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Identification and characterization of a B-type mannose-binding lectin from Nile tilapia (*Oreochromis niloticus*) in response to bacterial infection

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ABSTRACT

Lectins are a group of carbohydrate-binding proteins, which play an important role in innate immune system against pathogen infection. In this study, a B-type mannose-binding lectin (OnBML) was identified from Nile tilapia (*Oreochromis niloticus*), and characterized at expression patterns against bacterial infection and capability to promote phagocytosis by macrophages. The open reading frame of *OnBML* is 354 bp of nucleotide sequence encoding polypeptides of 117 amino acids. The deduced protein is highly homologous to other teleost BMLs, containing two repeats of the conserved mannose-binding motif QXDXNXVXY. Expression of *OnBML* was widely exhibited in all examined tissues, with the most abundance in spleen and following gill, peripheral blood, and head kidney. The *OnBML* expressions were significantly up-regulated following two major bacterial infections including a Gram-positive bacterium (*Streptococcus agalactiae*) and a Gram-negative bacterium (*Aeromonas hydrophila*) *in vivo* and *in vitro*. Recombinant OnBML protein possessed capacities of mannose-binding and calcium-dependent agglutination to *S. agalactiae* and *A. hydrophila*, and promoted the phagocytosis by macrophages. Taken together, the present study indicated that OnBML is likely to get involved in host defense against bacterial infection in Nile tilapia.

1. Introduction

Lectins are a group of proteins, rather than enzymes and antibodies, which can selectively recognize and combine a carbohydrate or a group of carbohydrates, resulting in a non-covalent interaction [1,2]. The lectin-carbohydrate interaction has been recognized as one of key components of innate immunity in animals [3]. Lectins can initiate the innate immune response by identifying pathogenic microorganisms [4,5]. Besides the recognition of the potential pathogens, lectins also participate in downstream effector functions, such as agglutination, immobilization, opsonic phagocytosis, complement activation and enhancement of natural killer cell activity [3,6–8]. In addition, lectins are widely distributed inside the body such as in plasma and cell cytoplasm or at cell surface, and present outside the body such as skin mucus [2].

Animal lectins are generally characterized by the presence of one or several carbohydrate recognition domain (CRD) [3,9]. On the basis of their CRD structures, motif types, sugar specificities, requirement of divalent cations and functions, animal lectins are broadly classified into several main families such as B-, C-, F-, P-, I-, M − , R-, S-type lectin, galectin, calnexin, pentraxins, and C-reactive protein [3,9,10]. The lectins of various types have been identified in many species and reported to play an important role in immune defense [3,11–13]. Among them, the Bulb-type (B-type) lectins are characterized by a two or three-fold internal repeat (beta-prism architecture) and the consensus mannose-binding motif QXDXNXVXY, which is associated with ligand interaction [9,10]. The B-type lectins are important components of innate immunity, which are able to identify and bind carbohydrate antigens of microorganisms and inhibit pathogen infection by direct neutralization, agglutination and opsonization [3,9,10].

The B-type lectins are predominantly reported in monocotyledonous plants, which selectively recognize mannose residues [10]. In teleost, B-type lectin genes were successfully cloned from pufferfish (*Takifugu rubripes* and *Takifugu niphobles*) [14,15], striped murrel (*Channa striatus*) [16], turbot (*Scophthalmus maximus*) [17] and tongue sole (*Cynoglossus semilaevis*) [9], and which protein was successful purified from pufferfish [14]. In these studies, the tissue distribution of the B-type lectins was determined, and their capacities combining with bacteria and function in the antibacterial immunity were examined. However, until now, the opsonization function and expression patterns of B-type lectin upon bacterial challenges *in vitro* at the cell level have

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not yet been reported in teleost.

Here we present the primary structure and function of a member of the B-type lectins from Nile tilapia, referred to as *OnBML*. The *OnBML* gene was successfully cloned and identified from Nile tilapia. The mRNA expression level of *OnBML* was investigated in various tissues of healthy fish and upon bacterial (*S. agalactiae* and *A. hydrophila*) infection. In addition, temporal patterns of *OnBML* expression *in vitro* after bacterial challenges were demonstrated. Moreover, the functional characterization of the (r)OnBML was performed using bacterial binding and phagocytosis assays. These findings indicated that OnBML, possessing agglutination and opsonization ability to bacterial pathogens, is likely to be involved in host defense against bacterial infection in Nile tilapia.

2. Materials and methods

2.1. Fish rearing and tissue sampling

Nile tilapia (*O. niloticus*), used for the cDNA clone, tissue expression analysis of *OnBML* and isolation of macrophages, were acquired by Guangdong Tilapia Breeding Farm (Guangdong, China), and kept a weight at 80 ± 10 g. Fishes were acclimated in the automatic filtering aquaculture system with a stocking rate of 10 g/L under 28 ± 2 °C for three weeks [18–20]. All animal protocols were reviewed and approved by the University Animal Care and Use Committee of the South China Normal University.

2.2. Immune challenges and tissue sampling

In the study of *OnBML* expression in healthy tilapia, the tissue samples including head kidney, thymus, spleen, liver, intestines, gill, skin, muscle, hind kidney and peripheral blood were collected, then immediately frozen by liquid nitrogen and storage at −80 °C for further use.

The samples in the challenge groups were injected with 100 μL (1×10^6 CFU/fish) live bacteria liquid (*S. agalactiae* (ZQ1901) or *A. hydrophila* (BYK00810)) in PBS (10 mM phosphate, 150 mM NaCl, pH 7.4) with a final concentration of 1×10^7 CFU/mL) [21,22], which were used in our previous studies [19,20]. The control group was injected with 100 μL of PBS. Tissue samples were harvested at the time of 0 h, 3 h, 6 h, 12 h, 24 h, 2 d, 3 d, 5 d and 7 d post-infection, then frozen by liquid nitrogen and transferred to −80 °C for further use.

