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Preparation of Water Soluble Yeast Glucan by Four Kinds of Solubilizing Processes

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ABSTRACT

(1→3)-β-D-glucan from the inner cell wall of Saccharomyces cerevisiae is considered a member of a class of drugs known as biological response modifiers (BRM). However the glucan was an insoluble polysaccharide, which could be the major barrier to the utilization of glucan. In this case, the insoluble glucan was convent into a soluble form by four kind of solubilizing processes. The yield, solubility, chemistry structure and immunoprophylaxis efficacy of the soluble products were compared. Our date suggest that urea has a significant effect on yield, and DMSO has a significant effect on solubility. FT-IR spectra, ^{13}C NMR spectra and helix-coil transition analysis demonstrate that the chemistry structure of native and solubilizing glucans have no significant difference. They still have the triple helical structure. The solubility and immunoprophylaxis efficacy assay indicate that the introduction of phosphate group not only enhanced the solubility of glucan, but also improved the survival rate of mice challenged with *E. coli*.

Keywords: Glucan; Solubility; FT-IR Spectra; 13C NMR Spectra; Immunoprophylaxis Efficacy

1. Introduction

Glucan is a β-linked polyglucose immune stimulant that can be isolated from the cell wall of *Saccharomyces cerevisiae* [1,2]. It can be used as fat replacer, gelling agent, thickening agent [3-6]. It is also considered a member of a class of drugs known as biological response modifiers (BRM) [7]. It has been reported that (1→3)-β-glucan enhances the innate host defense response by binding to a specific receptor on the plasma membrane of macrophages, resulting in their activation, and this activity is associated with anti-tumor, anti-bacterial and wound-healing activities [8-12]. However, the native glucan was an insoluble polysaccharide, which became a major barrier to the utilization of (1→3)-β-glucan as BRMs. If (1→3)-β-glucan is to become clinically applicable, it has to be converted into biologically effective, water-soluble form that can be safely administered via the systemic route. Numerous studies have therefore focused on converting these glucans into a water-soluble form through chemical modification such as amination [13], sulfation [14] and phosphorylation [15]. It has also been demonstrated that such modified polysaccharides exhibit anti-inflammatory, anti-tumor, anti-viral and immunomodulatory activities [16-18].

In this study, we have attempted to convert insoluble yeast glucans into a soluble form through phosphorylation process. However, in this process we found that only DMSO or DMSO plus urea (without H_3PO_4) could convert insoluble glucan into a soluble form. Therefore, four kind of preparation of soluble glucan process were investigated. And four different soluble products were compared by solubility, FT-IR spectra, ^{13}C NMR spectra, and helix-coil transition analysis and immunoprophylaxis efficacy assay.

2. Materials and Methods

2.1. Materials

Isolated cell walls from Baker's yeast were supplied as "spray-dried" powder by Angel Yeast LLC, China. Male ICR/HSD mice (18-20 g) were supplied by the Experimental Animal Center of the Academy of Military Medical Science (AMMS), Beijing, China. Mice were housed in a specific pathogen-free (SPF) environment. All other major reagents were purchased from Sigma (St. Louis, MO, USA).

2.2. Preparation of Particulate Glucans

Particulate glucans were isolated from the cell wall of *S. cerevisiae* by a modification of the method of Williams et al [15]. Briefly, the cell wall of *S. cerevisiae* was dispersed in 0.3 L of 0.75 M (3 %) sodium hydroxide (NaOH), and extracted in water bath at 75 ºC for 2 hours. The suspension was cooled to room temperature and centrifuged (4000 rpm, 10 min), the resulting pellet collected and this material then washed three times using 0.5 L distilled water. The residue was then mixed with 0.2 L of 0.75 M NaOH, and extracted in boiling water for 3 hours. Distilled water was then added to the cooled suspension, the pH adjusted to 7 with HCl, and the supernatant after centrifugation discarded. This water wash was repeated until the residue became white and flocculent, and finally the pellet was washed with absolute ethanol until the supernatant became colorless. Particulate glucan was obtained by drying the washed pellets.

2.3. Preparation of Soluble Glucans

Different soluble glucans were prepared by using the improved

method of Williams et al [15]. Briefly, particulate glucan (1 g) was suspended in 60 ml dimethyl sulfoxide (DMSO) containing 15 g urea. H_3PO_4 (85%, 15 ml) was added dropwise to the solution at room temperature (total volume 75 ml, volume ratio between H_3PO_4 and DMSO 1:4). The solution was then heated to 100 ºC for 5 hours, while the solution was constantly stirred, to bring about the reaction. A crystalline precipitate (presumably urea phosphate) formed after about 1.5 hours. The reaction mixture was then cooled to ambient temperature, and 500 ml distilled water added to dilute the solution. The solution was then dialyzed against deionizer water for 5 days to remove Me_2SO, H_3PO_4, urea and any other small compounds, and then filtrated, concentrated, and freeze-dried to obtain the pure samples. Different soluble glucans were generated by varying the dosage of urea and H_3PO_4, which were tabulated in **Table 1**.

2.4. Determination of Water Solubility

The water solubility of glucans was measured using a modification of the method of Chang et al [19]. Briefly, each sample (1 g) was suspended in distilled water (5 ml) and the resulting suspension agitated at 25 ºC for 24 hours. The sample was centrifuged at 1600 × g for 15 min. The supernatant (2 ml) was mixed with three volumes of ethanol to precipitate the glucan. The precipitates were recovered by centrifugation at 1600 × g for 15 min, vacuum-dried at 60 ºC, and weighed. The solubility was calculated using the formula below:

$$\text{Solubility}(\%) = 100 \times (2.5 \times \text{Dry precipitate weight}) / (\text{Dry sample weight})$$

2.5. Infrared Spectroscopy

IR spectra were recorded using the KBr-disk method with a Bruker VECTOR 22 Fourier transform infrared (FTIR) spectrometer (Bruker, Germany) in the range 400 to 4000 cm^{-1}. Sixteen scans at a resolution of 4 cm^{-1} were averaged and referenced against air.

