

Fig. 3. Stimulation of proliferation of spleen cells with **2** and its fractions at 31.25 µg/ml, ■ and 62.5 µg/ml, ■. The controls were untreated cells and cells challenged with 20 µg/ml LPS; they are denoted by □. (n = 3).

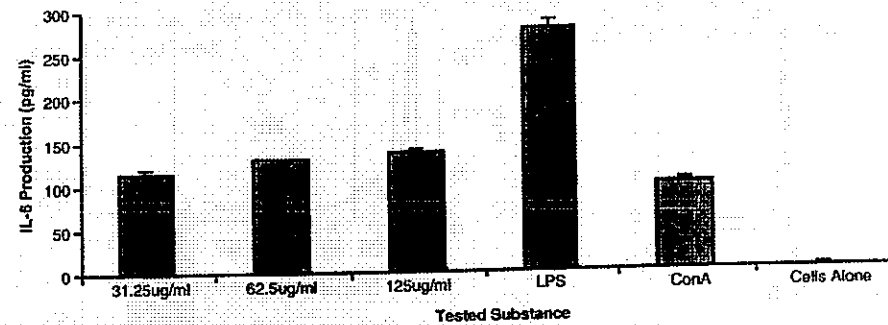


Fig. 4. IL-6 production by BALB/c mouse peritoneal macrophages. The positive controls, concanavalin A (10 µg/ml) and LPS at 20 µg/ml were included in the experiment (n = 3).

and decrease their solubility and hydratability resulting in the formation of non-covalent aggregates of differing stability.

Similar to carbohydrate glued protein aggregates (Matsubara and Ebina, 1997), components of Respondin™ aggregate over time leading to changes in molecular mass distribution. Since ultrafiltration shifts equilibrium between free molecules and their aggregates in favor of free entities, molecular masses estimated from ultrafiltration are more realistic than those determined from gel filtration in combination with MALS.

The immunomodulatory activity of Respondin™ is not affected by high temperature but digestion with pronase resulted in a decrease in the immunoreactivity, e.g. digestion of **2d** fragment resulted in 30–50% reduction in the immunoreactivity (data not shown). Thermostability is a very important feature for any nutraceutical or a functional food.

Immunostimulatory principles of higher molecular mass contained a higher proportion of protein. Interestingly, materials of higher purity such as **2a** or **2d** do not display markedly higher immunostimulatory activity than parent material **2** (Fig. 3). These fractions are likely composed of arabinogalactan-proteins combined

with glucorhamnans, as suggested by monosaccharide composition and the high percentage of hydroxyproline (Gane et al., 1995).

One benefit of B cell immunomodulators is that they can stimulate immune function in subjects who may have an impaired antibody response to an antigen. A B cell stimulator might increase the rapidity of the antibody immune response when presented with a new infection (Hadden, 1992). **1** (Respondin™, ONC-107) enhanced the antibody response to the influenza vaccine (Halperin, 2003). Macrophages are also important components of the initial response to infection. B cell proliferation and macrophage activation assays are used routinely in our laboratory, however, there are many other suitable immunotests that could be used to evaluate immunostimulatory products (Wagner et al., 1999). For instance, we plan to investigate other systems, particularly phagocytosis, since many high molecular weight polysaccharides stimulate this component of the immune system (Wagner et al., 1988).

Mizuno et al. (1980) isolated α-L-arabino-α-L-rhamno-α-D-galactan with a molecular mass of around 105 kDa from *Chlorella* and this material may resemble materials composing **2**. White and Barber (1972) isolated a complex 88 kDa acidic heteroglycan containing

mainly rhamnose, but also arabinose, galactose, xylose, mannose and glucuronic acid. A glycoprotein (218 kDa) studied by Konishi et al. (1985) is a non-polysaccharide-based immunomodulator from *Chlorella*. Only a few authors reported larger than 100 kDa immunomodulators; e.g. extraction of *Chlorella pyrenoidosa* cells with 70% ethanol resulted in a product with an estimated molecular mass of 10,000 kDa (Pugh et al. 2001).