2.3. RNA extraction and cDNA synthesis

Total RNA from the spleen was extracted by using Trizol Reagent (Invitrogen, USA) according to the protocol, and the template was synthesized with PrimerScript™ RT reagent kit with gDNA Eraser (TaKaRa, Japan) to amplify the open reading frame (ORF) of *OnBML*. The total RNA of the collected samples and template synthesized were also extracted as before, then diluted 10-fold and store at −80 °C for quantitative real time PCR (qRT-PCR). The gene of *OnBML* with complete ORF was cloned based on the predicted sequence of *Oreochromis niloticus* bulb-type mannose-binding lectin mRNA (GenBank accession XM_013276458.2). Each PCR amplification described below was conducted in a total volume of 20 μL containing 10 μL 2 × TaKaRa Ex Taq (TaKaRa, Japan), 8 μL dd H_2O, 1 μL cDNA template (100 ng) and 0.5 μL (100 nM) each of forward (BML-F) and reverse primer (BML-R). The reaction conditions were 3 min at 94 °C followed by 35 cycles of 94 °C for 30 s, 52 °C for 30 s, and 72 °C for 60 s, the last step was 72 °C for 10 min. All of the primers used in the present study were designed by Primer Premier 5.0 and summarized in Table 1. The PCR products were test by a 1% agarose gel electrophoresis (BIOWEST, Spain).

Table 1
Primers used in the present study.

Primers	Nucleotide Sequence (5′-3′)	Purpose
BML-F	ATGAGCAGAAACTTCTTGTCCAAAA	Full cDNA
BML-R	TCACTTCATGCCTTTGGACTTTTCA	Full cDNA
EBML-F	CGCGGATCCATATTTCAGGGTGATGGCAACT	Protein expression
EBML-R	CGCAAGCTTTCACTTCATGCCTTTGGACTTTTCA	Protein expression
qBML-F	TGTCATCTATGGCTGGAA	RT-qPCR
qBML-R	TTGACTGACTACTGCCGT	RT-qPCR
β-actin-F	CGAGAGGGAAATCGTGCGTGACA	RT-qPCR
β-actin-R	AGGAAGGAAGGCTGGAAGAGGGC	RT-qPCR
M13-F	TGTAAAACGACGGCCAGT	Sequencing
M13-R	CAGGAAACAGCTATGACC	Sequencing

2.4. Identification and molecular characterization of OnBML

The potential open reading frame (ORF) was analyzed with the Finder program (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The protein analysis was conducted with ExPASy tools (http://expasy.org/tools/). Multiple alignment of BML amino acid sequences was performed with the Clustalw2 program (http://www.ebi.ac.uk/Tools/clstalw2/) and the DNAMAN software. The similarity analyses of the determined nucleotide sequences and deduced amino acid sequences were performed by BLAST programs (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Location of domains was predicted using the InterProScan program (http://www.ebi.ac.uk/Tools/pfa/iprscan).

2.5. Quantitative real-time PCR

The relative expression of *OnBML* mRNA in healthy and stimulated fish tissues was performed on the 7500 Real Time PCR System (Life Technologies, USA). The PCR reaction volume was containing 10 μL 2 × TaKaRa Ex Taq™SYBR premix, 4 μL of diluted cDNA, 2 μL of each primer (2 μM), 1.5 μL DEPC treated water (Invitrogen, USA) and 0.5 μL Rox Reference Dye II (TaKaRa, Japan). The PCR program was initial denaturation 95 °C for 3 min, then repeat 95 °C for 15 s and 60 °C for 1 min for 40 cycles. The relative expression of *OnBML* was calculated using tilapia β-actin as a reference, and the results were compared to respective control group to determine the expression change. The *OnBML* relative expression levels were calculated by means of the $2^{-\Delta\Delta Ct}$ method [23] and the quantitative expression data were presented as the means ± standard deviation (SD).

2.6. Isolation and culture of monocytes/macrophages in vitro

The isolation of Nile tilapia head kidney monocytes/macrophages was performed according to the previous methods [18,24]. Briefly, head kidney leukocytes were separated from the cell suspension by density gradient centrifugation. A total volume of 15 mL Histopaque® 1077 (Sigma, USA) was assimilate to a 50 mL centrifuge tube, and then put it on ice. The leukocyte suspension was diluted to an equal volume and overlaid to the surface of Histopaque® 1077 gently, then centrifuged at 500 × *g* for 40 min at 4 °C [25]. The interface leukocytes were collected and washed by L-15 medium (Gibco, USA) for three times, and assessed the quantity and viability of the cell by 0.4% trypan blue. The cells were re-suspended in L-15 medium supplemented with 5% fetal bovine serum (Gibco, USA), 1% penicillin/streptomycin (Hyclone, USA), then regulated the cell concentration to 1×10^7 cells/mL and incubated at 25 °C for 2 h in 96-well microplates (Corning, USA) (100 μL/well). The non-adherent cells were removed and use trypsin (Gibco, USA) to digest the adherent cells [26]. The concentration of monocytes/macrophages was adjusted to 1×10^6 cells/mL with fresh L-15 medium including 10% fetal bovine serum and 1% penicillin/streptomycin. The treatment group was challenged with formalin-inactivated *S. agalactiae* (1×10^7 CFU/mL) and *A. hydrophila*

(1×10^7 CFU/mL), and another group with an equal volume PBS was as control. The monocytes/macrophages total number was 3×10^8 in a volume of 30 mL and the use of each bacteria was 1×10^8 CFU, the concentration of the original bacteria cell was 1×10^9 CFU/mL, use a total volume of 100 μL. All the groups were maintained at 25 °C, and cells were lysed with Trizol Reagent for RNA extraction at the time of 0 h, 3 h, 6 h, 12 h, 24 h, 2 d, 3 d, 5 d and 7 d post-challenges.

2.7. Plasmid construction, expression and D-mannose binding assay of OnBML

In order to express the recombinant OnBML, the open reading frame was amplified by using the cloning primers BML-F and BML-R are sequenced to confirm that no PCR error was introduced. The expressing primers were designed in front of the first QXDXNXVXY domain (EBML-F) and the terminal of amino acid sequence (EBML-R). The *Eco*R I and *Hin*d III sites were introduced at the 5′ and 3' ends of each primer. The PCR products were purified and inserted into the pMD-18T vector, positive recombinant vector constructs were identified by colony PCR and subsequently confirmed by sequencing (BGI, China). The recombinant pMD-18T plasmid and pET-32a (+) empty vector plasmid were digested with *Eco*R I and *Hin*d III at 37 °C overnight. After agarose gel electrophoresis, the digested DNA sequence was extracted from the gel using a TIAN GEN Gel Extraction kit (Tiangen, China). T4 DNA ligase (Invitrogen, America) was then used to insert the fragment into the digested pET-32a (+) vector and response overnight at 4 °C. The plasmid DNA was sequenced to confirm that the deduced primary structure was composed of OnBML and pET-32a (+) with 6 × His tag at the C-terminus. Finally, this positive recombinant vector and pET-32a (+) empty vector plasmid were transferred into *E. coli* BL21 (DE3) Competent Cells (Tiangen, China), respectively. Then cultured in LB-ampicillin at 37 °C in the shaker with a speed of 200 rpm [27]. When the culture optical density reached an O.D. 600 of 0.6, isopropyl-β-D-thiogalactopyranpside (IPTG) induction was added in a final concentration of 1 mM and induced at 37 °C for 3–5 h, then centrifuged at 6000 rpm for 30 min at 4 °C. Cells were re-suspended in PBS and purified with the His Band Resin columns (Novagen, Germany) according to the protocol. The eluted fractions containing (r)OnBML were dialyzed against PBS, and the protein concentration was measured by NanoDrop 2000 spectrophotometer (Thermo, USA). The purified recombinant OnBML protein and Trx-pET-32a protein (expressed by only the pET-32a (+) vector) were separated on a 12% SDS-PAGE gel electrophoresis.

