranes in Coomassie Blue R-250/10% acetic acid/ 40% methanol in water for 30 minutes. Membranes were

destained in 10% acetic acid/ 40% methanol until background was gone. Lectinochemistry consisted of blocking membranes in 5% BSA/PBS followed by probing with rMS15: HRP. Bound galectin was detected with Stable-DAB (Invitrogen).

Bacterial Strains: The bacterial strains used in this study are listed in Table 5.1. The liquid medium used for growing bacteria was Leurer Broth (LB) broth (Difco). The agar medium was LB with 1.5% agar. The bacteria were grown at 30^{o}C or room temperature (20-25^{o}C). Bacterial strains were kept in liquid medium at –70^{o}C. *Escherichia coli* strains were grown in LB broth or agar medium, and stored in LB liquid medium with 20% glycerol at –70^{o}C. Bacterial strains of *Aeromonas hydrophila, A. veronii, A. trota, Bacillus subtilis, Carnobacterium piscicola, Edwardsiella tarda, Photobacterium damselae, Plesiomonas shigelloides, Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus faecalis, Vibrio anguillarum, V. cholerae, V. mimicus, V. parahemolytica,* and *V. vulnificus* were obtained from collections of Dr. R. Colwell and Dr. G. Vasta of the University of Maryland. Bacteria were cultured on defined media, as described at the DSMZ - Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (German Collection of Microorganisms and Cell Cultures), supplemented with mucin, as described in Jonsson, *et al.*, 2001.

Preparation of Whole Cells: Log-phase bacteria were harvested from agar spread plates plates in PBS, pH 7.4. After centrifugation, first at 300 rotations per minute (RPM) for 10 minutes and at 4500 RPM for 15 minutes, the bacterial cells were resuspended in PBS to 0.5 OD_{550} before reactions with MS15. Alternatively, 1.5 mL of each strain at 0.5 OD_{550} of was incubated overnight with 0.05% formalin to gently fix cells.

Lectin Agglutination Assay: Bacterial suspensions (40 μl) were mixed with 10 μl of 100 μg/ml MS15 in PBS in U-shaped microtitration wells for 5 seconds or with 10 μl of PBS (negative control) and allowed to settle, undisturbed overnight at 20^{o}C (Hynes *et al.*, 1999). Results were read by visual inspection. A positive result was indicated by a carpet of aggregated cellular material on the bottom of the wells, whereas a negative result was indicated by a dot of cellular material in the center of the well. Negative results were confirmed by tilting wells at 45^{o} angle and observing movement of cellular material. Auto-agglutination was confirmed by observing positive result in negative control well. As positive controls, MS15 were shown to agglutinate a 0.5% v/v solution of human type O erythrocytes after incubation at 4^{o}C for overnight. To test *M. saxatilis* skin mucus (filter-sterilized and galectin-depleted) as competitor, MS15 was incubated with mucus for one hour at 4^{o}C, and used in previously described agglutination assay.

Bacterial Challenge of *M. saxatilis* skin and muscle: Based on results of bacterial agglutination and galectin absorption tests, two species of bacteria were chosen for challenge experiments. *Vibrio anguillarum* and *V. parahemolyticus* grown as previously described in broth to 0.5 OD_{600} of. An aliquot of this culture was serially diluted and plated on duplicate LB agar plates, grown at 30^{o}C for 24 hours. Colony forming units (CFU's) were counted, averaged between duplicate plates, and multiplied by the dilution factors to determine CFU/ml of original culture. By this method, a dilution was made to have 1.0 X 10^{6} CFU/ml. The final injected amount of bacteria was ~1.0 X 10^{5} bacteria/ injection site. Striped bass were injected into the dermis of the head skin and into the muscle just posterior to the dorsal fin. One side of the fish was

used for bacterial challenge, and the other side was used for the control "sham" injection of PBS only. Following four days of no further handling, fish were sacrificed as previously described, and sites of injection were recovered with a scalpel. Samples were immediately placed in neutral buffered formalin and following 8 hours of fixation, were dehydrated and paraffin-embedded as described in Chapter IV. Finally, samples were sectioned and mounted on APES-treated slides for histochemical and immunocytochemical staining. H&E and PAS/H staining were performed as previously described, and endogenous MS15 was detected as described in Chapter IV.

V.C. Results

Interactions with mucus: Mucus collection yielded approximately 20 ml mucus/fish. It is difficult to control how much tank water associated with the fish was included with the mucus removed from the surface of the animal. After centrifugation, mucus had separated into three distinct layers: a "clarified", slightly viscous top layer, a cloudy, extremely viscous middle layer, and a brown pellet, approximately 5% w/v, which microscopically appeared to be food debris, sloughed cells, and bacteria. The "clarified" mucus showed no discernable suspended particles when analyzed microscopically, and the cloudy layer had some debris in it. When blotted to PVDF and probed with MS15:HRP, the cloudy mucus strongly bound galectin, and the clarified mucus bound comparatively much less galectin (Figure 5.1). This interaction was dependent on the presence of lactose, the competitive inhibitory sugar for this protein, suggesting MS15 and mucus interactions are protein-carbohydrate interactions.