The most compelling evidence for oral effectiveness of large immunotherapeutics comes from two of the most clinically successful immunostimulants, PSK and SPG (Nio et al., 1992; Furue, 1987). Similar to these polysaccharide-based immunostimulants, the initial effect of *Chlorella* biopolymers is likely triggered on intestinally situated immune cells. Structure-activity studies do not disclose why some polysaccharides are orally immunoactive, while others are only active parenterally (e.g. lentinan, Maeda and Chihara, 1999) or not active at all. Our findings confirmed structural complexity of *Chlorella*-based immunomodulators and further resolution of some of the fractions has already been achieved (Reyes-Suarez et al., 2005). Studies are in progress to determine how the new data can assist in the design of nuclear magnetic resonance-based quality control of these immunomodulators.

Acknowledgements

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500–1000 kDa region and was maximal for molecules with mass greater than 1000 kDa (Fig. 1).

Chromatography

Sephadex G100 resolved **2** into two distinct portions, a large molecular mass peak **2a** eluting in the void volume and smaller molecular mass regions **2b** and **2c** (Fig. 2A). Sephacryl S1000 FF resolved **2a** in a void volume peak **2d**, and broad regions **2e** and **2f** (Fig. 2B). A portion of **2a** that had a stronger affinity to Q Sepharose was liberated using 2 M NaCl and labelled as **2h** (Fig. 2C). Hydrophobic interaction chromatography of **2h** resulted in fractions **2i** and **2j**, resolved **2d** into three major regions: **2k**, **2l** and **2m** (Fig. 2D), however peaks **2e** and **2f** could not be resolved.

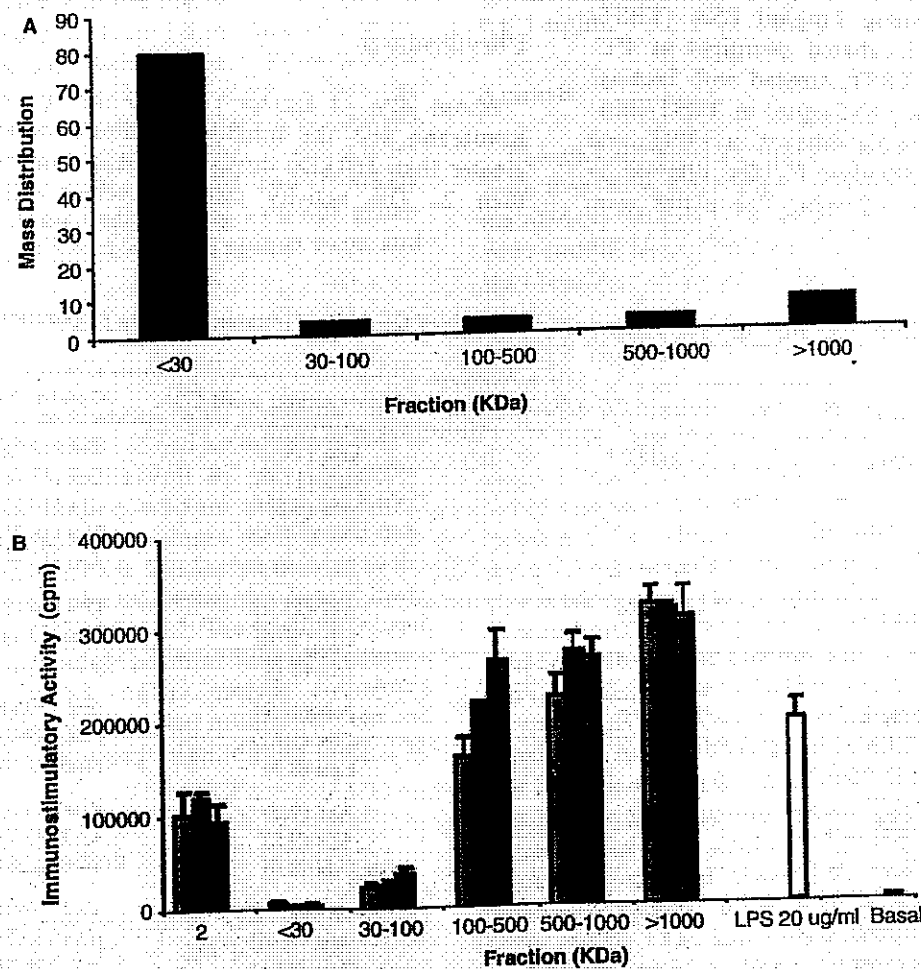


Fig. 1. Ultrafiltration of **1** (A) and immunostimulatory activity of the obtained fractions (B). Immunostimulating properties were measured by [³H]-thymidine incorporation by mouse splenocytes at 31.25 μg/ml, ■; 62.5 μg/ml, ■; 125 μg/ml, ■. The controls were untreated cells and cells challenged with 20 μg/ml LPS; they are denoted by □. (n = 3).