The (r)OnBML supernatant eluted from the His Band Resin columns was collected (by solution containing EDTA or different concentration of imidazole (100 and 250 mM) with a relative high content of OnBML) and dialyzed with 1 L TBS at 4 °C for 2 h every time. Then this supernatant was incubated with approximately 1 ml of mannose agarose (Sigma Aldrich, America) at 4 °C overnight with gentle rocking, after which the beads were transferred into an empty column. The unbound proteins were washed with 20 mL TBS and the beads were incubated with TBS containing 200 mM D-mannose at 4 °C overnight [15]. The eluted fraction was concentrated using a PEG 20000 and subjected to SDS-PAGE and Western blotting as described above.

2.8. ELISA

The *S. agalactiae* or *A. hydrophila* were diluted with coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) and coated in 96-well plates (Costar, 3590, USA) (100 μl/well, 1×10^7 CFU/mL). Then the plates were blocked in 200 μL/well TBS/Ca^{2+}/Em with 0.5% BSA for 1 h. The (r)OnBML and purified Trx-pET-32a protein were incubated in a three times dilution in the following well to each other, the blank well was as control. The primary antibody was anti-6 × His tag mouse monoclonal antibody (BBI, USA), and the biotinylated antibody was used in combination with streptavidin-HRP (Southern Biotech, USA). Between each step, the plates were washed with TBS/Ca^{2+}/Em for three times, unless otherwise stated, all the steps were made in 37 °C. Plates were developed using 0.5 mg/mL o-phenylenediamine (Sigma, America) in substrate buffer with 0.03% H_2O_2, according to the manufacturer's protocol. Development was stopped with 1 M H_2SO_4 and plates were read by a Microplate Reader (Thermo, USA) at O.D. 450 nm as subtractive reference [28].

2.9. Agglutination assay

Agglutination assay was performed according to the method of Zhang et al. [29]. The bacteria including *S. agalactiae* and *A. hydrophila* were labeled with fluorescein isothiocyanate (FITC) (Sigma, America) according to the previous reports [19,30]. Ten microliters of the labeled bacteria was mixed with 25 μL of purified (r)OnBML (80 μg/mL), purified Trx-pET-32a protein (80 μg/mL), heat-denatured (by boiling at 100 °C for 20 min) (r)OnBML (80 μg/mL), and TBS buffer, respectively, and incubated at 25 °C for 1 h. In order to examine the effect of ethylene diamine tetraacetic acid (EDTA) in Ca^{2+} on agglutination, the FITC-labeled bacteria were incubated with the (r)OnBML as described above in TBS-Ca^{2+} buffer containing 4 mM EDTA. To determine OnBML was a mannose-specific lectin, bacteria were incubated with the (r)OnBML as described above in TBS-Ca^{2+} buffer containing 200 mM D-mannose (Sigma, America). The Agglutination was observed with a fluorescence microscope (Leica, Germany).

2.10. Assay for effect of (r)OnBML on phagocytosis

To investigate the effect of (r)OnBML on phagocytosis, flow cytometric analysis was performed according to the method of Yang et al. [31]. The macrophages were prepared as described above. The *S. agalactiae* and *A. hydrophila* were labeled with FITC as the above description in 2.9. Briefly, the FITC-labeled bacteria were adjusted to 1×10^8 CFU/mL before use. Aliquots of 200 μL FITC-labeled *S. agalactiae* or *A. hydrophila* suspensions were mixed with 100 μL of (r) OnBML (100 μg/mL). The mixtures were incubated in the presence of 2 mM calcium by adding $CaCl_2$ in the dark for 60 min. After centrifugation at 5000 × *g* for 6 min, the pelleted bacterial cells were re-suspended in 300 μL TBS, mixed with 300 μL of the monocytes/macrophages suspension (2×10^7 cells/mL), and incubated at room temperature for 60 min, shaking every 5 min to prevent sedimentation of the monocytes/macrophages and microbes. For control, (r)OnBML solution was substituted by TBS (pH 7.4) or Trx-pET-32a protein solution and processed similarly. To remove the non-ingested bacteria from the monocytes/macrophages, the cell suspensions were centrifuged over a cushion of 3% BSA in TBS supplemented with 4.5% D-glucose at 100 × *g* at 4 °C for 10 min. The cell pellets were re-suspended in TBS (pH 7.4), and the fluorescence of the extra cellular bacteria was quenched by adding 1 μL of ice-cold trypan blue (0.4%) per sample [31]. The samples were immediately mixed gently, acquired and analyzed in FACS Aria III (BD, America) flow cytometer with an argon-ion laser adjusted to 488 nm. For each sample 10,000 individual cells were analyzed. Data were analyzed with the software Flowjo X.0.7. The phagocytic ability was defined as the percentage of the macrophages with one or more engulfed bacteria within the total cell population.

2.11. Statistical analysis

All of the experiments were performed at least three times and statistical analyses were carried out with SPASS 17.0 software. The data analyzed using one-way ANOVA were represented as mean ± standard deviation, statistical significance was defined as $*0.01 \leq p < 0.05$, $**p < 0.01$. The figures in this study were made by Sigma Plot 10.0 software.

