THE ROLE OF MANNOSE SPECIFIC ANTIBODIES IN INNATE IMMUNITY

A Senior Thesis

By

Abhay Srinivasan

1996-97 University Undergraduate Research Fellow

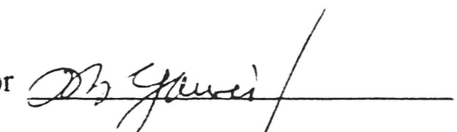
Texas A&M University

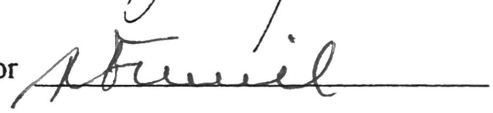
Group: BIOCHEMISTRY/VAPH

The Role of Mannose Specific Antibodies in Innate Immunity

Abhay Srinivasan
University Undergraduate Fellow
Texas A&M University
Department of Veterinary Pathobiology

APPROVED

Fellows Advisor 

Honors Advisor 

THE ROLE OF MANNOSE SPECIFIC ANTIBODIES IN INNATE IMMUNITY.

Abhay Srinivasan, Biochemistry/VAPH, Texas A&M University

The innate immune system is a non-specific, first-line of defense against invaders. It works by mediating complement-dependent cell lysis. Components of innate immunity include macrophages, complement, natural killer cells, carbohydrate-specific antibodies, and mannose binding protein (MBP), Ca^{2+}-dependent lectin. MBP has been shown to be important in preventing bacterial and viral infections. In addition to MPB, however, serum also has mannose-specific antibodies (MSA) whose role in innate immunity has not been characterized. This research sought to evaluate the respective roles of MBP and MSA in innate immunity by comparing relative amounts present in bovine sera. Inhibition assays with different sugar inhibitors showed yeast mannan to have the greatest inhibiting effect, suggesting MSA recognize a highly conformational epitope. Affinity chromatography using a mannan-conjugated matrix was then performed with elutions based on pH, charge, and Ca^{2+} interactions. SDS-PAGE showed a dominant presence of IgG and IgM. A 30 kDa lectin-like band was initially observed but was not subsequently confirmed. Elution with mannan provided the greatest protein yield, consistent with the previous characterization. Western blot analysis confirmed the presence of IgG and IgM. The biological effect was evaluated by agglutination with homogenized yeast particles. A positive result was achieved. Overall results did not show a strong presence of MBP in bovine sera. Mannose-specific antibodies were present in larger amounts, suggesting that they play a significant role in innate immunity.

INTRODUCTION

Within the past decade, researchers have learned much about carbohydrates and their pivotal part in the immune system. They have been found to serve both a structural and biological purpose. In the immune system, different factors interact with each other to generate a desired defensive effect, such as phagocytosis, cell adhesion, tumor metastasis, and migration. It has recently been shown that carbohydrates are essential for mediating these interactions. The research of new and important biological carbohydrate functions beyond traditional structural and dietary research has established the field of glycobiology. Carbohydrates are distributed throughout animal tissues and the surfaces of microorganisms. They can exist in various structures, such as pure carbohydrates, glycolipids, and glycoproteins.

Lectins are defined as the ligands of carbohydrates or carbohydrate-binding proteins excluding enzymes or antibodies. Most lectins are oligomers and are able to agglutinate by binding to their ligands on various cells. Mammalian lectins can be subdivided into C-, S-, P-, and I-types, based on the structure of their carbohydrate recognition domain (CRD). Those of the C-type, or calcium-dependent type (calcium needs to be present for proper function), are intensely studied, and are a very diverse group.

Members of a group of soluble C-type lectins called collectins (Fig. 1) are found in the plasma and other body fluids. They are oligomers of trimeric subunits, and their polypeptide subunit sizes vary from 28 to 47 kDa. The C-terminus contains the CRD, and the N-terminus contains a collagen-like domain, which allows three chains to twist into triple helices. Fully assembled collectins are 600-1000 kDa in size. Subunits of the collectin are bound through disulfide cystine bridges or non-covalent bonding. These calcium-dependent lectins are probably secreted by the intracellular hepatocytes in mammals (Summerfield and Taylor, 1986).

Fig. 1. A collectin, Mannose Binding Protein
From Science, 21 July 1995

The five collectins are conglutinin, pulmonary surfactant proteins SP-A and SP-D, CL-43, and mannose-binding protein (MBP), and each has been found to play an important part in the innate immune system. Innate immunity is non-specific and was found in animals to help ward off infection until more specific immune responses (generated by antigen presenters and T cells) are developed. The innate immune system includes natural killer cells, plasma proteins (inclusive of collectins and carbohydrate-specific antibodies), and complement. This system is the first line of defense to infection or injury. The reaction of collectins to injury is immediate, unlike that of antibodies, which takes at least one day.

MBP reacts by binding to mannose or N-acetylglucoseamine residues on microbial cell walls through the CRD, and then causing complement activation (via the classical pathway) through its N-terminal collagen domain (Tabona 1995). The organization of MBP is much like that of complement protein C1q, a key component of the pathway. C1q binds to IgG and IgM and then, through interaction with the $C1r_2C1s_2$ complex, activates cell lysis (Fig. 2) (Thiel, 1992). The collagen domain of MBP can also bind to C1q receptors found on most leukocytes, platelets, fibroblasts, and endothelial cells. The interaction of these receptors with mannose residues on microbial surfaces facilitates phagocytosis.

