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Identification of Mannose-Binding Protein from Milkfish (*Chanos chanos* F.) Serum

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Abstract: Lectins are integral part of the innate immunity of vertebrates performing roles in cell recognition and the neutralization of pathogens through protein-carbohydrate interactions. Mannose-binding protein, a type of lectin, isolated from the sera of milkfish by affinity chromatography was subjected to molecular weight analysis using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The protein bands showed molecular weights of 55 kDa, 30kDa and 12 kDa under reducing condition. Non-reducing and native PAGE conditions suggest multimeric structure of the 30 kDa MBP. The 55kDa and 12 kDa MBPs are possibly other mannose-binding proteins or co-eluting proteins that require further analyses to understand their characteristics and properties. A 50 kDa MBP showed strong agglutinating activity against *Candida albican*s suggesting the presence of mannan moieties on the surface of the yeast cell.

Keywords: Fish innate immunity, Mannose-binding protein, Affinity chromatography, SDS-PAGE, *Candida albicans*

1. Introduction

Lectins are carbohydrate-binding proteins which are involved in many biological functions including cell adhesion, phagocytosis, complement activation and innate immunity (Vasta et al., 2011). These are found and expressed in different organs, particularly those that are related to immune defense. Lectins, particularly in vertebrates, have been a focus of study in the past as many recognition systems of innate immunity are reliant on these proteins that have a specific affinity to carbohydrate moieties (Guo et al., 1998; Matsushita & Fujita 1992; Summerfield et al., 1997).

Mannose-binding lectin (MBL) is a C-type lectin, as the name suggests, binds to terminal mannose and N-acetylglucosamine moieties present on surfaces of certain pathogens and activates the classical complement

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pathway and maintains the innate immunity of the body via the circulation of serum (Matsushita & Fujita, 1992).

The crystal structure of the carbohydrate-recognition domain of a vertebrate mannose-binding lectin reveals an unusual fold consisting of two distinct regions, one of which contains extensive nonregular secondary structure. The structure explains the conservation of 32 residues in all C-type carbohydrate-recognition domains, suggesting that the fold seen here is common to these domains (Weis et al., 1991).

This study focused on mannose-binding proteins in milkfish *Chanos chanos* F. which are found in both the serum and liver. Mannose-binding lectin plays a vital role in the innate immunity of fishes by activating complementary pathways and works intandem with C1q in order to function as an opsonin. A lack of activity of MBL and opsonin in children usually results to an increase in fungal and bacterial infections (Summerfield et al., 1997).

Microbial recognition is a reported property of several fish lectins. A number of these lectins were identified from rainbow trout serum and was found to have calcium dependent binding to *Aeromonas salmonicida* lipopolysaccharide (Ewart et al., 2001). A galactose-binding lectin was successfully purified and characterized from the eggs of coho salmon which proved capable of bacterial recognition and binding to *Aeromonas salmonicida* bacterial cells (Yousif et al., 1999). The mannose-binding lectin isolated from serum of Atlantic salmon provided the first evidence of lectin-enhanced macrophage activity in fish which shown to bind *Vibrio anguillarum* and *A.salmonicida*.

This study aimed to isolate and characterize an MBP from milkfish serum. Mannose-binding protein was characterized by its molecular weight, its component subunits, the subsequent aggregation of these subunits and the bonds involved in the interactions of the protein. Knowledge of these factors contributes to the understanding of mannose-binding proteins in the serum and provides evidence of the possible contribution of this protein to the innate immunity of *C. chanos*.

2. Materials and Methods

Collection of Samples

Twenty milkfish with weight ranging 50-100g were obtained from the Bureau of Fisheries and Aquatic Resources-National Integrated Fisheries Technology Development Center (BFAR-NIFTDC), in Dagupan City, Pangasinan and from Paombong, Bulacan, and from Bais City, Negros Oriental Philippines. Blood extraction was carried out using hypodermic needles (gauge 23) with sterile vacutainers and by puncturing the caudal vein of the fish. The blood was allowed to clot for 5 minutes and was centrifuged at 3,000 rpm for 3 mins. The clear serum upper layer was pipetted out and was stored in a tank with liquid nitrogen (80^0C).

