Carbohydrate Polymers 92 (2013) 241–248

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

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

Soluble β-1,3/1,6-glucan in seaweed from the southern hemisphere and its immunomodulatory effect

Francisca Bobadilla[a,*], Carolina Rodriguez-Tirado[b], Mónica Imarai[b], María José Galotto[a], Roger Andersson[c]

[a] Facultad Tecnológica, Universidad de Santiago de Chile, Av. Libertador Bernardo, O'Higgins, 3363 Estación Central, Santiago, Chile
[b] Facultad de Química y Biología, Universidad de Santiago de Chile, Av. Libertador Bernardo, O'Higgins, 3363 Estación Central, Santiago, Chile
[c] Swedish University of Agricultural Sciences, Department of Food Science, P.O. Box 7051, SE-750 07 Uppsala, Sweden

ARTICLE INFO

Article history:
Received 8 June 2012
Received in revised form
25 September 2012
Accepted 26 September 2012
Available online 4 October 2012

Keywords:
Durvillaea antarctica (Chamisso) Hariot
Polysaccharide
Polymer structure
β-1,3/1,6-Glucan
Immunological action

ABSTRACT

Five types of macroalgae from the southern hemisphere were analysed for the presence of β-1,3/1,6-glucan and its immunostimulant properties. We were able to extract soluble β-1,3/1,6-D-glucan from *Durvillaea antarctica* (Chamisso) Hariot (DA). The morphology of the brown algae influenced extraction, and the highest percentage of β-glucan was found in the fronds. The content of β-glucan in the stipes and holdfast was on average 33% and <5%, respectively, of that in the fronds. A simple laboratory extraction process was developed. A highly pure water-soluble polysaccharide, mainly composed of glucose residues, was obtained with a dominant average molecular weight of 6.9 kDa. NMR spectroscopy confirmed the polysaccharide structure to be of β-1,3/1,6-glucan type, comprising a β-1,3-glucan backbone and 21% degree of branching of β-1,6-glucan side chains. Mouse cells were exposed to four DA extract concentrations in water (50, 100, 250 and 500 μg/mL) and no adverse effects on survival were noted. Remarkably, the β-glucan induced a 16.9% increase in activated CD19+ B lymphocytes compared with the control sample. The optimal concentration for maximum activity was 100 μg DA extract/mL.

1. Introduction

Naturally occurring non-specific immunostimulants, such as β-glucan, have been proved to have immunomodulatory effects in aquaculture (Bohn & BeMiller, 1995; Kumari & Sahoo, 2006; Robertsen, Rorstad, Engstad, & Raa, 1990; Sahoo & Mukherjee, 2001). Prophylactic immunomodulators might be of use as a prophylactic treatment to prevent seasonal outbreaks and also as a suppressive treatment for latent and sublethal pathogens (Gannam & Schrock, 2001, chap. 12). Compared with antibiotics, the advantage of naturally occurring non-specific immunostimulants is that they do not cause safety concerns in terms of residual contaminant, and particularly water quality damage, when used as prophylactics.

β-Glucans are long-chain polysaccharides found in the cell wall of fungi, yeasts and certain bacteria and plants (Gannam & Schrock, 2001, chap. 12; Rice et al., 2005). Yeast glucans are the most commonly used immunomodulators (US Patent 6444448, US Patent 5401727, US Patent 6476003, WO 2004030613, CN 101117357, *etc.*). Soluble β-1,3/1,4-glucan from barley (US Patent 7462607) and soluble β-1,3/1,6-glucan from fungi (KR 2007 1037035) have been developed for use in food and medicinal applications (US Patent 7462607). These include use as quasi drugs for cancer therapy, developed for the purpose of enhancing the efficacy of antibodies (Modak, Koehne, Vickers, O'Reilly, & Cheung, 2009). To a large extent, the applications are orientated towards supplementing diets to generally improve resistance against several diseases of bacterial, fungal and parasitic origin (Mantovani et al., 2008), but they also have great potential as an adjuvant complementing fish vaccination to increase antibacterial response (Shoemaker, Klesius, & Lim, 2001, chap. 7; Siwicki et al., 2004). However, the main drawback limiting the development of many of these polysaccharides is the lack of efficient processes for their extraction and purification and their cost (Laroche & Michaud, 2007).

To a large extent, commercial applications have been developed to incorporate immunomodulators as a dietary supplement in feed and food production. However, Gannam and Schrock (2001, chap. 12) noted that the use of glucans in a bath treatment for fish has not been examined in depth. Furthermore, Soltanian, Stuyven, Cox, Sorgeloos, and Bossier (2009) indicated that oral administration of water-soluble β-glucan preparations has several advantages compared with other routes of administration (*i.e.* intravenous, intraperitoneal, and peroral). Sandula, Kogan, Kacurakova, and

* Corresponding author. Permanent Postal Address: Kristinebergs Strand 33, Stockholm 11252, Sweden. Tel.: +56 25709642.
E-mail addresses: francisca.bobadilla@usach.cl (F. Bobadilla), carolina.rodriguezt@usach.cl (C. Rodriguez-Tirado), monica.imarai@usach.cl (M. Imarai), maria.galotto@usach.cl (M.J. Galotto), Roger.Andersson@slu.se (R. Andersson).

0144-8617/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.carbpol.2012.09.071

Machova (1999) reported problems with insoluble, or sparingly soluble, β-glucan causing significant adverse effects (*e.g.* granuloma formation, microembolisation, inflammation, and pain) when administered by parenteral routes and indicated that oral administration of the water-soluble glucan preparations, when tested in mice, had several advantages.

The use of natural immunostimulants in decapods and bivalves to protect them, especially from infectious diseases, is promising, since these animals do not have in their system the capacity for specific immunity against infectious agents (Klesius, Shoemaker, Evans, & Lim, 2001, chap. 17). In addition, the innate immune system is of primary importance in combating infections in fish and the reason is basically the intrinsic inefficiency of the acquired system due to its evolutionary status (Magnadóttir, 2006). Furthermore, it is well known that most larval marine fish will not readily accept formulated feeds and thus when rearing marine species it is often necessary to supply nutrient and health supplements by other methods (Weirich & Reigh, 2001, chap. 15). One of these methods is the absorption of prophylactics dissolved in bath treatments through the skin (Strand and Dalmo, 1997). Therefore, fish in the early stages of development and inland cultures of crustaceans are in urgent need of a soluble β-glucan bath treatment application and it has been suggested that soluble β-glucan from algae may have the potential to enhance immunity in early life stages before the development of acquired immunity (Gannam & Schrock, 2001, chap. 12).