Collectins' natural high-affinity ligands are sugars linked together in oligosaccharide chains (these are found on bacterial surfaces). They are able to subtly recognize and differentiate such foreign carbohydrates from those of the host, but are not specific for a certain type of invader. The C-terminal region of the collectin mediates

binding to various microorganisms (such as rough strains of *Escherichia coli*, *Candida albicans*, and *Salmonella montevideo*) to exert the biological effect (Ni and Tizard, 1996) (Tabona, 1995).

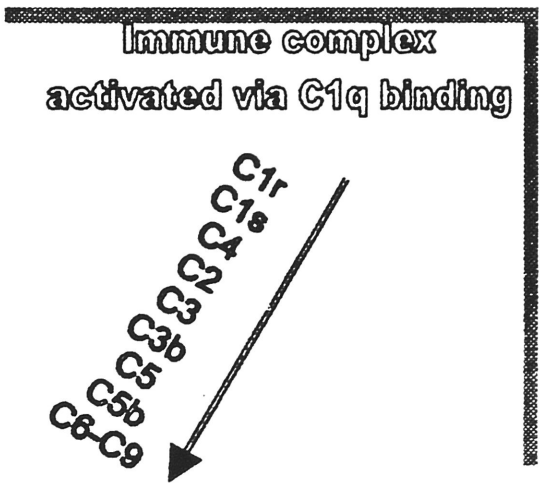


Fig. 2. The complement cascade via the classical pathway.

Immunoglobins (Fig. 3) are responsible for antigen-specific responses in the body. They are bivalent (Y-shaped) at the N-terminus, and are tetrameric protein molecules made of two light chains and two heavy chains. Carbohydrate subsititutions are often found along the heavy chain (Brock et. al. 1994).

Fig. 3. IgG, approx. 146 kDa, a common immunoglobin.
From Zinser Microbiology, Appleton and Lange, 1988

Carbohydrate-specific antibodies (CSA) recognize sugar-protein complex antigens much in the same way as MBP--their Fc region interacts with Fc receptors on marcophages and other cells. But their function is calcium-independent. CSA are also naturally present in humans and animals. Their presence is believed to be due to humoral

immune response to normal environmental antigens such as normal bacterial flora. One example of CSA is the anti-gal antibodies, the most abundant naturally occurring antibodies in humans. Anti-gal antibodies are mostly responsible for rejection of xenotransplanted tissues. Carbohydrate-specific antibodies in mammals can be IgG, IgM (a pentamer immunoglobin), or IgA (Ni and Tizard, 1996).

A defect in the innate immune system such as a loss of MBP could result in an increased susceptibility to infection by reducing complement activity. A defect in the ability to phagocytize yeast, often observed in children with "puzzling" immunodeficiencies, chronic diarrhoea, and otitis media, is often the result of defective MBP. It is currently estimated that up to 25% of cases of such deficiencies are a result of mutations in the MBP gene which occur in amino acid 54 (a glycine to aspartate shift) or 57 (Thiel 1992) (Thompson, 1995). Since CSA have the same effect as collectins, i.e. opsonization of invading microbes, they too are postulated to be an important part of the innate immune system. Increasing serum concentrations of certain monosaccharide-specific anitbodies through immunization with conjugated polysaccharides or monosaccharides may enhance innate immunity (Ni and Tizard 1996). A deficiency in innate immunity may become especially important in individuals where other responses towards polysaccharides are sub-optimal (Thiel 1992).

To date, MBP has been researched extensively. The role of mannose-specific antibodies (MSA) in innate immunity has not been extensively studied. The relative concentrations or significance to innate immunity of MSA versus MBP is not known. This research seeks to determine whether MSA also play important roles as counterparts of MBP. It will involve purification of MBP and MSA present bovine sera. Bovine sera was chosen due to its broader significance; bovines have three lectins--conglutinin, MBP, and CL-43--which may compete with MSA and provide a limiting case. Isolated proteins will be characterized biochemically and biologically, and their relative amounts will be compared.

EXPERIMENTS

ELISA

Indirect enzyme-linked immunosorbent assay (ELISA) was performed in 96-well nunc microtiter plates coated overnight at 4C with yeast mannan in 35 mM $NaHCO_3$, 15 mM Na_2CO_3 buffer, pH 9.6. In general protocol, plates were washed in PBS, and samples were diluted in blocking buffer (3% BSA, 0.05% tween-20 in PBS). Pooled and individual (bovine, chicken, mouse, and human) sera were incubated for 1 hour at room temperature. Sera used in these experiments were either obtained from Texas A&M University College of Veterinary Medicine or ordered from company catalogs. Secondary antibodies were affinity isolated, alkaline phosphatase conjugated, goat anti-bovine IgG whole molecule (K&P); goat anti-human IgG γ chain specific (K&P); goat anti-mouse IgG whole molecule (Sigma); and rabbit anti-chicken IgG whole molecule (Sigma). Anti-IgM was μ chain specific (K&P, Sigma). Incubation was performed for 30 minutes at room temperature. The substrate was P-nitrophenylphosphate disodium salt in 10 mM diethanolamine, 0.5 mM Mg^{2+} buffer (Pierce); reaction time was 15 minutes. All incubation and reaction volumes were 100 μl. Plates were read at 410 nm in a Dynatech MR600 microplate reader.

