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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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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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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.

(Table 1). Beta-glucan and its eight derivatives were added to the basal diet at two doses: 0.1% and 0.2%, respectively, to prepare 18 experimental diets. The basal diet was used as a control. All the dietary ingredients were ground into a fine powder through a 149 μm mesh sieve, and mixed thoroughly with fish oil and cold water. Then the mixture was passed through an extruder with a 1.5 mm die to produce 'spaghetti-like' strings. After drying, the diets were cut into the appropriate pellet and stored at −20 °C.

2.4.2. Animals and experimental procedure

Shrimp juveniles were obtained from a commercial farm in Zhanjiang, Guangdong, China and acclimated in a re-circulated seawater system for 2 weeks. Then, 2280 shrimps (initial mean weight 0.65 ± 0.05 g) were randomly distributed to 76 tanks (200 l). Four tanks were used as four replicates per treatment. There were 30 shrimps in each tank. The shrimps were fed to apparent satiation four times daily at 06:00, 12:00, 18:00 and 24:00, respectively. During the 35-day feeding trial, water temperature was maintained at 29–31 °C, pH 7.8–8.2, and salinity 30. Based on the previous study on the optimal feeding strategy for *L. vannamei* (Bai et al., 2010), the shrimps were fed with dietary immunostimulants (β-glucan or one of its derivatives) for two days and then with basal diet for five days alternately, and so on.

At the end of the feeding trial, all shrimps were weighted and five shrimps in the intermolt stage from per tank were randomly chosen for immune parameter assay. For each shrimp, 100 μl hemolymph was withdrawn from the ventral sinus with a 1-ml sterile syringe containing 900 μl anticoagulant solution (30 mmol l^{-1} trisodium citrate, 10 mmol l^{-1} EDTA, 0.34 mmol l^{-1} sodium chloride 0.12 mmol l^{-1} glucose, adjust pH to 7.55 and osmotic pressure to 780 m Osm kg^{-1}). The hemolymph from five shrimps per tank was pooled as a replicate for immune parameter determination. There were four replicates in each treatment. The molt stage was determined by the examination of uropoda, in which partial retraction of the epidermis could be distinguished (Robsertson et al., 1987).

The challenge test was conducted by feeding with WSSV-infected shrimp tissue (Tan et al., 2001; Escobedo-Bonilla et al., 2006). Briefly, the WSSV filtrate was prepared from the hemolymph of WSSV-infected shrimps. The hemolymph was checked for the presence of WSSV by polymerase chain reaction (PCR) using published primers of Yoganandhan et al. (2003). Then, it was centrifuged at 3000 ×*g* for 20 min at 4 °C. The supernatant fluid was re-centrifuged at 8000 ×*g* for 30 min at 4 °C. The final supernatant fluid was filtered to prepare WSSV filtrate. After being injected with WSSV filtrate, the moribund shrimps were collected and their meat was minced, mixed, checked for the presence of WSSV.

Twenty-five shrimps per tank were challenged by feeding WSSV-infected shrimp meat at the dose of 2% of body weight (Durand et al., 2003; Rajeshkumar et al., 2009). The mortality was recorded every day until no death happened. The 7-day cumulative mortality was used to express the immune protection.

2.4.3. Immune parameter analysis

Immune parameters were measured according to Chen et al. (2012) with some modifications. First, a drop of the anticoagulant–hemolymph mixture was placed in a hemocytometer to measure the total hemocyte count (THC) under the optical microscope (XPS-BM-2GA, Shanghai BM optical institution manufactures Co. Ltd). The left hemolymph–anticoagulant mixture was equally placed into four tubes for subsequent analyses. Each tube contained more than 1000 μl of hemolymph–anticoagulant mixture, and was used to measure phenoloxidase (PO) activity, respiratory burst (RB), superoxide dismutase (SOD) activity and lysozyme (LZM) activity, respectively.