Research on β-glucan bioactivity has shown increased macrophage and neutrophil numbers, enhanced macrophage phagocytic activity, elevated serum lysozyme and up-regulated macrophage respiratory burst activity (Guselle, Markham, & Speare, 2006). Furthermore, it is possible that the treatment of fish with β-glucan may enhance adaptive immunity, as demonstrated in *Labeo rohita* by Misra, Das, Mukherjee, and Pradhan (2006).

This study sought to characterise the properties of a soluble β-1,3/1,6-glucan extracted from brown algae samples from the southern hemisphere. Percentage recovery was measured and the content monitored throughout the process by sugar and β-1,3/1,6-glucan analysis. A purified extract was characterised chemically by sugar residue composition, molecular weight and molecular structure. Biological activity as a natural immunostimulant was tested *in vitro* with a model system based on murine splenic cells using a flow cytometry technique.

2. Experimental

2.1. Materials

Five species within the red (Gracilariaceae) and brown (Phaeophyceae) algae were studied. These were *Gracilaria chilensis*, *Durvillaea antarctica* (DA), *Macrocystis pyrifera*, *Lessonia trabeculata* and *Lessonia nigrescens*, all of which originated from the intertidal zone of the Chilean coast. The samples were washed, freeze-dried, milled and stored frozen at −20 °C inside vacuum-sealed bags until analysis. All chemicals and reagents used were of analytical grade. Furthermore, samples of DA algae were collected from two locations: A (34°34′S, 72°4′W and B (34°46′S, 72°6′W), and tissues of frond, stipe and holdfast were analysed separately.

2.2. Extraction

Extraction was carried out for the five types of algae in order to determine the content of β-1,3/1,6-glucan and its behaviour during the extraction process. Milled algal material (1 g) was placed in a 80 mL beaker with 20 mL 85% ethanol solution and soaked overnight at room temperature (Fig. 1). On the following day, the sample was mixed in an Ika ultraturrax T-25 homogeniser at 8000 × *g* (Janke and Kunkel) for 1 min, transferred to a 15 mL capped tube and placed in a water bath at 70 °C for approximately 3 h.

The unextractable residue was collected by centrifugation at 3000 × *g* (Heraeus Multifuge 3S) for 10 min. The pellet was extracted with 20 mL $CaCl_2$, homogenised by ultra-turrax (Kinematica Polytrom PT 3000) at 20,000 × *g* for 1 min and incubated at 70 °C with continuous mixing for 1 h. The sample was centrifuged at 3000 × *g* (Heraeus Multifuge 3S) for 10 min and the supernatant collected. The extraction cycle was repeated twice with 10 ml of $CaCl_2$ each.

Pooled supernatant was concentrated in a conical flask with a Büchi evaporator at 40 °C. The sample was defatted with 2 mL chloroform, mixed and centrifuged. The supernatant was dialysed in 29 mm diameter regenerated cellulose tubing membrane (12,000–14,000 MWCO, Spectrum Spectra/Por® 4). Dialysis was performed over 16 h against deionised water before freeze-drying and storage in a desiccator. DA extracts from locations A and B followed the same extraction protocol, but with the chloroform purification step omitted.

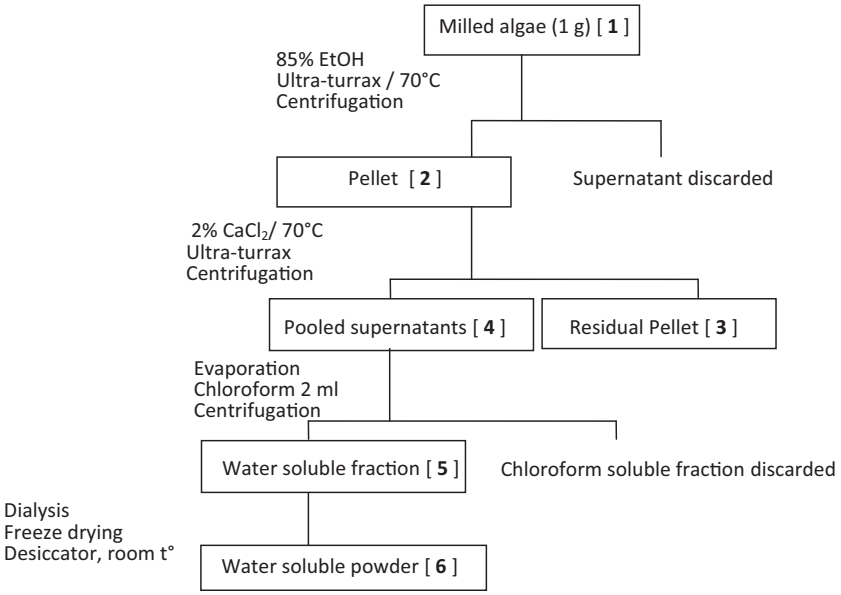

Fig. 1. Outline of extraction procedure. Fractions are indicated with bold numbers.

2.3. Determination of β-1,3/1,6-glucan content

Approximately 200 mg of the dried and milled algae were accurately weighed, in triplicate, into 25-mL screw cap tubes for determination of β-1,3/1,6-glucan content. The mushroom and yeast β-glucan enzymatic kit and assay procedure (K-YBGL 04/2008, Megazyme, Bray, Ireland) was used, following the manufacturer's instructions.

2.4. Sugar analysis and determination of extraction percentage recovery

Dried and milled samples of the algae types were analysed in triplicate by the Uppsala method for dietary fibre content and composition (Theander, Åman, Westerlund, Andersson, & Pettersson, 1995). Three batches of DA were analysed. The residual pellet after extraction (fraction 3 in Fig. 1) was freeze-dried and analysed by the Uppsala method, omitting the starch removal step. The extracted fraction (fraction 4 in Fig. 1, called the non-purified fraction) was brought up to 50 mL volume with 2% $CaCl_2$ (aq.) solution. A 1-mL aliquot of this volume was taken for sugar analysis according to the Uppsala method, with the following modifications: the starch removal step was omitted and hydrolysis was performed with 500 μL of 1.6 M sulphuric acid and 500 μL water containing 0.5 mg of myo-inositol as the internal standard. The samples were autoclaved at 125 °C for 1 h and analysed as in the original method. Fraction 5 was dialysed, freeze-dried and analysed for sugar composition by gas–liquid chromatography.