Conditions for ELISA experiments were initially established. Titration with varying concentrations of antigen--yeast mannan, a highly branched α(1-1) and α(1-2) linked mannose polysaccharide attanched to cell wall amino acid chains of *Saccromyces cervesiae* (Fig. 4)--showed optimum concentration to be 100 μg/mL. In addition, the mouse sera samples showed consistently high MSA concentrations (Fig. 5).

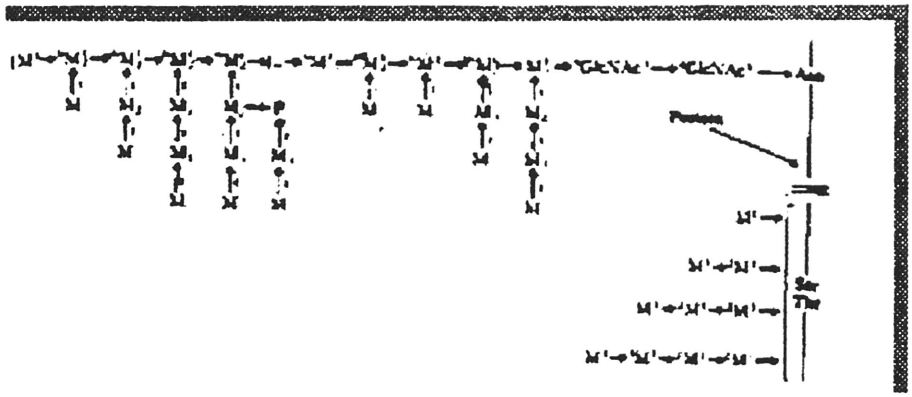


Fig. 4. A typical yeast mannan structure, with branches on a protein chain. "M" denotes mannose residues, and "P" phosphate.

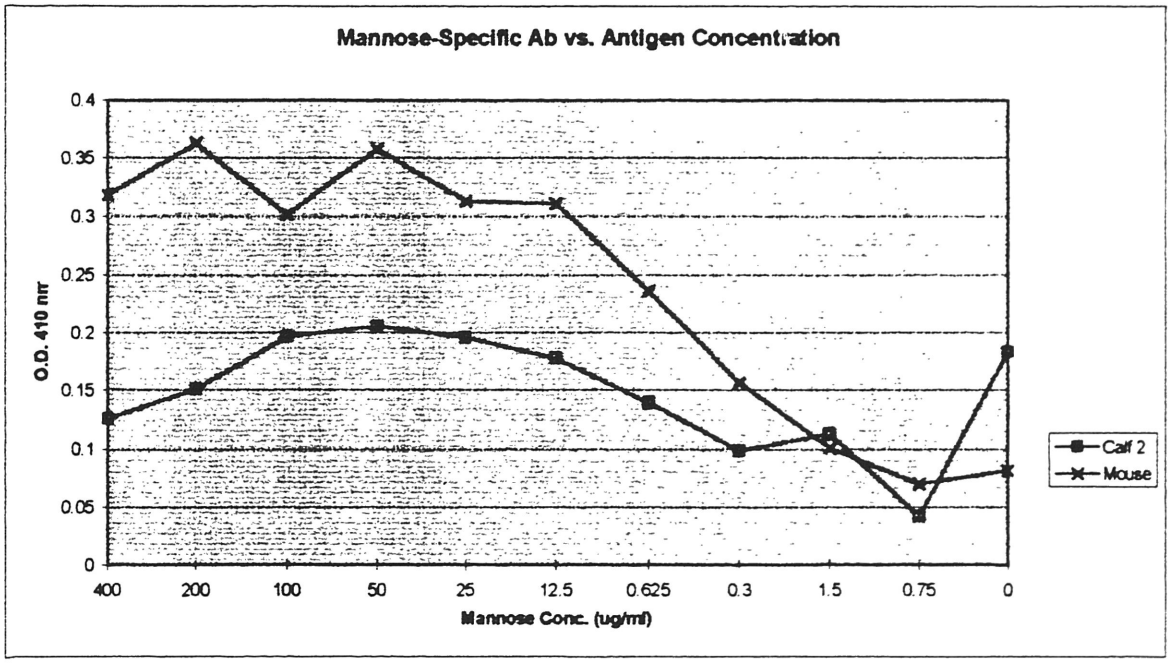


Fig. 5. Standardizing ELISA for antigen concentration. Optimum concentration was chosen at 100 μg/mL.