Affinity chromatography

Five mL mannan-agarose affinity column matrix (M9917, Sigma-Aldrich, St. Louis, MO) was used to

separate the mannose-binding protein by its affinity to the carbohydrate. The extracted serum was diluted with 7.5 mL TCS (10mM $CaCl_2$, 10 mMTris-HCl, 150 mMNaCl, pH 7.4). The columns were equilibrated by letting 200-300 mL of TCS buffer to pass through. The absorbance of the washed and unwashed buffer was determined using Bradford protein assay (Bradford, 1976). Five ml of serum sample was mixed with 15 mL of TCS buffer and 20 μL of phenylmethanesulfonylfluoride (PMSF) (0.05mM). This solution was loaded into the column and incubated for an hour. In order to remove unwanted proteins leaving the protein of interest bound to the beads of the column, 1-2 L of TCS buffer was then made to pass through the column. Bradford protein assay was carried out to make sure that all unwanted proteins have been washed out as indicated when the absorbance of the washed buffer and the unwashed buffer are equal. Eluting was then done by passing 15 mL of 200 mM methyl-α-D-mannopyranoside (M6882, Sigma-Aldrich, St. Louis, MO) through the column while collecting the eluates in 30 fractions of 0.5 mL each.

Ultrafiltration through the use of centrifugal filter unit (UFC801024, Merck, White House Station, NJ) was done to remove excess sugars and other unwanted compounds. Ultrafiltration was done in two steps, pooling and washing. Pooling was carried out by placing 4 mL of sample at a time in the centrifugal unit followed by centrifugation at 4000 rpm until 500 μL of sample remains in the smaller tube. Washing was then done by placing 4 ml of TCS buffer in the filter tubes followed by centrifugation until 500 μL of solution remained in the smaller tube. Washing was done three times using previously chilled rotors and cooled set-up.

Protein Concentration Measurement

The protein concentration of the fractions was determined through the Nanodrop 2000® spectrophotometer (ThermoFisher Scientific, Waltham, MA) by loading 2 μL of each elution fraction onto the loading pedestal of Nanodrop 2000® at a wavelength of 280 nm. TCS buffer was used as a blank.

Determination of Molecular Weight

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in order to characterize the molecular weight of the protein. This was done by following the protocol for a 15% acrylamide gel (Laemmli, 1970) and using a wide range molecular marker (M3788, Sigma-Aldrich, St. Louis, MO, USA) and bovine serum albumin (BSA) (A2153, Sigma-Aldrich, St. Louis, MO, USA).

Reducing SDS-PAGE was carried out by including β-mercaptoethanol (β-ME) in the sample buffer. Non-Reducing SDS-PAGE was performed by omitting the detergent (SDS) from the sample buffer. Two heat treatments were applied to both reducing and non-reducing SDS-PAGE; one was heated to 100 degrees Celsius for 10 minutes, while the second heat treatment had no heat applied. Native PAGE was done by omitting SDS from the gel mixture as well as from the tank buffer. The markers used were α-lactalbumin from bovine milk (~14.2 kDa) (L5385, Sigma-Aldrich, St. Louis, MO, USA); albumin bovine serum (~66 kDa) (A2153, Sigma-Aldrich, St. Louis, MO, USA); albumin from chicken egg white (~45 kDa) (A5253,

Sigma-Aldrich, St. Louis, MO, USA). The gels were stained using silver nitrate staining protocol (Laemmli, 1970). The molecular weight of the protein bands were calculated by forming a regression line by plotting the logarithm of the molecular weight of the wide range marker against its relative mobility (Rf) value.

Microbial Agglutination Assay of CcMBP

The fungal isolate *C. albicans* (catalogue no.: ATCC 10231) was obtained from the culture collection of the University of the Philippines, Manila. Bacterial isolates were grown and maintained in medium containing nutrient agar and incubated at 37 ºC. Maintenance of *C. albicans* was done using Potato Dextrose agar medium incubated at room temperature. *C. albicans* cells (10^6) were suspended in 1 ml TCS buffer was used for agglutination assay. Twenty (20) microliters of the purified mannose-binding lectin was mixed to an equal volume of the bacterial and fungal suspension and was examined for agglutination. The agglutination was checked under a phase contrast microscope. As control, TCS buffer was used in the same manner instead of the lectin solution.