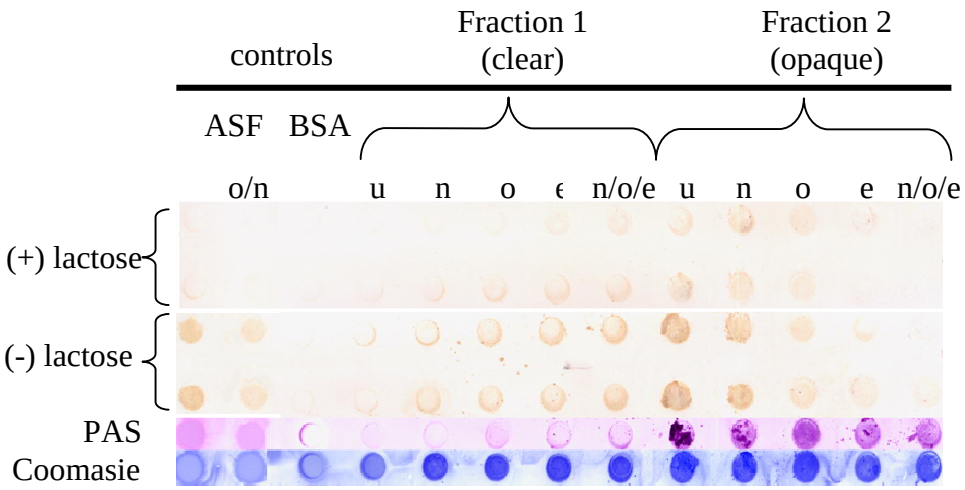


Figure 5.1. MS15 binds striped bass skin mucus: Crude mucus was fractionated by centrifugation. Each fraction was enzymatically treated to remove glycans. Treated samples of each fraction were blotted to PVDF, and probed with MS15 with or without lactose as competitor. PAS and Coomasie show relative amount carbohydrate and total protein per dot. ASF and BSA are glycosylated and non-glycosylated controls. *u*= untreated; *n*=N-glycosidase; *o*=O-glycosidase; *e*= EndoH.

Enzymatic cleavage of carbohydrate in clarified mucus increased the amount of galectin bound, but the same treatment in the cloudy mucus sharply decreased the amount of galectin bound. Pre-incubation of MS15 with lactose mostly/completely prevented binding to mucus fractions. Coomasie staining revealed equal amounts of mucus in each blot, and PAS staining revealed that the clarified mucus had much less carbohydrate that the cloudy mucus per blot. PAS staining also revealed a decrease in the carbohydrate content of cloudy mucus with each enzymatic treatment.

Interactions with bacteria: Lectin Agglutination Assay- Interpretation of this lectin agglutination assay was unambiguous (Figure 5.2). “Negative” agglutination observed as an even lawn of bacteria spread across the bottom of the wells. “Positive”

agglutination was a tight clump of cells at the bottom of the wells. Some bacteria species auto- or self-agglutinate, giving a potential false positive. Bacteria that were chosen for these studies included both gram negative and gram positive, and were known pathogens and non-pathogens for striped bass. There was no pattern for autoagglutination based on gram stain or pathogenicity in striped bass. There was no pattern of agglutination with MS15 based on gram stain or pathogenicity in striped bass. Bacteria that autoagglutinated under these conditions were *Photobacterium damselae, Staphylococcus aureus, Vibrio cholerae, and Aeromonas hydrophila.* Bacteria that agglutinated with MS15 under these conditions were *Carnobacterium piscicola, Edwardsiella tarda, Plesiomonas shigelloides, Streptococcus faecalis, Vibrio anguillarum, V. cholerae, V. mimicus, V. parahemolytica, V. vulnificus.* Bacteria that neither agglutinated with MS15 or autoagglutinated under these conditions were *A. veronii*, *A. trota*, *Bacillus subtilis*, and *Pseudomonas aeruginosa* (Table 5.1).

Immunohistochemistry of skin challenge: Injection of striped bass head skin with either bacteria or PBS (mock) revealed that skin reacted similarly to injections with either species of bacteria, and with or without bacteria. The reaction appeared to be that of wound healing, and no bacteria could be localized in the bacteria-challenged skin. There was a slight increase in MS15 observed in the ECM around fibroblasts in the dermis, but no increase in mucus-producing cells or epithelial cells (Figure 5.2).

Bacterial strain	agglutination	self-agglutination
Aeromonas hydrophila	+	+
A. veronii	-	-
A. trota	-	-
Carnobacterium piscicola	+	-
Edwardsiella tarda	+	-
Vibrio anguillarum	+	-
V. mimicus	+	-
Photobacterium damselae	+	+
Bacillus subtilis	-	-
Pseudomonas aeruginosa	-	-
Plesiomonas shigelloides	+	-
Staphylococcus aureus	+	+
Streptococcus faecalis	+	-
V. cholerae	+	+
V. parahemolytica	+	-
V. vulnificus	+	-

Table 5.1. MS15 agglutination of bacteria: MS15 agglutinated bacteria that are known striped bass pathogens (first seven) and non-pathogens (last eight). *P. damselae* is fish pathogen not yet associated with striped bass. Some bacteria auto (self) agglutinate, but positive self-agglutination does rule out binding of MS15. *V. anguillarum* and *V. parahemolytica* were chosen for bacterial challenges.