Activity of PO was measured spectrophotometrically by recording the formation of dopachrome produced from L-dihydroxyphenylalanine (L-DOPA) (Hernández-López et al., 1996). One thousand microliters of hemolymph–anticoagulant mixture was centrifuged at 800 ×*g* and 4 °C for 20 min. The supernatant was discarded, and the pellet was rinsed, re-suspended gently in 1 ml of cacodylate-citrate buffer (0.01 M sodium cacodylate, 0.45 M sodium chloride, and 0.10 M trisodium citrate; pH 7.0), and then centrifuged again. The pellet was then re-suspended in 200 μl of cacodylate buffer (0.01 M sodium cacodylate, 0.45 M sodium chloride, 0.01 M calcium chloride, and 0.26 M magnesium chloride; pH 7.0), and the aliquot of cell suspension was placed equally into two tubes. Tube A was used for measuring PO activity and Tube B was for measuring background PO activity. Therefore, each tube contained 50 μl of original hemolymph. Tube A was added 50 μl of trypsin (1 mg ml^{-1}) which served as the elicitor and was incubated for 10 min at 25–26 °C. Fifty microliters of L-DOPA was added, followed by 800 μl of cacodylate buffer 5 min later. For Tube B, trypsin was replaced by 50 μl of cacodylate buffer and other conditions were same with Tube A. After the incubation, 200 μl of the reaction liquid in the tube was placed on microplates (96-wells) and optical density at 490 nm of the shrimp's PO activity was measured using a microplate reader (Model Multiskan spectrum, Thermo, MA, Waltham, USA). The PO activity was expressed as increase of dopachrome formation per 50 μl of hemolymph.

Respiratory burst of hemocytes was quantified using the reduction of nitroblue tetrazolium (NBT) to formazan as a measure of the superoxide anions produced (Song and Hsieh, 1994). Briefly, 100 μl of diluted hemolymph–anticoagulant mixture was placed in triplicate on microplates (96-wells) previously coated with 100 μl of a poly-L-lysine solution (0.2%) to improve cell adhesion. Therefore, each plate contained 10 μl of original hemolymph. Microplates were centrifuged at 800 ×*g* for 20 min and 4 °C. Plasma was removed, and 100 μl zymosan (0.1% in Hank's balanced salt solution) was added and allowed in reacting for 30 min at room temperature. One hundred microliters of the NBT solution (0.3%) was added, and incubated for 30 min at room temperature, and then 100 μl of 100% methanol was added to stop the reaction, after which the mixture was discarded. The microplates were washed three times with 100 μl 70% methanol and air-dried for 30 min. One hundred and twenty microliters of 2 M KOH and 140 μl of dimethy sulfoxide (DMSO) were added to dissolve the insoluble formazan crystals formed by the reduction of NBT. The optical density at 630 nm for the shrimp's RB was measured in triplicate using a microplate reader (Model Multiskan spectrum, Thermo, MA, Waltham, USA). RB activity was expressed as NBT-reduction in 10 μl of hemolymph.

Superoxide dismutase (SOD) activity was measured by its ability to inhibit superoxide anion generation by xanthine and xanthine oxidase reaction system using the kit from Nanjing Jiancheng Bioengineering Institute (Bai et al., 2010). Briefly, 1000 μl of hemolymph–anticoagulant mixture (1:9) was centrifuged at 800 ×*g* and 4 °C for 20 min. Plasma was removed, and the pellet was re-suspended with 1 ml of 0.85% sodium chloride solution, and centrifuged again. The supernatant was discarded, and the pellet was re-suspended in 100 μl of distilled water at 4 °C for 15 min. A 30-μl aliquot was placed in a microplate that contained 170 μl of the reaction mixture (the amount of cell suspension and reaction mixture was based on primary experiment following the instruction book of the kit). The microplate was incubated in 37 °C for 5 min and the optical density was measured at 505 nm (Model Multiskan spectrum, Thermo, MA, Waltham, USA). A reference standard of SOD was supplied with the kit. One unit of SOD was defined as the amount required inhibiting the rate of xanthine reduction by 50%. The specific activity was expressed as SOD units ml^{-1}.

Lysozyme activity was conducted following the method described by Lin et al. (2010). Briefly, 500 μl of hemolymph–anticoagulant mixture was centrifuged at 800 ×*g* and 4 °C for 20 min. The precipitate was mixed with 1 ml (0.02%) of *Micrococcus lysodeikticus* solution (provided by the kit from Nanjing Jiancheng Bioengineering Institute). The reaction was carried out at room temperature and the absorbance at 530 nm was measured 30 s and 4.5 min after adding bacterial solution, respectively. A unit of lysozyme activity was defined as the amount of sample causing decrease in absorbance of 0.01 per min.