3. Results and Discussion

Affinity Chromatography

Mannose-binding protein purified by affinity chromatography was obtained from the serum extracted from *C. chanos*. The highest protein concentration obtained was at fraction 10 containing 0.196 mg·mL^{-1} of buffer (Figure 1). The total MBP of the 500 μL pooled solution after ultrafiltration was 0.138 mg·mL^{-1} per 15 mL sera from C. *chanos.*

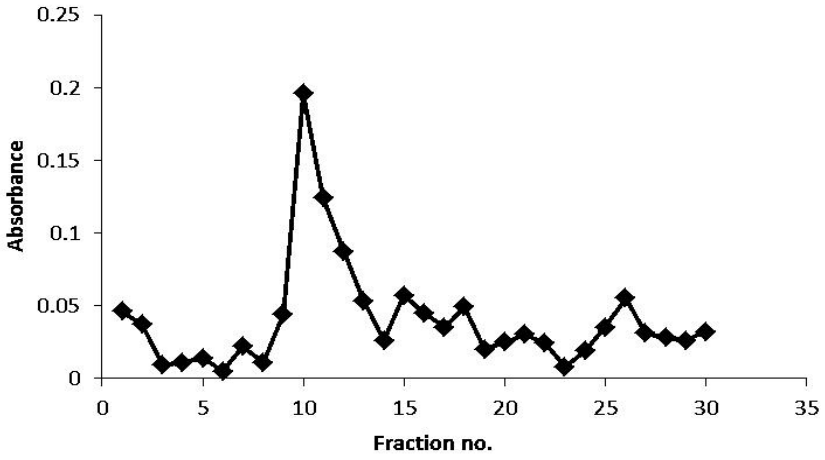

Figure.1. Absorbance of each fraction of MBP purified by affinity chromatography at 280nm obtained by using Nanodrop® spectrophotometer.

Polyacrylamide Gel Electrophoresis

SDS-PAGE was carried out by loading 1.38 μg of protein sample into a 15% acrylamide gel matrix. Standard conditions for SDS-PAGE include the addition of SDS onto the gel mixture and sample buffer, the addition of β-ME onto the sample buffer and heating for 10 minutes at 100^{o}C. Figure 2 shows the result of SDS-PAGE analysis of purified MBP under standard conditions. Major bands observed were characterized to have the following molecular weights, 67 kDa, 55 kDa, 30 kDa and 15 kDa (Figure 2A). SDS-PAGE was then repeated under various conditions of denaturing to characterize the bonds that participate in protein interactions. Heat was removed to determine its effect on the integrity and aggregation of the protein segments. Figure 3 shows the result of reducing SDS-PAGE (15%) analysis with β-ME in the sample buffer, without the application of heat. The molecular weights of major bands observed were determined to be 67 kDa, 55 kDa, 30 kDa and 15 kDa (Figure 2B). Non-reducing SDS-PAGE was carried out by removing β-ME. Non-reducing SDS-PAGE was performed to maintain the disulfide bonds that contribute to protein integrity and aggregation. Figure 4 shows the SDS-PAGE (15%) analysis after heating without the application of β-ME. The molecular weights of the major bands detected were 100 kDa, 58 kDa and 28 kDa. The molecular weights of the minor bands obtained were determined to be 76 kDa and 91 kDa (Figure 3A). Non-reducing SDS-PAGE was repeated without the application of heat to observe the aggregation of proteins in the absence of reducing agent and heat. High molecular weight protein bands were observed. The molecular weights of the 2 major bands obtained were 110 kDa and 55 kDa (Figure 3B) while a minor band at 107 kDa was also detected.

Native PAGE was done by removing sodium dodecyl sulfate from the gel solutions as well as the running tank buffer. This allows the proteins to remain in their native state. Only one major band was observed with a molecular weight equal to 66 kDa. Two minor bands were also observed above 66 kDa and one with a molecular weight of 28 kDa (Figure 4).