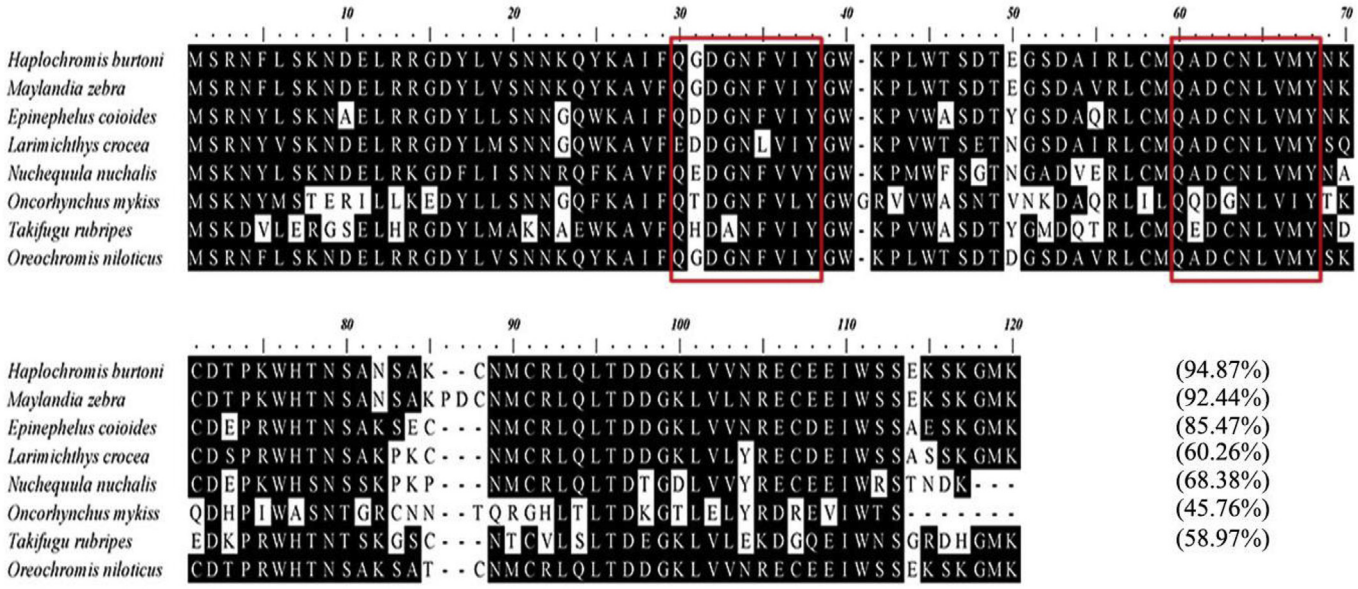

Fig. 1. Multiple sequence alignment of the deduced amino acid sequences of bulb type mannose binding lectin among different species. The conserved and identical residues are represented by black shading and the conserved mannose binding sites are boxed. The GenBank accession numbers of genes involved are as below, *Haplochromis burtoni*, XP_005922607; *Maylandia zebra*, XP_012774751; *Epinephelus coioides*, AEG78370; *Larimichthys crocea*, KKF19757; *Nuchequula nuchalis*, BAE79275; *Oncorhynchus mykiss*, NP_001117986; and *Kryptolebias marmoratus*, XP_017261926.

3. Results

3.1. Molecular features and sequence analysis of OnBML

The cDNA sequence of *OnBML* was identified from tilapia with an ORF of 354 bp (including the stop codon), and encoded 117 amino acid residues with an estimated molecular weight of 13.44 kDa and theoretical isoelectric pI of 7.98. The OnBML included two repeats of the consensus mannose-binding site QXDXNXVXY (Fig. 1). No potential N-glycosylation site was detected from the amino acid sequence, and signal peptide and transmembrane region were not exhibited.

Multiple sequence alignment analysis of the deduced amino acid sequence of OnBML showed that the gene was homologous to other teleost BMLs. The deduced amino acid sequence of OnBML shared 58.97%, 60.26%, 68.38%, 85.47%, 92.44% and 94.87% identities to the B-type lectins of teleost species including *Takifugu rubripes*, *Larimichthys crocea*, *Nuchequula nuchalis*, *Epinephelus coioides*, *Maylandia zebra* and *Haplochromis burtoni*, respectively (Fig. 1).

3.2. Tissue expression patterns of OnBML in healthy and challenged tilapia

The tissue expression of *OnBML* in healthy tilapia tissues was determined by qRT-PCR. Under normal conditions, the expression of *OnBML* transcript was widely distributed in all examined tissues (Fig. 2). The most abundant expression of the *OnBML* was in the spleen, which was more than 80-fold higher than the lowest expression in hind kidney (Fig. 2).

In order to examine effects of bacterial infection on *OnBML* expressions in tissues, temporal expression patterns of *OnBML* upon both bacterial challenges (*S. agalactiae* and *A. hydrophila*) were investigated by qRT-PCR. As shown in Fig. 3, upon *S. agalactiae* and *A. hydrophila* infections, the expressions of *OnBML* transcript were significantly up-regulated post-challenge in all examined tissues including spleen, intestines, gill and skin. Generally, the up-regulations of *OnBML* expression after *S. agalactiae* challenge was more remarkable than those following *A. hydrophila* infection. Upon *S. agalactiae* infection, in spleen tissue, the expression of *OnBML* transcript was significantly up-regulated with a 16-fold increase at 24 h p.i.; while after *A. hydrophila* challenge, its expression was also significantly up-regulated with a

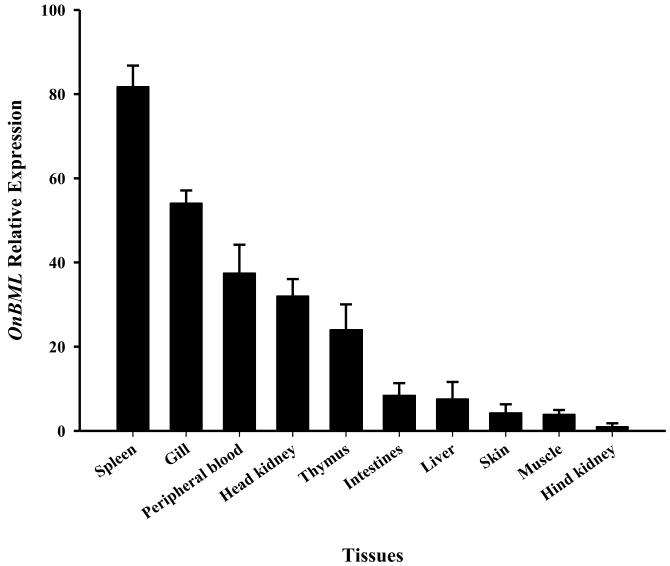

Fig. 2. Tissue distribution of *OnBML* mRNA in normal Nile tilapia. The ratio refers to the gene expression in different tissues relative to that in hind kidney and target gene expression is normalized against β-actin. The results were mean ± SD of three replicate samples.

slighter increase of 7-fold at 6 h p.i. (Fig. 3A). In intestines, both bacterial infections caused significant increases of *OnBML* expression, with 36-fold and 26-fold increase by *S. agalactiae* and *A. hydrophila*, respectively (Fig. 3B). In gill, the maximum expression occurred at 12 h p.i. by *S. agalactiae* (8-fold) and 2 d p.i. (6-fold) by *A. hydrophila* (Fig. 3C). Further, the *OnBML* expressions in skin were also significantly up-regulated by *S. agalactiae* and *A. hydrophila* challenges (Fig. 3D).