2.5. Statistics analysis

Results are presented as mean ± S.D.M. (standard deviation of means). Software of JMP (Version 9) was used for the statistical analyses. Firstly, one-way analysis of variance (ANOVA) and Tukey HSD test were used to compare the mean values from the all treatments. Secondly, data on CMGs or SEGs treated shrimps were submitted to two-way ANOVA. After that, Tukey HSD test was used to evaluate the influence of DS, and *T*-test was used to evaluate the influence of dietary contents of CMGs or SEGs. Finally, *T*-test was conducted to compare the highest value of each immune parameter between CMGs and SEGs treated shrimps. All tests used a significance level of $p < 0.05$.

3. Results

3.1. Degree of substitution and solubility

The yeast β-glucan (with glucan content 89.22%, protein 1.63%) was prepared in the present study and used as substrate for preparation of β-glucan derivatives. The DS of CMGA, CMGB, CMGC and CMGD was 0.325, 0.449, 0.771 and 0.945, respectively (Table 2). The DS of SEGA, SEGB, SEGC and SEGD was 0.235, 0.414, 0.652 and 0.875, respectively (Table 3).

The solubility of β-glucan and its derivatives was listed in Tables 2 and 3. Solubility of β-glucan was significantly lower than all the derivatives. For the derivatives, the solubility increased with the increasing of DS. The highest solubility for carboxymethylglucans was 86.56% (CMGD). While for sulfoethylglucans, the highest solubility was 81.70% (SEGD).

3.2. Survival of shrimp

After the 35-day feeding trial, the final body weight of shrimp was 4.25 ± 0.29 g. No significant difference in growth was detected among all treatments. Meanwhile, no dead shrimp was found during the feeding trail.

Seven days after the WSSV challenging, however, all shrimps in the control group died (Fig. 1). The cumulative mortality of shrimps fed with 0.2% of dietary β-glucan was significantly lower than those fed with 0.1% of dietary β-glucan ($p < 0.05$). However, shrimps fed with 0.1% of dietary β-glucan derivatives showed lower mortality than those fed with 0.2% of derivatives. Besides dietary contents, DS of the derivatives also significantly influenced the mortality of shrimps ($p < 0.05$). For shrimps fed with 0.1% of dietary CMGs, the mortality increased with the increasing of DS. Similar trend was found in the shrimps fed with 0.2% of dietary SEGs. The shrimps fed with 0.1% of dietary CMGA showed the lowest cumulative mortality among all groups (6.25 ± 3.61%).

Table 2
The degree of substitution, solubility and harvest rate of glucan and carboxymethylglucan (CMG).[1]

	Degree of substitution	Solubility %	Harvest rate [2]
Glucan	–	4.07 ± 0.02[a]	10.3 ± 0.06
CMGA	0.325 ± 0.012[a]	33.76 ± 0.17[b]	90.09 ± 1.15[d]
CMGB	0.449 ± 0.021[b]	41.06 ± 0.21[c]	83.41 ± 0.98[c]
CMGC	0.771 ± 0.015[c]	62.69 ± 0.32[d]	76.02 ± 1.06[b]
CMGD	0.945 ± 0.008[d]	86.56 ± 0.31[e]	67.67 ± 1.12[a]

[1] Values are expressed as means ± S.D.M (n = 10). Values in one column with the different superscript letters are significantly different ($p < 0.05$).

[2] Harvest weight of glucan is the dry weight of glucan divided by dry weight of yeast. Harvest weight of CMG is the dry weight of CMG divided by the amount of substrates (glucan and sodium monochloroacetic acid).

Table 3
The degree of substitution, solubility and harvest of glucan and sulfoethylglucans (SEG).[1]

	Degree of substitution	Solubility %	Harvest rate[2]
Glucan	–	4.07 ± 0.02[a]	10.3 ± 0.06
SEGA	0.235 ± 0.011[a]	27.67 ± 0.45[b]	89.09 ± 1.74[d]
SEGB	0.414 ± 0.021[b]	36.09 ± 0.51[c]	85.26 ± 0.98[c]
SEGC	0.652 ± 0.009[c]	62.76 ± 0.24[d]	81.33 ± 1.15[b]
SEGD	0.875 ± 0.014[d]	81.69 ± 0.21[e]	78.72 ± 1.21[a]

[1] Values are expressed as means ± S.D.M (n = 10). Values in one column with the different superscript letters are significantly different ($p < 0.05$).