Standardizing ELISA for secondary antibody concentration with the above samples gave preferable antibody dilutions of 1 to 1000 in dilution buffer for chicken and mouse. Human samples showed a preferable dilution of 1 to 5000. Serum titrations of the above samples showed normal profiles, with mouse and human samples showing highest MSA concentrations. The titration curve for bovine samples is relevant to the majority of the research. The titration end-point was taken at the point of optical density

twice that of background for sample #1. This value, corresponding to an optical density of 0.32, was defined as a "unit" for quantifying MSA (Fig. 6). Serum dilution for subsequent assays was chosen to be 1 to 20.

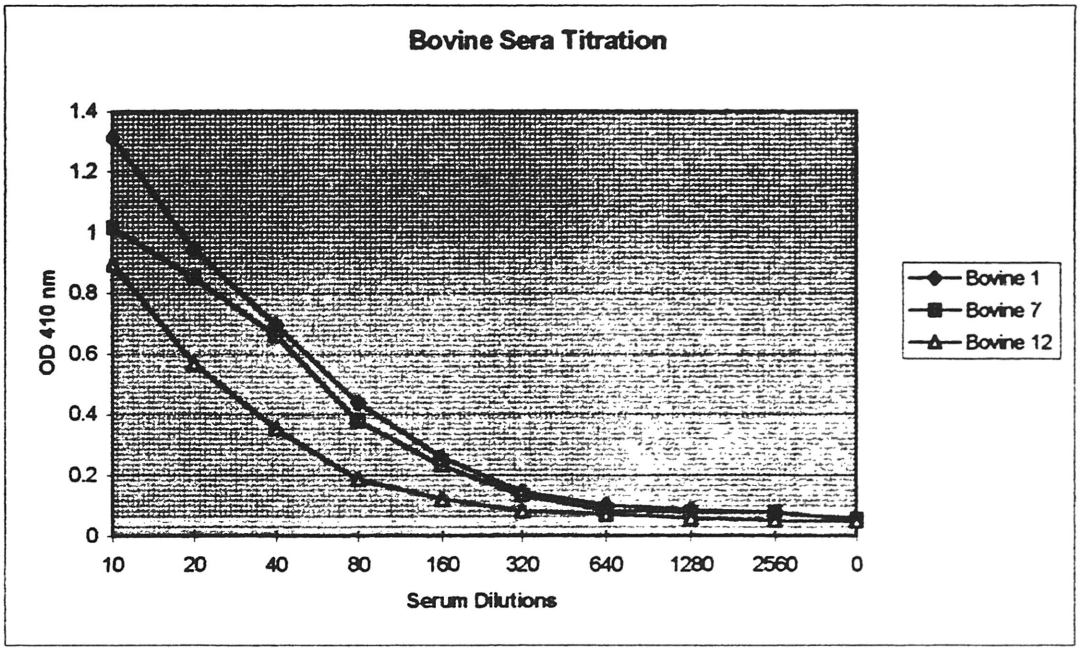


Fig. 6. Titration of bovine sera samples. The first sample was take as standard, with the end-point at the 320 dilution. Samples in ELISA assays were diluted 1 to 20.

Various bovine samples were tested for MSA content using the described established protocol (Fig. 7). Results, after standardization, indicated an age-dependent presence of MSA in bovine sera (Fig. 8).

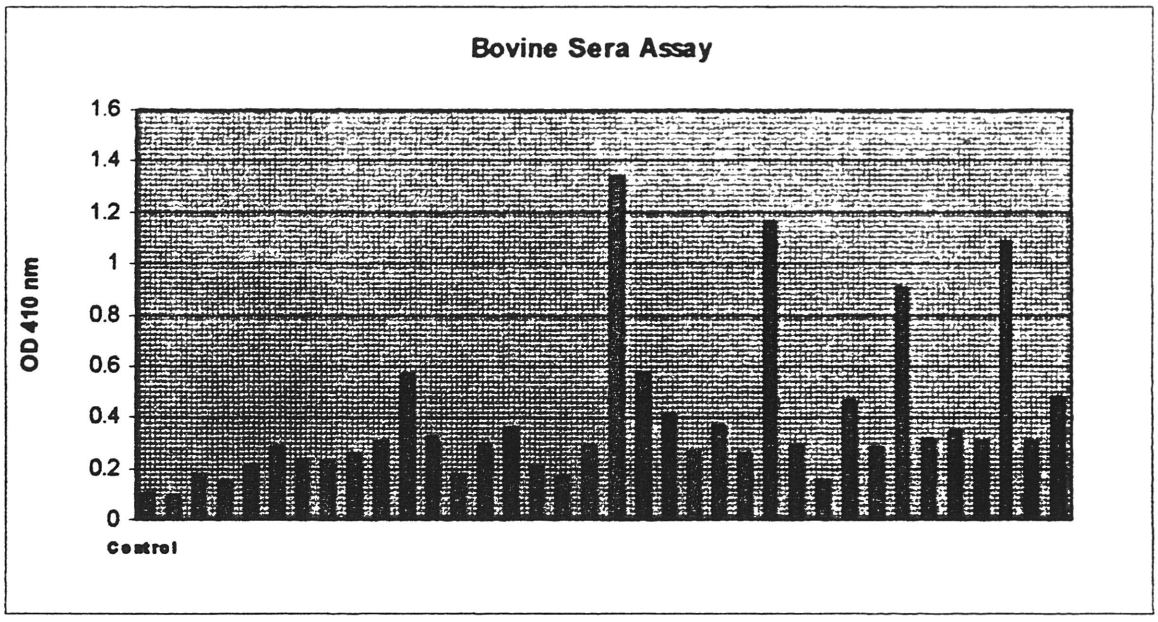


Fig. 7. Levels of MSA in bovine sera samples. Experimental conditions for the ELISA were previously established. The first is fetal bovine serum. Samples 2 through 4 are from newborn calves (pooled, 1-10 days old); sera 5 through 9 are from calves (pooled, 6-12 months old). All subsequent samples are from mature cattle (individual, > 1 year old).