2.5. ^1H NMR spectroscopy

Proton nuclear magnetic resonance (^1H NMR) spectra of extracted polysaccharide from DA batch 2 were obtained with a NMR spectrometer DRX-400 (Bruker-Switzerland). Samples (5–10 mg) were dissolved in 1 mL D_2O and instantly frozen with a Benchtop Shell Labconco freezer, and then freeze-dried. After completing 3 cycles of solvent replacement, the samples were dissolved in dimethyl-d_6 sulphoxide:D_2O 6:1. Spectra were recorded at 80 °C, using 90° radio frequency pulse angle with 64 scans, with and without water presaturation. Chemical shifts were determined with dimethyl sulphoxide as reference. Crude extracts of DA from locations A and B were analysed in a similar way, but dissolved in D_2O without dimethyl sulphoxide and with the water signal as chemical shift reference.

2.6. Molecular weight determination

Dried extracts (10–20 mg) were dissolved in 1 mL deionised water, microfiltered and injected through a combined size exclusive chromatography-multiangle laser light scattering system (Wyatt Technology Inc.) consisting of three serially connected columns (OHpak SB-806 MHQ, OHpak SB-804 HQ and OHpak SB-803 HQ, Shodex, Showa Denks KK, Miniato, Japan), a light scattering detector (Dawn DSP, Wyatt Technology Corp., Santa Barbara, CA) and a refractive index detector (model 250, Viscotex Corp., Houston, TX).

2.7. Immunomodulatory activity of the β-glucan

Biological activity, specifically modulation of the immune system by the β-glucan, was evaluated through *in vitro* analysis on murine splenic cells from Balb/c mice. Animals between 6 and 8 weeks of age were killed by cervical dislocation and spleen cells were obtained by disaggregation on passage through a sterile, 100-gauge stainless steel mesh. The whole process was carried out in Roswell Park Memorial Institute supplemented medium (RPMI) (Gibco) (1 mM L-glutamine, 1 mM sodium pyruvate and 100 U penicillum/100 μg streptomycin, and 10% foetal bovine serum). The cell suspension was centrifuged for 10 min and then treated with erythrocyte ammonium chloride potassium lysis buffer (ACK; 0.15 M NH_4Cl, 0.1 M $KHCO_3$ and 0.1 M EDTA) for 2 min. The cells were washed and placed in culture plates with supplemented RPMI medium and kept at 37 °C, 5% CO_2 and 75% relative humidity.

Cells (2×10^5) were then treated with the crude β-glucan extract from DA batch 2. After 12 h of β-glucan treatment, the cells were recovered in 1.5-mL Eppendorf tubes and centrifuged at 2000 × *g* for 10 min. The supernatant was removed and the cells were resuspended in a 2% SFB-PBS buffer (saline phosphate buffer supplemented with 2% foetal bovine serum). The viability was determined by staining with 10 μg/mL propidium iodide. Labelled (death) and unlabelled (live) cells were analysed on a FACSCanto II Flow Cytometer with FACS Diva software (both from Becton Dickinson, San Jose, CA).

For activity analysis, immunofluorescence staining was performed for both non-stimulated and β-glucan-stimulated splenocytes using phycoerythrin (PE)-labelled anti-CD4 antibody (T helper cell marker), PE-labelled anti-CD8 antibody (T cytotoxic cell marker) or allophycocyanin-cyanine dye (APC-Cy7)-labelled anti-CD19 antibody (B cell marker). Double staining was performed by adding a fluorescein isothiocyanate (FITC)-labelled anti-CD69 antibody, which is an activation marker. Based on the differential spectral properties of the fluorescent molecules (PE, APC-Cy7 and FITC), simultaneous detection of fluorescence permitted discrimination of different cellular populations (CD4+, CD8+ or CD19+), which were activated (CD69+). In brief, cells were incubated in phosphate-buffered saline (PBS)–2% foetal calf serum (IF buffer) for 30 min at 4 °C. Incubation with antibodies was then performed for a further 30 min, after which samples were centrifuged at 600 × *g* for 5 min at 4 °C and cell pellets were washed in IF buffer. To guarantee the response to activation from the splenic cells, polyclonal activators such as phytohaemagglutinin (PHA) or lipopolysaccharide (LPS) were used as positive controls. Fluorescence was determined in a FACSCanto II Flow Cytometer with FACS Diva software (Becton Dickinson). The equipment was set up to quantify 30,000 simultaneous acquiring events (cells).

3. Results and discussion

3.1. Screening of the raw material

Screening of the five species of algae within the Phaeophyceae and Gracilariaceae using the Megazyme β-1,3/1,6-glucan analysis kit revealed the presence of β-glucan (Table 1). Of particular interest was the high content of β-1,3/1,6-D-glucan (14.4%) found in the species *Durvillaea antarctica* (DA). In addition, *Lessonia trabeculata* and *L. nigrescens* showed 0.4% β-glucan or lower contents, similar to the low levels reported by Matsuhiro and Zambrano (1990) and Chandia, Matsuhiro, Ortiz, and Mansilla (2005) for these species. Samples generally had low levels of α-glucan.

Parallel sugar residue composition and dietary fibre analysis showed a high content of glucose residues among the majority of the macroalgae examined. The glucose content was in the range 5.5–7.6% for both *Lessonia* species and *Macrocystis pyrifera*, suggesting that the glucose residue did not represent β-glucan but other storage carbohydrates. Furthermore, varying presence of fucose, mannose and galactose was also found among the algae species. No rhamnose was detected. Analysis of the sugar composition in *Lessonia* species showed higher fucose content in *L. trabeculata* (4.33%) than in *L. nigrescens* (1.03%). A similar content of mannose (1.5%) and galactose (0.6%) was found in both *Lessonia* species.

Table 1
Content of β-1,3/1,6-glucan, α-glucan and sugar residues (% of dry matter) in the whole algae.

Algae	β-Glucan	α-Glucan[a]	Polysaccharide sugar residues				
			Glucose	Fucose	Mannose	Galactose	Uronic acids
Macrocystis pyrifera	0.6	0.1	7.6	4.0	1.4	0.8	12.6
Lessonia trabeculata	0.4	0.2	5.5	4.3	1.2	0.6	10.1
Lessonia nigrescens	0.6	0.1	6.6	1.0	1.8	0.5	14.4
Gracilaria chilensis	3.5	0.4	4.8	n.d.[b]	1.0	15.9	2.2
D. antarctica, batch 1	3.7	0.2	3.9	1.7	2.0	0.3	21.1
D. antarctica, batch 2	14.5	0.6	14.7	6.8	7.9	1.1	19.6
D. antarctica, batch 3	5.5	0.4	6.4	2.8	5.1	0.4	19.1

[a] Starch maltosaccharides, glucose in sucrose and free glucose.
[b] Not detected.