[2] Harvest weight of glucan is the dry weight of glucan divided by dry weight of yeast. Harvest weight of SEG is the dry weight of SEG divided by the amount of substrates (glucan and sodium 2-chloroethane sulfonate).

3.3. Immune parameters

All the analyzed immune parameters (THC, PO, RB, SOD and LZM) of shrimps fed with dietary β-glucan were significantly higher than those in the control ($p < 0.05$) (Tables 4 and 5).

Except for the effects of dietary SEGs on the THC, all the analyzed immune parameters were significantly influenced by the contents of dietary glucan derivatives ($p < 0.05$, Tables 3 and 4). All the immune parameters of shrimps fed with 0.2% of dietary glucan derivatives were lower than those fed with 0.1% of derivatives. The THC, PO activity, RB and SOD activity were significantly influenced by the DS of dietary CMGs ($p < 0.05$, Table 4). They were decreased with the increasing of DS. For the shrimps fed with dietary SEGs, only RB was significantly influenced by the DS ($p < 0.05$, Table 5). Furthermore, the RB was

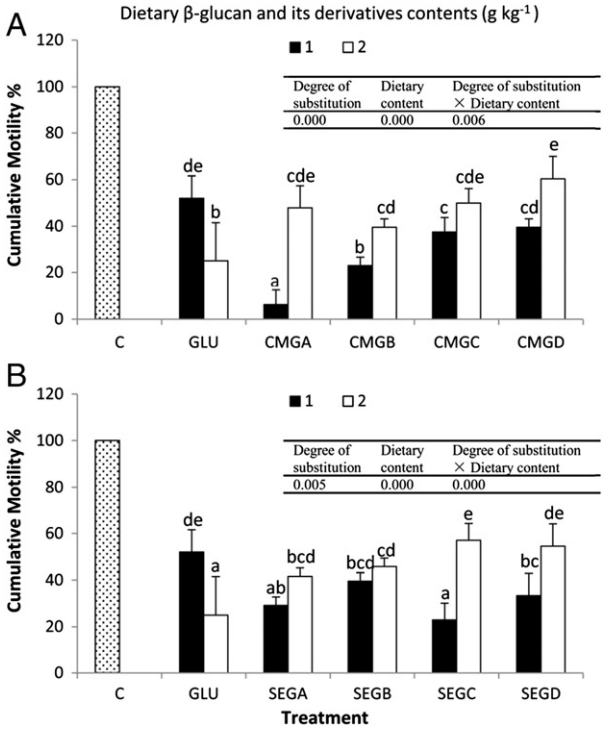

Fig. 1. Seven days cumulative mortality of *L. vannamei* fed with basal diet (C) and diets contain β-glucan (GLU), carboxymethylglucan (CMG, Fig. A) or sulfoethylglucan (SEG, Fig. B) at two contents: 0.1% (black column) and 0.2% diet (white column) for 35 days and challenged with WSSV. Each kind of β-glucan derivative has four derivatives with different degrees of substation (DSs). The DSs of CMGA, CMGB, CMBC and CMGD are 0.325, 0.449, 0.771 and 0.945, respectively. The DSs of SEGA, SEGB, SEGC and SEGD are 0.235, 0.414, 0.652 and 0.875, respectively. Values are expressed as means ± S.D.M (n = 4). Different letters in the same figure (A or B) mean that the values are significantly different with each other. The table in the figure lists the effects of degree of substitution, dietary contents and their interaction of CMG or SEG on the cumulative mortality after challenge. All the comparisons were conducted by Tukey HSD tests, using a significance level of $p < 0.05$.

Table 4
Effects of degree of substitution and dietary contents of carboxymethylglucan on the immune responses of white shrimp *Litopenaeus vannamei*.[1]

