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# Alkali insoluble glucan extracted from *Acremonium diospyri* is a more potent immunostimulant in the Indian White Shrimp, *Fenneropenaeus indicus* than alkali soluble glucan

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## Abstract

Effect of an extraction method on the structure of glucan and its immunostimulatory response in *Fenneropenaeus indicus* was investigated. Here we extracted alkali insoluble glucan (AIG) and alkali soluble glucan (ASG) from a filamentous fungi *Acremonium diospyri* following alkali–acid hydrolysis and the sodium hypochlorite oxidation and dimethyl sulphoxide extraction method respectively. Structural analysis showed that 85% of glucan in AIG was a (1 → 3)-β-D-glucan and it increased the prophenoloxidase and reactive oxygen intermediate activity when administered to *F. indicus*. On the other hand, ASG, which contained 93% (1 → 3)-α-glucan, did not induce significant immune response in shrimp. Here we report that the difference in immunostimulatory potential between AIG and ASG is due to the difference in the percentage of (1 → 3)-β-D-glucans present in each preparation, which varies with the method of extraction employed. Also our observations suggest that glucan can be used as a potential immunostimulant to shrimp, provided it contains (1 → 3)-β-D-glucan as the major fraction.

**Keywords:** glucan, *Fenneropenaeus indicus*, immunostimulant, prophenoloxidase, reactive oxygen intermediate, *Acremonium diospyri*

## Introduction

β-Glucans are widely used in aquaculture to stimulate the non-specific immune system of animals in order to indirectly increase resistance to pathogenic invasions and plausibly for growth enhancement (Williams 1997; Dalmo 2000; Chang, Su, Chen & Liao 2003; Leung, Liu, Koon & Fung 2006; Dalmo & Bogwald 2008; Mar Costa, Novoa & Figueras 2008). β-Glucans have a common structure, a main chain consisting of (1 → 3)-linked β-D-glucopyranosyl units along which are randomly dispersed single β-D-glucopyranosyl units attached by (1 → 6)-linkages giving a comb-like structure. The non-specific immune system of crustaceans identifies β-glucan as a pathogen-associated molecular pattern, and initiates the recognition of these molecules by specific 'pathogen recognition receptors' (Medzhitov & Janeway Jr 1997), β-glucan-binding proteins (Roux, Pain, Klimpel & Dhar 2002; Cheng, Liu, Tsai & Chen 2005). The binding of pathogen-associated molecular patterns with its specific receptor initiates an array of immune response in the host animals, including clotting cascade, the synthesis of a wide array of antimicrobial peptides and the phenoloxidase (PO)-activating system (Hoffmann, Reichart & Hetru 1996; Sritunyalucksana & Soderhall 1999). Chang, Chen, Su and Liao (2000) evaluated the effectiveness

of a water soluble β-(1 → 3)-glucan derived from *Schizophyllum commune* in enhancing shrimp survival as well as haemocyte phagocytosis and superoxide anion production in brooders of *Penaeus monodon*. The brooders showed enhanced survival rate, haemocyte phagocytic activity, cell adhesion and superoxide anion production when glucan was administered through feed. Additionally, glucans reportedly protected shrimps from whitespot syndrome virus (WSSV) and *Vibrio* infections (Sung, Kou & Song 1994; Song, Liu, Chan & Sung 1997; Chang *et al.* 2003; Dalmo & Bogwald 2008). Although glucans are reported as a specific activator of prophenoloxidase (proPO) enzyme cascade in several investigations, Sritunyalucksana, Sithisarn, Withayachumnarnkul and Flegel (1999) did not observe any change in the proPO, agglutinin and antibacterial activities in *P. monodon* on administering glucan under *in vivo* and *in vitro* conditions, the reason for which is not known.