Physicochemical characterization of the isolated materials

Although the molecular mass range of biopolymers comprising **1** extends from a few kDa to about 30,000 kDa, the weight average molecular mass for the entire extract is determined to be around 90 kDa. The protein content of fractions **2a**, **2d**, **2e-f** and **2h** ranged from 9.4% to 37%. Glutamic and aspartic acid residues were major constituents and their content was similar in all preparations (Table 1). Fractions **2e** and **2f** contained a significant amount of 4-hydroxyproline. All fractions of **2a** contained about 1% aminoethanol, a known building block of *Chlorella* cells (Kneifel, 1979) suggesting the presence of a lipid component.

Hydrolysates produced from **2** contained ribose (small RNA fragments were present—data not shown), galactose, glucose, rhamnose and arabinose. Only about

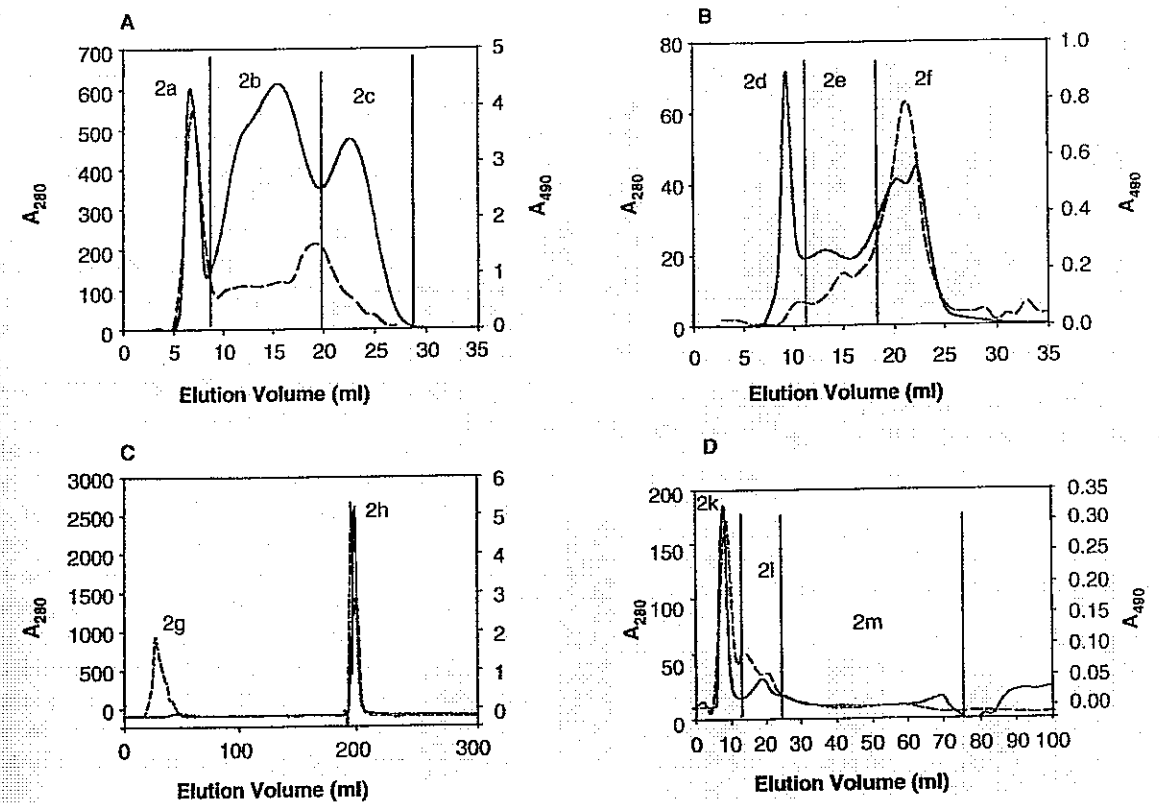


Fig. 2. Chromatography of **2** using Sephadex G100 (A), **2a** using Sephacryl S1000 FF (B) or Q Sepharose (C), and **2d** and **2f** using a HiPrep 16/10 Butyl FF column (D). The fractions were monitored at 280 nm (—), and at 490 nm (---) after mixing with phenol-sulfuric acid reagent.