Treatment	Degree of substitution	Dietary contents (%)	THC	PO activity	RB	LZM activity	SOD activity
Individual treatment means (one-way ANOVA) [2]							
C			1.52^{a}	0.023^{a}	0.171^{a}	1.27^{a}	14.90^{a}
Glu0.1		0.1	2.06^{bc}	0.033^{bc}	0.241^{b}	2.12^{b}	16.43^{b}
Glu0.2		0.2	2.10^{c}	0.039^{cd}	0.360^{cd}	2.53^{b}	18.54^{bcd}
CMGA0.1	0.325	0.1	2.62^{d}	0.052^{e}	0.418^{d}	2.95^{c}	21.84^{d}
CMGA0.2	0.325	0.2	2.24^{c}	0.040^{cd}	0.408^{d}	2.23^{b}	18.87^{bcd}
CMGB0.1	0.449	0.1	2.26^{c}	0.047^{de}	0.389^{d}	2.38^{bc}	20.89^{cd}
CMGB0.2	0.449	0.2	1.75^{ab}	0.033^{bc}	0.384^{d}	2.17^{bc}	18.93^{bcd}
CMGC0.1	0.771	0.1	2.10^{c}	0.037^{c}	0.361^{cd}	2.48^{bc}	20.31^{cd}
CMGC0.2	0.771	0.2	1.65^{a}	0.036^{c}	0.288^{bc}	2.07^{b}	17.70^{abc}
CMGD0.1	0.945	0.1	2.05^{bc}	0.033^{bc}	0.365^{cd}	2.63^{bc}	18.38^{bc}
CMGD0.2	0.945	0.2	1.53^{a}	0.024^{ab}	0.253^{ab}	2.10^{b}	16.82^{ab}
Pooled S.E.M			0.06	0.002	0.017	0.14	0.66
Mean of main effect (two-way ANOVA) [3]							
	0.325		2.43^{z}	0.046^{z}	0.413^{y}	2.59	20.36^{y}
	0.449		2.02^{y}	0.040^{y}	0.386^{y}	2.28	19.91^{y}
	0.771		1.88^{xy}	0.036^{x}	0.324^{x}	2.28	19.00^{xy}
	0.945		1.80^{x}	0.028^{x}	0.309^{x}	2.37	17.60^{x}
		0.1	2.26^{q}	0.042^{q}	0.383^{q}	2.61^{q}	20.35^{q}
		0.2	1.80^{p}	0.033^{p}	0.333^{p}	2.14^{p}	18.08^{p}
p-Value (two-way ANOVA)							
Degree of substitution			0.000	0.000	0.000	0.162	0.010
Dietary contents			0.000	0.001	0.001	0.000	0.000
Degree of substitution × dietary contents			0.729	0.201	0.018	0.436	0.784

[1] Shrimps were fed with basal diet (C) and diets contain β-glucan (GLU) or carboxymethylglucan (CMG) at two contents for 35 days. Abbreviation and unit: THC, total hemocytes counts, $\times 10^{7}$ cells ml^{-1}; PO, phenoloxidase, O.D. 490 nm; RB, respiratory burst, O.D. 630 nm; LZM, lysozyme, units ml^{-1}; SOD, superoxide dismutase, units ml^{-1}.
[2] Values represent the mean of four replicates per treatment. Subscription letters (a–f) indicate significant difference ($p < 0.05$) as determined by Tukey HSD test.
[3] Means followed by the different letters are significantly different ($p < 0.05$) as determined by Tukey HSD test for degree of substitution (x–z) or by *T*-test for dietary contents (p,q).

decreased with the increasing of DS. In all groups, the highest values of THC ($2.62 \pm 0.03 \times 10^{7}$ cells ml^{-1}), PO activity (O.D.490 nm 0.052 ± 0.010), SOD activity (21.84 ± 0.22 units ml^{-1}) and LZM activity (2.95 ± 0.23 units ml^{-1}) were found in the shrimps fed with 0.1% of dietary CMGA. The highest RB (O.D.630 nm 0.458 ± 0.032) was found in the shrimps fed with 0.1% of dietary SEGA. The THC and SOD activity of shrimps fed with dietary CMGs were significantly higher than those fed with dietary SEGs ($p < 0.05$, Table 6).