The results from the parallel enzymatic kit and the sugar residue analyses showed that there was a clear correlation between the glucose residue content and the β-glucan percentage detected in DA. However, the results showed a high variability in the levels of glucose residues obtained from three batches of whole DA. This variability can be explained in part by the different levels of β-glucan that are allocated to different parts of the algae (Table 2). Thus, the results of the β-glucan analysis of samples separated by morphology indicated a higher concentration of β-glucan allocated to the fronds. The content of β-glucan in the stipes and holdfast was on average 33% and less than 5%, respectively, of the content in the fronds. The geographical origin of the samples (location A, B) was found to have no effect on β-glucan content. The species DA is abundantly present on the central and southern Chilean coast. It is harvested from rocky intertidal zones and recollected from offshore. Fronds of this species can reach 10 m in length and represent the largest component, comprising around 90% of the total mass (Lawrence, 1986), while their biomass reaches up to 80 kg per m^2 (Santelices, Castilla, Cancino, & Schmiede, 1980).

The other species that had interesting levels of β-glucan was *Gracilaria chilensis* (3.5%). The *Gracilaria* had galactose as the major sugar residue constituent, corresponding to 15.9%. The high content of galactose indicates that this algal residue may be relevant for industrial extraction of agar. However, the presence of galactose has serious drawbacks as it interferes with β-glucan isolation due to ion interaction and high hygroscopicity. In the present case, during the selective extraction procedure it was not possible to concentrate the extracts from *Gracilaria chilensis* in an evaporator, which made the extract unavailable for the enzymatic kit assay. Furthermore, obtaining β-glucan as a sub-product of agar extraction may have an impact on its quality, since the industrial extraction of agar requires a lengthy process of boiling (and sometimes also an alkaline digestion). This could have an impact on the structural conformation of the β-glucan, for instance decreasing its biological activity (Gannam & Schrock, 2001, chap. 12; Zekovic & Kwiatkowski, 2005; Wood, 2010).

The analyses of sugar residue composition in the algae (Table 1) clearly showed that the glucose residues cannot always be attributed to the presence of β-glucan. Therefore, it is necessary to verify the presence of β-1,3/1,6-glucan with a confirmatory test specific for quantification of this polymer, such as the K-YBGL 1,3/1,6-glucan enzymatic kit.

Table 2
β-1,3/1,6-Glucan and α-glucan content of fond, stipe and holdfast of *Durvillaea antarctica* from two locations. Values are $\bar{x} \pm SD$.

Tissue	Location	β-Glucan[a]	α-Glucan[b]
Frond	A	6.2 ± 0.1	0.5 ± 0.0
	B	7.1 ± 0.1	0.6 ± 0.0
Stipe	A	1.0 ± 0.0	0.3 ± 0.0
	B	3.4 ± 0.1	0.3 ± 0.0
Holdfast	B	0.3 ± 0.0	0.2 ± 0.0

[a] Recollection from location A (34°34′S, 72°4′W) and B (34°46′S, 72°6′W).
[b] Includes starch maltosaccharides, glucose in sucrose and free glucose.

3.2. Evaluation of selective extraction and selection of the raw material

A selective extraction method with 2% $CaCl_2$ for algae containing laminaran was used for the five macroalgae species, as shown in Fig. 1 (described in Section 2.2).

Table 3 shows the percentage of neutral sugar residues from extracted fractions of DA batch 1 before and after dialysis. The largest ratio of glucose residues was obtained during the first extraction cycle, ranging between 30 and 40% of glucose content. Because of the $CaCl_2$ salt precipitating the alginate constituent of DA (Matsuhiro, Zúñiga, Jashes, & Guacucano, 1996), a higher β-glucan concentration was also obtained in the first cycle. For the following cycles, the majority of the $CaCl_2$ salt used during the extraction seemed to accompany the extract, as it had a highly diluting effect on the non-purified extracts (cycles 2 and 3). After dialysis, the remaining salt was efficiently eliminated. For example, as shown in Table 3, the glucose concentration increased from about 30–40% to 60–70% in the first cycle.

The purification process also showed an interesting ability to remove manno-proteins, which were mostly present (around 11% of mannose) in the first fraction obtained from the non-purified fraction (Table 3). This removal was most likely due to precipitation together with complex proteoglycan-like material in the chloroform phase. This material is commonly found in seaweed carbohydrate structures (Duarte, Cardoso, Noseda, & Cerezo, 2001). Similarly, during purification of the first cycle, low molecular weight fucose and galactose residues appeared to have been removed by the dialysis, as shown by the decreased concentration of these sugar residues in the purified extracts.

As the recovery percentage values in Table 3 show, the purification step greatly contributed to concentrating the β-glucan and eliminating the salt added during extraction. It is clear that increasing the number of cycles of extraction with $CaCl_2$ would probably not improve the extracted β-glucan significantly, while also increasing the amount of salt to be removed in the pooled extract.

The results showed that β-glucan was the dominant polysaccharide in the $CaCl_2$ extract from DA (Table 4). In contrast, for the other the algae investigated (*Lessonia* spp., *Macrocystis* and *Gracilaria*, the results showed a low recovery with the use of the selective β-1.3/1,6-glucan extraction procedure. As mentioned earlier, due to a problem with hygroscopicity of the sample during evaporator concentration, the extract from *Gracilaria* was unavailable for β-glucan analysis.

DA showed increasing percentage recovery of β-glucan with the procedure outlined here. Mechanical disruption of the sample performed twice with ultra-turrax, both during the alcohol soaking

Table 3
Content of neutral sugar residues (% of dry matter) and relative percentage of glucose residues (%) in freeze-dried extracts of *Durvillaea antarctica* (batch 1).

Extract	Fraction cycle[a]	Sugar residues (%)				Glucose residues[b]
		Glucose	Fucose	Mannose	Galactose	Relative %
Non-purified extract	1	34.0	2.6	10.6	2.4	68.6
	2	2.6	1.0	1.0	0.4	53.2
	3	1.2	0.6	0.5	0.2	48.3
Purified extract	1	62.7	1.7	1.4	1.3	93.5
	2	49.6	1.6	1.6	1.5	92.0
	3	29.8	1.8	1.8	0.2	88.9

[a] Fraction cycles during extraction with $CaCl_2$; 1 is the first cycle, 2 the second cycle and 3 the third cycle.
[b] Expressed as percentage of total neutral sugar residues.