3.3. Expression patterns of OnBML after stimulation in vitro

In order to investigate effects of stimuli on *OnBML* expressions *in vitro*, Nile tilapia head kidney macrophages were isolated and stimulated with inactivated *S. agalactiae* and *A. hydrophila*. Upon both

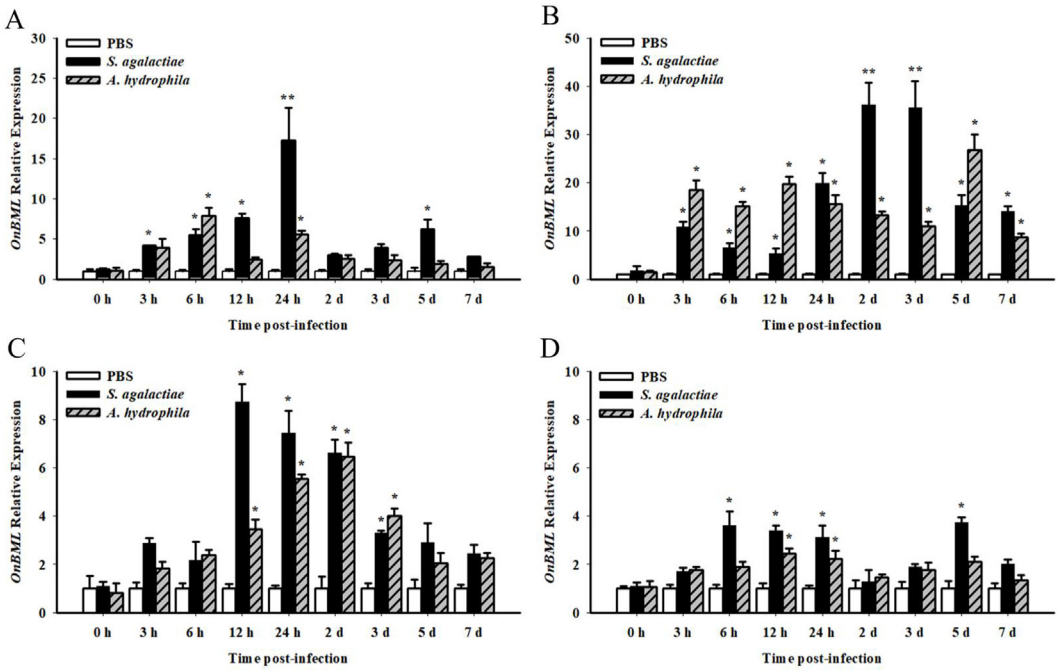

Fig. 3. Temporal mRNA expression of *OnBML* transcript in the spleen (A), intestines (B), gill (C) and skin (D) after *S. agalactiae* and *A. hydrophila* challenges. The mRNA level of *OnBML* gene was normalized to that β-actin and fold units were calculated deciding the values of the vaccinated tissues by PBS. In each case, the expression level of the control fish was set as 1. The error bars represent standard deviation (n = 3) and significant difference was indicated by asterisks (*0.01 ≤ p < 0.05, **p < 0.01).

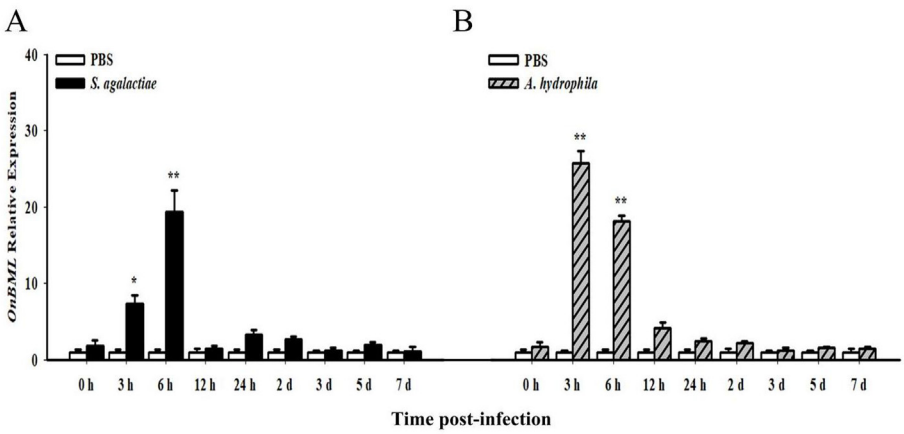

Fig. 4. The mRNA expression of *OnBML* in the head kidney macrophages. Nile tilapia head kidney macrophages were treated with *S. agalactiae* (1×10^7 CFU/mL), *A. hydrophila* (1×10^7 CFU/mL) and PBS. The mRNA level of *OnBML* gene was normalized to that β-actin and fold units were calculated deciding the values of the PBS treated cells. In each case, the expression level of the control fish was set as 1.The error bars represent standard deviation (n = 3) and significant difference was indicated by asterisks (*0.01 ≤ p < 0.05, **p < 0.01).

bacterial challenges, the expressions of *OnBML* mRNA were significantly up-regulated at 3–6 h p.i., with a 19-fold increase at 6 h p.i. following *S. agalactiae* challenge (Fig. 4A) and an increase of 25-fold at 3 h p.i. after challenge of *A. hydrophila* (Fig. 4B).

3.4. Recombinant OnBML expression, purification and western blotting analysis

The *OnBML* ORF was cloned into pET-32a (+) vector and transformed into BL21 (DE3), and the recombinant protein fused with His-tag was purified and analyzed by SDS-PAGE and western blotting. On SDS-PAGE gel (Fig. 5A), a distinct band was identified with size approximately 32 kDa, corresponding to the OnBML-His fusion protein. The detection of a specific positive band at 32 kDa on western blotting membrane (anti-His tag mouse monoclonal antibody as the primary antibody) confirmed the predicted molecular weight of (r)OnBML protein.