The presence of distinct protein bands in the different denaturing conditions was tabulated to illustrate the effect of the denaturants to protein aggregation and structure (Table 1). The 55-58 kDa proteins were present in all reducing and non-reducing conditions applied to the protein. High molecular weight bands (~90-100+ kDa) were only obtained in the absence of β-ME. The protein bands obtained with the application of β- ME were quite similar with or without heat. The 15 kDa, ~28-30 kDa bands appeared consistently in the reducing conditions.

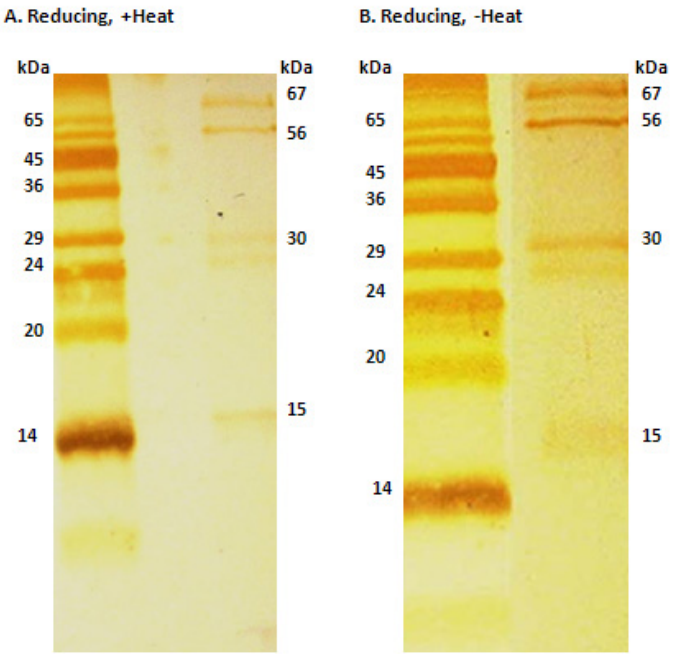

Figure.2. Representative reducing SDS-PAGE (15%) (withβ-ME) analysis of MBP derived from serum extracted from *C. chanos*, with molecular weight marker (MW). A: Reducing with heat, B- Reducing without heat.

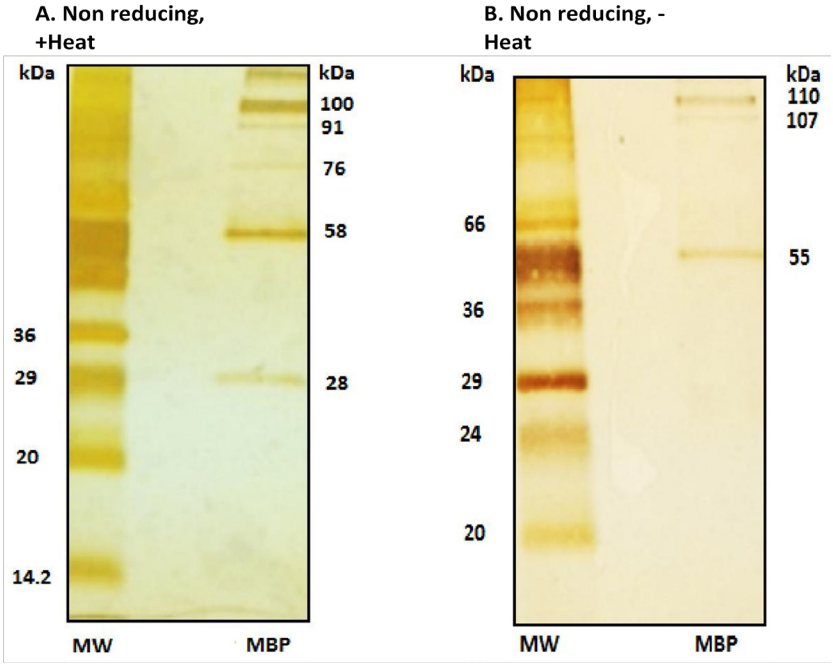

Figure 3. Representative non-reducing SDS-PAGE (15%)(without β-ME) analysis of MBP derived from serum extracted from *C. chanos*, with molecular weight marker (MW). A: Non-reducing with heat, B: Non-reducing without heat.