Table 4
β-1,3/1,6-Glucan, α-glucan and neutral sugar residues content (% of dry matter) and relative percentage of glucose residues in pooled fractions of non-purified algae extracts and purified *Durvillaea antarctica* extracts with the recovery expressed as percentage of β-glucan in the original algae.

Sample	β-Glucan	α-Glucan[a]	Sugar residues				Glucose residues[b]	Recovery[c]
			Glucose	Fucose	Mannose	Galactose	(Relative %)	%
Non-purified extracts								
Macrocystis pyrifera	0.1	0.0	0.1	0.1	1.0	0.1	3.5	0.7
Lessonia trabeculata	0.1	0.0	0.1	1.9	0.3	0.1	2.4	1.0
Gracilaria chilensis	–	–	0.3	n.a.[d]	0.1	0.7	24.8	5.4
Durv. antart – batch 2	13.5	0.1	14.6	n.a.[d]	0.8	n.a.[d]	95.0	93.0
Purified extracts of *Durvillaea antarctica*								
Batch 1	60.7	0.6	64.3	1.7	1.4	1.2	93.3	92.3
Batch 2	56.9	0.7	76.2	1.3	1.3	0.7	96.0	74.6
Batch 3	59.3	0.7	72.7	1.2	1.4	1.8	94.3	86.0

[a] Including starch maltosaccharides, glucose in sucrose and free glucose.
[b] Expressed as percentage of total neutral sugar residues.
[c] Recovery of glucose residues in extract as percentage of glucose residues in the dry algae.
[d] Not available.

treatment and in the 2% $CaCl_2$ solution, increased extraction. As shown by a study on β-glucan in cereals reported by Wood (2010), β-glucans are limited to few species within a food group and the content can vary quite widely within the same species. The content of glucose residues in our DA extracts determined by compositional analysis was similar to that obtained with the enzymatic kit. Thus, DA can be considered an excellent source of soluble β-1,3/1,6-D-glucan, unlike the levels of laminaran reported in other brown algae studies (Honya, Mori, Anzai, Araki, & Nisizawa, 1999; Zvyagintseva et al., 2003). Furthermore, similar contents of β-glucan in the final extract after purification were obtained from samples of different batches (batch 1 and 2), indicating that consistent results can be obtained regardless of the initial content in the raw material (Table 4).

Kelly and Brown (2000) studied the content of alginate in DA and reported high variability between individuals and over time, but no significant seasonal variation in the composition of the various tissue fractions. In addition, Haug, Larsen, and Smidsrød (1974) studied alginates in seven types of brown algae and concluded that the differences between the alginates from different species are mainly due to alginates from older parts of the plant. At this point in our studies we examined variables such as the morphology of the algae and seasonality. While maturity was not investigated in depth, our results indicated that the amount of uronic acid in batch 1 (juvenile DA) was 21.1% (Table 1), while older DA batches (2 and 3) showed lower contents of uronic acids.

As reported by Lawrence (1986), the level of TCA-insoluble carbohydrates is similar in the fronds of juvenile and adult DA, so while alginate content is higher in juveniles, the content of non-alginate structural carbohydrates, including β-glucan, increases in the adult fronds.

3.3. Characterisation of the soluble β-1,3/1,6-glucan product

The extraction procedure confirmed that significant β-glucan extractability was possible with the 2% $CaCl_2$ solution, with recovery ranging from 75 to 92% (Table 4). Thus, the percentage recovery was nearly 90% of the β-glucan content originally found in the DA algae, with the remaining 10% of the β-glucan being left in the pellet. After purification, the glucose concentration increased to 65-75% even for a range of raw material batches with varying β-glucan content. The salt used during the extraction was efficiently removed, as confirmed by conductivity tests during dialysis and the increase in glucose residues content.

The resulting product can be described as a fine non-dense powder with an off-white colour. Solubility tests carried out on the final fraction in water resulted in 100% solubility as assessed by centrifugation.

The β-glucan purified extract from DA macroalgae was predominantly composed of polysaccharides (63–79%), in which glucose residues were the main sugar component (80–92%) (Tables 3 and 4). The content of uronic acids in the final extract ranged between 5 and 8% after the purification procedure. Mannose (1.4%), fucose (1.4%) and galactose (0.8%) were also present in minor quantities and the presence of sulphate was indicated.

3.4. ^1H NMR analysis

^1H NMR spectroscopy of the purified β-glucan from DA batch 2 extracts dissolved in 6:1 DMSO:D_2O confirmed the presence of a $(1 \rightarrow 3)$,$(1 \rightarrow 6)$-linked β-glucan (Fig. 2). Anomeric signals from 1,3- and 1,6-linked residues were found at 4.56 and 4.28 ppm, respectively (Størseth, Kirkvold, Skjermo, & Reitan, 2006), along with

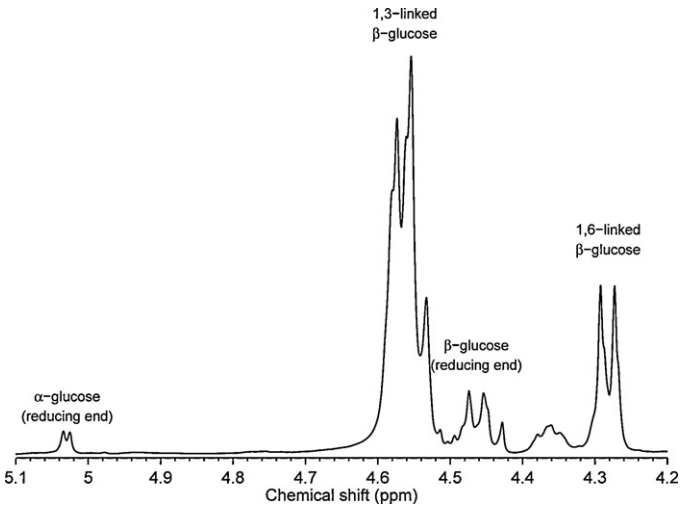

Fig. 2. Anomeric region of the ^{1}H NMR spectrum of (1 → 3),(1 → 6)-linked β-glucan extracted from *Durvillaea antarctica*.

α-glucose and β-glucose reducing ends found at 5.03 and 4.46 ppm, respectively. The complexity of the signals, especially those from 1,3-linked residues, indicates heterogeneity in the branching pattern. The 1,6-linked residues were estimated to comprise 21% based on anomeric signal areas. This structural analysis provides evidence of the potential of this polysaccharide to be biologically active and to exert an immunostimulant enhancing action (Kim et al., 2000; Zekovic & Kwiatkowski, 2005). Similarly, Bohn and BeMiller (1995), among others, reported that (1,3)-β-D-glucans that have β-D-glucopyranosyl units attached by (1,6) linkages as single unit branches show great potential for systematic enhancement of the immune system. In the present study the structure was confirmed by NMR analysis of crude extracts from the different morphological parts of the algae and different locations. The content of 1,6-linked residues ranged from 18.4 to 18.7% in the crude extracts.