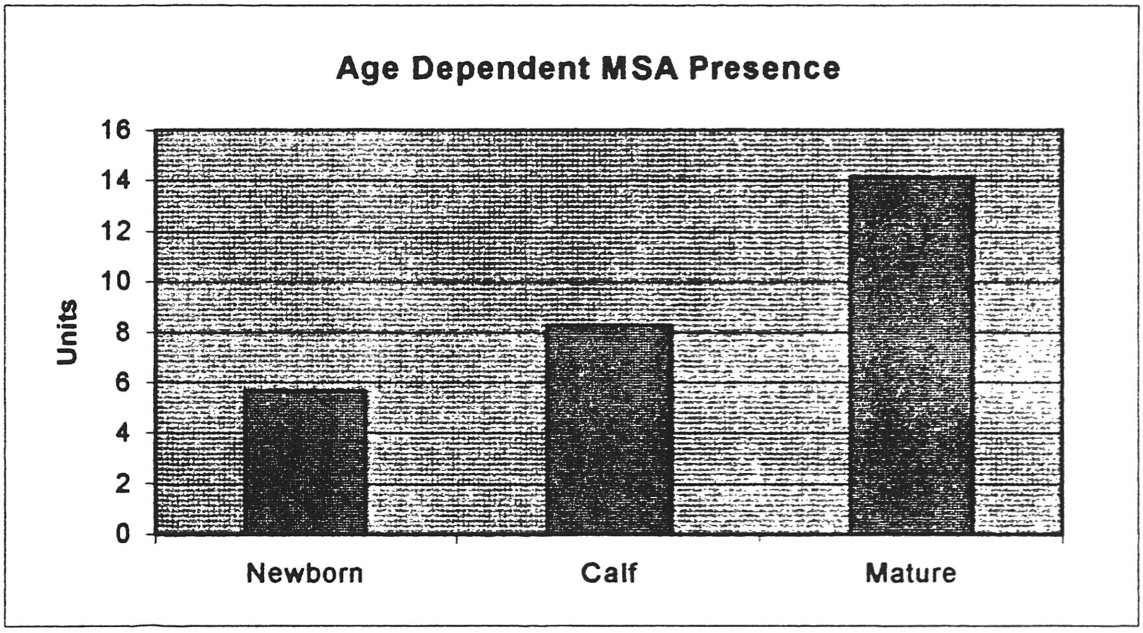


Fig. 8. Age-dependent increase of MSA in bovine sera given in standard units, based on mean values of those reported in figure 7. Results for the fetal bovine serum were negative. Samples 2 through 4 were designated newborn; sera 5 through 9 as calf. All subsequent samples were in the mature category.

Inhibition assays with yeast mannan, D-mannose, man-α(1-3)-man disaccharide, and man-β(1-4)-man disaccharide were performed to test the specificity of MSA previously assayed (Fig. 9). Results indicate that MSA have greatest specificity for yeast mannan, consistent with previous standardization and assay results. Alpha and beta linked mannose disaccharides showed approximately equal extents of inhibition of antigen binding. D-mannose, however, showed poor inhibition even at high concentrations. Results suggest that MSA epitopes are highly conformational. This characteristic is not observed with carbohydrate binding proteins such as MBP.