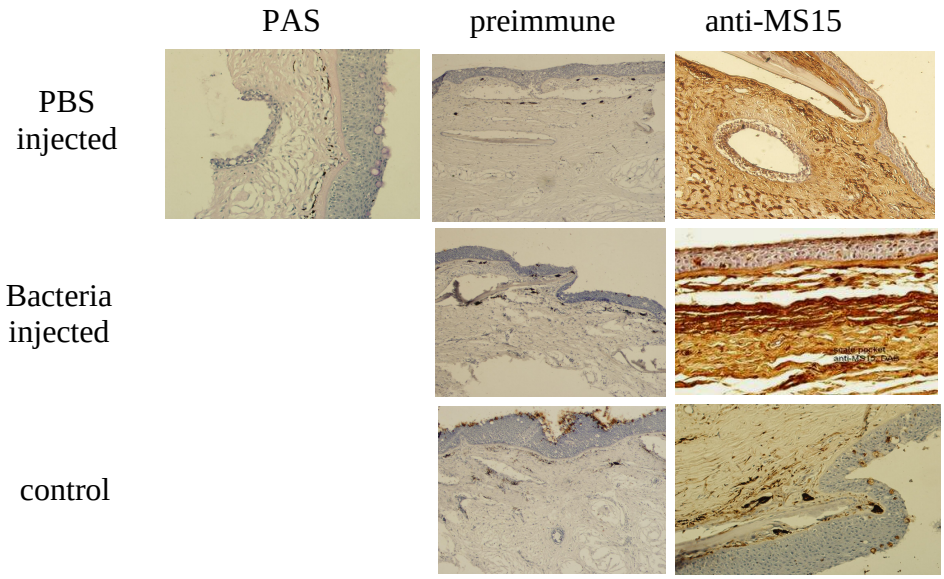


Figure 5.2. Bacterial challenge of striped bass skin: *V. parahemolyticus* injected, 4 days post-injection. There is an increase in MS15 in and around wound, but no apparent bacteria. As compared to muscle challenge, specific cells staining for MS15 appear to be fibroblasts. An increase in MS15 also appears in the dermis. Sham vs. bacteria revealed no significant difference.

Immunohistochemistry of muscle challenge: Injection of striped bass muscle with either bacteria or PBS (mock) revealed that muscle with different treatments reacted similarly. The reaction appeared to be that of wound healing, and no bacteria could be localized in the bacteria-challenged muscle. There was an increase in MS15+ cells in the wound site (Figure 5.3), in necrotic muscle fibers (Figure 5.3), and in the ECM immediately surrounding the MS15+ cells. The morphology of the MS15+ cells in the wound site (mostly peripheral blood cells) suggests that they are monocytes and/ or heterophils. The MS15+ cells in the necrotic muscle tissue morphologically and functionally appears to be macrophages or fibroblasts. There was no false positive with pre-immune serum controls, and PAS/H staining was comparable to the control (no

handling) fish staining.