1% of aminosugars and 6% of uronic acids were detected. Hydrolysates of fraction **2a** did not contain ribose or glucose but contained galactose, rhamnose and arabinose. **2d** had three times more galactose than arabinose, while **2e** and **2f** contained galactose, rhamnose and arabinose in almost equal amounts. Methyl 3-O-methyl- α -L-rhamnopyranoside and methyl 2-O-methyl- α -L-rhamnopyranoside, monosaccharides identified in hydrolysates of *Chlorella vulgaris* by others (Ogawa et al., 1997), were not detected in our materials.

Stimulation of different immune cell types

The immunostimulatory activity of various fractions is summarised in Fig 3. Proliferation of BALB/c splenocytes challenged with dialyzed extract **2** or its fractions revealed that the highest immunostimulatory activity was associated with high molecular masses. Further chromatographic resolution based on molecular size did not increase bioactivity but ultrafiltration revealed a strong relationship of bioactivity with molecular size within the >100 kDa range. The larger molecular masses composing **2a** and represented by **2d** are protein/polysaccharide complexes, whereas the low-

er molecular fractions **2e** and **2f** are mainly polysaccharides. Material **2h** (fraction of **2a**) had a strong affinity for Q Sepharose anion exchanger, was composed mainly of polysaccharide/protein complexes and exhibited strong immunoactivity. B lymphocyte proliferation was markedly stimulated with **2** and its components, whereas T cell proliferation was not stimulated significantly (data not shown). Treatment of mouse peritoneal macrophages by **2** stimulated IL-6 production (Fig. 4). Production of IL-10, IL-12, and γ -IFN was also stimulated (data not shown).

Discussion

Correlation between molecular mass and immunoactivity of *Chlorella*-based biopolymers resembles findings observed for glucans, e.g. SPG (Yamaki et al., 1983). A need for significantly smaller amounts of guanidine-based eluent compared to water (data not shown) for effective ultrafiltration of **1** suggests the existence of strong non-covalent assemblies. Resolution by size exclusion chromatography is difficult because salts compete with polysaccharides for the water of hydration,

were obtained from Taiwan *Chlorella* Manufacturers Ltd. (Taipei, Taiwan). Chromatography methods were developed using the AKTA FPLC system (Amersham Pharmacia Biotech) at a linear flow rate of 0.1 ml/min. and scaled up for preparative runs. Samples were dissolved in running buffers and filtered through 0.45 µm filter. RPMI 1640 and tetramethyl benzidine substrate media were obtained from Gibco-BRL (Invitrogen Life Technologies, Burlington, ON). Costar 3950 ELISA and 3596 tissue culture plates were purchased from Fisher Scientific (Halifax, NS). Cytokines and the corresponding antibodies for evaluation of activity of macrophages were obtained from Pharmigen Canada (Mississauga, ON). Anti-Thy-1.2- and anti-B220-coated (anti-CD45R) Miltenyi microbeads, MidMACS columns and magnets were purchased from Miltenyi Biotec (Auburn, CA).

Extraction/ultrafiltration

Chlorella cells were extracted with five volumes of 0.003% benzoic acid at 80 °C for 1 h, centrifuged and the pellet washed twice with distilled water. The combined liquids were evaporated to 1/3 of the volume, a third was directly lyophilized to obtain 1, a third was dialyzed against water and lyophilized to obtain 2, and a third was fractionated using 30, 100, 500 and 1000 kDa ultrafiltration membranes after mixing with 4 M guanidine hydrochloride (1:1 v/v), dialyzed against water and lyophilized.

Chromatography

Material 2 was fractionated with Sephadex G100 column equilibrated with 0.1 M sodium acetate in 0.02% sodium azide, pH 4.5. Fraction 2a was chromatographed under the same conditions using Sephacryl S1000 FF and a Q Sepharose column equilibrated with 20 mM ethanolamine, pH 8.0. Species not binding to the anion exchanger were cleared before the bound compounds were eluted with 2 M NaCl. The fractions 2d and 2h were treated using a HiPrep 16/10 Butyl FF column equilibrated with 0.1 M sodium acetate, containing 0.02% sodium azide. Non-binding species were cleared before the bound compounds were eluted using 1 M Na₂SO₄.