Recombinant OnBML was expressed in *E. coli*, and its binding activity to D-mannose was evaluated using affinity chromatography. As shown in Fig. 5B, a single band with the predicted size was detected in the fraction bound to mannose-agarose and eluted with D-mannose, and the band was also detected by Western blotting using an anti-His tag antibody. The Trx-pET-32a protein was also expressed in *E. coli*, a specific positive band at ~21 kDa on western blotting membrane (anti-His tag mouse monoclonal antibody as the primary antibody) confirmed the predicted molecular weight of Trx-pET-32a protein (Fig. 5C).

3.5. Binding of (r)OnBML to bacteria pathogens

The ability of recombinant OnBML protein binding with bacteria was detected by ELISA assay. The results showed that the (r)OnBML was able to bind *S. agalactiae* and *A. hydrophila*, and the binding was in a concentration-dependent manner (Fig. 6). However, the purified Trx-pET-32a protein had no binding ability to both bacteria (Fig. 6), even with the increase of protein concentration to remarkably high one (100 μg/mL). In addition, no significant binding was detected in the

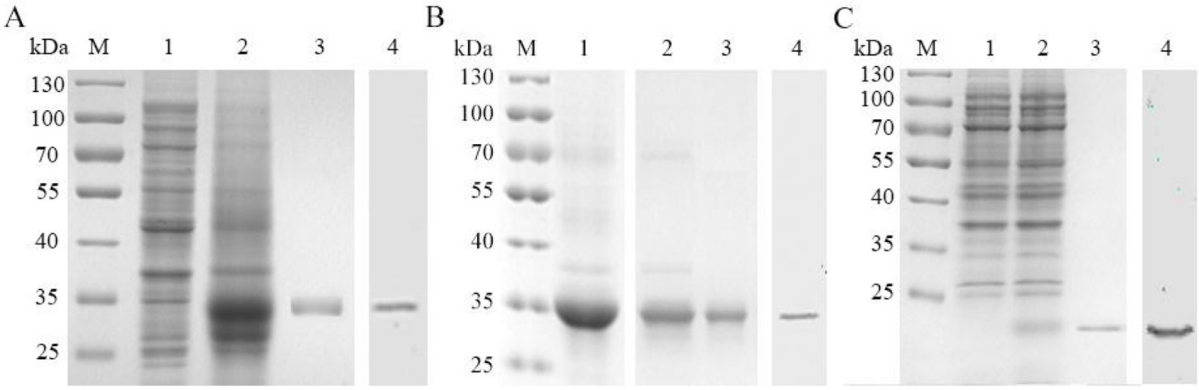

Fig. 5. Purification of (r)OnBML and Trx-pET-32a protein. Lane M, markers; Lane 1, the bacterial cells before IPTG induction (OnBML); Lane 2, the (r)OnBML was induced with 1 mM IPTG at 37 °C for 5 h; Lane 3, purified (r)OnBML fusion protein; Lane 4, western blot analysis of (r)OnBML (A); Lane M, markers; Lane 1, the mixed (r)OnBML sample (eluted by solution containing EDTA or different concentration of imidazole (100 and 250 mM)); Lane 2, the protein that unbound with the mannose; Lane 3, the (r)OnBML was purified with mannose agarose; Lane 4, western blot analysis of (r)OnBML (B); Lane M, markers; Lane 1, the bacterial cells before IPTG induction (pET-32a (+) empty vector); Lane 2, bacterial cells was induced with 1 mM IPTG at 37 °C for 5 h; Lane 3, Trx-pET-32a protein; Lane 4, western blot analysis Trx-pET-32a protein (C).

absence of coating.

3.6. Agglutinating activity of (r)OnBML

The agglutinating activity of (r)OnBML protein, according to its binding activity with *S. agalactiae* and *A. hydrophila*, was further examined using FITC-labeled bacteria. In the presence of Ca^{2+}, the results indicated that (r)OnBML could agglutinate *S. agalactiae* and *A. hydrophila* (Fig. 7A; 7G). However, the agglutinating ability of (r)OnBML was completely lost when the protein was heat-denatured before the reaction (Fig. 7B; 7H). When D-mannose or EDTA was added to the reaction system, no agglutination was observed (Fig. 7C; 7D; 7I; 7J). The control Trx-pET-32a protein showed no agglutination activity for both bacteria (Fig. 7E; 7K), similar as the TBS control group (Fig. 7F; 7L).

3.7. Enhancement of phagocytosis by (r)OnBML

The ability of (r)OnBML to promote the phagocytosis of bacteria by monocytes/macrophages was examined by phagocytosis assay employing flow cytometer. Since only the fluorescence of ingested bacteria in the macrophages could be detected by flow cytometer, according to the part of the macrophages without any phagocytosis shown in Fig. 8A, the other part was shown as phagocytosis part. In Fig. 8B, the phagocytic percentage of the macrophages was detected by phagocytosing the (r)OnBML or Trx-pET-32a protein treated bacteria. Statistical analyses showed that the phagocytic percentages of the macrophages engulfing (r)OnBML-treated *S. agalactiae* and *A. hydrophila* were all significantly increased in comparison with those of Trx-pET-32a protein treated or non-treated bacteria (Fig. 8C). The result showed that tilapia (r)OnBML could enhance the phagocytosis of bacteria by macrophages.

4. Discussions

Lectin, a key component of innate immune system, is able to identify and interact with pathogenic bacteria and play an important role in host defense against bacterial infection in teleost [3,9,32]. In this study, we presented the identification and characterization of a *BML* gene from Nile tilapia, which indicated that the OnBML possesses apparent agglutination and promotes phagocytosis ability to tilapia bacterial pathogens, and is likely to get involved in host defense against bacterial infection.