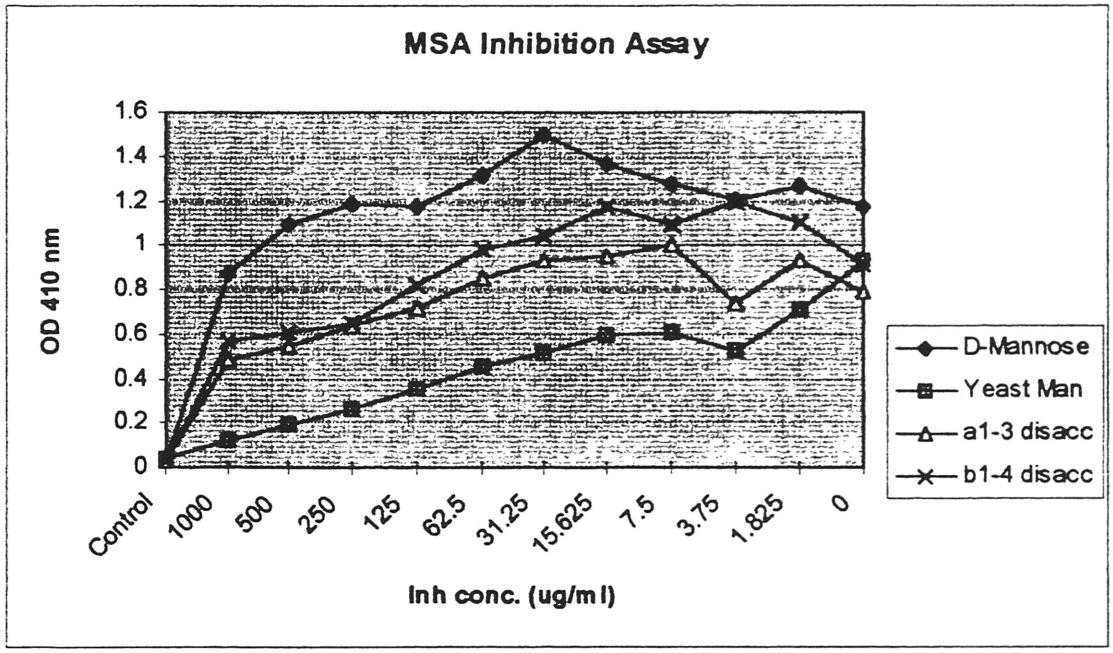


Fig. 9. Inhibition ELISA. Inhibitors were added at various concentrations in volumes of 50 µl concurrently to bovine serum (50 µl). Results show a high specificity of MSA for yeast mannan.

A subsequent ELISA performed to determine relative concentrations of mannose-specific IgG and IgM indicated that IgG had an overwhelmingly dominant presence, by a

factor of more than 10. These results, however, were not consistent with the results of the remaining research, and may be an artifact of inefficient anti-IgM antibody binding.

Isolation and Characterization

Mannose-specific proteins were isolated using affinity chromatography. The matrix was Ultralink™ immobilized mannan on 3M Emphasize™ biosupport (sepharose) medium AB-1 (Sigma). The column, from the ImmunoPure® IgG purification kit (Pierce), was 10 mL. Running speed and elution volume were set to allow efficient binding and elution. A serum (pooled #42N2651, Gibco) showing intermediate MSA levels from the ELISA phase was chosen for study. Also, this sample was chosen because it is used to study anti-gal antibodies in related research. The column equlibriation was performed with 20 mL of a wash buffer (0.2M NaCl, 20 mM tris-HCl, pH 7.4). 20 mL serum samples were diluted in an equal volume of buffer (0.2M NaCl, 5 mM CaCl2, 20 mM tris-HCl, pH 7.4). A peristalitic pump was calibrated to run at 15 mL/hr and was used to load samples. The column was washed with 200 mL of the wash buffer, and elution was performed with 20 mL of the specific solution. Column regeneration was achieved with a 50 mL solution of 2.5 mM NaCl and 10 mM ethylenediaminetetraacetate (EDTA) and a final wash with 100 mL wash buffer. The eluent was dialysed overnight in de-ionized water and was concentrated to a small volume (1-3 mL) with polyvinyl pyrrolidone (Sigma). This was followed by overnight dialysis in PBS and analysis for concentration using BCA protein assay (Pierce).

The initial column run (Sample 1) was eluted with 2.5 mM NaCl and 10 mM EDTA. This salt concentration was high enough to decrease the bio-specific component of the binding without increasing hydrophobic (non-specific) forces between the sample and the matrix. This elution provided a yield of approximately 0.95 mg of protein. In a

second experiment (Sample 2), the column was eluted with a 10 mg/mL solution of yeast mannan. This provided highest yield of roughly 1.25 mg. A key differentiating factor between MBP and MSA is Ca^{2+} dependency; under the premise that EDTA (a divalent cation chelator) should preferentially elute MBP, column elution with 10 mM EDTA was then performed (Sample 3), and a protein assay of this eluent showed a low yield of 0.42 mg. A fourth column (Sample 4) was run and eluted with a 0.5 M glycine, 0.15 M NaCl, pH 2.5 solution; yield was approximately 1.20 mg, indicating that the column matrix had not degenerated. Eluent protein concentrations are reported in Table 1.

Table 1
Protein concentrations from affinity chromatography

Run	Protein conc. (mg/mL)	Yeild (mg)
1	0.53	0.95
2	0.73	1.25
3	0.28	0.42
4	0.61	1.20

1. Values achieved through regression analysis of BCA protein assay titration curves.
2. Samples are denoted by their eluting solutions.

Characterization of the eluents was achieved through denaturing gel elecrophoresis (SDS-PAGE) using a BioRad minigel electrophoresis apparatus was used. Polyacrylamide concentration was 10%, and gels were run at 60 mA and high voltage. Gels were stained with a 0.1% Coomasie Blue, 50% methanol, 10% acetate solution and were destained with a 10% ethanol, 7% acetate solution. The following bands may be observed from affinity elution from a mannan matrix:

32 kDa------ MBP (?)
55 kDa------ IgG heavy chain
28 kDa------ IgG light chain
80 kDa------ IgM heavy chain
28 kDa------ IgM light chain
66 kDa------ Serum albumin

PAGE results are shown in Figure 10. Samples 1, 2, and 4 showed a strong presence of mannose -specific IgM and IgG. These samples were eluted based on ionic strength and pH. IgM concentration in these samples was significant, in contrast to the results of the reported ELISA. Sample 3, eluted on Ca^{2+} interaction only, however, consistently showed bands of low intensity, consistent with the results of the protein assay. In addition, a slight 32 kDa band was observed in Sample 1, indicating the presence of MBP. This, however, was not confirmed in Sample 3, which should have had a higher concentration of Ca^{2+} dependent lectins. Literature on related research cites elution of MBP form affinity columns with similar EDTA concentrations (Andersen, 1992). Sample binding and column elution showed high specificity; no contamination was observed in any samples.

