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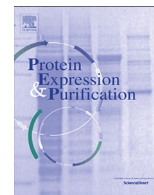

Optimal conditions for expressing a complement component 3b functional fragment (α2-macroglobulin receptor) gene from *Epinephelus coioides* in *Pichia pastoris*

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ABSTRACT

The α_2-macroglobulin receptor (α_2MR) is a major domain of complement component 3b, which may play an important role in regulating the downstream complement system in teleosts. In order to characterize the domain thoroughly larger than currently available quantities are required. Thus, in this study the *Epinephelus coioides* α2MR (Ec-α2MR) was expressed and secreted by the methylotrophic yeast *Pichia pastoris* with variations in pH and induction time to identify optimal production conditions. At pH 5.5 with 48 h induction 13 mg of Ec-α2MR (ca. 90% purity) was obtained from 500 ml of culture. The Ec-α2MR protein product was validated by MALDI-TOF MS sequence analysis, and both Western blotting and ELISAs demonstrated that it possessed the expected activity, binding to C3b or C3b homolog antibodies, and thus can be used for future studies of the interactions and functions of complement proteins in teleosts.

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Introduction

The orange-spotted grouper, *Epinephelus coioides*[2], is widely cultured in China and Southeast Asian countries because of its excellent seafood quality and high market value [1]. However, the increasingly large grouper populations resulting from aquaculture could elicit a surge in infectious diseases, posing threats for their survival [2]. In addition, environmental degeneration and various pathogenic diseases have affected grouper aquaculture, resulting in considerable economic losses [2,3]. The major threats include bacterial challenges, which may result in suppression of the groupers' immune defenses and thus compromise their disease resistance [4]. Furthermore, populations of grouper pathogens are closely associated with human activity, and microbiological contamination of the aquaculture systems could adversely affect other marine organisms [5]. Nevertheless, despite the potential severity of these threats, little is known about the grouper's immune defenses against invasion by bacteria.

In mammals, the complement system plays vital roles in immune defenses against bacterial invasion, mediating responses of the innate immunity system and linking them to responses of the adaptive system [6]. The complement system can be activated by acrophage-activating cytokines, resulting in increased phagocytosis [7]. A key component is complement component 3 (C3), which can be activated by cleavage (catalyzed by C3-convertase) to C3a and C3b, which initiates cascades of further cleavage and activation events, via the classic, alternative and/or lectin pathways. C3b plays a key role in the formation of membrane attack complexes (which mediate lytic responses to bacterial invasion) and may recruit other proteins or serve as an opsonin [8]. C3 also directly binds to immunoglobulin M (IgM) and to low-density lipoprotein-receptor-related protein 1/α2-macroglobulin receptor (LRP-1/α2MR), responsible for the clearance of α2-macroglobulin-protease complexes [16]. Furthermore, conversion of C3 to C3b is crucial for complement activation and the feedback amplification triggered by the complement pathways [9]. A C3 amplification loop is also essential for the complement system, as it is a key element of all three activating pathways and balances two

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[2] *Abbreviations used: P. pastoris, Pichia pastoris; E. coioides*, EC, *Epinephelus coioides*; C3b, complement component3b; C3, complement component3; α2MR, α2-macroglobulin receptor; CR3, complement-like repeat 3; LRP, lipoprotein receptor-related protein; YPD, yeast extract peptone dextrose medium; BMGY, buffered minimal glycerol-complex medium, BMMY, buffered minimal methanol-complex medium; YPDS, yeast extract peptone dextrose sorbitol medium; ELISA, enzyme linked immunosorbent assay; MALDI-TOF MS, matrix-assisted laser desorption/ionization time of flight mass spectrometry.

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competing cycles that regulate C3 [10]. Following amplification, C3 synthesis may also be induced, thereby further enhancing immune responses, including downstream cytokine production [11,12].

An early suggestion regarding the origin of C3 is that it shares a common ancestor, containing an α_2-macroglobulin receptor (α_2MR) domain, with α_2-macroglobulin [13]. More recent findings show that calcium complexes of the complement-like repeat 3 (CR3) of the low density lipoprotein receptor-related protein (LRP) have high affinity for the α_2MR binding domain [14]. LRP and α_2MR are now recognized as the same multifunctional receptor (LRP/α_2MR), which binds various biological ligands, thereby modulating various downstream biological processes [15], including metabolism of activated C3 [16].

A complement system also plays a crucial role in immune defenses of teleosts (bony fishes) [17], which apparently includes all three complement pathways known in mammals [18]. Their complement proteins also share significant sequence homology with their counterparts in mammals [19]. However, there is limited information on the functions and activities of teleost complement proteins. In a previous study we characterized the cDNA sequence of the C3 gene from *E. coioides* [20]. However, in order to characterize the protein thoroughly larger quantities than are currently available are required. Thus, in this study the *E. coioides* α2MR (Ec-α2MR) was expressed and secreted by the methylotrophic yeast *Pichia pastoris* with variations in pH and induction time to identify optimal production conditions.

