Fish & Shellfish Immunology 54 (2016) 46–53

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

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

Full length article

Innate immune-stimulating and immune genes up-regulating activities of three types of alginate from *Sargassum siliquosum* in Pacific white shrimp, *Litopenaeus vannamei*

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ARTICLE INFO

Article history:
Received 23 December 2015
Received in revised form
6 March 2016
Accepted 11 March 2016
Available online 15 March 2016

Keywords:
Alginate
Immune response
Genes expression
Litopenaeus vannamei
Sargassum

ABSTRACT

The Total Haemocyte Count (THC), phenoloxidase (PO), Superoxide Dismutase (SOD) activity, Phagocytic Activity/Index and Total Protein Plasma (TPP) were examined after feeding the white shrimp *Litopenaeus vannamei* with diets supplemented with three different types of alginates (acid, calcium and sodium alginates). Immune–related genes expression was evaluated by quantitative Real Time PCR (qRT-PCR). Results indicated that the immune parameters directly increased according to the doses of alginates and time. The 2.0 g kg^{-1} of acid and sodium alginate treatments were gave better results. Four immune-related genes expression i.e. LGBP, Toll, Lectin, proPO were up regulated. It is therefore concluded that the supplementation of alginate of *Sargassum siliquosum* on the diet of *L. vannamei* enhanced the innate immunity as well as the expression of immune-related genes. It is the first report on the simultaneous evaluation of three alginate types to enhance innate immune parameters and immune-related genes expression in *L. vannamei.*

1. Introduction

Up to now, the Pacific white shrimp (*Litopenaeus vannamei*) is still a prosperous commodity in Indonesia due to the fact of the high price. Unfortunately, the world production of shrimp were faced with several problems caused by viral disease [1]. The mechanism of the innate immune system of shrimp in response to the inevitably encounter pathogens requires the recognition of the pathogen-associated molecular patterns (PAMPs) by a set of well defined receptors or pathogen-recognition receptors (PRPs) from host [2]. Recognition of the unique patterns of PAMPs eventually results in the widespread innate immune activation, including via the release of soluble molecules and the circulating haemocyte mediated humoral and cellular components which work together [3]. The key of enzymatic reaction is catalyzed by enzyme phenoloxidase (PO) and this step is considered as the rate-limiting melanin formation [4]. The humoral responses include the prophenoloxidase (proPO) system, the clotting cascade and a wide array of antimicrobial peptides, while the cellular immune responses include apoptosis, encapsulation, phagocytosis and nodule formation.

A brown algae, *Sargassum siliquosum* is abundant in Indonesia which is still under exploited. In fact, this brown algae is rich in alginate ie. 24% [5]; 37.91% [6]; 30.5% [7]; 32.57% [8]. Alginate is a linear hetero polysaccharide that consist of two unit monosaccharides, D-manuronat and L-glucoronat. Alginate has an abundance of free hydroxyl and carboxyl groups distributed along the polymer chain backbone, and it, therefore, unlike neutral polysaccharides has two types of functional groups that can be modified to alter the characteristics in comparison to the parent compounds [9]. Na_2CO_3 is an alkaline compound that can be used to modify the carboxyl groups and alter the alginate types. $CaCl_2$ which is added to the alginate extraction will build the “egg box” and stabilized the structure [10]. EDTA will act as a booster to get the better yield in extraction process.

Sodium alginate is effectively enhance the immunomodulator of

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http://dx.doi.org/10.1016/j.fsi.2016.03.022
1050-4648/

shrimp innate immune of penaeid shrimp by oral administration. Liu et al. [11] reported that the *Penaeus monodon* diet supplemented with 2 g kg^{-1} commercial sodium alginate enhances the cellular phagocytic activity. Cheng et al. [12] demonstrated the application of commercial alginate at 2 g kg^{-1} feed enhances the immune parameters and survival rate of *L. vannamei*. There were some information about the succeed of extracted alginate from tropical *Sargassum* sp. [13] and extracted fucoidan from tropical *S. cristaefolium* [14] to enhance the non-spesific defense in *Clarias* sp. and *Oreochromis niloticus*. However, there is still lack of information about the most effective extracted alginate, especially from local brown seaweed. The local *Sargassum siliquosum* were extracted into three different types and pursued to enhance the immune response and the immune-related genes expression in Pacific white shrimp.

The objectives of this study were to evaluate the effect of alginate in three different types on immune parameters and immune-related genes expression in Pacific white shrimp. Accordingly, in this study, we examined 1) the immune parameters, 2) the expression of related-immune genes when shrimp had been fed diets supplemented with different alginates for 15 days.

2. Materials and methods

2.1. Preparation of acid, calcium and sodium alginate of S. siliquosum and the experimental diets

Sargassum siliquosum was collected from seabed of Sundak Coast, Gunungkidul, Yogyakarta, Indonesia. The acid, calcium and sodium alginate extract was prepared based on the methods of [5,15,16], respectively. The acid alginate was prepared by 2 h waterbath extraction of depigmented *S. siliquosum* with 2% Na_2CO_3 at 70 °C. After filtration, the extract was then precipitated with HCl at pH < 1, followed by centrifugation at 3500 rpm for 20 min. The supernatant was then discharged and the pellet washed in 96% ethanol (1:1) and then filtered. The sodium alginat was collected by overnight magnetic stirrer extraction with 5% Na_2CO_3/50 μM EDTA. The pellet was then filtered and 0.13 M KCl was added and followed by 96% ethanol in 1:1 vol and stirred well. Centrifugation at 3500 rpm for 5 min was then performed.