Table 5
Effects of degree of substitution and dietary contents of sulfoethylglucan on the immune responses of white shrimp *Litopenaeus vannamei*.[1]

Treatment	Degree of substitution	Dietary contents (%)	THC	PO activity	RB	LZM activity	SOD activity
Individual treatment means (one-way ANOVA) [2]							
C			1.52^{a}	0.023^{a}	0.171^{a}	1.27^{a}	14.90^{a}
Glu0.1		0.1	2.06^{b}	0.033^{bc}	0.241^{b}	2.12^{bc}	16.43^{b}
Glu0.2		0.2	2.10^{b}	0.039^{c}	0.360^{cde}	2.53^{bcde}	18.54^{bc}
SEGA0.1	0.235	0.1	1.62^{a}	0.041^{c}	0.458^{f}	2.73^{de}	20.77^{de}
SEGA0.2	0.235	0.2	1.57^{a}	0.026^{ab}	0.407^{ef}	2.08^{b}	18.66^{cd}
SEGB0.1	0.414	0.1	1.60^{a}	0.039^{c}	0.413^{ef}	2.70^{cde}	20.03^{cde}
SEGB0.2	0.414	0.2	1.56^{a}	0.029^{b}	0.393^{def}	2.18^{bcd}	18.76^{cd}
SEGC0.1	0.652	0.1	1.56^{a}	0.040^{c}	0.388^{def}	2.73^{de}	21.36^{e}
SEGC0.2	0.652	0.2	1.36^{a}	0.032^{b}	0.351^{cde}	2.30^{bcde}	19.34^{cde}
SEGD0.1	0.875	0.1	1.57^{a}	0.041^{c}	0.311^{bcd}	2.80^{e}	20.53^{cde}
SEGD0.2	0.875	0.2	1.56^{a}	0.033^{b}	0.288^{bc}	2.15^{bcd}	18.67^{cd}
Pooled S.E.M			0.08	0.001	0.017	0.12	0.42
Mean of main effect (two-way ANOVA)[3]							
	0.235		1.59	0.034	0.433^{z}	2.41	19.71
	0.414		1.58	0.034	0.402^{yz}	2.44	19.39
	0.652		1.56	0.036	0.369^{y}	2.52	20.35
	0.875		1.46	0.037	0.299^{x}	2.48	19.61
		0.1	1.59	0.040^{q}	0.392^{q}	2.74^{q}	20.67^{q}
		0.2	1.51	0.030^{p}	0.360^{p}	1.80^{p}	18.86^{p}
ANOVA: p-value (two-way ANOVA)							
Degree of substitution			0.456	0.076	0.000	0.821	0.208
Dietary contents			0.252	0.000	0.015	0.000	0.000
Degree of substitution × dietary contents			0.726	0.100	0.781	0.753	0.787

[1] Shrimps were fed with basal diet (C) and diets contain β-glucan (GLU) or sulfoethylglucan (SEG) at two contents for 35 days. Abbreviation and unit: THC, total hemocytes counts, $\times 10^{7}$ cells ml^{-1}; PO, phenoloxidase, O.D. 490 nm; RB, respiratory burst, O.D. 630 nm; LZM, lysozyme, units ml^{-1}; SOD, superoxide dismutase, units ml^{-1}.
[2] Values represent the mean of four replicates per treatment. Subscription letters (a–f) indicate significant difference ($p < 0.05$) as determined by Turkey HSD test.
[3] Main effect means followed by the different letter are significantly different ($p < 0.05$) as determined by Tukey HSD test for degree of substitution (x–z) or by *T*-test for dietary contents (p, q).

Table 6
The comparison of carboxymethylglucan and sulfoethylglucan.[a]

Parameters	Highest value in all CMGs groups	Highest value in all SEGs groups	Probability
THC ($\times 10^7$ cells ml^{-1})	2.62 ± 0.03	1.61 ± 0.09	0.000*
PO activity (O.D. 490 nm)	0.052 ± 0.010	0.041 ± 0.008	0.210
RB (O.D. 630 nm)	0.418 ± 0.017	0.458 ± 0.033	0.135
LZM activity (units ml^{-1})	2.95 ± 0.23	2.73 ± 0.43	0.489
SOD activity (units ml^{-1})	21.84 ± 0.22	20.76 ± 0.61	0.046*
	Lowest value in all CMGs groups	Lowest value in all SEGs groups	Probability
Cumulative mortality (%)	6.25 ± 3.61	22.91 ± 7.21	0.039*

[a] Values labeled with * are statistically significant at $p < 0.05$. Abbreviation: CMG, carboxymethylglucan; SEG, sulfoethylglucan; DS, degree of substitution; THC, total hemocyte counts; PO, phenoloxidase; RB, respiratory burst; LZM, lysozyme; SOD, and superoxide dismutase.