Materials and methods

Reagents

EcoRI, XbaI, MssI and T4 DNA ligase were obtained from TaKaRa (Dalian, China), and Zeocin™ from Invitrogen (Carlsbad, CA, USA). Mouse anti-Histidine (anti-His) and peroxidase-conjugated horse anti-mouse antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). Horseradish peroxidase (HRP)-labeled goat anti-rabbit antibody was purchased from Protein Tech (Chicago, IL, USA). Yeast extract peptone dextrose medium (YPD: 1% yeast extract, 2% peptone, 2% dextrose, 1.5% agar), buffered minimal glycerol-complex medium (BMGY: 1% yeast extract, 2% peptone, 1% glycerol, 1.34% Yeast Nitrogen Base (YNB), 4 × 10–5% biotin and 100 mM potassium phosphate, pH 6.0), buffered minimal methanol-complex medium (BMMY; 1% yeast extract, 2% peptone, 0.5% methanol, 1.34% YNB, 4×10^{-5}% biotin and 100 mM potassium phosphate, pH 6.0), and yeast extract peptone dextrose sorbitol medium (YPDS: 1% yeast extract, 2% peptone, 2% dextrose, 1 M sorbitol, 2% agar containing 0.5 mg/mL Zeocin™) were all prepared according to the supplier's guidelines (Invitrogen, Carlsbad, CA, USA).

P. pastoris strains and vectors

Pichia pastoris (*P. pastoris*) strain X-33, *Escherichia* coli (*E. coli*) strain DH5α and Plasmid pPICZαA were purchased from Invitrogen (Carlsbad, CA, USA).

Construction of the expression vector

The Ec-α_2MR sequence was obtained from a plasmid established in our lab using gene-specific primers. An EcoRI restriction site sequence was added to the N-terminal using the primer 5′GCGAATTCATGGAAGCAACGGTGAAAA3′ and a XbaI restriction site sequence was added to the C-terminal using the primer 5′GCTCTAGCTAAATGATGATGATGATGATGTCTTTCTGACAGGACTG3′. The sequence was amplified by PCR in a 25 μl mixture, including 10 μl of PrimeSTAR HS (Premix) (TaKaRa, Dalian, China), 1.5 μl of each primer (10 μM), 11 μl of PCR-grade water and 1 μl of plasmid cDNA. The PCR program consisted of 95 °C for 3 min, 35 cycles of denaturation at 94 °C for 30 s, annealing at 65 °C for 30 s, and extension at 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. The PCR products were analyzed by 1% agarose gel electrophoresis and purified using an AxyPrep extraction kit (Sigma, USA) according to the manufacturer's instructions. The purified DNA fragment and pPICZαA vector were digested with EcoRI and XbaI. After digestion and gel purification, the target DNA fragment was ligated into the pPICZαA expression vector by T4 DNA ligase. The ligation products were transferred into *E. coli* DH5α and the transformants were selected on Low Salt LB plates with Zeocin (0.5 mg/mL). A single clone was selected and incubated in Low Salt LB medium at 37 °C for 12 h then the recombinant plasmid pPICZαA-Ec-α2MR was extracted using a Plasmid Extraction Kit (Omega, China) and linearized by incubation with MssI at 37 °C for 3 h.

P. pastoris transformation and screening of transformants

A colony from a culture of a single *P. pastoris* clone spread on a YPD plate incubated at 30 °C for 72 h was propagated to an OD600 of 1.2 in 100 ml YPD medium incubated at 30 °C. The culture was centrifuged at 1500*g* at 4 °C for 5 min (these conditions were also used for all subsequent centrifugations for preparation of transformants) and the cells were resuspended in 50 ml of ice-cold sterile water. Following rinses in 5 ml portions of ice-cold sterile water and ice-cold 1 M sorbitol, by further rounds of centrifugation and suspension, the cells were centrifuged and resuspended in 200 μl of ice-cold 1 M sorbitol. Following Invitrogen's recommendations they were then incubated with the linearized pPICZαA-Ec-α2MR plasmid on ice for 5 min, subjected to electroporation at 2000 V for 5 ms, incubated in YPD medium at 30 °C for 60 min and spread on YPDS plates containing Zeocin (0.5 mg/ml) to select transformants.

Induction of Ec-α_2MR expression

The selected *P. pastoris* transformants were incubated in BMGY medium at 30 °C for 24 h and grown to an OD_{600} of 6.0. The cells were then centrifuged at 3000*g* for 5 min, resuspended and incubated in BMMY medium to induce expression of Ec-α_2MR, re-centrifuged, and the supernatants were collected for SDS–PAGE and Western blot analysis.

Protein concentrations in the supernatants were determined by the Bradford method. Samples containing 20 mg of total protein were then loaded onto 12% SDS–PAGE gels and electrophoretically separated. The SDS–PAGE gels were washed in TBS (50 mM Tris–HCl pH 8.0, 150 mM NaCl) containing 0.05% Tween-20 (TBST) for 10 min, and the separated proteins were transferred to PVDF membranes on ice at 100 V for 60 min. The membranes were washed in TBST for 5 min, and incubated in 20 mM TBS containing 3% BSA at 4 °C overnight. The PVDF membranes were then washed in TBST for 5 min, and incubated with mouse anti-His monoclonal antibody diluted 1:1000 in 0.5% BSA-TBST for 60 min at room temperature with gentle shaking. Following four 10-min rinses in TBST the membranes were incubated with peroxidase-conjugated horse anti-mouse antibody diluted 1:2000 for 60 min at room temperature. Following a further four 10-min rinses in TBST, the development and visualization procedure.

Optimization of Ec-α_2MR expression

To identify optimal conditions for Ec-α2MR expression, transformants were first incubated in buffered minimal methanol-complex medium with variations in pH (4.0, 4.5, 5.0, 5.5, 6.0, 6.5 and

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