The available evidence indicates that the physicochemical properties of glucan such as structure, molecular weight, degree of branching and solution conformation are the major determinants of its immunostimulatory activity in animals (Falch, Espevik, Ryan & Stokke 2000; Tokunaka, Ohno, Adachi, Tanaka, Tamura & Yadomae 2000; Kimura, Sumiyoshi, Suzuki, Suzuki & Sakanaka 2007). The physicochemical properties vary considerably with the source and the extraction protocol employed (Ishibashi, Miura, Adachi, Tamura, Tanaka & Ohno 2004). Remarkably, there is little information available on the relationship between the physicochemical properties of glucan and the induction of immune response in shrimp. Majority of the methods developed for glucan extraction are based on the alkali–acid hydrolysis (Misaki, Johnson, Kirkwood, Scaletti & Smith 1968; Williams, McNamee, Jones, Pretus, Ensley, William Browder & DiLuzio 1991; Muller, Ensley, Pretus, McNamee, Jones, McLaughlin, Chandley, Browder, Lowman & Williams 1997; Leung *et al.* 2006). The alkali insoluble glucan (AIG) extracted by this method is chemically pure with no other carbohydrates, proteins or residual lipids (Muller *et al.* 1997). Solubility of drugs being a pharmacological need for effective administration, an alkali soluble glucan (ASG) was prepared using sodium hypochlorite oxidation and dimethyl sulphoxide (NaClO–DMSO) extraction from the cell wall of yeast and fungi (Ohno, Uchiyama, Tsuzuki, Tokunaka, Miura, Adachi, Aizawa, Tamura, Tanaka & Yadomae 1999). Ohno *et al.* (1999) reported that the glucan extracted by this method was structurally similar to the ones extracted by alkali–acid hydrolysis. Here we report that difference in physicochemical properties and immunostimulatory response does exist between the glucan samples extracted from *Acremonium diospyri* using alkali–acid hydrolysis and the NaClO–DMSO extraction methods. Here we selected *A. diospyri* as the source of glucan, considering its comparatively higher glucan yield. Also, previous studies in our lab had demonstrated that glucan from *A. diospyri* significantly enhanced the survival rate of *Macrobrachium rosenbergii* larvae (Vici, Singh & Bhat 2000; Anas & Singh 2003).

We extracted AIG and ASG following alkali acid hydrolysis and the NaClO–DMSO extraction method respectively. Structure of AIG and ASG were compared using proton nuclear magnetic resonance (NMR) spectra and their immunostimulatory response in *Fenneropenaeus indicus* was quantified using haemocyte count, proPO and reactive oxygen intermediate (ROI) assays. Finally, the differences in the immunostimulatory response of AIG and ASG were correlated with their structure and the extraction protocol used.

## Materials and methods

### Production of *A. diospyri* biomass

*Acremonium diospyri* (MTCC 1316) was obtained from the Microbial Type Culture Collection at Institute of Microbial Technology, Chandigarh, India. It was inoculated into 500 mL Sabouraud's dextrose broth (dextrose 20 g; mycological peptone 10 g; distilled water 1000 mL, pH 6.5 ± 0.2, autoclaved at 110 °C for 10 min) and incubated at 28 ± 1 °C for 30 days. On incubation, it developed into a mucilaginous mat on top of the medium and started settling down on attaining maximum growth and weight. Culture broth was incubated for 30 days for developing several such mats, the biomass was separated by filtering through a muslin silk and washed twice with phosphate-buffered saline ($NaH_2PO_4$ 6.42 g; $Na_2HPO_4$ 34.316 g; NaCl 10 g; distilled water 1000 mL; pH 7.5 ± 0.2), dried at 80 °C for 48 h in a hot air oven and the weight stabilized in a desiccator over silica gel at room temperature.

### Extraction of glucan from *A. diospyri*

Alkali insoluble glucan and ASG were prepared from *A. diospyri* following the methods of Williams *et al.* (1991) and Ohno *et al.* (1999), respectively, with minor

modifications. Alkali insoluble glucan was prepared as follows: 1 g dried *A. diospyri*, suspended in 20 mL 3% (w/v) aqueous sodium hydroxide (HiMedia, Mumbai, India) was refluxed at 100 °C for 6 h in a serological water bath. Filtering through muslin silk and re-extracting with aqueous sodium hydroxide resulted in separation of alkali insoluble material. The insoluble material was again separated and extracted with 20 mL 0.5 M acetic acid (SRL, Mumbai, India) at 75 °C for 6 h. The resultant insoluble residue was separated by filtration through muslin silk and refluxed repeatedly with ethanol until the filtrate became colourless. The remaining precipitate was washed with distilled water and vacuum dried over silica gel at 28 ± 1 °C and designated as AIG.