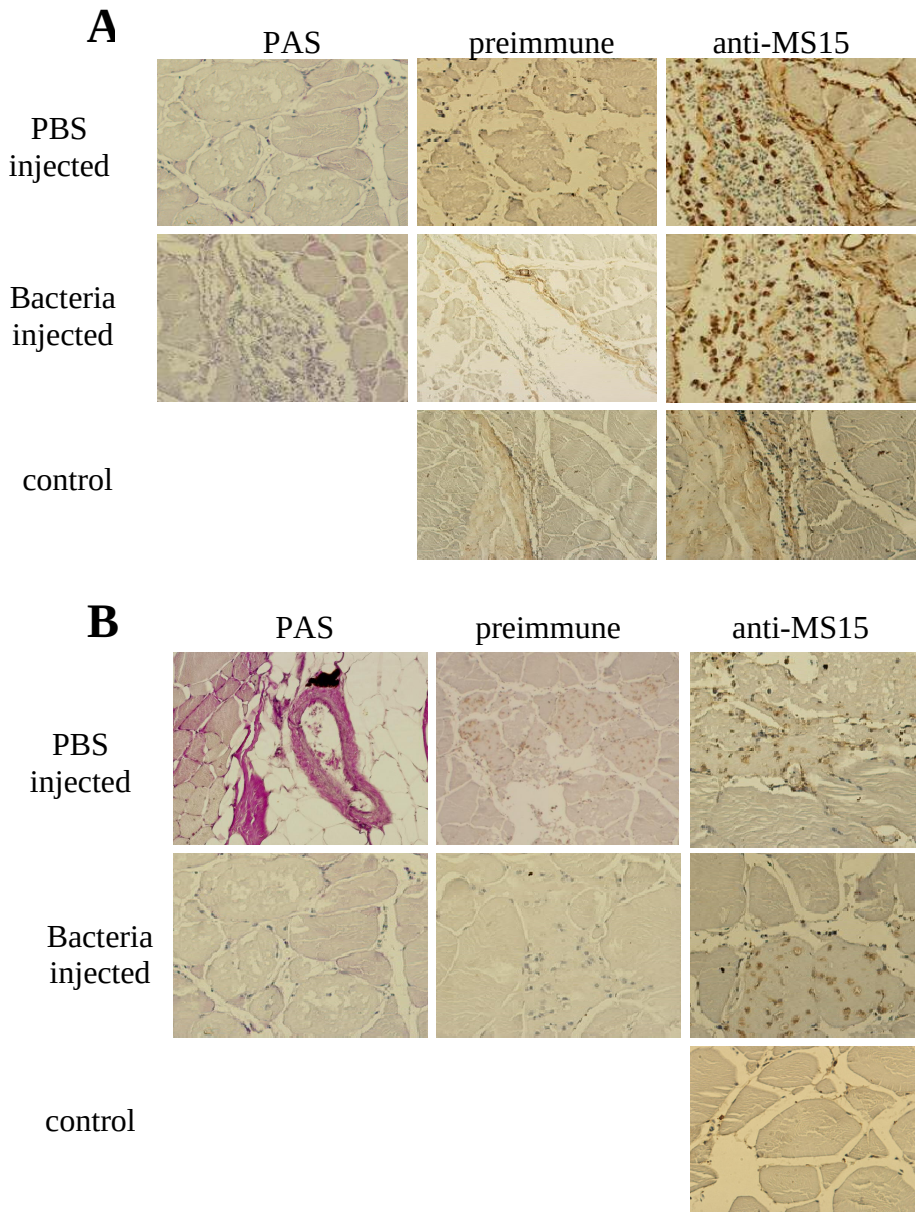


Figure 5.3. Bacterial challenge of striped bass muscle: *A*) *V. anguillarum* injected, 4 days post-injection *B*) *V. parahemolyticus* injected, 4 days post-injection. Both reveal an increase in MS15 in and around wound, but no apparent bacteria. Specific cells staining for MS15 appear to be heterophils and/ or monocytes, and macrophage in necrotic muscle fibers. An increase in MS15 also appears in ECM immediately surrounding wound/ infection site.

V.D. Discussion

Some mammalian mucins possess N-linked and O-linked glycans, and some of these mucins have been demonstrated to be counter receptors for galectin-1 (Seelenmeyer *et al.*, 2003) and galectin-3 (Bresalier *et al.*, 1996). The structure of these mucins is considered to be similar to fish mucins. The enzymes used to treat mucus were PNGase F, which releases virtually all known N-linked oligosaccharide structures, O-Glycosidase, which releases the disaccharide Galβ(1–3)GalNAc from O-glycans attached to serine or threonine; and Endo H, which show considerable specificity for N-linked structures such as oligomannosyl ("high mannose") and "hybrid"-type oligosaccharides. Some glycopeptides are refractory to one or more of these enzymes. For example, PNGase F cannot cleave oligosaccharides from amino- or carboxy-terminal asparagine residues. It had been previously noted that skin mucus from striped bass fractionated into two distinct layers following centrifugation. The upper layer was slightly viscous and clear. The lower layer was highly viscous, and opaque white. These layers were about 50/50, but the amount of water collected with mucus may have an effect on the proportions. There was also a small brown pellet beneath the lower layer, and analysis by light microscopy suggested that it consisted of particulates suspended in the mucus, i.e., food, bacteria, and cellular debris. The pattern of MS15 binding to treated mucus revealed that the upper layer had low carbohydrate content, but binding of MS15 improved slightly with O-glycosidase and Endo H treatment. The lower layer, though, possessed a relatively much greater amount of carbohydrate. MS15 bound both untreated and neuraminidase-treated lower fraction strongly, but further deglycosylation prevented binding of MS15 to mucus. All binding of MS15 was

dependent on the presence of lactose, which blocked MS15 binding. These data suggest that mucus possesses ligands for MS15, and that these ligands are not equally distributed in mucus, but rather, are associated with distinct substituents of mucus. In addition, MS15- mucin interactions are not protein-protein, but rather protein-carbohydrate in nature.

Bacterial association with host mucosal surfaces involves several stages. Successful negotiation of each of these requires -- or is at least facilitated by -- the development of a distinct set of characteristics (virulence factors) by the bacterium (Freter, 1981). The major stages include: (a) chemotactic attraction of motile bacteria to the surface of the mucus, (b) penetration of and trapping within the mucus (passive or active by bacterial motility and chemotaxis), (c) adhesion to receptors in the mucus or to mucus-associated layers of the indigenous microflora, (d) adhesion to epithelial cell surfaces, and (e) multiplication of the mucus-associated bacteria. Each reaction is further modified-or reversed-by substan *et al.*, 1999), inhibitors of adhesion, competitive bacterial species (McEldowney and Fletcher, 1987) and substrates for bacterial growth that are present in the mucosal microenvironment. Association with the mucus is often important for bacterial colonization but can also lead to more effective elimination of the bacterium by the host. This is where galectins, present in mucus and able to interact with bacteria, may play an important role. Whether this is through competition for binding sites, agglutination of bacteria and/or mucus, altering bacterial surface characteristics (hydrophobicity, Absolom *et al.*, 1983), or another mechanism is not known.

