Aquaculture 426–427 (2014) 66–73

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aqua-online

Effects of β-glucan derivatives on the immunity of white shrimp *Litopenaeus vannamei* and its resistance against white spot syndrome virus infection

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ARTICLE INFO

Article history:
Received 28 August 2013
Received in revised form 2 January 2014
Accepted 13 January 2014
Available online 26 January 2014

Keywords:
Shrimp
Glucan
Derivative
Carboxymethylglucan
Sulfoethylglucan

ABSTRACT

Yeast β-glucan is widely used as an immunostimulant in aquaculture. However, the insolubility of β-glucan limited its immunoenhancing effects. In order to increase the solubility, in the present study, carboxymethylglucans (CMGs) and sulfoethylglucans (SEGs) were made. Both CMG and SEG had four derivatives with different degrees of substitution (DSs). Then, β-glucan and its eight derivatives were added to the diets at the contents of 0.1% or 0.2%, respectively, to prepare 18 experimental diets. Shrimps (initial average weight 0.65 g) were fed with experimental diets for 35 days, and then were sampled for immune analyses and WSSV challenging test. The results showed that the types of derivatives and their DS as well as contents in diet significantly influenced the immunity and resistance against WSSV infection of *Litopenaeus vannamei*. For the shrimps fed with CMGs, the total hemocyte count (THC), phenoloxidase (PO) activity, respiratory burst (RB), superoxide dismutase (SOD) activity and resistance against WSSV challenge significantly decreased with the increasing of DS. The DS of SEGs significantly influenced the RB, SOD activity and WSSV resistance. Diets with 0.1% β-glucan derivative resulted in higher immunity and resistance than those with 0.2% β-glucan derivative, regardless of the derivative type. Moreover, the shrimps fed with 0.1% of dietary CMG with DS 0.325 showed the highest immunity and WSSV resistance. This CMG could be a better immunostimulant for *L. vannamei*.

1. Introduction

During the last decade, the worldwide shrimp culture is threatened by viral diseases. Among the viral pathogens, white spot syndrome virus (WSSV) is highly pathogenic and fatal (Chang et al., 1998). Yeast β-glucan could reduce the mortality of shrimps challenged with WSSV (Sajeevan et al., 2009; Sukumaran et al., 2010). The reason is that β-glucan could trigger immune system of shrimps and increase their immunity (Dalmo and Bøgwald, 2008). The efficacies of yeast β-glucan have been reported using different commercially available β-glucan brands in the market (Ringø et al., 2012).

However, the efficacy of yeast β-glucan against WSSV is also limited. Sukumaran et al. (2010) reported that the survival rate of the shrimps fed with yeast β-glucan was only 4% under the challenge of WSSV. Even after optimizing the administrative does and frequency, there were still more than 60% of shrimps died. Beta-glucan is insoluble in water due to its hydroxyl. It is hard for dietary β-glucan to release from the intake feed by shrimp. So, its immunoenhancing effects to act with hemocytes after passing through intestine were limited (Šandula et al., 1995).

Making polysaccharide derivatives is an efficient way to enhance the solubility. During the derivative preparation, the hydroxyl is replaced by certain chemical groups (Wang et al., 2004). It was suggested in previous studies that the bioactivity of derivatives was higher than those of the original polysaccharides. This bioactivity was influenced by the added chemical groups and the degree of substitution (DS) (Zekovic et al., 2005). However, there is no published data on the effects of β-glucan derivatives on the survival, immune response and disease resistance of crustacean.

In the present study, β-glucan was carboxymethylated or sulfoethylated to prepare two families of derivatives. They were carboxymethylglucan (CMG) and sulfoethylglucan (SEG). Each of them had four DSs. Effects of dietary β-glucan and its derivatives on the survival, immune responses and resistance against WSSV of white shrimp *Litopenaeus vannamei* were analyzed.

2. Materials and methods

2.1. Preparation of yeast β-glucan

Yeast β-glucan was prepared by the method of Suphantharika et al. (2003) with some modifications. Briefly, dry yeast *Saccharomyces cerevisiae* (purchased from Angel Yeast Co., Ltd, Yichang, Hubei Province, China) was adjusted to 15% (w/v) solid content with deionized

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http://dx.doi.org/10.1016/j.aquaculture.2014.01.019

water and incubated at 55 °C for 24 h with agitation at 120 rpm. During the incubation, 3% sodium chloride solution was added and pH was adjusted to 5.0 (Liu et al., 2008). The autolysate was then heated at 80 °C for 15 min, cooled to room temperature and centrifuged at 4500 ×g for 10 min (Sorvall Legend RT, Germany) to yield the supernatant (yeast extract) and solid residue (yeast cell wall).

The yeast cell wall was suspended in 1 mol l^{-1} sodium hydroxide solution with a 1:5 (w/v) ratio. The mixture was heated at 90 °C for 1 h with continuous stirring and then cooled to room temperature. The mixture was centrifuged (as described above) and the supernatant was poured off. The solid residue was washed several times with deionized water until the neutral results. The wet β-glucan was lyophilized, ground and stored in 4 °C until use. The glucan content was determined according to Dallies et al. (1998).