Alkali soluble glucan was isolated by the NaClO–DMSO extraction method (Ohno *et al.* 1999). Briefly, 1 g dried *A. diospyri* biomass was suspended in 100 mL 0.1 M NaOH and 12.5 mL of the NaClO (Merck, Mumbai, India) oxidizing agent was added immediately, incubated for 1 day at 4 °C in a glass-stoppered conical flask and filtered through muslin silk to separate the insoluble fraction. The insoluble fraction was washed with distilled water, dried first in ethanol and then in acetone (HiMedia), and recovered by decanting the fluid. The recovered insoluble fraction was macerated with DMSO (Merck) and extracted for 60 min at 90 °C with intermittent sonication at full power in an ultrasonicator (VCX500, Sonics, Newtown, CT, USA). From this suspension, the insoluble fraction was specifically removed by centrifugation at 1000 *g* for 15 min. By treating the supernatant with four volumes of ethyl alcohol, glucan was precipitated from the soluble fraction and designated as ASG.

### NMR spectroscopy

The proton NMR spectra of AIG and ASG were recorded on a JEOL Model Eclipse+600 NMR spectrometer (Peabody, MA, USA) in 5 mm OD NMR tubes at 80 °C with perdeuterated DMSO (DMSO-$d_6$) as the solvent. A few drops of trifluoroacetic acid-d were added to the solution to shift the resonance from the exchangeable protons downfield (Ross & Lowe 2000). Spectra were collected for either 4 or 24 h depending on the amount of isolate available.

### Immunostimulatory potential of AIG and ASG

The immunostimulatory potential of AIG and ASG was assessed using *F. indicus* as the target animal. The experimental diets were prepared by surface coating the commercially available pellet feed (Higashimaru, Cochin, India) with 0.2% (w/w) AIG and ASG separately. Sixty animals were maintained in six tanks with 10 animals each. Animals maintained in two tanks were fed AIG-coated pellets while animals in a second set of two tanks were fed ASG-coated feed for 7 days. The remaining animals in two tanks were fed control diet (without glucan) during the same period. The experimental animals were returned to normal diet (without glucan) after completing the experimental feeding. Haemolymph of the animals from one tank in each feeding regimen was withdrawn on the first day following cessation of the experimental feeding for haematological assays. Haemolymph from the remaining animals of each feeding regimen was withdrawn on sixth day following cessation of the experimental feeding for haematological assays.

### Haemolymph collection

Haemolymph was collected from the rostral sinus situated beneath the rostral spine and between the eyestalks by inserting a capillary tube, after drying this region with sterile cotton swabs. The withdrawn haemolymph was transferred into 2 mL capacity microcentrifuge tubes each pre-filled with 200 µL ice cold anticoagulant (Tris HCl 0.01 M, sucrose 0.25 M, trisodium citrate 0.1 M prepared in double distilled water, autoclaved and adjusted to pH 7.6) (Song & Hsieh 1994).

### Haematological assays

The collected haemolymph was subjected to haematological assays such as haemocyte count and activities of proPO and ROI. The total haemocyte count was determined using Neubaeur's haemocytometer (Perazzolo & Barracco 1997). The proPO activity was estimated spectrophotometrically by measuring the formation of dopachrome from L-3-4-dihydroxyphenylalanine at 490 nm (Smith & Soderhall 1983; Chang *et al.* 2000). Reactive oxygen intermediate activity was measured based on the ability of haemocytes to adhere to plastic centrifuge tube and to reduce nitroblue tetrazolium chloride to formazan (Munoz, Cedeno, Rodriguez, Van der Knaap, Mialhe & Bachere 2000). The proPO activity and ROI were expressed as absorbance per milligram haemolymph protein per minute and absorbance per milligram

haemocyte protein respectively. Total proteins in haemolymph and haemocytes were estimated using the Bradford method with bovine serum albumin standard (Bradford 1976).

### Statistical analysis

All results were statistically analysed using one-way analysis of variance, and significant differences were established at the 95% confidence interval, $P < 0.05$ (Bailey 1995).

## Results

The insoluble (AIG) and soluble (ASG) glucans extracted from *A. diospyri* had markedly different physical properties. The AIG was only partially soluble in DMSO even at temperature up to 150 °C, whereas the ASG completely dissolved in DMSO at ambient temperature. This facilitated only a partial structural characterization of AIG while ASG could be analysed more completely.