There are many species of bacteria associated with striped bass in their natural

environment and in an aquaculture tank. Not all interact with teleost skin, gut and mucus, and of the ones that do, not all cause disease in striped bass. This investigation focused on these bacterial-mucosal-skin interactions from the perspective of colonization, but not on whether the interactions led to a disease state. Bacterial challenge by injection of live bacteria was used to introduce bacteria in a controlled manner to a particular tissue, followed by recovery of the challenged tissue for analysis. The bacterial challenges in striped bass did not lead to discernable infection or disease, but it did produced changes in the distribution of galectin in the injection site. Peripheral blood cells filling the wound site included MS15+ cells that morphologically appeared to be heterophils and/ or monocytes. The ECM surrounding the wound site had a greater level of MS15 than did ECM away from the wound, but in similar tissue. Controls revealed no background staining present to complicate interpretation of results. The epidermis has mucus, and functions in wound healing and covering (osmotic barrier), the dermis has scales, pigments, and binds the epidermis, and the hypodermis has fat and holds the dermis to muscle. In fish, it has been shown that wound healing begins with a rapid closure of the wound by movement of the epidermal cells surrounding the wound into the wound cavity. This was done with the cells remaining in blocks, and maintaining their desmosomal contacts (Iger and Abraham, 1990). After wounding in mammals, the wound space fills with a fibrin clot, consisting of matrix components including fibronectin (Clark *et al*., 1981; Yamada and Clark, 1996). This forms a scaffold for the inward migration of the cells involved in early wound repair (Clark, 1996, Clark, 1997) The importance of fibronectin in early wound repair may lie in is ability to bind both cells and the extracellular matrix proteins

simultaneously (Engvall *et al*., 1978, Yamada and Clark, 1996). Interactions between human galectin-1 and laminin have been demonstrated, with galectin competing for fibronectin binding sites. MS15, sharing similar localization and carbohydrate specificity as mammalian galectin-1, may be involved in skin repair/remodeling by modulating migration of fibroblasts and cells of the wound edges into the wound.

V.E. SUMMARY

MS15 binds striped bass mucus and some species of bacteria in a carbohydrate-dependent manner. MS15 is localized in loose connective tissue of the skin (dermis), in macrophage and heterophils throughout the body, and in rodlet cells. This localization coupled with the interactions with bacteria and mucus suggests that MS15 plays a role in innate defense. The increase in the local concentrations of MS15 and MS15(+) cells following bacterial challenge and wounding of striped bass skin and muscle further supports a role in innate defense and possibly in wound healing.

CHAPTER VI: GENERAL DISCUSSION AND FUTURE DIRECTIONS

Striped bass, *Morone saxatilis*, possesses a 15 kDa β-galactoside binding protein that can be grouped with the galectin family of proteins, based on biochemical characteristics such as carbohydrate specificity and primary structure, and on molecular characteristics such as gene organization. Striped bass galectin, called MS15, can be modeled to structures of other prototype galectins with few predicted conformational conflicts. The purified protein and its recombinant form agglutinate some species of bacteria, and bind to components in mucus. The protein localizes to the dermis and lamina propia (loose connective tissue) throughout the body, to smooth vascular muscle (veins, arteries), to large circulatory cells (heterophils, monocytes), to peripheral leukocytes (tissue resident macrophages, heterophils), to rodlet cells (possible immune cell in alimentary canal), and to leukocytes throughout the gills. These are all tissues and cells that protect the animal from invasion and infection by bacteria.

The data collected to date has been used to generate three models of function for MS15. There were many possible interactions to consider for these models. These include wound repair (stabilizing mucus to exposed dermis), complement (recognizing bacteria), heterophil-endothelium-ECM interactions (extravasation), macrophage-ECM interactions (migration), heterophil-macrophage interactions (phagocytosis of activated heterophils), macrophage-T cell interactions (apoptosis of activated T-cells), and tissue remodeling (macrophage, fibroblast migration). During bacterial infection or aseptic inflammatory processes, galectins are produced and released by e.g. infected epithelium, activated tissue-resident macrophages and endothelial cells. These

extracellular galectins may facilitate binding of heterophils to the endothelium by cross-linking carbohydrates on the respective cells. Further, the galectins improve binding of the neutrophil to the extracellular matrix proteins laminin and fibronectin, and are potential chemotactic factors, inducing migration through the extracellular matrix towards the inflammatory focus (Matsushita *et al*., 2000; Sano *et al*., 2000). In mammals, both galectin-1 and galectin-3 have the capacity to induce a respiratory burst in neutrophils, if the cells have been primed by degranulation and receptor upregulation. The reactive oxygen species produced may be destructive to the invading micro-organisms as well as to the surrounding host tissue, pointing out the possible role of galectins, not only in defense toward infection, but also in inflammatory-induced tissue destruction (Almkvist and Karlsson, 2004). In trout, results show that the macrophages are the resident phagocytes of the peritoneal cavity, while heterophils are present in that body cavity in significant numbers only in situations of inflammation and only as long as the inflammation persists (Afonso *et al.*, 1998)

