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Characterization of mannose-binding protein isolated from the African catfish (*Clarias gariepinus* B.) serum

Anacleto M. Argayosa *, Rieziel Ann D. Bernal, Adrian U. Luczon, Jason S. Arboleda

Institute of Biology, College of Science, University of the Philippines, Diliman, Quezon City 1101, Philippines

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ABSTRACT

Lectins are carbohydrate-binding proteins which are involved in many biological functions including cell adhesion, phagocytosis, complement activation and innate immunity. This paper reports the first isolation of mannose-binding protein (MBP), designated as CgMBP or *kumpolin1*, from the serum of the African catfish, *Clarias gariepinus* (Burchell, 1822), a robust freshwater fish farmed extensively in Africa and South East Asia. Mannan-agarose affinity column was used to purify the mannose-binding protein from the serum. Molecular weight determination using reducing SDS-PAGE and non-reducing SDS-PAGE analyses revealed a single band close to 35 kDa and 28 kDa protein, respectively. Positive microbial cell agglutination activities of the African catfish mannose-binding protein were observed against *Candida albicans, Saccharomyces cerevisiae, Aeromonas hydrophila, Bacillus subtilis, Enterococcus faecalis, Escherichia coli,* and *Pseudomonas aeruginosa.* The functional properties of the MBP were tested under various conditions by using *S. cerevisiae* as the test organism. Agglutination activity of MBP was found to occur at pH 3–7. It required 0.5 mM calcium and was inhibited by 1 mM EDTA.

1. Introduction

Fishes are exposed to a complex array of infectious agents like viruses, bacteria, fungi and parasites that inhabit and exploit them as abundant source of nutrients (Janeway et al., 2001; Yano, 1996). Bony fishes protect themselves against pathogens by expressing immune relevant proteins, such as lectins, which can neutralize and help kill pathogens weeks before antibodies are produced (Yano, 1996). Recognition and agglutination of pathogenic microorganisms are some of the vital biological properties of fish serum lectins and are considered to be an innate immune response against infection (Ewart et al., 2001). Lectins bind glycans or carbohydrate moieties on viral and cell surfaces mainly through hydrogen bonds (Yano, 1996) resulting in the restriction of bound and agglutinated pathogens from spreading, multiplying, and infecting other tissues and organs. Lectins can also opsonize or coat viral and cell surfaces for phagocytosis (Matsushita et al., 1996). There is growing evidence in humans and other vertebrates that serum lectin deficiency is strongly correlated to high susceptibility to infection. Hence, mannose-binding lectin has been proposed as a biomarker for disease resistance in vertebrates (Ezekowitz, 2003; Laursen et al., 1998; Ourth et al., 2007; Yano, 1996). Although mannose-binding proteins (MBPs) have been isolated and characterized from other teleosts (Ewart et al., 1999; Fock et al., 2000; Gercken and Renwrantz, 1994; Ourth et al., 2007), isolation of an MBP from the serum of the African catfish or *Clarias gariepinus* has not been reported yet.

This paper aims to isolate and characterize the mannose-binding protein from the African catfish serum. *C. gariepinus,* locally known as "hito", is farmed extensively in the Philippines, and other countries in Africa and South East Asia (Teugels, 1986).

2. Materials and methods

2.1. Extraction of catfish serum

A total of 30 pieces of live African catfish (*C. gariepinus*) weighing at least 1 kg pc^{-1} were acquired from a fish farm in Bustos, Bulacan, Philippines. The fish were anesthetized by immersion in distilled water containing ethyl 3-aminobenzoate methanesulfonate salt (MS-222) (Sigma Chemical Company, St. Louis, MO, U.S.A.) at a concentration of 100 mg l^{-1}. Sterile Vacutainer® blood collection tubes and sterile injection needles were used in puncturing the caudal vein of the catfish to extract the blood (Schẽaperclaus et al., 1992). The blood was allowed to clot for 15 min at room temperature and centrifuged at 1000 rpm for 5 min to separate the serum from the clotted blood components. The sera were collected into 50 ml centrifuge tubes and flash frozen using liquid nitrogen in preparation for storage and transport.