Proton NMR spectra of AIG (top) and ASG (middle) are shown in Fig. 1 along with a spectrum of pure (1 → 3)-β-D-glucan (bottom) for comparison. Spectra of AIG and ASG indicate that the samples are mixtures of α- and β-(1 → 3)-D-glucan primarily. Alkali insoluble glucan (top) is a mixture containing 33% glucose and 67% glucan. The major glucan component is (1 → 3)-β-D-glucan, present at 85% of the total glucan composition, while (1 → 3)-α-D-glucan comprises the remaining 15%. Anomeric proton resonance assignments for these components are indicated in Fig. 1 (top). Alkali soluble glucan (middle) is predominantly (1 → 3)-α-D-glucan (93%) with 7% (1 → 3)-β-D-glucan. Proton resonance assignments of (1 → 3)-α-D-glucan and the anomeric proton of (1 → 3)-β-D-glucan are indicated in Fig. 1 (middle). Proton resonance assignments for (1 → 3)-β-D-glucan are indicated in Fig. 1 (bottom). Assignment of the structure for ASG was accomplished by 2D COSY, TOCSY and HMQC NMR spectra (data not shown) as well as comparison with resonance assignments for (1 → 3)-α-D-glucan extracted from *Ganoderma incidium* and characterized by Chen, Zhou, Zhang, Nakamura and Norisuye (1998). Proton resonance assignments for (1 → 3)-β-D-glucan are based on the work of Ensley, Tobias, Pretus, McNamee, Jones and Browder and Williams (1994). Chemical shift assignments for ASG, (1 → 3)-α-D-glucan from Chen *et al.* (1998) and (1 → 3)-β-D-glucan from Ensley

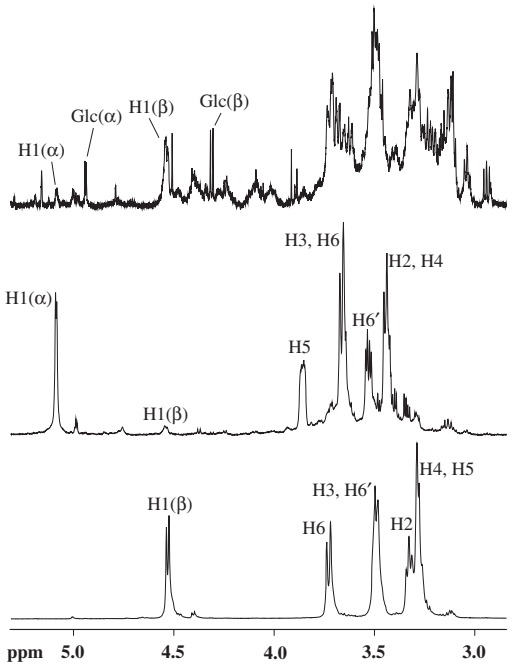


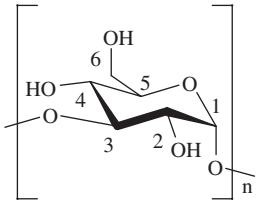

**Figure 1** Comparison of proton nuclear magnetic resonance spectra for alkali insoluble glucan (AIG; top), alkali soluble glucan (ASG; (middle) and a pure β-(1-3)-D-glucan (bottom). Anomeric proton resonance assignments for AIG (top) and ASG (middle) are indicated. See the text for a discussion of the composition of AIG and ASG.

*et al.* (1994) are summarized in Table 1. Structures for the α- and β-conformer repeat units of these glucans are shown in Table 1.

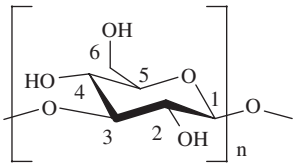
Among the three immunological parameters measured in response to feeding with AIG and ASG, significant variations were observed in the proPO and ROI activity but not in the haemocyte count (Tables 2 and 3). The group of animals fed with AIG exhibited approximately fivefold increased proPO activity over the control group and nearly twofold increase over the animals fed with ASG ($P < 0.05$) on sixth day of post-experimental feeding. No significant difference could be seen between the group fed with AIG and ASG on the first day of post-experimental feeding ($P < 0.05$). In a similar pattern on the first day of post administration of AIG and ASG, ROI did not show any significant variation between groups. However, on sixth day, the group of animals fed with AIG exhibited significantly higher ROI activity compared with the control groups and the one fed with ASG. Strikingly, no significant variation could be recorded between the control and the ASG-fed group.