Model I: Macrophage in epithelial mucus- Macrophages that reside in skin can be found on surface of epithelium. In this model, macrophage release MS15 upon contact/ activation by bacteria (close range); bacteria are agglutinated and/or crosslinked with mucins, slowing or preventing binding to the epithelium. Bacteria are cleared as mucus is naturally discarded, or phagocytized by macrophage. (Fig 6.1)

The experimental evidence found to support this model is: Resident macrophage of skin can be found on epithelial surfaces, in the skin mucus; macrophage have cytoplasmic MS15; MS15 binds mucus and bacteria in carbohydrate-dependent manner; and MS15 is sensitive to oxidation, therefore can be inactivated when

extracellular and unbound to ligand, so probably does not exist extracellularly in mucus.

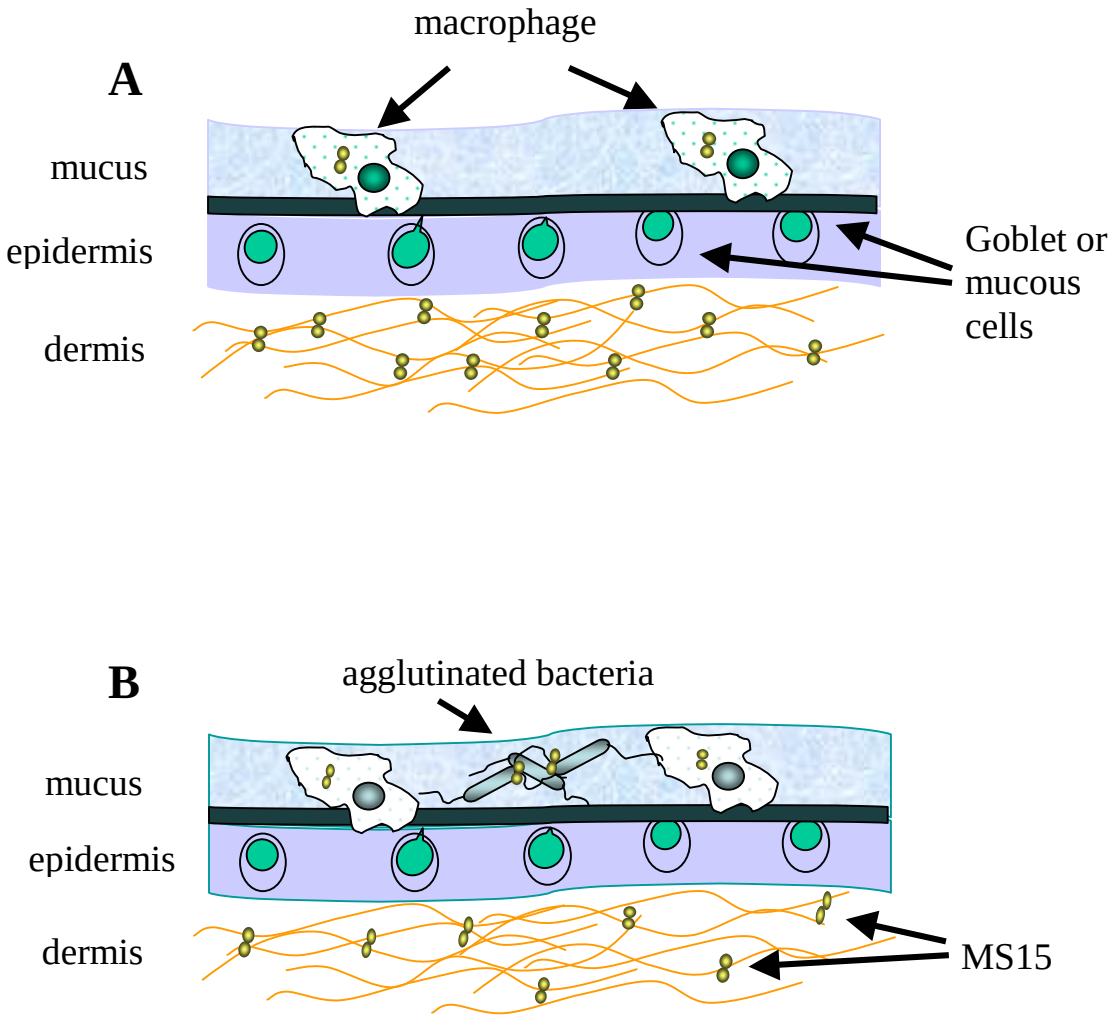


Figure 6.1. Model I-Intact skin defense: *a*) Galectin in mucus released by macrophage,and *b*)agglutinates bacteria by crosslinking bacteria with mucus or other bacteria, or opsonizes bacteria for macrophage phagocytosis.