According to the cDNA sequence, the predicted OnBML protein has a B-type mannose-binding lectin domain and two repeats of the conserved motif QXDXNXVXY. The result was consistent with previous reports in pufflection of *Takifugu rubripes* [14] and *Takifugu niphobles* [15]; however, it was different from the findings in striped murrel and tongue sole with three repeat motifs [9,16]. In the B-type lectins, the

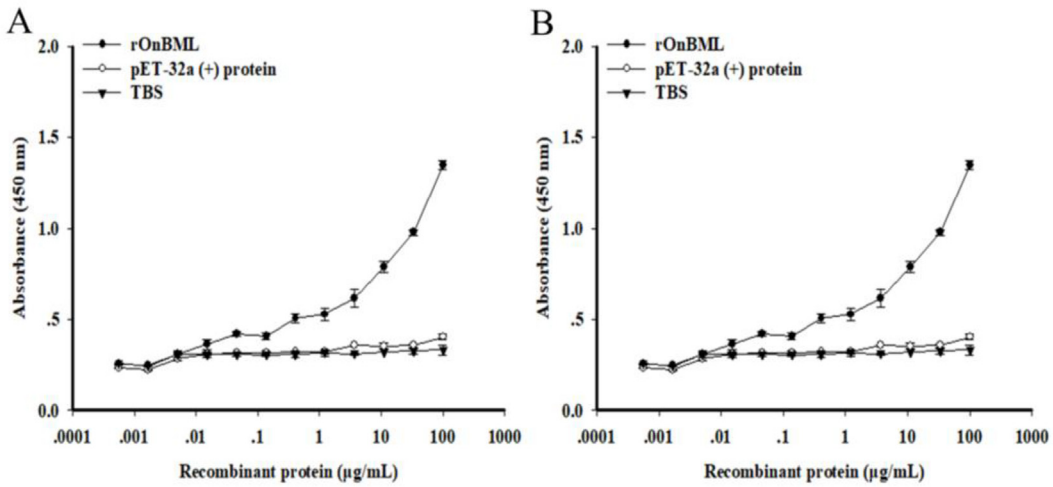

Fig. 6. Binding of (r)OnBML to *S. agalactiae* (A) and *A. hydrophila* (B). Data are presented as means ± SD of three triplicate experiments.

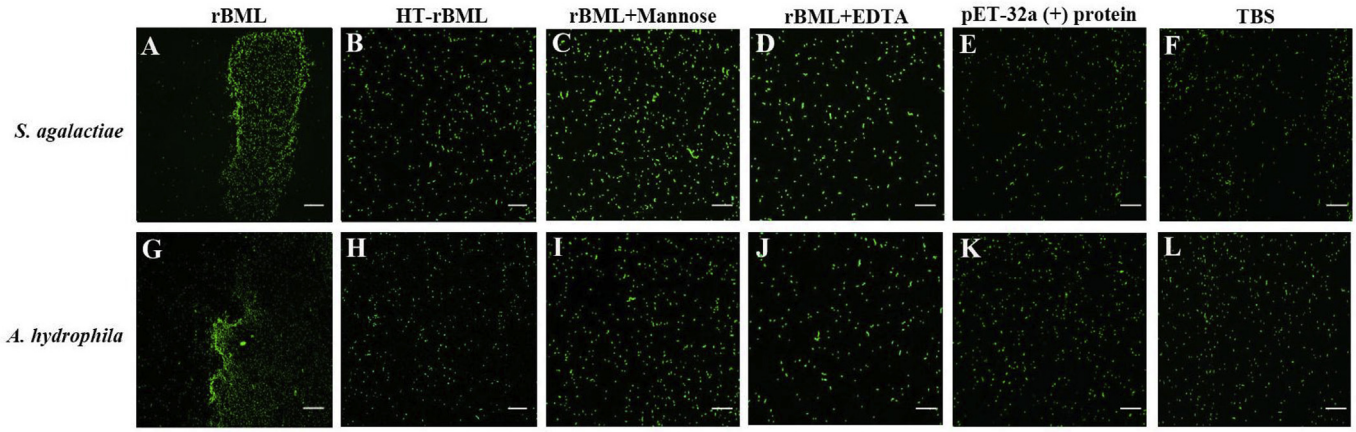

Fig. 7. Agglutination of *S. agalactiae* and *A. hydrophila* by (r)OnBML. FITC-labeled *S. agalactiae* and *A. hydrophila* was incubated with native (r)OnBML (A, G), heat-denatured (r)OnBML (B, H), (r)OnBML with D-mannose (C, I), (r)OnBML with EDTA (D, J), Trx-pET-32a protein (E, K) and TBS control (F, L), respectively. The agglutination was observed with a fluorescence microscope (Objective 10 ×).

QXDXNXVXY motif possibly acts as a specific binding site for D-mannose [9,16]. The homology modeling showed that three or four of the amino acid residues were located at the same positions and with the same angles to the corresponding D-mannose [15]. According to its function and role in antibacterial resistance, further investigation concerning the function of the mannose-binding site is necessary.

Tissue distribution analysis showed that the *OnBML* transcript was widely expressed in all examined tissues of healthy Nile tilapia (Fig. 2). The *OnBML* transcripts were abundantly expressed in the spleen, gill and peripheral blood (Fig. 2). This tissue distribution pattern is similar to that of *Takifugu niphobles* and *Channa striatus* with abundant expression in the gill [14,16], and *Cynoglossus semilaevis* with significant high level in the spleen and blood [9]. However, different from the *OnBML*, the *BML* expression was abundant in the liver in *Cynoglossus semilaevis* [9], and expressed highly in the intestine in *Scophthalmus maximus* [17]. Thus, for the same B-type lectin, its tissue distribution in different teleost species might be not similar with each other [9,17]. In order to examine the change of *OnBML* expressions in tissues after bacterial infection, temporal expression patterns of *OnBML* upon different tissues (spleen, gill, intestine and skin) were investigated by qRT-PCR. The spleen is an important immune organ in teleost and one main target organ attacked by bacterial infection, especially the main pathogen *S. agalactiae* of tilapia [19,33,34]. In addition, as the first barrier of body defense, mucosal tissues such as gill and skin are involved in a continuous water exchange from outside environment in fish and are more susceptible to pathogen infection, and the intestine is continuously exposed to microbial-rich environment [16,17,35,36]. Thus, the spleen and the three mucosal tissues are important organs of the immune system in teleost fish [36,37]. From the results of *OnBML* mRNA expression at a 7-days period after infection, the spleen, intestine, gill and skin were up-regulated, and the intestine raised more prominent with the increase of 36-fold (2 d, *S. agalactiae*) and 26-fold (5 d, *A. hydrophila*) (Fig. 3). The findings were coincided with the results of the turbot, puffferish and tongue sole [9,15,17]. Moreover, the expression of *OnBML* in mucosal tissues such as gill and intestines sharply rose in early commitment (3 h) after *S. agalactiae* infection. The