**Table 1** H-1 NMR chemical shift assignments for glucan isolate alkali soluble glucan (this work) compared with the literature-reported chemical shifts for α- and β-(1-3)-D-glucans




α-Glucan




β-Glucan

| | Chemical shifts (ppm) ($^3J_{HH}$, HZ) | | |
|---|---|---|---|
| **Proton assignment** | **This work** | **(1-3)-α-D-glucan** | **(1-3)-β-D-glucan** |
| H-1 | 5.09 (3.6) | 5.13 | 4.52 (8) |
| H-2 | 3.42 | 3.40 | 3.28 |
| H-3 | 3.66 | 3.68 | 3.46 |
| H-4 | 3.45 | 3.44 | 3.25 |
| H-5 | 3.86 | 3.86 | 3.25 |
| H-6 | 3.66 | 3.64 | 3.7 (6,11) |
| H-6′ | 3.53 | 3.53 | 3.46 |
| OH-2 | 4.45 | ∼ 4.73 | 4.87 (3.5) |
| OH-4 | 4.85 | ∼ 5.28 | 4.4 |
| OH-6 | 4.15 | ∼ 4.58 | 4.29 (6) |
| Solvent | DMSO-$d_6$ | 0.25 M LiCl in DMSO-$d_6$ | DMSO-$d_6$ |
| Temperature (°C) | 80 | 60 | 80 |
| Reference | – | 32 | 33 |

Coupling constants, $^3J_{HH}$, are shown in parentheses.
DMSO-d6, perdeuterated dimethyl sulphoxide.

**Table 2** Neither AIG or ASG altered immunologic parameters 1 day following completion of the feeding schedule*

| Treatment | Haemocyte count† | ProPO‡ | ROI§ |
|---|---|---|---|
| Control | 6.83 ± 0.11 | 0.051 ± 0.02 | 0.4 ± 0.26 |
| AIG | 6.84 ± 0.19 | 0.041 ± 0.03 | 0.43 ± 0.31 |
| ASG | 6.91 ± 0.15 | 0.061 ± 0.04 | 0.35 ± 0.12 |

*Values expressed as mean ± SD, $N = 10$ group$^{-1}$.
†Log of haemocyte count.
‡Absorbance mg$^{-1}$ protein min$^{-1}$.
§Absorbance mg$^{-1}$ protein.
AIG, alkali insoluble glucan; ASG, alkali soluble glucan; proPO, prophenoloxidase; ROI, reactive oxygen intermediate.

**Table 3** AIG enhanced haematological parameters on day 6 after completion of the feeding schedule*

| Treatment | Haemocyte count† | ProPO‡ | ROI§ |
|---|---|---|---|
| Control | 6.72 ± 0.24 | 0.014 ± 0.006 | 0.423 ± 0.26 |
| AIG | 6.85 ± 0.23 | 0.083 ± 0.05¶ | 0.858 ± 0.39¶ |
| ASG | 6.93 ± 0.21 | 0.028 ± 0.013¶ | 0.412 ± 0.236 |

*Values are expressed as mean ± SD, $N = 10$ group$^{-1}$.
†Log of haemocyte count.
‡Absorbance mg$^{-1}$ protein min$^{-1}$.
§Absorbance mg$^{-1}$ protein.
¶$P < 0.05$ versus control.
AIG, alkali insoluble glucan; ASG, alkali soluble glucan; proPO, prophenoloxidase; ROI, reactive oxygen intermediate.

## Discussion

In the present study, the ASG extracted from *A. diospyri* using the NaClO–DMSO extraction method primarily contained (1 → 3)-α-linkages, while the alkali–acid hydrolysis provided AIG composed primarily of (1 → 3)-β-linkages. The immunostimulatory potential of ASG and AIG were assayed on first and sixth day following cessation of experimental feeding. This assay strategy was adopted based on our previous experience on immunization of *F. indicus* with WSSV vaccine, where the immune response was initiated on fifth day after completion of experimental feeding (Singh, Manjusha & Pai 2005). In the present study, ASG did not induce a significant immune response in *F. indicus*. However, the small increase in proPO activity observed in ASG-treated animals on sixth day of post-experimental feeding

may be due to the low per cent (7%) of (1 → 3)-β-D-glucan present in ASG. In striking contrast, the AIG exhibited significant immune response in *F. indicus* confirming the inevitability of β-linkages of glucan for eliciting immune response in *F. indicus*. Furthermore, it indicated that the method of extraction is also critically important because it determined the type of glucan extracted (α versus β) and thereby its biological potency. There were other differences between the two preparations. The α-linked ASG is alkali soluble, while the β-linked AIG is alkali insoluble. It is possible that the differences in physical state could account for some of the differences in biological activity. However, there is a substantial literature documenting the biological activity of both soluble and insoluble (1 → 3)-β-D-glucans (Browder, Sherwood, Williams, Jones, McNamee & DiLuzio 1987; Pretus, Ensley, McNamee, Jones, Browder & Williams 1991; Williams 1997; Chang *et al.* 2000; Tokunaka *et al.* 2000; Engstad, Engstad, Olsen & Osterud 2002; Kimura *et al.* 2007). Thus, we consider that the primary determinant of immune response in our experiments was the structure of the glucan polymer.