Model II- MS15 in shallow wound defense: MS15 in skin helps in defense and healing by stabilizing mucus to MS15-rich dermis. As the epidermis is removed by mechanical (abrasion) or biological (infection) means, mucus flowing across skin is stabilized to exposed dermis, creating mucus "bandage". This would help in homeostasis (osmotic balance) and in defense.

Experimental evidence found to support this model is: MS15 is abundant in dermis, not epithelial cells; MS15 binds some components of crude mucus (Fig 6.2).

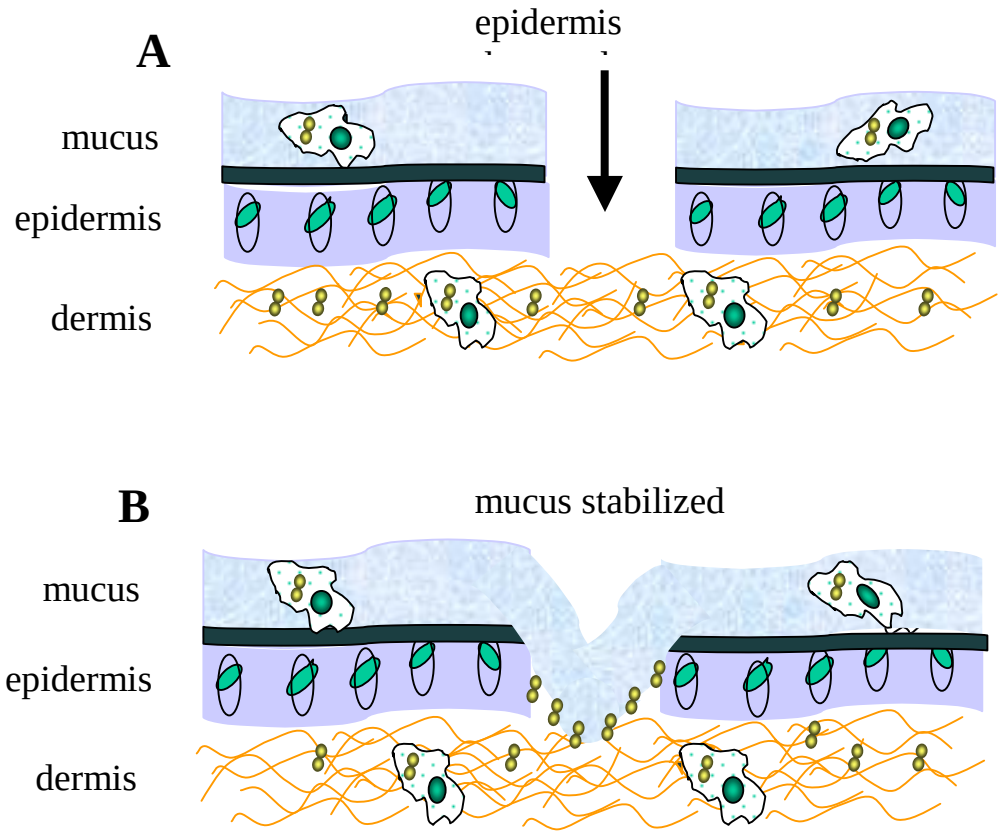


Figure 6.2. Model II- Shallow wound defense: *a*) Damage to the epidermis exposes the dermis, which is rich in MS15. *b*) Mucus flowing over wound is stabilized by crosslinking of mucins of mucus and loose connective tissue of dermis.

Model III- MS15 in deep tissue wound/ infection: MS15 in deep tissues helps in wound defense by agglutinating bacteria, which improves phagocytosis by creating larger particle sizes (Fig 6.3). MS15 may have additional role in healing, influencing fibroblast migration and tissue remodeling.

Experimental evidence found to support this model is: agglutination of bacteria by MS15; MS15 found in dermis, loose connective tissue throughout body; macrophage and fibroblast express MS15 in challenged and unchallenged striped bass; and MS15(+) macrophage collect in wounded/ challenged muscle fibers.

The three models proposed are not mutually exclusive, and could be occurring concurrently at any given time. Function of MS15 localized to other tissues and organs, such as the liver, spleen, and brain, and in non-mammalian cell types, such as rodlet cells, remains to be investigated but no doubt will prove interesting on how a small globular protein can fill so many roles.