4. Discussion

4.1. Glucan derivative preparation

In the present work, four CMGs and four SEGs were made with different DSs. Each kind has four derivatives with increasing DS. The chemical reactions to prepare derivatives were based on the Williamson's ether synthesis, whereby the polysaccharide alkoxides were substituted by carboxymethyl or sulfoethyl groups under alkaline condition. There are two steps of this reaction. The first step is an equilibrium reaction between sodium hydroxide and the hydroxyl groups. And the second step is the actual formation of the carboxymethyl group or sulfoethyl group by the substitution. The amount of formed chemical groups is indicated by DS, which is defined as the average number of substituents per anhydroglucose unit, the monomer unit of β-glucan. According to researches on the β-glucan from *Ganoderma Lucidum* (Zhang et al., 2001) and *Poria cocos* (Wang et al., 2009), the DS was determined by several reaction parameters, such as molar ratio of reagents, reaction time, reaction temperature and ratio of organic solvent to water. In the present work, many pre-tests were conducted first and all the parameters were fixed, except for the amount of sodium chloroacetate or sodium 2-chloroethane sulfonate. Preparation of the β-glucan derivatives with different DSs was performed by increasing the amount of sodium chloroacetate or sodium 2-chloroethane sulfonate.

4.2. Comparison of carboxymethylglucan and sulfoethylglucan

Mucksová et al. (2001a) observed that both CMG and SEG could enhance the reductase activity of neutrophils of piglets after weaning. Moreover, CMG showed greater enhancing effect than SEG. However, when comparison was investigated in mitogenic and co-mitogenic tests on rat thymocytes, SEG was more active than CMG (Šandula et al., 1995). Slameňová et al. (2003) also found that SEG's ability to reduce oxidative DNA damage, which was induced by H_2O_2 and visible light-excited methylene blue in V79 hamster lung cells, was higher than CMG. However, Mucksová et al. (2001b) showed that there is no significant difference between the impacts of CMG and SEG on the function (peroxidase activity and nitric oxide synthesis) of murine peritoneal adherent cells. It can be concluded from above that the comparison of CMG and SEG depended on the experimental animal (or cell line) and analyzed parameters. The comparison of CMG and SEG in the present study also has its own feature. Among the four CMGs, only the CMGA showed the significantly higher immune protection than β-glucan. Meanwhile, all the SEGs did not showed significantly higher protection than β-glucan. The THC of shrimps fed with SEGs, regardless of DS or dietary contents, was not significantly higher than that of control. This means that SEG, dislike CMG and β-glucan, cannot active the proliferation of hemocytes. In penaeid shrimp, circulating hemocytes play crucial roles in the innate immune system and are involved in a pattern recognition, phagocytosis, reactive oxygen species formation, prophenoloxidase activating system, encapsulation, nodule formation, and the release of antimicrobial peptides and lysozyme (Jiravanichpaisal et al., 2006). With more hemocytes, the shrimps had more immune substances, which finally lead to the lower mortality after challenge.

4.3. The effects of substitution degree

The introduction of ionic groups to polysaccharide would change the intramolecular and intermolecular hydrogen bonding and strengthen the effect of electrostatic repulsion, which enabled the polymer chain to adopt a certain structure (Adachi et al., 1989; Zhang et al., 2001). After chemical modification, the structure of polysaccharides changed and the structure differed with the changing of DS (Zhang et al., 2001, 2004). The solubility and bioactivity also changed with the changing of the polysaccharide structure (Bohn and BeMiller, 1995). The DS, as the reflection of polysaccharide derivatives structure, is the most important factor to determine the solubility and bioactivity of polysaccharide derivatives (Zekovic et al., 2005). However, it is difficult to draw a conclusion on the optimal DS for β-glucan derivatives. Šandula et al. (1995) prepared five CMGs with different DSs (0.56, 0.75, 0.89, 1.08 and 1.15). The results showed that the CMG with 0.75 of DS exhibited the highest activity in mitogenic and co-mitogenic tests on rat thymocytes. Furthermore, the CMGs with DS more than 1.00 were inactive. Bao et al. (2001) prepared seven CMGs with the DS ranging from 0.17 to 1.44, and then they investigated the impacts of CMGs on lymphocyte proliferation and antibody production in vitro and in vivo. The results showed that CMGs with lower DS (<0.28) had higher immunostimulating activity than those with higher DS. Similarly, Zhang et al. (2001) also found that the CMG with lower DS (0.38) showed higher antitumor activity against Ehrlich ascites carcinoma than those with higher DS (0.43, 0.54). For the candidate optimal DS of CMG in the previous studies, the lowest one was 0.17 and the highest one was 1.44 (Bao et al., 2001). The experimental animals in those literatures were not aquatic. Meanwhile, the present experimental condition is limited. So four medium candidate DSs (0.325, 0.449, 0.771 and 0.945) were chosen between 0.17 and 1.44. Though the designed DS levels could be lower or higher, the present results still provided some valuable information. Carboxymethylated β-glucan was able to enhance the immunity of shrimp. Meanwhile, further work is needed to clarify whether the CMG with the DS less than 0.325 had higher efficacy in shrimp.

