Fish & Shellfish Immunology 65 (2017) 235–243

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Plasma immune protein analysis in the orange-spotted grouper *Epinephelus coioides*: Evidence for altered expressions of immune factors associated with a choline-supplemented diet

Ya-Li Shiu [a], Kuo-Hsun Chiu [b], Truong-Giang Huynh [a, c], Ping-Chung Liu [d], Chun-Hung Liu [a, *]

[a] Department of Aquaculture, National Pingtung University of Science and Technology, Pingtung, Taiwan
[b] Department of Aquaculture, National Kaohsiung Marine University, Kaohsiung, Taiwan
[c] College of Aquaculture and Fisheries, CanTho University, CanTho, Vietnam
[d] Department of Aquaculture, National Taiwan Ocean University, Keelung, Taiwan

ARTICLE INFO

Article history:
Received 1 April 2017
Received in revised form
21 April 2017
Accepted 24 April 2017
Available online 25 April 2017

Keywords:
Choline
Disease resistance
Epinephelus coioides
Immune-related protein
Proteomics

ABSTRACT

This study aimed to unravel the regulatory roles of choline in activating immune responses and disease resistance of the orange-spotted grouper *Epinephelus coioides*. Fish were fed a choline-supplemented diet at 1 g kg^{-1} of feed for 30 days. Fish fed a fish meal basal diet without choline-supplement served as controls. At the end of the feeding trial, fish were challenged with *Vibrio alginolyticus*. Meanwhile, plasma proteomics of fish in each group were also evaluated by two-dimensional gel electrophoresis (2-DE), and differentially expressed proteins were identified by tandem mass spectrophotometry (MS/MS), then a Western blot analysis or real-time polymerase chain reaction was used to confirm differential expressions of immune-enhancing proteins. Results showed that choline significantly increased survival of *E. coioides* 48 days after being injected with *V. alginolyticus*. From maps of plasma proteins, a comparative analysis between the control and choline groups revealed that 111 spots matched, with 26 altered expression spots in the choline group. Of these 26 spots, 16 were upregulated and 10 downregulated. After protein identification by reverse-phase nano-high-performance liquid chromatography-electrospray ionization MS/MS analysis, eight of 26 proteins were found to be immune-related proteins, all of which were upregulated, including complement 3 (C3), alpha-2-macroglobulin-P-like isoform (A2M), fibrinogen beta chain precursor (FBG), and immunoglobulin heavy constant mu (Ighm) proteins. Expression of the A2M protein and A2M enzyme activity in plasma of fish fed choline significantly increased compared to the control group. Additionally, A2M messenger (m)RNA transcripts were also upregulated in the liver and kidneys. Significantly higher C3 expressions at both the mRNA and protein levels were detected in the liver of fish in the choline group. Moreover, FBG gene expressions in the liver and kidneys significantly increased, while Ighm increased in the kidneys and spleen of fish in the choline group. Our results suggest that dietary administration of choline can protect grouper against bacterial infections through activating the complement system, thereby inducing antiprotease activity and natural antibodies that play important roles in the innate immune system of fish.

1. Introduction

In 2014, fish harvested from aquaculture amounted to 73.8 million tons, with an estimated first-sale value of US$160.2 billion. Of these, marine aquaculture fish accounted for 6.3 million tons (8.5% of total global production). Asian countries contribute the highest proportion to global marine fish production with 3.4 million tons (53.8% of global marine fish production) [1]. Among maricultured fish, groupers *Epinephelus* spp. are reported to be an economically important sector in China and Southeast Asian countries [2], among which China produced 62% of all farmed grouper in 2012, followed by Taiwan with 19%, Indonesia with 10%, Malaysia with 5%, and Thailand with 1% [3]. The Food and

* Corresponding author. Department of Aquaculture, National Pingtung University of Science and Technology, Pingtung 91201, Taiwan.
E-mail address: chliu@mail.npust.edu.tw (C.-H. Liu).

http://dx.doi.org/10.1016/j.fsi.2017.04.022
1050-4648/

Agriculture Organization estimates that grouper demand will reach 500,000 tons with equal shares by fishing and aquaculture in 2020 [2]. This issue has opened great further opportunities and challenges for grouper aquaculture.

Obviously, the high-density intensive farming of groupers has had to serious disease outbreaks resulting from viral, bacterial, and parasitic infections [2,4]. Traditionally, to prevent or treat these endemic diseases, chemotherapeutic agents such as commercial antibiotics or disinfectants have been used, although this is not advisable due to costs, environmental hazards, and antibiotic resistance [5]. Recently, some remediable additives were suggested to enhance immune responses and disease resistance in farmed grouper *Epinephelus coioides*, such as herbal biomedicines [6] and probiotics [7]. Also of interest, choline chloride, known as an essential nutrition for vertebrates, was introduced as an effective additive that boosts freshwater fish immunity [8–10]. In grouper, dietary choline was demonstrated to improve growth performance, with a requirement of around 0.1% [11]. However, whether choline induces immune responses and disease resistance in *E. coioides* remains largely unknown.

