Letters in Applied Microbiology
Volume 35 Issue 4 Page 267 - October 2002

# Preparation of microparticulate B-glucan from *Saccharomyces cerevisiae* for use in immune potentiation

K.W. Hunter Jr, R.A. Gault and M.D. Berner

**Aims:** To develop a method for the preparation of an immunologically active, homogeneous, nonaggregated, microparticulate β-glucan-containing material from the budding yeast *Saccharomyces cerevisiae*.

**Methods and Results:** Using a combination of sonication and spray-drying, a homogeneous preparation of 1-2-μ diameter β-glucan-containing particles was made from alkali- and acid-insoluble yeast cell wall material. **This microparticulate β-glucan remained in suspension longer and, following oral administration at 0·1 mg kg $^{-1}$ for 14 d, enhanced phagocytosis of mouse peritoneal macrophages significantly better than did aggregated β-glucan particles.**

**Conclusions: A new sonication and spray-drying method can be employed to overcome the problem of aggregation of β-glucan microparticles in aqueous media.**

**Significance and Impact of the Study: A microparticulate form of β-glucan that remains in suspension longer for pharmaceutical applications and has superior immune potentiation characteristics has been developed.**

## Introduction

β-glucans are polymers of β-(1,3)-D-glucose [with or without β-(1,6)-D-glucose side chains] found in the cell walls of many bacteria, plants and yeasts. There is an extensive literature describing the immunomodulating effects of both water soluble and insoluble β-glucans, with macrophages as the principal target cells (Reynolds *et al*. 1980; DiLuzio 1983; Gallin1992; Cleary *et al*. 1999). While various soluble and particulate β-glucans have been used in pharmaceutical applications (Williams *et al*. 1992; Chihara 1992; Babineau *et al*. 1994) particulate β-glucan preparations derived from the yeast *Saccharomyces cerevisiae* are widely used as over-the-counter nutritional supplements. Examination of several commercially available products consistently revealed a predominant 'globular' morphology consisting of aggregated β-glucan particles ranging in size from 5 to 100-μ diameter, with some unaggregated single particles in the 1-2-μ range.

While globular β-glucan preparations have immune potentiating activity, it was thought that a homogeneous preparation of smaller particles would be more efficient at activating macrophages, as well as more suitable for incorporation into pharmaceutical and cosmetic

formulations. **As 1-2-μ diameter particles are optimally phagocytized by macrophages (**Tabata and Ikada 1988**), our goal was to increase the number of microparticles in this size range in the β-glucan preparations**.

However, even after extensive grinding and sieving of dried β-glucan extracted from yeast cell walls, it was discovered that the β-glucan particles formed aggregates when suspended in aqueous media. Therefore, **we devised a sonication and spray drying method that yielded a consistent 1-2-μ diameter particle that remained dispersed upon hydration. Although both aggregated and microparticulate glucans enhanced peritoneal macrophage activation when administered orally to mice, the microparticulate glucan was significantly better than the aggregated form.**

## Materials and Methods

### Processing of yeast glucan

The starting *S. cerevisiae* β-glucan material was obtained from Nutritional Supply Corporation (Carson City, NV, USA). This material was processed from common baker's yeast using the following procedure. Active dry yeast was added to 0·1 mol $l^{-1}$ of NaOH and stirred for 30 min at 60 °C. The material was then heated to 115 °C at 8·5 psi for 45 min and then allowed to settle for 72 h. The sediment was resuspended and washed in distilled $H_2O$ by centrifugation (350 g for 20 min). The alkali insoluble solids were combined with 0·1 mol $l^{-1}$ acetic acid and heated to 85 °C for 1 h, then allowed to settle at 38 °C. The acid insoluble solids were drawn off and centrifuged as above. The compacted solid material was mixed with 3% $H_2O_2$ and refrigerated for 3 h with periodic mixing. The material was then centrifuged and the pellet washed twice with distilled $H_2O$ followed by two washes in 100% acetone. The harvested solid material was dispersed on drying trays and dried under vacuum at 38 °C for 2 h in the presence of $Ca_2SO_4$, and then further dried overnight under vacuum at room temperature. This procedure yielded a white powder with less than 5% protein, lipid and nucleic acid. Carbohydrate analysis revealed 85% hexoses (using the anthrone method) with 4·5% chitin (measured as N-acetylglucosamine).