3.5. Molecular weight determination

The molecular weight analysis revealed two molecular weight distributions (Fig. 3). The low molecular weight population with retention volume 26–28 mL dominated, with a weight average molecular mass of 6.9×10^3 Da. The population at 17–22 mL was less abundant, but had a much larger weight average molecular mass of 6.4×10^6 Da.

It has been reported in the literature that products with immunological potential require a minimum molecular size of 1 kDa to be biologically active. For example, Elyakova et al. (2007) reported a proven biologically active molecule from laminaran with a molecular size of 6–8 kDa.

3.6. Immunomodulatory activity of the β-glucan

The cell viability test after 12 h of treatment with the β-glucan resulted in >95% of surviving cells from a total of 2×10^4 cells analysed with a β-glucan dose range of 50–500 μg/mL. Thus, the product was not toxic under these experimental conditions. The biological activity was subsequently determined as the capacity of β-glucan to induce lymphocyte activation. Activation was established by the expression of CD69, an early cell surface marker of B and T lymphocyte activation (Vilanova et al., 1996). The results showed that β-glucan induced a dose-dependent increase in CD69+ of the CD19+ B lymphocytes (Fig. 4). The effect was observed in the concentration range 1–500 μg/mL. After 100 μg/mL, there was a steady response of B lymphocytes, which may mean that the product reaches a plateau in effect (Fig. 4). β-Glucan treatment had no effects on the levels of CD69 of the CD4+ and CD8+ T lymphocytes (not shown). Therefore, it was established that DA extract containing β-glucan had a significant effect in activating spleen CD19+ B lymphocytes. The optimum dose at cellular level occurred at a concentration of 100 μg/mL.

Fisheries are currently struggling to find new and more efficient aids to production in terms of increasing fish immunity. By improving growth and survival at earlier stages of life, it is also clear that improved fish immunity would provide a good foundation for increasing production and food absorption, as healthier organisms can strengthen the production cycle and thereby lower its exposure to infectious diseases and contamination. As viruses are currently strongly affecting commercial aquaculture, there is an urgent need for commercial products capable of strengthening this area of production. A study on the use of soluble β-glucan in activation of cell immunological markers in fish at early stages of development is in progress, and will be reported in a later article.

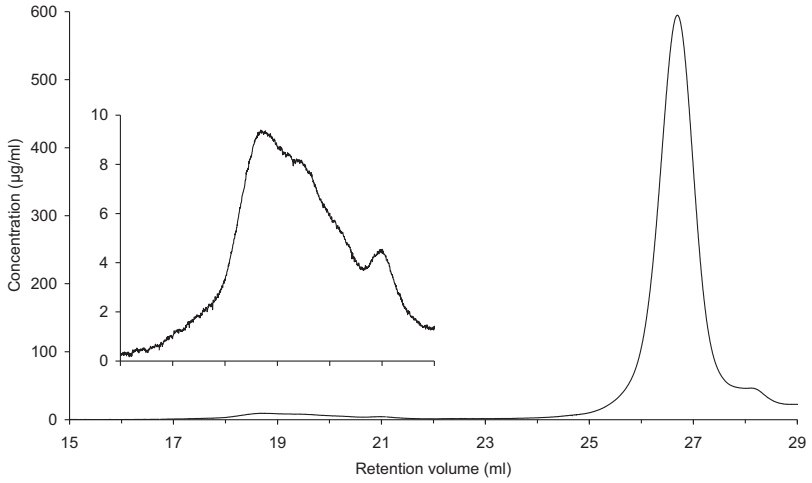

Fig. 3. High performance size exclusion chromatogram of extract from algae. Inlay show peak at 19 mL with a different scale.