2.2. Preparation of yeast β-glucan derivatives

2.2.1. Carboxymethylation of the β-glucan

Carboxymethylation of the β-glucan was performed with a modified procedure (Machová et al., 1995). Briefly, 10 g of β-glucan was suspended in a mixture of 12.4 ml of aqueous sodium hydroxide (300 g l^{-1}) and 125 ml of isopropanol. The suspension was vigorously stirred at 10 °C for 1 h. The subsequent procedure was then modified to prepare CMGs with different DSs. Based on the previous study on the influence of DS to the bioactivity of CMG (Šandula et al., 1995; Bao et al., 2001; Zhang et al., 2011), four DSs were set (i.e., 0.30, 0.50, 0.70 and 0.90). Accordingly, 2.25 g, 3.95 g, 7.15 g or 10.05 g of the sodium monochloroacetic acid dissolved in 14 ml of deionized water was added to prepare four CMGs with different DSs. They were named as CMGA, CMGB, CMGC and CMGD, respectively. The mixture was stirred at 70 °C for 2 h. Excessive sodium hydroxide was neutralized with 6 mol l^{-1} hydrochloric acid solution and the salts were removed by dialysis for 72 h against deionized water. The dialysis tube was purchased from Sigma-Aldrich Co. LLC (D2272) with pore size 2000 MWCO (molecular weight cut off). The non-dialyzable portion was lyophilized and stored in 4 °C until use.

The DS of the carboxymethylated β-glucan was determined by back titration (Stojanović et al., 2005). Ten grams of glucan or CMG sample were dispersed in acetone by stirring, and then converted to the acid form (H-CMG) by adding 30 ml of 6 mol l^{-1} hydrochloric acid solution with continued stirring for 30 min. The dispersion was filtered. The precipitate was washed with 80% aqueous methanol until neutral results. Then the precipitate (H-CMG) was dispersed in acetone, dried under vacuum at 50 °C and ground.

About 0.5 g of the H-CMG sample was dissolved in 20 ml of 0.2 mol l^{-1} sodium hydroxide solution, and then 50 ml of deionized water was added. The solution was transferred to a 100 ml volumetric flask, which was then filled up to the mark with deionized water. Then, 25 ml of the solution was transferred to an Erlenmeyer flask and diluted by the addition of 50–100 ml of deionized water. The excess of sodium hydroxide was back-titrated with standard 0.05 mol l^{-1} hydrochloric acid solution using phenolphthalein as the indicator. The titration was repeated three times and the average value of the hydrochloric acid solution volume was used for the calculations. A blank (only without H-CMG) was also titrated. The DS was calculated using equation:

$$DS = 162 \times N_{COOH}/(M - 58 \times N_{COOH})$$

where 162 (g mol^{-1}) is the molar mass of an anhydroglucose unit (AGU); 58 (g mol^{-1}) is the net increase in the mass of an AGU for each carboxymethyl group substituted; M (in g) is the mass of dry H-CMG sample. N_{COOH} (in mol) is the amount of COOH calculated from the obtained value of the equivalent volume:

$$N_{COOH} = (V_b - V) \times C_{HCL} \times 4$$

where V_b (in ml) is the volume of hydrochloric acid solution used for the titration of the blank; V (in ml) is the volume of hydrochloric acid solution used for titration of the sample; C_{HCL} (in mol ml^{-1}) is the hydrochloric acid solution concentration and 4 is the ratio of the total solution volume (100 ml) and the volume taken for titration (25 ml).

2.2.2. Sulfoethylation of the glucan

Sulfoethylation of the glucan was performed using the modified procedure (Šandula et al., 1995). Briefly, suspension of 10 g glucan in 12.5 ml of 2-propanol was stirred and slowly mixed with 15.5 ml of 30% sodium hydroxide solution at 10 °C for 1 h. Then, 2.75 g, 4.50 g, 8.05 g or 10.90 g of sodium 2-chloroethane sulfonate was added and kept at 70 °C for 3 h, respectively, to prepare four SEGs with different DSs (i.e., 0.20, 0.45, 0.65 and 0.90). They were named as SEGA, SEGB, SEGC and SEGD, respectively. The resulting mixture was neutralized with acetic acid (10% in ethanol), dialyzed (as describe above) against deionized water for 72 h, concentrated and lyophilized.

The DS was calculated according to Zhang et al. (2011). The calculation equation was as follow:

$$DS = (S\% \times 9)/(C\% \times 4 - S\% \times 3)$$

where S% and C% are contents of sulfur and carbon, respectively, determined by elemental analysis (CHNS/O Analyzer, Vario EI III, Perkin Elmer).

2.3. Solubility of β-glucan and its derivatives

Solubility of β-glucan and its derivatives were measured according to Byun et al. (2008). Briefly, 2 g of sample was put into a 50-ml glass tube with a cap, vortexed with 10 ml deionized water for 20 min, and centrifuged at 3500 ×g for 20 min (Sorvall Legend RT, Germany). The supernatant was dried at 105 °C. Then, the weight of the dried products was determined. The solubility was calculated as follows:

$$\text{Solubility}\ (\%) = \text{Weight of the dried supernatant}\ (g)\ /\text{Weight of initial sample}\ (g) \times 100.$$

2.4. Feeding trial

2.4.1. Experimental diets

Based on the recommended nutrient requirements of *L. vannamei* (Shiau, 1998), a basal diet was formulated without immunostimulants

Table 1
Composition of the basal diets (as percentage dry weight).

Ingredients	Percentage
Fish meal [1]	25
Shrimp head meal	5
Peanut meal	14
Squid visceral meal	5
Soybean meal	18
Fish oil	1
Soy lecithin	2
Wheat flour	27.58
Choline chloride (50%)	0.30
$Ca(H_2PO_4)_2$	0.37
Vitamin premix [2]	0.50
Mineral premix [2]	1
Antimycin [2,3]	0.10
Molt hormone [2]	0.10
Ethoxyquin [2]	0.05

[1] Crude protein 67.5% (dry weight basis), crude lipid 7.8% (dry weight basis).
[2] Provided by Qingdao Master Biotechnology Co. Ltd, Qingdao, China.
[3] Contained 50% calcium propionic acid and 50% fumaric acid.