### Preparation of microparticulate β-glucan

Examination of the β-glucan-containing powder dispersed in saline revealed aggregates ranging from 5 to 100 μ in diameter (20 μ on average). To make a uniform 1-2-μ diameter particulate preparation, the aggregated β-glucan material was first hydrated in distilled $H_2O$ overnight at 4 °C. A 1·5% suspension of the hydrated material was subjected to sonic energy via a 19-mm probe utilizing a 300-V/T Sonic Dismembrator (BioLogics, Gainesville, VA, USA). Using an ultrasonic output frequency of 20 kilohertz per s at 192 watts, the glucan suspensions were sonicated on ice for 12 min (12 48-s cycles of sonication with a 12-s pause between cycles). The sonicated β-glucan suspension was spray-dried using a Buchi 190 Mini-Spray Dryer (Buchi, Germany) with an inlet air temperature of 110-170 °C, an outlet air temperature of 90-120 °C and an atomizer pressure of 30-100 psi. Using flow cytometric analysis with an EPICS XL-MCL Flow Cytometer (Coulter Electronics, Hialeah, FL, USA), 1 mg of sonicated glucan consisted of $1{\cdot}81 \times 10^{11}$ microparticles.

### Morphology and sedimentation of the β-glucan preparations

β-glucan preparations were suspended in normal saline and viewed with a Nikon Eclipse E400 microscope under bright-field illumination. Photomicrographs were taken with a Kodak DC digital camera. Dried β-glucan samples were placed on an s.e.m. specimen holder and sputter-coated with gold to an approximately 200-Å thickness. Prepared samples were viewed on a JEOL TSM T300 Scanning Electron Microscope (s.e.m.).

Suspensions of aggregated β-glucan and microparticulate β-glucan were prepared in distilled $H_2O$ (1·5% w/v). Each suspension was vortexed for 10 s and allowed to settle in one gravity in a 15-ml test tube for 0, 2, 5, 10, 20, 30 or 60 min. Photographs were taken of the sedimentation using a Kodak digital camera.

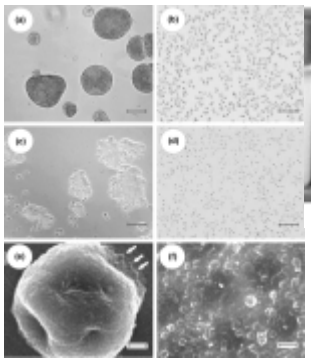
### Phagocytosis of bioparticles by peritoneal macrophages from mice treated orally with β-glucan preparations

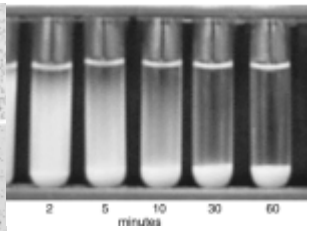
Six- to eight-week old female BALB/c mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA). The mice were housed and cared for under an approved protocol in accordance with NIH/USDA guidelines. They were given daily oral doses of 0·1 mg $kg^{-1}$ aggregated β-glucan or microparticulate β-glucan adsorbed on a food pellet (vehicle control was food pellet only) for 2 weeks. On day 14 the mice were killed and their resident peritoneal macrophages removed by lavage with cold RPMI-1640 medium without serum. Because Gram-negative bacterial endotoxin or lipopolysaccharide is a potent macrophage activator and inducer of proinflammatory mediators, all biochemical reagents and culture media were obtained as endotoxin-free or screened for LPS by the Limulus test (BioWhittaker, Walkersville, MD, USA). However, because β-glucan also triggers clotting in the standard Limulus test, we employed the LPS-specific Glucospecy test to screen for LPS in our β-glucan preparations (Shigegaku, Tokyo, Japan).

All reagents and β-glucan preparations had < 10 pg $ml^{-1}$ LPS. After 1 h of incubation at 37 °C in a 5% $CO_2$ in air atmosphere, nonadherent cells were removed by washing in warm culture medium and the adherent cells (> 85% macrophages by morphology) were cultured for 24 h in RPMI-1640 medium containing 10% fetal bovine serum. The macrophages were tested for their ability to ingest fluorescein isothiocyanate-labelled bioparticles using the Vybrant™ Phagocytosis Assay Kit (Molecular Probes, Eugene, OR, USA). The percentage of cells ingesting fluorescent bioparticles, and the number of fluorescent bioparticles per cell were determined using a Nikon Eclipse E400 fluorescent microscope. At least 100 cells in each of 10 replicate wells per treatment were counted after the fluorescence of noninternalized bioparticles was quenched with trypan blue. Differences between the means of the treatment groups were evaluated using Student's *t*-test for paired samples.

## Results and Discussion

β-glucan-containing material resulting from the chemical extraction process detailed in the Methods section was examined by light microscopy after hydration in distilled $H_2O$ or saline.