It is accepted that the immunostimulatory property of glucan is directly linked to its structural and physical properties such as degree of branching, conformation and molecular weight (Falch *et al.* 2000; Tokunaka *et al.* 2000; Ishibashi, Miura, Adachi, Ohno & Yadomae 2001; Kimura *et al.* 2007). Comparison of the impact of the extraction method on structural and physical properties and immunostimulatory potential of glucan in shrimp is interesting because of the debatable reports on the immunostimulatory potential of glucan in shrimp. For example Chang *et al.* (2000) reported enhanced phagocytic activity, cell adhesion and super oxide production in brooders of *P. monodon* following the administration of glucan extracted from *S. commune*, and Song & Hsieh (1994) reported a 2.5-fold enhancement of reactive intracellular oxygen production in *P. monodon* haemocyte following administration of glucan extracted from *Saccharomyces cerevisiae*. On the other hand, Sritunyalucksana *et al.* (1999) did not observe significant immunostimulatory property with the glucan preparation that they administered to shrimp. Similarly, Scholz, Diaz, Ricque, Suarez, Albores and Latchford (1999) observed no significant difference in the proPO activity in *Penaeus vannamei* administered with glucan prepared by rupturing cells of *S. cerevisiae* with enzymes, separating the soluble fraction from insoluble fraction, washing with ethanol, subjecting to acidification and drying.

On the basis of proton NMR spectra, immunological assays and literature reports, we attribute the difference in immunostimulatory potential between AIG and ASG to the difference in the percentage of (1 → 3)-β-D-glucans present in each preparation, which originally depends on the method of extraction. The (1 → 3)-β-D-glucan functions as a substrate for the activation of pattern recognition proteins such as β-glucan-binding proteins, and initiates a cascade of immune response in the target animal, includes clotting cascade, the synthesis of a wide array of antimicrobial peptides, and the PO-activating enzyme. An extension of current work on the effect of AIG and ASG on expression of immune genes and microbial invasion would give comprehensive information on the molecular mechanism of action of AIG. The basis of alkali–acid hydrolysis is the removal of contaminating substances such as proteins, lipids and other polysaccharides from β-glucans using repeated alkali and acid treatments and ethanol extraction, which leaves the (1 → 3)-β-D-glucan as the insoluble fraction. Whereas, in the NaClO–DMSO extraction method, the contaminating substances are removed by NaClO oxidation and the glucan is extracted by dissolving in DMSO. However, this process is greatly dependent on the solubility of the (1 → 3)-β-D-glucan. From the data generated, it appears that the (1 → 3)-β-D-glucan present in *A. diospyri* is insoluble, or sparingly soluble in DMSO under normal conditions. Therefore, it may not be feasible to extract (1 → 3)-β-D-glucan from *A. diospyri* using the NaClO–DMSO extraction method suggested by Ohno *et al.* (1999).

It is clear from this work that an analysis of the structure of isolated glucan is critical to our understanding of structure/property relationships in immune responses of shrimp to glucans. The β-linked insoluble glucan in AIG exhibited a greater immune response in shrimp than did the α-linked soluble glucan in ASG. We conclude that the presence of a higher concentration of (1 → 3)-β-D-glucan in AIG, versus the (1 → 3)-α-linked glucan in ASG, is responsible for differences in biological activity between the two isolates. Alkali insoluble glucan as a preparation is composed of 33% glucose and 67% glucan. The major glucan component is (1 → 3)-β-D-glucan present at 85% of the total glucan composition, while (1 → 3)-α-D-glucan makes up the remaining 15%. We attribute this structural difference to the method of extraction used. Because the (1 → 3)-β-D-glucan present in AIG is not soluble in DMSO, it may not be possible to separate employing the NaClO–DMSO

extraction method. Our observations suggest that glucan can be used as a potential immunostimulant to shrimp, provided it contains (1 → 3)-β-D-glucan as the major fraction.

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