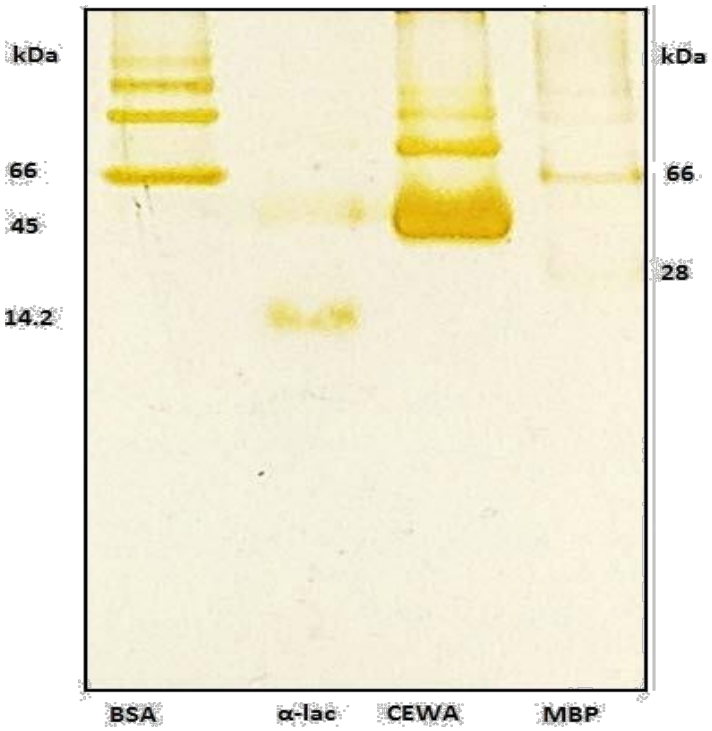

Figure 4. Representative native PAGE analysis of MBP derived from serum extracted from *C. chanos* (without sodium dodecyl sulfate), with bovine serum albumin (BSA) (66 kDa), α-lactalbumin (α-lac) (14.2 kDa) and chicken egg white albumin (CEWA) (45 kDa).

Table 1. Matrix of major protein bands under the different denaturing conditions and subjected to SDS-PAGE using a 15% acrylamide gel.

Molecular weight (kDa)	Reducing with heat	Reducing without heat	Non reducing reducing with heat	Non reducing reducing without heat
100-110			+	+
90-100			+	+
66	+	+		
55	+	+	+	+
30	+	+		
28	+		+	
15	+	+		

Microbial Agglutination Assay of CcMBP

Microbial agglutination assay revealed that with a concentration of 0.06 mg/ml, CcMBP can strongly agglutinate 10^6*C. albicans* cells.