This material was determined to be a heterogeneous mixture of individual microparticles (1-2 μ in diameter) and glucan particle aggregates ranging from 5 to 100-μ diameters (Fig. 1a). As there was evidence that **macrophages, key target cells for the immunopharmacological activity of β-glucans, preferentially ingest particles in the 1-2-μ diameter size range** (Tabata and Ikada 1988), we wanted to develop a method for making microparticulate glucan. However, initial attempts to disrupt the aggregates by vigorous vortexing, heating (100 °C), or treatment with strong acid (2 N HCl) or strong base (2 N NaOH) failed (data not shown). Because ultrasonic energy has been used to prepare microparticles in other systems (Hata *et al*. 2000), we investigated sonication as a method of disrupting the glucan aggregates.

Although disaggregation was accomplished by sonication using the optimized conditions outlined in the Methods section (Fig. 1b), when the sonicated material was air-dried (either directly or after addition of various organic solvents such as acetone) the resulting dry material had the consistency of cardboard. This material could be ground into a fine powder with a mortar and pestle, but upon hydration in distilled $H_2O$ or saline it demonstrated significant aggregation (Fig. 1c). To overcome this re-aggregation problem, we employed a spray-drying technique. The fine powder resulting from this spray-drying process when hydrated in distilled $H_2O$ or saline resulted in a homogeneous suspension of 1-2-μ diameter particles with very few small aggregates (Fig. 1d).

Interestingly, the addition of an excipient like maltodextrin did not significantly improve the process. A similar ultrasonic approach was used by Levis and Deasy (2001) to achieve particle size reduction of microcrystalline cellulose. These authors discovered that re-aggregation in aqueous media was substantially reduced by spray-drying, with or without the addition of a surfactant. Just how sonication and spray-drying alters the chemical or physical attributes of particles to mitigate against re-aggregation remains to be determined.

The aggregated β-glucan and the microparticles obtained following the sonication and spray-drying procedure were gold shadowed and examined with a s.e.m.. Figure 1(e) shows the morphology of a typical aggregate with a diameter of approximately 35 μ. Note that this aggregate appears to be composed of subunits in the 1-2-μ diameter size range (→). Sonication and spray drying results in separate and discrete microparticles in the 1-2-μ diameter size range (Fig. 1f). This analysis indicates that the aggregated β-glucan is composed of discrete subcomponents that can be disrupted into 1-2-μ diameter microparticles by a combination of sonication and spray-drying. The chemical composition (β-glucan and chitin) and size of the β-glucan microparticles suggest that they may be yeast bud scars (Bacon *et al*. 1969; Manners *et al*. 1973). We are presently investigating this notion.

To demonstrate that the microparticulate β-glucan preparation had a lower sedimentation rate, we performed the experiment shown in Fig. 2. As can be seen from this figure, after 1 h of sedimentation at 1 g the microparticulate β-glucan demonstrated very little settling, where as the aggregated β-glucan preparation had nearly sedimented fully. Indeed, some settling of the aggregated β-glucan was observed at even the earliest time point. **Because the microparticulate β-glucan remains in aqueous suspension longer, it can be more easily formulated into gels**

**and creams for dermatological applications.**

**β-glucans bind to glucan receptors on phagocytic cells** (Goldman 1988; Czop and Kay 1991; Brown and Gordon 2001) **and cause these cells to become 'activated'** (DiLuzio 1983). Earlier studies by Suzuki *et al*. (1990) in mice showed that oral administration of a β-1,3-glucan derived from the fungus *Sclerotinia sclerotiorum* enhanced the phagocytic activity of peritoneal macrophages. Therefore, we compared the ability of orally administered microparticulate and aggregated β-glucan preparations given at 0·1 mg $kg^{-1}$ daily for 14 d to enhance peritoneal macrophage phagocytosis. **Note that this dosage is equivalent to a 10-mg capsule of β-glucan given orally to a 75-kg human.** As shown in Table 1, cultured peritoneal macrophages taken from mice treated with either microparticulate β-glucan or aggregated β-glucan increased the percentage of peritoneal macrophages ingesting bioparticles over the vehicle control ($P< 0·05$).