Future Directions: The strongest data collected was the presence of a proto type galectin in cells with immune function, such as leukocytes and rodlet cells, and the location of these galectin positive cells in the dermis, gills, alimentary canal, and in wound/ infection sites. These cells and tissues should be further characterized to better understand the role of galectin found there. Though this teleost galectin was characterized in striped bass, further elucidation of function should take place in a teleost that is a well established model organism, one possessing established molecular tools and that is better characterized. The teleost zebrafish, *Danio rerio,* possesses many desirable traits. There exist cell lines and markers, including those for myeloid cells. There are mutants that can be studied, as well as the genome project that has

revealed several putative galectins. Three types of galectins have been identified, isolated, and partially characterized (Ahmed, *et al.*, 2004) in zebrafish, and the role of these proteins in zebrafish embryonic development is already being studied. The possibility of diverse roles of prototype galectins in fish must be considered, due to the early origins of this protein family, the vast number of extant fish species, the genomic duplication(s) that have occurred in fish, and the varied environmental conditions of fish (salt vs. fresh, temperature, pathogens). The teleost *Ictalurus punctatus*, the channel catfish, would serve well to further investigate the role of galectins in leukocyte functions. Possessing cell lines and cell markers, and with putative galectins identified in their genome, catfish may help to identify what type of leukocytes are producing galectin throughout the body as well as in wound sites and sites of infection. These models should be used for studying roles of galectin in immunity, but also in development and wound repair.

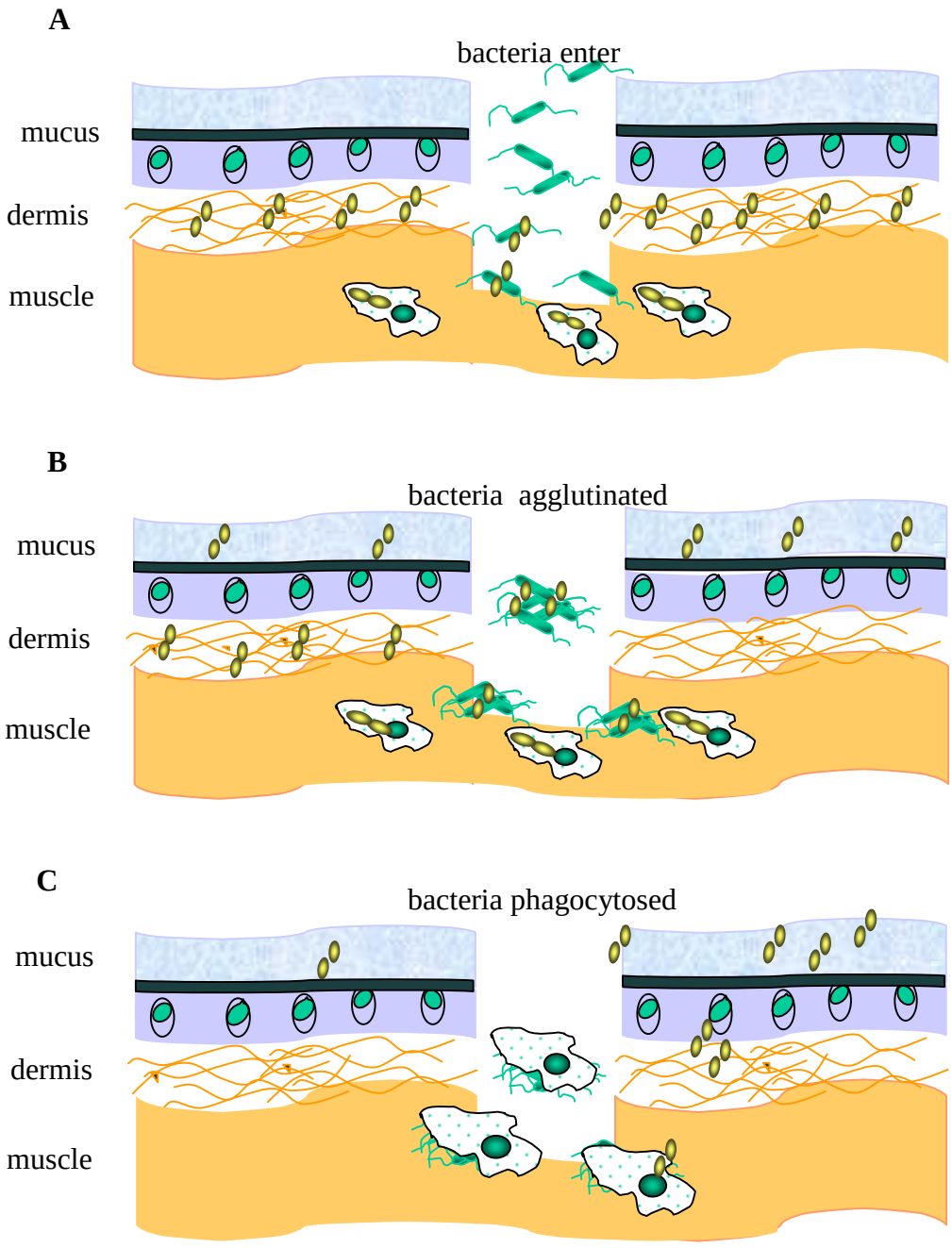


Figure 6.3. Model III-Deep wound defense: *a*) Damage to dermis and epidermis allows bacteria to bypass mucus and dermal layer. *b*) MS15 present in dermis and muscle agglutinates bacteria in deep wound, which *c*) improves phagocytosis by increasing particle size. This may work in conjunction with physiological processes proposed in Model I and Model II.

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