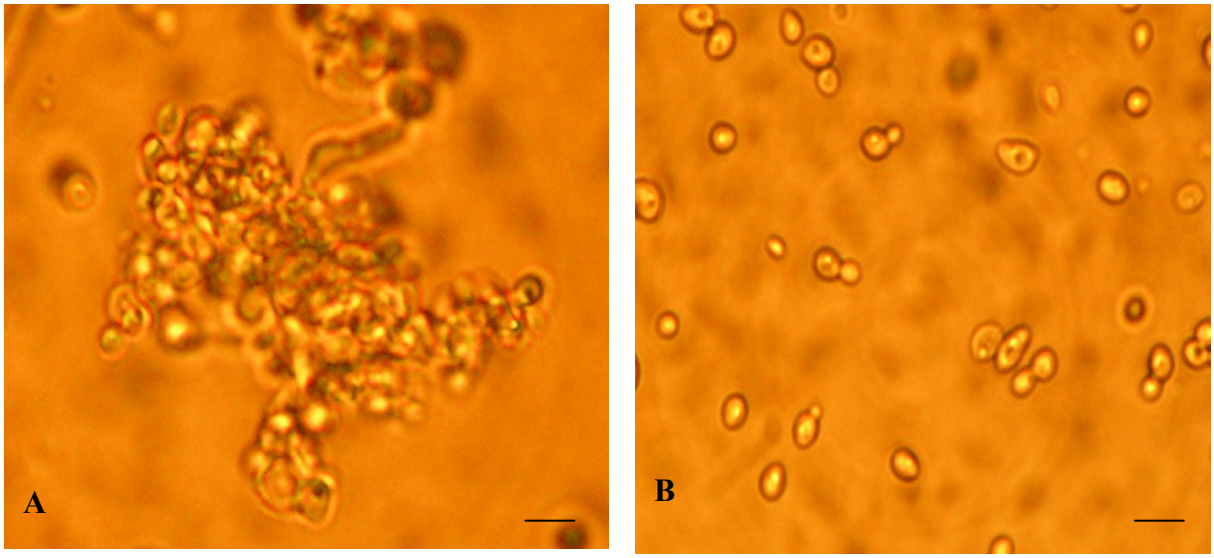

Figure 5. Agglutination of mannose-binding lectin of milkfish *Chanos chanos* against *Candida albicans*. Affinity-purified MBP from the serum of milkfish obtained from Negros Oriental was tested in an agglutination assay against *C. albicans* cells (10^6). A: yeast cells were mixed with CcMBP, B: yeast cells with TCS (B). The agglutination was observed in a slide and viewed at 400X magnification (bar = 10 μM).

In this study, the mannose-binding protein from the crude sera of milkfish (*C. chanos* F.) was isolated and characterized. The use of methyl-α-D-mannopyranoside as the eluant has been extensively studied by Dr. Shibuya and his colleagues (1988). They determined that MBP displayed a high affinity for methyl-α-D-mannopyranoside by performing affinity chromatography to test the affinity of MBP for various mannose-derived compounds (Shibuya et al., 1988). Shibuya and his colleagues observed that the addition of a methyl group at the α carbon of the glycoside inhibits the binding of the lectin to the agarose matrix which may impede the flow rate of the affinity column. Affinity chromatography has been successfully used to isolate MBL from *Petromyzon marinus* (Ourth et al., 2008), *Cyprinus carpio* (Nakao et al., 2006), *Clarias gariepinus* (Argayosa et al., 2011), *Ictalurus furcatus* (Ourth et al., 2007) and *Oncorhynchus mykiss* (Shiina et al., 2002). Affinity chromatography using methyl-α-D-mannopyranoside is an effective method to purify and isolate mannose-binding proteins from teleost sera.

Treatment of the protein sample with β-ME resulted to protein bands with molecular weights that are equal to 15 and 30 kDa while removal of the reducing agent resulted to the disappearance of these bands. Under reducing conditions with the application of β-ME, the disulfide bonds that allowed the formation of

multimers were cleaved. Mannose-binding protein in teleosts is a homopolymer composed of two to eight subunits, bound by disulfide bonds, each composed of a polypeptide chain with a molecular weight of 32 kDa (Lipscombe, 1995). This means that aggregations and denaturation of MBP should result to protein segments with molecular weights of 32 kDa and multiples of 32 kDa (Matsushita & Fujita, 1992; Shiina et al., 2002). Isolated MBL from *C. carpio* (Nakao et al., 2006) displayed the same protein bands when subjected to reducing SDS-PAGE. Native PAGE revealed no protein band at the 30 kDa mark suggesting that the protein formed aggregates in the absence of SDS. Native PAGE shows a major band with molecular weight ~66 kDa, which could be a dimer of the ~30 kDa protein. Non-reducing SDS-PAGE showed no major bands at the ~30 kDa mark. It is assumed that the monomers formed multimers as the disulfide bonds of the protein were retained. Non-reducing SDS-PAGE with the application of heat revealed major protein bands equal to ~58 kDa, which could be a dimer of the 32 kDa MBP, and ~91-100 kDa, which could be trimers of the 30 kDa MBP. Under non-reducing conditions with the application of heat, MBP with a molecular weight of ~28 was observed. MBP isolated from *C. gariepinus* was shown to have a major band close to 28 kDa under non-reducing conditions (Argayosa et al., 2011). When heat is not applied under non-reducing conditions, there was no observable band at the 28 kDa mark. Non-reducing conditions with heat treatment caused denaturation of the protein that led to faster migration of the protein in the gel matrix. Non-disulfide linkages that might be sensitive to heat could also be explored. The absence of heat under non-reducing conditions allowed for the protein to retain its globular form, impeding its progress through the gel matrix. Under non-reducing conditions, without the application of heat, MBP with molecular weights equal to ~110 kDa, which could be high molecular weight forms. Standard SDS-PAGE (15%) (with β-ME and application of heat) showed a major band with molecular weight equal to ~15 and 30 kDa. These same protein bands were observed under reducing conditions without the application of heat.