The Bradford was used to determine the protein concentration of the fish serum (Bradford, 1976). Absorbance of the protein samples was determined using a Statfax® 2100 microplate reader.

* Corresponding author. Tel.: +63 2 981 8500 3730; fax: +63 2 920 5471.
E-mail address: litoargayosa@science.upd.edu.ph (A.M. Argayosa).

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2.2. Affinity chromatography

A five ml mannan-agarose affinity column matrix (M9917, Sigma Aldrich Inc. St. Louis, MO, U.S.A.) was used. The affinity matrix is composed of agarose beads that are covalently linked to mannan-containing mannose moieties in a β1→4 linkage (Wang et al., 2006). The column was equilibrated with TCS buffer (10 mM $CaCl_2$, 10 mM Tris–HCl, 150 mM NaCl, pH 7.4) (Ewart et al., 1999) and loaded with 10 ml of catfish serum diluted at 1:4 with TCS buffer with 0.5 mM phenyl methane sulfonyl fluoride (PMSF). The unbound proteins were collected and stored in a freezer. The column was then washed extensively with TCS until no protein was detected using the Bradford protein assay. The bound mannose-binding proteins were eluted with 30–50 ml of 200 mM methyl α-D-mannopyranoside (Sigma Chemical Company, St. Louis, MO, U.S.A.) in TCS buffer. The protein content of each 500 μl fraction was detected using the Bradford. Eluted fractions containing the mannose-binding proteins were pooled and concentrated to a desired volume using Pall® Microsep 10 kDa Omega filter and centrifuged at 4000 rpm at 10 °C for 5 min. The remaining sugar in the retentate was removed by ultrafiltration with the use of mannose-free TCS buffer. The washed MBPs in the final retentate were stored in an ultra-low freezer for characterization.

2.3. Molecular weight analyses

Molecular weight analyses of the mannose-binding protein were done using reducing and non-reducing sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Dunn, 1989; Laemmli, 1970). Reducing SDS-PAGE assays were performed with 10% SDS-PAGE gels. About 210 ng of *C. gariepinus* MBP was loaded in the sample well. The standard molecular weight marker kit (M3788, Sigma Chemical Company, St. Louis, MO, U.S.A.) was used. Non-reducing SDS-PAGE analyses were also performed using 10% SDS-PAGE gels.

Native PAGE analyses were conducted using a 3% stacking and 5% separating gels. The sample buffer (2 ml glycerol, 2 ml Bromphenol blue, 1.25 ml Tris–HCl pH 6.8, diluted to 10 ml with dH_2O) was added to a 0.050 μg protein sample. A molecular weight marker kit MW ND5000 (Sigma Chemical Company, St. Louis, MO, U.S.A.) was used as standard.

Protein bands on SDS-PAGE and native PAGE gels were visualized using a silver staining protocol based on the Amersham Biosciences® PlusOne stain kit (Yan et al., 2000).

2.4. Microbial agglutination assay

Microbial strains (PNMCC, 2006) and isolated *Enterococcus faecalis* TE 107 from a diseased eye of Nile tilapia (Olondriz et al., 2009) were obtained from the Culture Collection of the Microbiological Research and Services Laboratory, Natural Sciences Research Institute, University of the Philippines at Diliman, Quezon City. The microbes were grown and subjected to agglutination activity with the *C. gariepinus* mannose-binding protein. Briefly, *Candida albicans* (UPCC 2168), *Saccharomyces cerevisiae* (UPCC 2115), *Aeromonas hydrophila* (Biotech 10089), *Bacillus subtilis* (UPCC 1295), *Escherichia coli* (UPCC 1195) and *Pseudomonas aeruginosa* (UPCC 1244) cultures were suspended in PBS buffer (pH 7.4) (Harris and Angal, 1989) with 0.5 mM $CaCl_2$ and diluted to obtain a McFarland No. 2 for bacteria and McFarland No. 3 standard for yeast.