In addition, **the microparticulate β-glucan was more stimulatory than the aggregated β-glucan** ($P=0·06$). Also, the number of bioparticles ingested/cell was increased over controls by both aggregated β-glucan and microparticulate β-glucan ($P< 0·05$), and macrophages from microparticulate β-glucan-treated mice ingested more bioparticles/cells than macrophages from mice treated with aggregated β-glucan ($P=0·06$). These data imply that both microparticulate and aggregated β-glucan can survive transit through the gastrointestinal tract in forms capable of being absorbed and ultimately of interacting with β-glucan receptors on the surfaces of resident peritoneal macrophages. It appears that **the same dose of microparticulate β-glucan is better able to enhance macrophage phagocytosis than aggregated β-glucan.**

In conclusion, we have developed a new method for preparing homogeneous, nonaggregated, 1-2-μ diameter β-glucan-containing particles from yeast cell walls. **Compared with the aggregated form of β-glucan, the β-glucan microparticles remain in suspension longer for pharmaceutical applications and are more effective at enhancing phagocytosis by peritoneal macrophages following oral administration.**

## Acknowledgements

We thank Brandon Carter for technical assistance. This work was supported by an Applied Research Initiative Matching Grant from the State of Nevada and Nutritional Supply Corporation, Carson City, Nevada, NV, USA.

## References:


- Babineau, T.J., Marcello, P., Swails, W., Kenler, A., Bistrian, B. and Forse, R.A. (1994) Randomized phase I/II trial of a macrophage-specific immunomodulator (PPG-glucan) in high-risk surgical patients. *Annals of Surgery* **220**, 601-609.
- Bacon, J.S.D., Farmer, V.C., Jones, D. and Taylor, I.F. (1969) The glucan components of the cell wall of Baker's yeast (*Saccharomyces cerevisiae*) considered in relation to its ultrastructure. *Biochemical Journal* **114**, 557-569.
- Brown, G.D. and Gordon, S. (2001) A new receptor for β-glucans. *Nature* **413**, 36-37.
- Chihara, G. (1992) Recent progress in immunopharmacology and therapeutic effects of polysaccharides. *Development of Biological Standards* **77**, 191-197.
- Cleary, J.A., Kelly, G.E. and Husband, A.J. (1999) The effect of molecular weight and β-1,6-linkages on priming of macrophage function in mice by (1,3)-β-D-glucan. *Immunology and Cell Biology* **77**, 395-403.

- Czop, J.K. and Kay, J. (1991) Isolation and characterization of β-glucan receptors on human mononuclear phagocytes. *Journal of Experimental Medicine* **173**, 1511-1520.
- DiLuzio, N.R. (1983) Immunopharmacology of glucan: a broad spectrum enhancer of host defense mechanisms. *Trends in Pharmacological Science* **4**, 344-347.
- Gallin, E.K., Green, S.W. and Patchen, M.L. (1992) Comparative effects of particulate and soluble glucan on macrophages of C3H/HeN and C3H/HeJ mice. *International Journal of Immunopharmacology* **14**, 173-183.
- Goldman, R. (1988) Characteristics of the β-glucan receptor of murine macrophages. *Experimental Cell Research* **174**, 481-490.
- Hata, H., Onishi, H. and Machida, Y. (2000) Preparation of CM-chitin microspheres by complexation with iron (III) in w/o emulsion and their biodisposition characteristics in mice. *Biomaterials* **21**, 1779-1788.
- Levis, S.R. and Deasy, P.B. (2001) Pharmaceutical applications of size reduced grades of surfactant co-processed microcrystalline cellulose. *International Journal of Pharmaceutics* **6**, 25-33.
- Manners, D.J., Masson, A.J. and Patterson, J.C. (1973) The structure of a b-(1,3)-D-glucan from yeast cell walls. *Biochemical Journal* **135**, 19-30.
- Reynolds, J.A., Kastello, M.D., Harrington, D.G., Crabbs, C.L., Peters, C.J., Jemski, J.V., Scott, G.H. and Di Luzio, N.R. (1980) Glucan-induced enhancement of host resistance to selected infectious diseases. *Infection and Immunity* **30**, 51-57.
- Suzuki, I., Tanaka, H., Kinoshita, A., Oikawa, S., Osawa, M. and Yadomae, T. (1990) Effect of orally administered beta-glucan on macrophage function in mice. *International Journal of Immunopharmacology* **12**, 675-684.
- Tabata, Y. and Ikada, Y. (1988) Effect of the size and surface charge of polymer microspheres on their phagocytosis by macrophages. *Biomaterials* **9**, 356-362.
- Williams, D.L., Pretus, H.A., Mcnamee, R.B., Jones, E.L., Ensley, H.W. and Browder, I.W. (1992) Development of a water-soluble, sulfated (1->3)-beta-D-glucan biological response modifier derived from *Saccharomyces cerevisiae*. *Carbohydrate Research* **235**, 247-257.