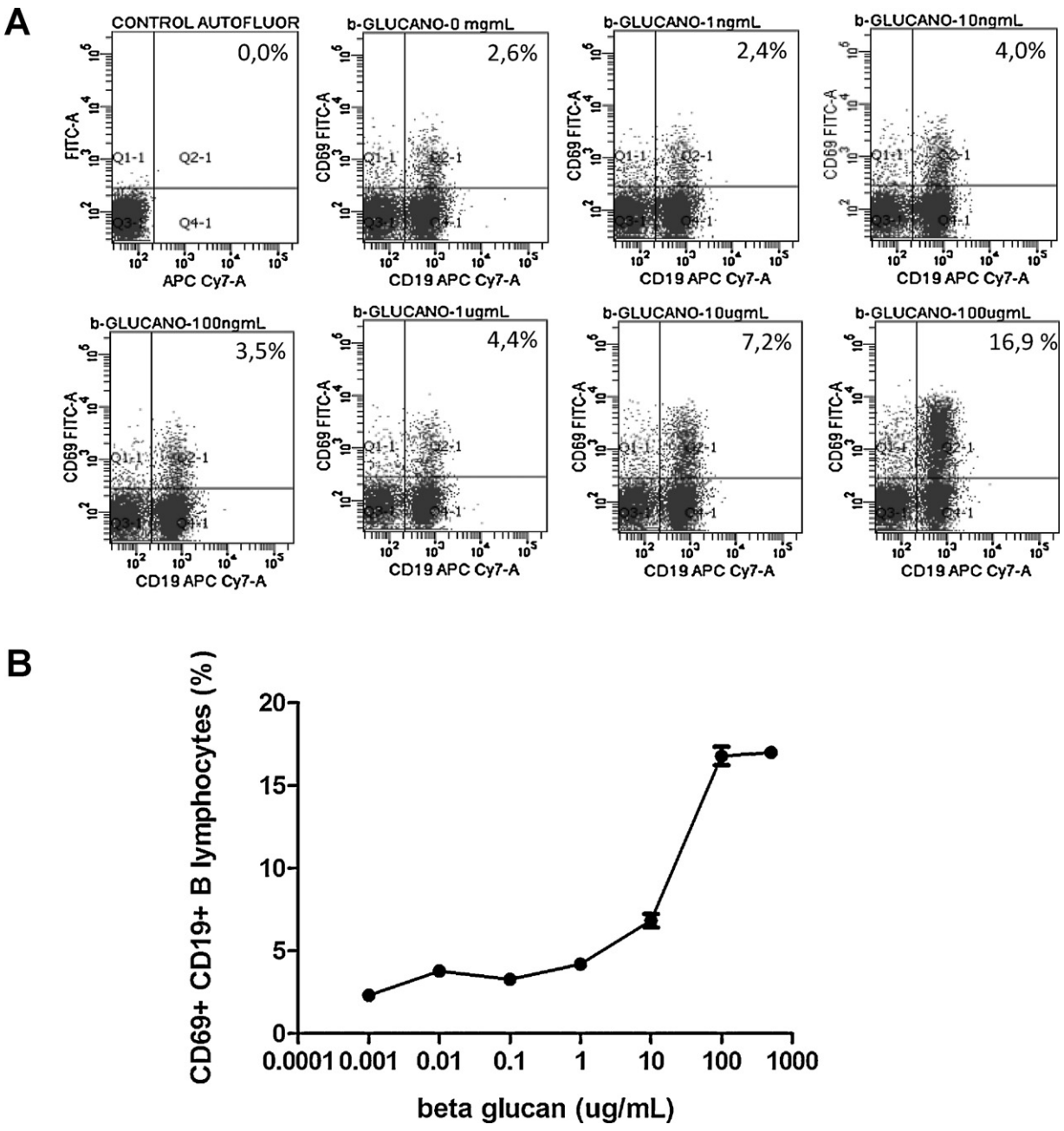

Fig. 4. Effect of β glucan extract from batch 2 of *Durvillaea antarctica* on activation of CD19+ B lymphocytes. CD69 expression on B lymphocytes was analysed by flow cytometry after 12 h of β-glucan treatment. (A) Dot plot displaying CD69+ label on the *y* axis and CD19+ label on the *x*-axis. Autofluorescence and cells without treatment (0 mg/L of β-glucan) are included. Percentage of activated CD69+CD19+ B cells is indicated in the upper right square. (B) Quantitative results of the effects analysed for three independent experiments.

4. Conclusions

This study sought to establish the presence of the polysaccharide β-(1-3)/(1-6)-D-glucan in seaweed from the southern hemisphere. The potential for immunostimulant activity of a β-1,3/1,6-D-glucan immunomodulator from a particular macroalgal species was demonstrated. The species *Durvillaea antarctica* (DA) was selected as the raw material for β-glucan extraction because of its high content (4–15%) of soluble β-1,3/1,6-glucan. The enzymatic kit and gas-liquid chromatography of the alditol acetates proved to be suitable analytical methods for investigating the content of β-glucan in DA sources.

Remarkably, the final product presented a high level of purity. The extract was predominantly composed of glucose residues (76%) with some presence of mannose (1.5%) and fucose (1.5%) residues. The extract also contained other non-sugar components such as uronic acids and protein (1.6%).

In addition to developing the procedure for the β-glucan isolation, important criteria for the extraction process were established. One of these concerns the use of algae with a low presence of interfering hydrocolloids, such as agar, which can hinder β-glucan extraction and isolation of the polysaccharide of interest. A second criterion is that both the enzymatic kit and gas–liquid chromatography methods must be used for investigating sources of

β-1,3/1,6-D-glucan, since chromatographic results concerning the polysaccharide of interest can only be confirmed with the more specific enzymatic YBGL kit method.

The simple extraction process investigated in this study has interesting economic potential when used for DA and may add value to current industry practices. As the structure of the molecule was consistent within the different samples, the biological activity is also likely to be consistent.

Acknowledgements

This work was supported by a collaborative participation of university institutions: Universidad de Santiago de Chile (Santiago, Chile), Swedish University of Agricultural Sciences-SLU (Uppsala, Sweden), Universidad de Los Lagos (Osorno, Chile) and private companies and the co-funding of Fondo de Cooperación Chile-Suecia (Utvecklingsfonden Sverige Chile) Project 207-0135. We also would like to thank Dr. Ali Moazzami for his help with the NMR analysis.

References

Bohn, J., & BeMiller, J. (1995). (1-3)-β-D-glucans as biological response modifiers: A review of structure-functional activity relationship. *Carbohydrate Polymers*, *28*, 3–14.

Chandia, N., Matsuhiro, B., Ortiz, J., & Mansilla, A. (2005). Carbohydrates from the sequential extraction of *Lessonia vadosa* (Phaeophyta). *Journal of the Chilean Chemistry Society*, *50*, 501–504.

Duarte, M. E. R., Cardoso, M. A., Noseda, M. D., & Cerezo, A. S. (2001). Structural studies on fucoidans from the brown seaweed *Sargassum stenophyllum*. *Carbohydrate Research*, *333*, 281–293.

Elyakova, L. A., Isakov, V. V., Lapshina, L. A., Nagorskaya, V., Likhatskaya, G., Zvyagintseva, T., et al. (2007). Enzymatic transformation of biologically active 1,3;1,6-β-D-glucan. Structure and activity of resulting fragments. *Biochemistry (Moscow)*, *72*, 29–36.

Gannam, A. L., & Schrock, R. M. (2001). Immunostimulants in fish diets. In C. E. Lim, & C. D. Webster (Eds.), *Nutrition & fish health* (pp. 235–266). New York: Haworth Press Inc.

Guselle, N. J., Markham, R. J. F., & Speare, D. J. (2006). Intraperitoneal administration of β-1, 3/1,6-glucan to rainbow trout, *Oncorhynchus mykiss* (Walbaum), protects against Loma salmonae. *Journal of Fish Diseases*, *29*, 375–381.

Haug, A., Larsen, B., & Smidsrød, O. (1974). Uronic acid sequence in alginate from different sources. *Carbohydrate Research*, *32*, 217–225.

Honya, M., Mori, H., Anzai, M., Araki, Y., & Nisizawa, K. (1999). Monthly changes in the content of fucans, their constituent sugars and sulphate in cultured *Laminaria japonica*. *Hydrobiologia*, *398/399*, 411–416.

Kelly, B. J., & Brown, M. T. (2000). Variations in the alginate content and composition of *Durvillaea antarctica* and *D. willana* from southern New Zealand. *Journal of Applied Phycology*, *12*, 317–324.

Klesius, P. H., Shoemaker, C. A., Evans, J. J., & Lim, Ch. (2001). Vaccines: Prevention of diseases in aquatic animals. In C. E. Lim, & C. D. Webster (Eds.), *Nutrition & fish health* (pp. 3–319). New York: Haworth Press Inc.

Kim, Y. T., Kim, E. H., Cheong, C., Williams, D. L., Kim, C. W., & Lim, S. T. (2000). Structural characterization of beta-D-(1 → 3, 1 → 6)-linked glucans using NMR spectroscopy. *Carbohydrate Research*, *328*, 331–341.