Ten (10) μl from each of the microbial suspensions were dropped on the glass slide and mixed with 10 μl of MBP (140 ng μl^{-1}). The treated cells were incubated in a humid chamber for at least 15 min and observed under an inverted phase contrast microscope. Negative controls contained 10 μl of each of the microbial suspension and PBS buffer. All assays were done in triplicate trials. The sera and purified *C. gariepinus* MBP were also tested against *C. albicans* and *A. hydrophila.*

2.4.1. Effect of calcium

To determine the cation requirements of the agglutinating activity of the fish serum lectin, 10 μl of *C. gariepinus* MBP in TCS was incubated with 10 μl of *S. cerevisiae* cell suspension with 20, 10, 0.5, 0.2, 0.02 and 0 mM $CaCl_2$ for 30 min in slide preparations.

2.4.2. Effect of EDTA

For the effect of EDTA, 10 μl of the lectin was incubated with 10 μl yeast cell suspensions having 100, 10, 1, 0.5 and 0 mM EDTA for 30 min using slide preparations.

2.4.3. Effect of pH

To examine the effect of pH, slide preparations of 10 μl of lectin incubated with 10 μl yeast cell suspensions in pH adjusted PBS buffer were made. The PBS buffer used in making yeast cell suspensions was adjusted to pH 3, 5, 7, 8 and 10.

3. Results

3.1. Affinity chromatography

The elution profiles corresponding to the three (3) batches of isolated mannose-binding protein from the serum of the African catfish, *C. gariepinus*, using mannan-agarose affinity chromatography are shown in Fig. 1. The affinity-purified *C. gariepinus* mannose-binding protein was designated as CgMBP or *kumpolin1*. Peaks of absorbance corresponding to the eluted mannose-binding proteins were observed at fractions 3–5 in the elution profiles observed. The absorbance was based on the Bradford assay of each of the fractions eluted with mannose-containing buffer.

3.2. Molecular weight analyses

Electrophoretic analyses of the molecular weight of the purified mannose-binding protein were conducted. Reducing SDS-PAGE analysis of the purified *C. gariepinus* MBP from mannan-agarose affinity column showed a single band of 35 kDa (Fig. 2). No co-eluting proteins that may correspond to the 75–80 kDa fish immunoglobulin heavy (IgM) chains were detected. The non-reducing SDS-PAGE of

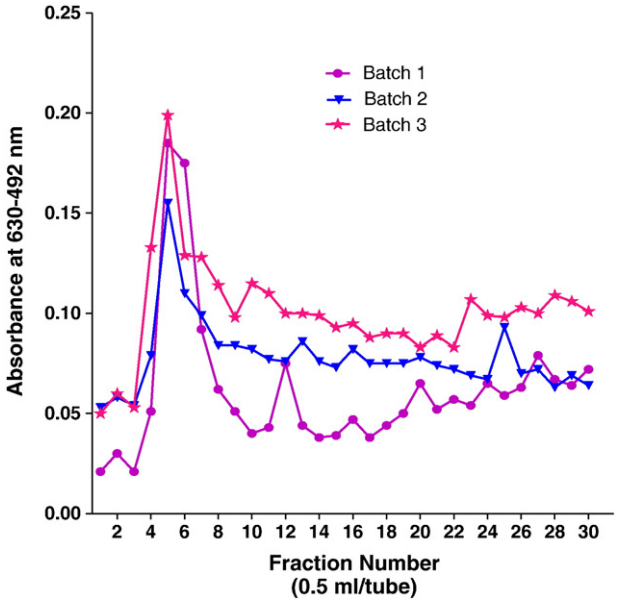

Fig. 1. Elution profiles of mannose-binding protein (CgMBP) from *Clarias gariepinus* B. serum using mannan-agarose affinity column. The CgMBP was eluted with 200 mM methyl α-D-mannopyranoside in TCS buffer at 0.5 ml/fraction. Fractions in the protein peak were subsequently concentrated and washed using 10 kDa cut-off filter tubes.