In mammals, involvement of choline chloride in the immune system is well documented, in which choline chloride is metabolized and transformed into free choline after absorption in the body [12]. Choline chloride is a precursor of acetylcholine (Ach) by reacting with acetyl-CoA under choline-acetyl transferase (ChAT) enzyme catalysis [13]. In humans [14], it was suggested that choline may directly modulate systemic immune responses to pathogenic invasion. From this, ACh significantly attenuates lipopolysaccharide (LPS)-stimulated release of cytokines such as interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α, and IL-18 in macrophages through nicotinic ACh receptors (nAChRs) that are expressed on immune cells, such as lymphocytes, macrophages, mast cells, dendritic cells, and basophils [15,16]. In addition, it was previously stated [17] that activation of human T cells enhances the synthesis of ACh and expression of muscarinic (m)AChRs. T cell adhesion to vascular endothelial cells and keratinocytes acts on mAChRs in an autocrine and/or paracrine fashion to regulate immune cell function. On the other hand, in mice, it is known that lymphocytes express both mAChRs and nAChRs, and stimulation of mAChRs and nAChRs produces various biochemical and functional changes. Most immune cells, such as T cells, B cells, and monocytes, express all five subtypes of mAChRs (M1~M5), and serum antibody production in M1 and M5-combined mAChR gene-knockout (KO) mice immunized with ovalbumin (OVA) revealed that M1/M5 mAChRs upregulate TNF-α, IFN-γ, and IL-6 production by spleen cells. These previous findings demonstrated that both mAChRs and nAChRs modulate production of cytokines, such as TNF-α, resulting in modifications of antibody production [18].

In aquaculture, it was reported that choline is a precursor of the neurotransmitter, ACh, the amino acid, methionine, and glycine betaine [19]. Mechanistically, several previous studies proved the immune regulatory function of choline in freshwater fish. For instance, dietary choline induced the expression of IL-10 in the spleen and head kidneys, target of rapamycin (TOR) in the spleen, and eIF4E-binding protein2 (4E-BP2) in head kidneys of Jian carp, *Cyprinus carpio* var. Jian [8,9]. Moreover, in grass carp, *Ctenopharyngodon idella*, dietary choline significantly upregulated IL-10, transforming growth factor (TGF)-β1, and Nrf2 in gills [10]. In marine fish, the requirement of choline for growth of cobia *Rachycentron canadum* was estimated [20], and in grouper, choline is involved in regulating lipid metabolism and stress tolerance in the giant grouper *E. lanceolatus* [21] and orange-spotted grouper *E. coioides* [11,22].

According to previous studies, we assumed that big gaps in our understanding of the roles of choline in activating the immune system of grouper still remain. We hypothesized that there is a link between choline and the mucosal immunity of fish. Therefore, the present work aimed to reveal some new insights into the actions of disease resistance in orange-spotted grouper *E. coioides* against *Vibrio alginolyticus* and to point out enduring altered plasma immune associated-protein expression patterns and expressions of immune-related genes in crucial organs, including the liver, kidneys, and spleen, that are involved in immunomodulation during cholinergic treatment.

2. Materials and methods

2.1. Preparation of the experimental diets

Two experimental diets were prepared, including a fish meal basal diet without choline supplement (control) and a 1 g kg^{-1} choline-supplemented diet, which was recommended to improve the stress tolerance of grouper [22]. The determined choline concentrations in the control and choline-supplemented diet were 2.7 ± 0.11 and 4.31 ± 0.54 g kg^{-1} of feed, respectively (Table 1). The diets were formulated as reported by Shiu et al. [22], in which choline chloride (A15828, Alfa Aesar, Heysham, UK) was used. Ingredients were ground in a hammer mill to pass through a 60-mesh screen. Experimental diets were prepared by mixing dry ingredients with fish oil and then adding water until a stiff dough resulted. The dough was then passed through a mincer with a die and the resulting spaghetti-like strings were dried in a drying cabinet using an air blower at 80 °C. After drying, the pellets were stored in plastic bins at 4 °C until use. The proximate analysis of the basal diet was 48.1% crude protein, 10.2% crude lipid, 12% ash, and 7.1% moisture according to AOAC methods [23].

2.2. Grouper

Orange-spotted grouper *E. coioides* juveniles obtained from a private farm in Pingtung, Taiwan were transported in sealed plastic bags with pure oxygen to the aquafarm of the Department of Aquaculture, National Pingtung University of Science and Technology, Taiwan. Prior to the experiment, fish were reared in a cement tank (6 × 2 × 1.2 m) containing 10 tons of 20-ppt brackish

Table 1
Ingredients in the feed (g kg^{-1}) used to culture orange-spotted grouper *Epinephelus coioides*.

Ingredients	Experimental diets (g kg^{-1})	
	Control	1
Fish meal[a]	600	600
Soya meal[b]	100	100
α-Cellulose	40	40
α-Starch	110	110
Choline chloride[c]	0	1
Squid meal[d]	50	50
Fish oil	50	50
Vitamin mix[e]	10	10
Mineral mix[e]	40	40
Proximate analysis		
Moisture (%)	7.12 ± 0.05	6.89 ± 0.22
Crude protein (%)	48.22 ± 0.11	48.11 ± 0.25
Crude lipid (%)	10 ± 0.11	10.15 ± 0.34
Ash (%)	12.12 ± 0.54	12.02 ± 0.22
Choline (g kg^{-1})	2.7 ± 0.11	4.31 ± 0.54

[a] Fish meal contains 3.57 ± 0.05 g kg^{-1} of choline (imported from South Africa).
[b] Soya meal contains 2.09 ± 0.05 g kg^{-1} of choline (a kind gift from Central Union Oil Corp., Taichung, Taiwan).
[c] Choline chloride purchased from Alfa Aesar (A15828).
[d] Squid meal contains 2.96 ± 0.07 g kg^{-1} of choline.
[e] Vitamin and mineral premix are following Shiu et al. [22].