Kumari, J., & Sahoo, P. K. (2006). Dietary β-1,3-glucan potentiates innate immunity and disease resistance of Asian catfish, *Clarias batrachus* (L.). *Journal of Fish Diseases*, *29*, 95–101.

Laroche, C., & Michaud, P. (2007). New developments and prospective applications for β (1,3) glucans. *Recent Patents on Biotechnology*, *1*, 59–73.

Lawrence, J. M. (1986). Proximate composition and standing crop of *Durvillaea antarctica* (Pheophyta) in the Bay of Morbihan, Kerguelen (South Indian Ocean). *Marine Ecology Progress Series*, *33*, 1–5.

Magnadóttir, B. (2006). Innate immunity of fish (overview). *Fish and Shellfish Immunology*, *20*, 137–151.

Mantovani, M. S., Bellini, M. F., Angeli, J. P., Oliveira, R. J., Silva, A. F., & Ribeiro, L. R. (2008). β-Glucans in promoting health: Prevention against mutation and cancer. *Mutation Research*, *658*, 154–161.

Matsuhiro, B., & Zambrano, D. (1990). Carbohydrate constituents of *Lessonia trabeculata*. *Journal of Applied Phycology*, *2*, 183–185.

Matsuhiro, B., Zúñiga, E., Jashes, M., & Guacucano. (1996). Sulfated polysaccharides from *Durvillaea antarctica*. *Hydrobiologia*, *321*, 77–81.

Misra, C. K., Das, B. K., Mukherjee, S. C., & Pradhan, J. (2006). Effect of long term administration of dietary β-glucan on immunity, growth and survival of *Labeo rohita* fingerlings. *Aquaculture*, *255*, 82–94.

Modak, S., Koehne, G., Vickers, A., O'Reilly, R., & Cheung, N-K. V. (2009). Rituximab therapy of lymphoma is enhanced by orally administered (1 → 3),(1 → 4)-β-glucan. *Leukemia Research*, *29*, 679–683.

Rice, P. J., Adams, E. L., Ozment-Skelton, T., Gonzalez, A. J., Goldman, M. P., Lockhart, B. E., et al. (2005). Oral delivery and gastrointestinal absorption of soluble glucans stimulate increased resistance to infectious challenge. *The Journal of Pharmacology and Experimental Therapeutics*, *314*, 1079–1086.

Robertsen, B., Rorstad, G., Engstad, R., & Raa, J. (1990). Enhancement of non-specific disease resistance in Atlantic salmon, *Salmo salar* L., by a glucan from *Saccharomyces cerevisiae* cell walls. *Journal of Fish Diseases*, *13*, 391–400.

Sahoo, P. K., & Mukherjee, S. C. (2001). Effect of dietary β-1,3-glucan on immune responses and disease resistance of healthy and aflatoxin B_1-induced immunocompromised rohu (*Labeo rohita* Hamilton). *Fish & Shellfish Immunology*, *11*, 683–695.

Sandula, J., Kogan, G., Kacurakova, M., & Machova, E. (1999). Microbial (1 → 3)-β-D-glucans, their preparation, physico-chemical characterization and immunomodulatory activity. *Carbohydrate Polymers*, *38*, 247–253.

Santelices, B., Castilla, J., Cancino, J., & Schmiede, P. (1980). Comparative ecology of *Lessonia nigrescens* and *Durvillaea antarctica* (phaeophyta) in Central Chile. *Marine Biology*, *59*, 119–132.

Shoemaker, C. A., Klesius, P. H., & Lim, C. E. (2001). Immunity and disease resistance in fish. In C. E. Lim, & C. D. Webster (Eds.), *Nutrition & fish health* (pp. 149–163). New York: Haworth Press Inc.

Siwicki, A. K., Kazun, K., Głabski, E., Terech-Majewska, E., Baranowski, P., & Trapkowska, S. (2004). The effect of beta-1.3/1.6-glucan in diets on the effectiveness of anti-Yersinia Ruckeri vaccine—An experimental study in rainbow trout (*Oncorhynchus mykiss*). *Polish Journal of Food and Nutrition Sciences*, *13*, 59–61.

Soltanian, S., Stuyven, E., Cox, E., Sorgeloos, P., & Bossier, P. (2009). Beta-glucans as immunostimulant in vertebrates and invertebrates. *Critical Reviews in Microbiology*, *35*, 109–138.

Størseth, T. R., Kirkvold, S., Skjermo, J., & Reitan, K. I. (2006). A branched β-D-(1 → 3,1 → 6)-glucan from the marine diatom *Chaetoceros debilis* (Bacillariophyceae) characterized by NMR. *Carbohydrate Research*, *341*, 2108–2114.

Strand, H. K., & Dalmo, R. A. (1997). Adsorption of immunomodulating β-(1-3)-glucans in yolk sac larvae of Atlantic halibut *Hippoglossus hippoglossus* (L.). *Journal of Fish Diseases*, *20*, 41–49.

Theander, O., Åman, P., Westerlund, E., Andersson, R., & Pettersson, D. (1995). *AOAC Method 994.13, Official Methods of Analysis* (16th ed.). , 1st supplement.

Vilanova, M., Tavares, D., Ferreira, P., Oliveira, L., Nóbrega, A., Appelberg, R., et al. (1996). Role of monocytes in the up-regulation of the early activation marker CD69 on B and T murine lymphocytes induced by microbial mitogens. *Scandinavian Journal of Immunology*, *43*, 155–163.

Weirich, C. R., & Reigh, R. C. (2001). Dietary lipids and stress tolerance 1 of Larval Fish. In C. E. Lim, & C. D. Webster (Eds.), *Nutrition & fish health* (pp. 301–302). New York: Haworth Press Inc.

Wood, P. J. (2010). Oat and rye β-glucan: Properties and function. *Cereal Chemistry*, *87*, 315–330.

Zekovic, D. B., & Kwiatkowski, S. (2005). Natural and modified (1 → 3)-β-D-glucans in health promotion and disease alleviation. *Critical Reviews in Biotechnology*, *25*, 205–230.

Zvyagintseva, T. N., Shevchenko, N. M., Chizhov, A. O., Krupnova, T. N., Sundukov, E. V., & Isakov, V. V. (2003). Water-soluble polysaccharides of some far-eastern brown seaweeds, distribution, structure, and their dependence on the developmental conditions. *Journal of Experimental Marine Biology and Ecology*, *294*, 1–13.