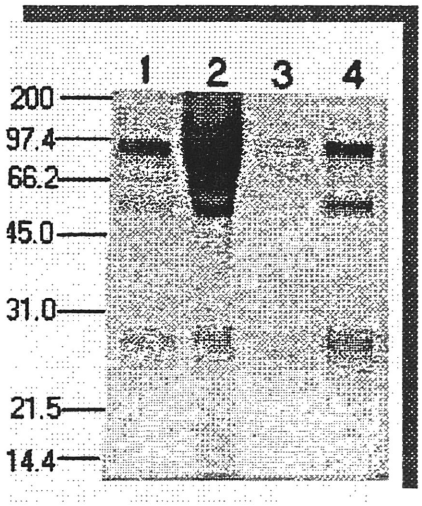


Fig. 10. SDS-PAGE analysis affinity chromatography eluents. A slight 32 kDa band was observed in Sample 1. Protein sizes: MBP,32 kDa; IgG heavy chain, 55 kDa; IgG light chain, 28 kDa; IgM heavy chain, 80 kDa; IgM light chain, 28 kDa; BSA, 66 kDa.

Western blot (immunoblot) analysis using a Biorad minigel apparatus confirmed conclusion observed from the SDS-PAGE. Membrane transfer following SDS-PAGE was performed at 20 mA, 10 V overnight. Non-specific binding sites on the membrane were blocked using a solution of 25 mM Tris, 150 mM NaCl, 0.02% Tween-20, and 3% blotto. Enzyme-conjugated anti bovine IgG and IgM secondary antibodies (described in the ELISA phase) were diluted (25 mM Tris, 150 mM NaCl, 0.02% Tween-20, and 0.05% blotto) and were applied to the membrane. The substrate was a 10 mL 25 mM Tris, 150 mM NaCl, pH 7.5 solution with a 330 µl:33 µl ratio of 25 mg 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in 0.5 mL dimethylformamide; and 10 mg nitro blue tetrazolium (NBT) in 1 mL water (Sigma). The reaction gives an insoluble colored product. Reaction time was 15 minutes. The IgG and IgM bands indicated in Figure 11 were highlighted in the IgG and IgM reactions, respectively.

The biological effect of the Samples was evaluated through agglutination assay. A 10 mg/mL solution of yeast cell wall extract, or Zymosan (Sigma), was homogenized with a Polytron homogenizer and washed repeatedly in PBS to reduce aggregation. Samples were added in a 1:1 volume ratio to petri plates and agglutination was observed under the microscope (Fig. 11). The most positive sample was Sample 4, and this was possibly due to its freshness.