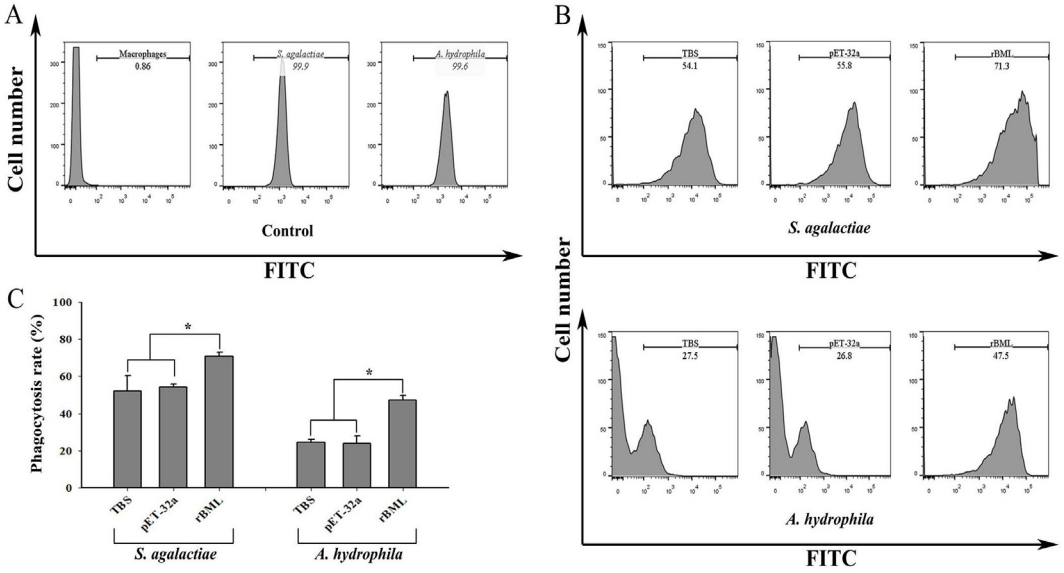

Fig. 8. Effects of (r)OnBML on phagocytosis of tilapia macrophages. Flow cytometric analyses of the macrophages phagocytosing *S. agalactiae* and *A. hydrophila*. Data show analyses of 10,000 events. (A) The histogram of the macrophages, *S. agalactiae* and *A. hydrophila* alone. The marker represented phagocytosis part. (B) The histogram of flow cytometric analyses of the macrophages phagocytosing *S. agalactiae* and *A. hydrophila* pre-incubated with TBS, Trx-pET-32a protein or (r)OnBML. The phagocytosis rates were shown near the marker. The results shown here were from one experiment out of three independent experiments. (C) The histogram of the phagocytosis rates. The symbol shows a significant difference from control ($*0.01 \leq p < 0.05$, $**p < 0.01$).

results suggested that tilapia BML might serve as important parts of the first line of defense against microbial infection and therefore be likely to play an essential role in the mucosal immune system [3,14,35]. In addition, comparison with those following *A. hydrophila in vivo* infection, the up-regulations of *OnBML* expression upon *S. agalactiae* challenge was generally more dramatic, indicating that the *S. agalactiae* is the main pathogen to tilapia [19,33,34]. To examine effects of stimuli on the expressions of *OnBML* at cell level, the macrophages isolated from head kidney were challenged *in vitro*. It showed that *OnBML* expression was rapidly and significantly up-regulation post-infection (Fig. 4), revealing that the expression of *OnBML* was quite active against pathogenic bacteria by macrophages. Overall, the qRT-PCR analyses indicated that Nile tilapia BML might be involved in the immune response against pathogen infection.

B- type lectins play important roles in host defense against bacterial infection, due that they are able to recognize, combine and interact with wide variety of microorganisms and allergen [3,38,39]. In this study, we successfully obtained the recombinant protein (r)OnBML of Nile tilapia, through the construction of prokaryotic expression vector and induced expression of recombinant protein (Fig. 5). Then through the bacteria and recombinant proteins combined experiment, it demonstrated that (r)OnBML could combine with *S. agalactiae* and *A. hydrophila* in the presence of Ca^{2+} (Fig. 6). This was in accordance with the previous reports showing that the MBL was able to recognize bacterium [19,31], indicating that it may play a pattern recognition role via interaction with bacteria. Moreover, from the agglutination experiment, we found that the two bacterial pathogens (*S. agalactiae* and *A. hydrophila*) were agglutinated by (r)OnBML (Fig. 7A; 7G). It agreed with the previous findings showing that the tongue sole BML and turbot LTL were able to agglutinate bacteria [9,17]. However, the thermal denaturation (r)OnBML was disabled to agglutinate with bacteria (Fig. 7B; 7H). It may due that high temperature destroyed the specific spatial conformation of (r)OnBML, resulting in the change of its physical and chemical properties, and the loss of biological activity, which was consistent with the MBL study [19,29]. The added D-mannose and EDTA inhibited the agglutination, indicating that OnBML was a Ca^{2+} dependent mannose-specific lectin.

Lectins are able to interact with the surface specific receptors of macrophages to enhance pathogen phagocytosis [4,38,40]. We found that the recombinant protein (r)OnBML could promote the phagocytosis of pathogenic bacteria by Nile tilapia macrophages (Fig. 8), implicating it was likely as an opsonin to promote phagocytosis function. This was similar to the previous report showing that the MBL was able to enhance the phagocytosis [31]. However, there are also some evidences that other lectins can directly affect the phagocytosis by non-opsonization, such as SP-D [41] and SP-A [42].These results indicated that the OnBML might perform a dual-function in both pattern recognition and opsonization.

In summary, a B-type lectin OnBML was successfully identified and characterized from Nile tilapia. It shares important structural elements with other BML lectins. The mRNA encoding *OnBML* was predominantly expressed in the spleen. Following bacterial challenges, the expression of *OnBML* was significantly up-regulated *in vivo* (spleen, intestine, gill and skin) and *in vitro* (macrophages). This study indicated that OnBML might be involved in host defense against bacterial infection. Moreover, the recombinant protein (r)OnBML can combine and agglutinate with *S. agalactiae* and *A. hydrophila*, and promote the phagocytosis of bacteria by macrophages. This study indicated that OnBML, possessing apparent agglutination and opsonization ability to pathogenic bacteria, is likely to get involved in host defense against bacterial infection in Nile tilapia.

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