Calcium alginate was prepared by 60 °C waterbath extraction with 0.2 N HCl for 2 h. The pellet was then filtered and maintained at pH = 7. This was then followed by washing with 96% ethanol and then centrifuged at 3000 rpm for 15 min and added with aquadest until pH = 2. Finally, Ca-alginate was collected and then dried overnight in the oven at 60 °C.

2.2. FT-IR spectroscopy

The characterization of alginates were determined by fourier transformed-infra red spectroscopy. The FT-IR spectra of the samples were mixed in KBr pellets (10% w/w) and were recorded in the 4000–500 cm^{-1} region using a *Thermo Nicolet 380 FTIR* (Germany).

2.3. Experimental animals and the experimental design

Shrimp obtained from the pond of Brackishwater Aquaculture Development Center were reared in indoor fiberglass tanks (1 m^3), and acclimated to room temperature (26–28 °C) for 2 weeks. During the acclimation period, shrimp were fed with the control diet. A polymerase chain reaction (PCR) technique was applied to identify and confirm that the shrimp were not infected with WSSV. Shrimp were then transferred to 7 plastic tanks. Each tank (144 L) with 120 L filtered seawater was stocked with 7 shrimp with an average initial weight of 17.2–22.9 g. The factorial completely randomized design with two level was applicated. The first level was the alginate type (acid, sodium and calcium), while the second level was the dose of alginate (1 g kg^{-1} and 2 g kg^{-1}). One tank was designed as a control. Tanks received continuous aeration, and water quality were managed by changing seawater at 50% daily. During the experimental period, water temperature ranged 26–28 °C, pH 7.3–7,9, salinity 33–37, and dissolved oxygen (DO) concentration 4.48–4.83 mg L^{-1}. Concentrations of ammonia-N were 0.08 mg L^{-1}, measured by the indophenol methods. Shrimp were fed their respective diets at a daily rate of 3% of body weight at 06.00, 11:00, 17.00 and 22.00.

A commercial diets (*CP, Thailand*) containing different types and different levels of alginate were prepared by coating 2–3% progol® (PT. INDOSCO, Surabaya, Indonesia), diluted with aquadest and sprayed all over the diets. Alginates were added to the three different test diets at level of 0, 1.0, and 2.0 g kg-^{1}.

2.4. Evaluation of immune parameters of L. vannamei

2.4.1. Haemolymph sampling

Only shrimp in the intermoult stage were used for the haemolymph sampling [17]. The haemolymph sampling, preparation of haemolymph, and counting of haemocytes were conducted by the previously described procedures [18]. Briefly, haemolymph (300 μl) was individually withdrawn from the ventral sinus of each shrimp using a 1-ml sterile syringe fitted with a 25-gauge needle. Prior to this, the syringe was coated with anticoagulant solution (10% sodium citrate). The haemolymph was placed in six tubes. Each tube contained 20, 20, 100, 40, 15 and 25 μl of haemolymph, and was used to measure 1) haemocyte counts 2) phagocityc activity (PA) and phagocytic index (PI) 3) Phenol oxidase (PO) activity, 4) Superoxide Dismutase (SOD) activity 5) Total Protein Plasma and 6) gene expression, respectively.

Two micro liter of the haemolymph was diluted with 8 μl of 0.8% NaCl in distilled water then was placed in a haemocytometer to measure the total haemocyte count (THC) using a light microscope (*Axioskop, Zeiss, Germany*). The remainder of the diluted haemolymph was used for subsequent tests.

2.4.2. Phagocytic activity (PA) and phagocytic index (PI) tests

Phagocytic activity and phagocytic index was determined by mixing 20 μl haemolymph and 20 μl of PBS in a micro well-plate. The mixture then were added with 20 μl of 10^8 cells ml^{-1} formalin killed *Bacillus subtilis*. Seven microliter of these mixture was then smeared gently and followed by fixing with 95% ethanol, and staining with 10% Giemsa for 20 min. The slides were then rinsed with tap water and then dried up. The slides were observed under a light microscope (*Axioskop, Zeiss, Germany*) and some photographes were taken.

2.4.3. Phenol oxidase (PO) activity test

PO activity was measured spectrophotometrically by recording the formation of dopachrome produced from L-dihydroxyphenylalanine (L-DOPA) following the procedure of [19] with some modification. Briefly, 100 μl of haemolymph was mixed with 100 μl phosphate buffer saline, then centrifuged at 700 g, 4 °C for 20 min. The pellet was then added with 100 μl of cacodylate buffer and recentrifuged at same condition. The one hundred micro liter of buffer cacodylate was then added and 100 μl of trypsin was mixed and resuspended, incubated for 10 min. Finally, 50 μl of L-DOPA was mixed. The optical density at 490 nm was measured using a spectrophotometer (*R-Biopharm Well Reader, Germany*).

2.4.4. Superoxide dismutase (SOD) test

SOD activity was measured by recording the formation of riboflavine by NBT, spectrophotometrically [20]. Briefly, 40 μl of