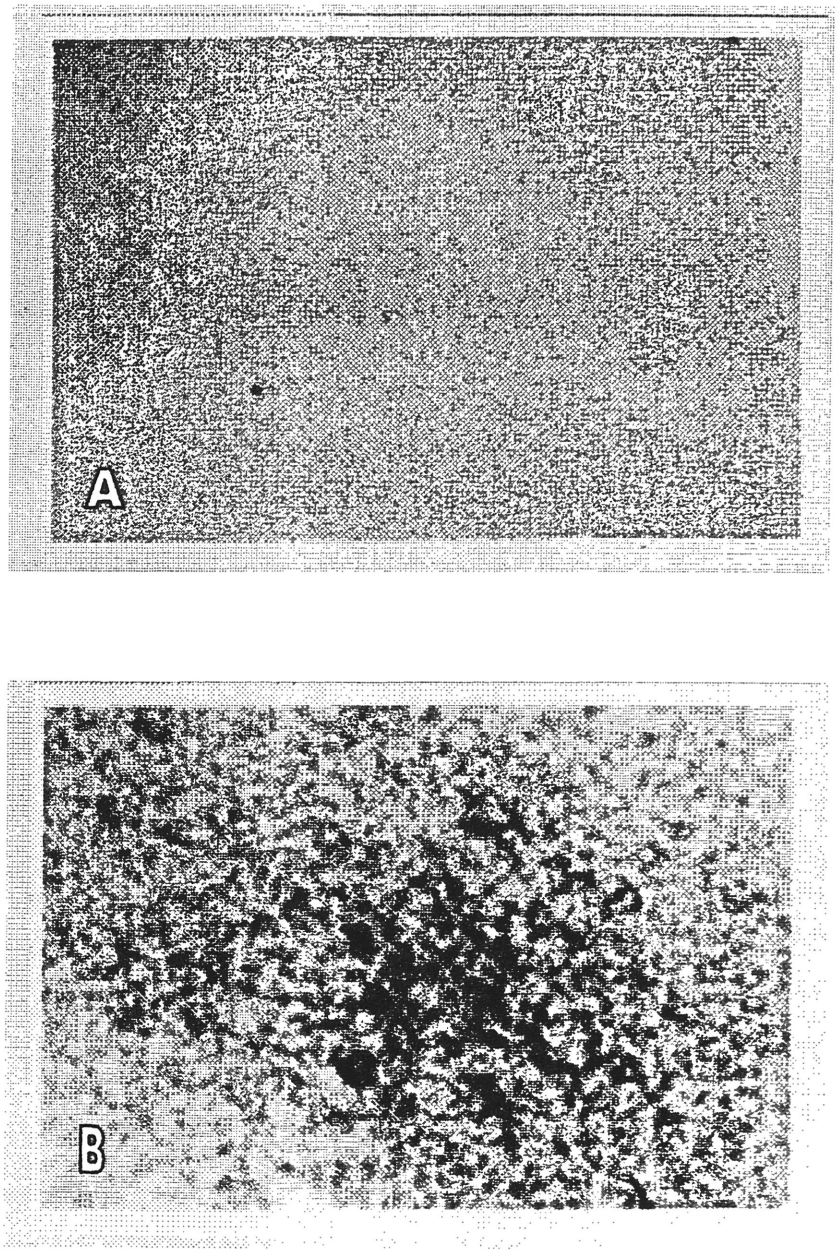


Fig. 11. (A) Negative control for Zymosan agglutination assay. (B) Positive result.

DISCUSSION

The cumulative results of this study show a dominant presence of mannose-specific IgG and IgM in mammalian sera. Bovine serum was shown to have a stronger presence of MSA than MBP. Although possible presence of lectins was observed in one sample (Fig. 10), this was not confirmed by SDS-PAGE of other samples. Serum MSA concentration was age-dependent; the antibodies reported in the newborn bovine serum sample (Fig. 7) possibly originated from the mother's colostrum. Carbohydrate-specific antibodies are produced in response to normal flora, against fungi and bacteria. The mannose-specific antibodies, probably developed upon exposure to non-disease causing agents, later may be used to combat pathogenic microorganisms.

Inhibition ELISA and the agglutination assay indicate that the antibodies were specific to yeast mannan. This suggests that the epitopes of MSA are highly conformational, unlike that of MBP. The dominant class of immunoglobins in MSA was not established. Although ELISA indicated an overwhelming dominance of IgG, SDS-PAGE did not confirm the observation. The high IgG count from ELISA may be a result of anti-IgG, specific to the whole molecule, recognizing IgM as well. Results from ELISA, SDS-PAGE, western blot analysis, and agglutination assays suggest that MSA may play a significant role in innate immunity as a counterpart to mannose binding protein. Extensions of this work may involve further characterization of MSA, in vivo assays of complement activity (C3 hemolysis), and a direct comparison MBP and MSA concentrations after infection.

ACKNOWLEDGEMENT

I thank Dr. Yawei Ni for the hard work and many hours of his time he invested in guiding me through this work. His patience and ability to make things understandable should be acknowledged. None of this would have been possible without him. In addition, I would like to thank Dr. Ian Tizard for graciously providing me the opportunity to participate in this program.

REFERENCES

Andersen, O et al. "Purification, Subunit Characterization, and Ultrastructure of Three Soluble Bovine Lectins: Conglutinin, Mannose-Binding Protein, and the Pentraxin Serum Amyloid P-Component." *Scand. J. Immunol*, vol 36, pp. 131-141, 1992.

Ausubel, Frederick M. et al., eds. Current Protocols in Molecular Biology. Canada: John Wiley and Sons, 1995.

Brock, Thomas et al. The Biology of Microorganisms. Englewood Cliffs, NJ: Prentice Hall, pp. 441-443, 1993.

Kemeny, D. M. A Practical Guide to ELISA. Oxford: Pergamon Press, 1991.

Ni, Yawei and Ian Tizard. Lectin-carbohydrate interaction in the immune system. *Veterinary Immunology and Immunopathology*. vol. 55, pp. 205-223,1996.

Scopes, Robert K. Protein Purification, Principles and Practice. New York: Springer-Verlag, Inc., pp. 127-141, 1988.

Summerfield, John A. and Maureen E. Taylor. Mannose-binding proteins in human serum: identification of mannose-specific immunoglobins and a calcium-dependent lectin, of broader carbohydrate specificity, secreted by hepatocytes. *Biochimica et Biophysica Acta*, vol. 883, pp. 197-206, 1986.

Tabona, P., A. Mellor, and J. A. Summerfield. Mannose-binding protein is involved in the first-line host defence: evidence from transgenic mice. *Immunology*, vol. 85, pp. 153-159, 1995.

Thiel, Stephen. "Mannan Binding Protein: a Complement Activating Animal Lectin." *Immunopharmocology*, vol. 23, pp. 91-99, 1992.

Thompson, Clare. "Protein Proves to Be a Key Link in Innate Immunity." *Science*, vol. 269, 21 July 1995.

Note: the format of this thesis follows that of *Biochimica et Biophysica Acta*