In mice, the different mechanisms for glucans with different solubility have been analyzed. Qi et al. (2011) reported the different pathways of particle/insoluble β-glucan and soluble β-glucan to regulate the immune system. Moreover, Hino et al. (2012) reported that the particle β-glucan could be phagocytized and solubilized by the macrophage cells. Furthermore, they pointed out that reactive oxygen species (ROS) played a very important role in the solubilizing of β-glucan. Fuentes et al. (2011) also proved that the phagocytizing and solubilizing of particle β-glucan could be inhibited by soluble β-glucan in a dose-depended manner. Though there was no report on how the immune system of shrimps to response to the β-glucans with different solubility, results in the present study could be explained by the mechanisms

reported in some previous studies. With the increasing of DS, the proportion of the soluble glucan increased. Compared with the β-glucan without modification, soluble glucan and insoluble glucan in the modified β-glucan with lower DS could active different parts of immune systems. In the present study, values of THC, PO activity, RB, LZM activity and SOD activity in CMG with 0.325 of DS were higher than those in the treatment of β-glucan (Table 4). However, the increasing amount of the soluble glucan might inhibit the insoluble glucan to react with the immune system. In the present study, values of the analyzed immune parameters decreased with the increasing DS of CMG (Table 4). Further study is needed to clarify the mechanisms of β-glucan derivatives with different DSs reacting with the immune system of shrimp.

4.4. The effects of dietary derivatives contents

The present study showed that dietary contents of β-glucan or its derivatives significantly affected the immunity and resistance against WSSV of *L. vannamei*. As can be seen from previous study, 0.2% was the most common contents of dietary β-glucan for shrimps (Chang et al., 1999; Chang et al., 2000; Thanardkit et al., 2002; Anas et al., 2009). Sajeevan et al. (2009) proved that the post-challenge survival of shrimps fed dietary β-glucan at the dose of 0.2% was higher than those fed with higher (0.3% or 0.4%) or lower (0.05 or 0.1%). The present study confirmed that 0.2% of dietary glucan is better than that of 0.1% to stimulate the immune system of shrimp. However, for the β-glucan derivatives, the better content is 0.1%. Because of higher solubility of β-glucan derivatives, it can be assumed that it is easier for derivatives than β-glucan to leave from the feed, pass through the intestine of shrimps and react with the immune system of shrimps. Studies on mice showed the general route of dietary β-glucans to host immune system (Hong et al., 2004). Beta-glucans were first captured by the intestinal macrophages and then were internalized and fragmented into smaller sized β-glucan fragments within the macrophages. After releasing from macrophages, small β-glucan fragments were eventually taken up by the circulating granulocytes, monocytes or macrophages and turned on the immune response (Hong et al., 2004). Moreover, special route for dietary soluble glucans was found by Rice et al. (2005). Besides macrophages, intestinal epithelial cells and gut associated lymphoid tissue cells can also bind and internalize the soluble β-glucans and transfer them to circulating system. Further work is needed to investigate whether the absorbance efficiency of β-glucan increases with the increasing of its solubility in *L. vannamei*.

5. Conclusion

The present study proved that under the optimal feeding strategies, dietary β-glucan could keep the immunity of shrimps at high level up to one month and efficiently protect the shrimps against the WSSV challenge. Beta-glucan derivatives could provide even greater protection than β-glucan. Meanwhile, the added chemical group, DS and dietary contents all significantly affected their protective effects. The shrimps fed dietary CMG with 0.325 of DS at the contents of 0.1% showed the highest immunity and the lowest mortality after challenge. This CMG has the potentialities to be a more efficient immunostimulant for *L. vannamei*.

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