Another protein of note that appears despite treatment of β-ME and heat are proteins with molecular weights equal to ~55 kDa. Under non-reducing conditions, without the application of heat, a major band equal to ~110 kDa is observed, which could be a possible homodimer of the 55 kDa MBP. Further analysis of the 55 kDa MBP is recommended.

The agglutination activity of CcMBP against *C. albicans* was also observed in this study. Microbial recognition is a characteristic of lectins and a significant mechanism in teleost innate immune system. Innate immune responses are mediated by specific pattern recognition receptors or proteins that recognize microbe-specific pathogen-associated molecular pattern molecules such as LPS of Gram negative bacteria, lipoteichoic acid of gram positive bacteria, mannan and beta glucans of yeast and fungal cell walls (Mirelman et al., 1980). This process also occurs in mammals where secreted pattern recognition proteins such as mannose-binding lectin (MBL) and ficolins bind to microbial cells constituting a group of soluble pattern recognition proteins that can stimulate the lectin pathway of the complement following binding to

several ligands, such as mannan, lipoteichoic acids, lipopolysaccharides on bacteria, fungi and other invading microorganisms (Mirelman et al., 1980). The major carbohydrate components of the cell wall of *C. albicans* are mannans or polymers of mannose covalently associated with proteins to form glycoproteins, also referred to as mannoproteins; b-glucans; that are branched polymers of glucose containing b-1,3 and b-1,6 linkages and chitin, which is an unbranched homopolymer of *N*-acetyl-D-glucosamine (Martinez et al., 1998). It was found that agglutination of intact yeast cells was achieved by activities of mannose-binding lectin isolated in the serum of Atlantic salmon (*S. salar*) (Shiina et al., 2002).

Immunity is an essential physiological mechanism for protection against infection and preservation of internal homeostasis among animals. Generally, the immune response is mediated by innate and acquired systems. Although acquired immunity is found only in vertebrates, innate immunity is present in all multicellular organisms. Non-self-recognizing proteins involved in innate immunity recognize mainly carbohydrate moieties on pathogens such as lipopolysaccharides (LPS) of Gram negative bacteria, lipoteichoic acids of gram positive bacteria, glycolipids of mycobacterium and mannan components of yeasts (Shiina et al., 2002).

Serum mannose-binding lectin is a C-type lectin which recognizes certain carbohydrates such as mannose or *N*-acetylglucosamine on the surfaces of pathogens and plays a crucial role in innate immunity. Mannose binding lectin acts as an opsonin and also activates the complement system (Takahashi et al., 1999).

4. Conclusion

This study showed the isolation of MBP from the serum of *C. chanos*. The molecular weight of 30 kDa as well as its behaviour to denaturing by application of β-mercaptoethanol (Shiina et al., 2002) provides strong evidence for the isolation and characterization of the intramolecular bonds of MBP. A 55 kDa and 15 kDa MBP were also detected. It was also observed that these proteins forms aggregates under non-reducing conditions. Without further analysis however, we can only conclude that they display affinity for the mannose sugar moiety. This study shows preliminary results to the analysis and characterization of the behaviour of MBP in *C. chanos*. Also this study reported the strong agglutination activity of *C. chanos* MBP against yeast cells, *C. albicans*. Opsonization and microbial recognition are some of the properties of lectins in innate immunity. A thorough understanding and investigation of these mechanisms is highly recommended as the potential of using lectin as markers of teleost innate immunity is deemed significant to further improve fish health and subsequently improve the aquaculture industry.

Acknowledgments

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