Carbohydrate composition

Briefly, samples were stirred in 1 M trifluoroacetic acid (3.0 mg/ml) under N₂ at 100 °C for 3 h, dried, reduced with 0.5 M NaBH₄ in 0.6 ml of 1 M NH₄OH and acetylated with 3.0 ml of acetic anhydride. The retention times and mass spectra of the tested samples were compared with commercially available mixtures of

alditol acetates (Supelco, Inc. (St. Louis, MO). Determination of uronic acid and *N*-acetylamino sugar content was performed as described by Blumenkrantz and Asboe-Hansen (1973) and Reissig et al. (1955), respectively.

Amino acid analysis and protein content

Samples were hydrolyzed in 6 M HCl under vacuum at 110 °C for 20 h. The protein content was determined with the BCA method (Smith et al., 1985) or from amino acid analysis.

Molecular mass distribution by MALS

Samples (2 mg/ml) in 88 mM sodium acetate buffer, containing 40 ppm of sodium azide, pH 4.5 were subjected to HPLC prior to light scattering and RI analysis. The flow rate was 0.60 ml/min. and the *dn/dc* values were determined using a series of known concentrations of sample covering a range of 0.1–1 mg/ml.

Animals

Female BALB/c mice aged 6–8 weeks were purchased from Charles River Canada (Saint-Constant, PQ) and maintained under a 12 h light/dark cycle. The studies were carried out in full compliance with regulations of the Canadian Council on Animal Care.

Immune cell isolation and proliferation assay

Mouse splenocytes were isolated as described (Wong et al., 1988). Enriched preparations of B or T cells were prepared using a negative selection procedure. Briefly, a 1.0 ml suspension of splenocytes (3 × 10⁷ cells/ml) was incubated for 20 min. in the presence of 100 µl of Miltenyi microbeads coated with anti-Thy-1.2-antibody (for B cell preparation) or anti-B220 antibody (for T cell preparation) and applied to a midiMACS column/magnet system. The column was rinsed with 5.0 ml of PBS containing 2 mM EDTA and 0.5% bovine serum albumin, pH 7.4. This was centrifuged and the cells resuspended to 5 × 10⁶ cells/ml in cRPMI and plated at 3 × 10⁵ cells/well in 0.1 ml of cRPMI medium in 96-well flat bottom tissue culture plates. The samples dissolved in the cell medium were added to triplicate wells to give a final volume of 0.2 ml/well and the plates were incubated at 37 °C under a humidified atmosphere of 95% O₂/5% CO₂. Lipopolysaccharide (LPS, 20 ng/ml) and concanavalin A (10 ng/ml) were added to triplicate wells as a positive control. After 48 h, the cells were pulsed with [³H]-thymidine (1 µCi/well in 10 µl cRPMI), incubated for 18 h, harvested onto glass fiber filters and

the radioactivity determined by liquid scintillation counting.

Stimulation of cytokine production by mouse macrophages

Peritoneal macrophages, obtained by lavage with 10 ml of ice-cold cRPMI medium injected into the peritoneal cavity, were cultured in Costar 3596 culture plates in the presence of the samples or controls. The culture media were collected after 48 h and stored at –80 °C. EIA plates were coated with the appropriate anti-cytokine monoclonal antibody in carbonate buffer (0.05 M, pH 9.6) at 4 °C overnight, washed with TBS (Tris 20 mM, NaCl 28 mM, and KCl 5.5 mM, pH 7.4), coated with 2 mg/ml BSA in TBS (200 µl/well) for 2 h at room temperature, and washed with TBST (TBS, containing 0.05% Tween 20). Culture media from the various treatments (1/200, 1/400, 1/1600 and 1/3200 dilutions in TBST containing 1 mg/ml BSA (TBST/BSA)) were applied and incubated overnight at 4 °C. Plates were washed with TBST, treated with biotinylated anti-cytokine antibody (0.5 µg/ml in TBST/BSA; 100 µl/well) for 1 h, washed six times with TBST, and treated with extravidin-peroxidase (1/1000 dilution in TBST/BSA; 100 µl/well) for 30 min. The plates were

washed, developed using tetramethyl benzidine substrate solution (100 µl/well) and read at 450 nm.

Results

Extraction

Material 1 was produced with an average yield of 10.2%. Dialysis followed by lyophilization provided 2 in a 6.8% yield with specific immunostimulatory activity increased by about 25%. The extraction could be executed at temperatures ranging from 0 to 100 °C for up to 2 h without compromising the immunostimulatory activity (data not shown). Although longer extraction times at 80 °C resulted in higher yields, the immunostimulatory activity was diminished. The yield, immunostimulatory activity and other physicochemical characteristics of the basic immunostimulatory preparation 2 and selected fractions are listed in Table 1.

Ultrafiltration

About 70% of extract 1 passed through a 30 kDa membrane and the fractions with molecular masses greater than 100 kDa displayed higher immunostimulatory activity than 2. The activity started levelling off in the

Table 1. Yields, immunoactivities and other physicochemical characteristics of the selected immune boosting preparations isolated from *Chlorella pyrenoidosa*

Material	Yield (%)	RISA (% of 2)	Protein (%) (BCA/AAA)	Major amino acids (%)	Monosaccharides (%)
2	6.8	100.0	27.6/37.7	Glx (14.7) Asx (11.5) Ala (9.8)	Rib (23.6) Gal (22.6) Glu (18.5) Rha (18.3) Ara (13.9)
2a	13.5	169.7	28.6/24.7	Asx (11.2) Glx (10.6) Ala (8.2)	Gal (38.0) Rha (33.7) Ara (23.3)
2d	18.5	176.4	37.3/37.7	Asx (11.9) Glx (10.7) Leu (8.5)	Gal (50.6) Rha (29.6) Ara (17.5)
2e	40.9	85.8	9.4/10.5	Asx (9.5) His (9.2) Glx/Ala (8.6)	Rha (35.5) Gal (33.7) Ara (25.9)
2h	48.4	148.6	11.4/13.3	Glx (11.2) Asx (10.8) Leu (8.8)	Gal (39.4) Rha (30.7) Ara (20.4)

Samples (125 µg/ml) were compared to the activity of 2 (taken as 100%) and are expressed in terms of relative immunostimulatory activity (RISA) as determined using both the BCA method and amino acid analysis (AAA). Major amino acids are listed as percentages of total amino acid content.

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Immunostimulatory principles from *Chlorella pyrenoidosa*—Part 1: Isolation and biological assessment in vitro

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Abstract

Our proprietary preparation obtained by extraction of *Chlorella pyrenoidosa* cells, ONC-107 (RespondinTM), was recently found to selectively boost antibody response to the influenza vaccine in a human clinical trial. RespondinTM is a potent stimulator of mouse B cell proliferation and an activator of macrophages. Bioactivity-guided resolution concluded that RespondinTM is composed of a mixture of immunostimulatory principles of different chemical nature. A combination of size exclusion, anion exchange and hydrophobic interaction chromatography revealed that the bulk of the immunostimulatory activity resides in polysaccharide/protein complexes with molecular masses larger than 100 kDa that are composed primarily of galactose, rhamnose and arabinose.

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Keywords: *Chlorella* extract; Bioassay-guided fractionation; Immunostimulatory; B cells; Macrophages

Introduction

Chlorella is a unicellular green alga found in both fresh and marine water. It is a valuable source of protein and essential amino acids, relatively low in cellulose and exhibiting a remarkable spectrum of physiological properties. *Chlorella* is popular in Japan and was introduced in the USA as a health food when novel technology made it more digestible (Mitsuda et al., 1977). It is largely associated with immune system-mediated effects on cancer and bacterial, fungal and viral infections (Kojima et al., 1973; Hasegawa et al., 1990; Tanaka et al., 1998). Distinct *Chlorella*-based immunostimulatory materials have been reported (e.g. Mizuno et al., 1980; Pugh et al., 2001; White and Barber, 1972; Umezawa and Komiyama, 1985). A

proprietary *Chlorella*-based preparation (RespondinTM, ONC-107; Kralovec et al., 2003), was found to boost antibody response to the influenza vaccine in a human clinical trial (Halperin et al., 2003). Bioassay-guided fractionation revealed that the active principles of RespondinTM are of distinct chemical nature and here we report on initial work on fractionation, chemical evaluation and in vitro assessment of the immunomodulator.

Materials and methods

General

Chemicals (analytical-grade) and 12 kDa dialysis membranes were purchased from Sigma Aldrich (St. Louis, MO). Lyophilized *Chlorella pyrenoidosa* cells

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