2.6. Nuclear Magnetic Resonance Spectroscopy (NMR)

^{13}C NMR spectra were recorded on Bruker AVANCE 600 MHz spectrometers (Bruker BioSpin, Swiss) at room temperature. All samples were dissolved in Me_2SO-d_6 (heat at 80 ºC for 30 min) to a final concentration of 80 mg/ml. Tetramethylsilane (TMS) was used as internal standard.

2.7. Analysis of Helix-coil Transition

The conformational structure of the glucans in solution was determined by characterizing Congo red-polysaccharide complexes. The transition from a triple-helical arrangement to the single-stranded conformation was examined by measuring the λ_{max} of Congo red-polysaccharide solutions at NaOH concentrations ranging from 0.0025 to 0.5 M. Polysaccharide aqueous solutions (0.5 mg/ml) containing 2 ml of 312 μM Congo red were treated with different concentrations of NaOH (2 ml). Visible absorption spectra were recorded with a UV/visible spectrophotometer (type 2401PC) at each alkali concentration.

Table 1. Dosage of urea and H_3PO_4.

Group	Glucan name	Urea (g)	H_3PO_4 (ml)
A	Glucan A	0	0
B	Glucan B	18	0
C	Glucan C	0	10
D	Glucan D	18	10

2.8. Immunoprophylaxis Efficacy Assay

Fifty ICR/HSD mice were divided into five groups. Groups A to D were injected intraperitoneally with Glucan A, Glucan B, Glucan C and Glucan D respectively at a dose of 200 mg/kg (4 mg per mouse) 24 hours prior to intraperitoneal challenge with 1×10^9 viable *E. coli*. While group O was injected with isovolumetric dextrose 24 hours prior to challenge with *E. coli* served as control. The same clinical isolate of *E. coli* was used in all cases, and was maintained in our laboratory. *E. coli* was cultured in trypticase soy broth for 18 hours at 37 ºC in an air bath shaker at 180 rpm. The culture was then centrifuged (3500 rpm, 8 min), the cell pellet washed three times, and this mixture diluted to provide 1×10^9 *E. coli*/ml. Survival of each group of mice was monitored hourly during the 24 hour period.

3. Results and Discussion

3.1. The Yield and Solubility of Glucan Samples

The yield and solubility of glucans were shown in **Figure 1**. The yield of glucan A and C was very low (~18%). However, the yield of glucan B and D was higher. This could be concluded that DMSO and urea had effects on the yield of glucans. DMSO and urea was used as denaturant could facilitate the breakdown of the intermolecular hydrogen bonds between the glucan chains. As a result, more hydroxyl group exposed, which was beneficial to the solubility of glucan. Meanwhile the influence of urea on the yield was more powerful than DMSO. The solubility of glucan C and D was significantly higher than glucan A and B, which could be attribute to the introduction of phosphate group.

3.2. FT-IR Spectra of Different Glucan Samples

The FT-IR spectra of the glucans are shown in **Figure 2**. The

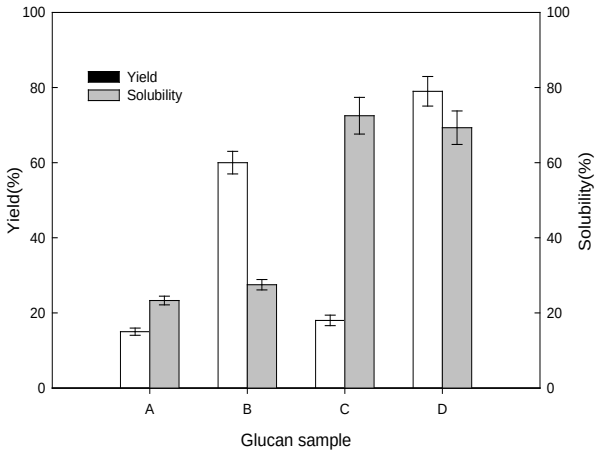

Figure 1. The yield and solubility of glucan samples.

peak assignment of native glucan is as follows (cm-1): 3390 (O-H stretch), 2919, 1383, and 1305 (C-H stretch), 1645 (intra-molecular hydrogen bonds), 1372 (CHOH stretch), 1254 (CH2OH stretch), 1159 (bridge O stretch), 1076 and 1041 (C-O stretch), and 890 (characteristic of β-linked polymer). Compared with native glucan, the absorption peaks of glucan A and glucan B have no change. While the new absorption peaks of glucan C and glucan D appeared at 930 cm^{-1} and 857 cm^{-1} (C-O-P and O-P-O stretch). These data confirmed that the phosphate groups had been successfully introduced into the glucan molecule by esterification. And the new absorption peak of glucan D appeared at 1719 cm^{-1} originating from C=O vibration due to the carboxyl group, which indicated that there were COOH moieties present in the glucan D.

3.3. 13C NMR Spectra of Different Glucan Samples

^{13}C NMR spectra from glucans are shown in **Figure 3**. Chemical shifts from all the glucans were observed at 103.5, 86.7, 76.8, 73.3, 68.9 and 61.4 ppm, which correspond to C-1, C-3, C-5, C-2, C-4, and C-6, respectively. In addition, another signal was observed at 70.3 ppm (C6'), which is a characteristic peak due to β-(1→6) branching. Therefore, all the glucan appeared to be composed of 1→3 -β-glucans with (1→6) -linked branches. This indicated that the placement of all the glucans did not change.

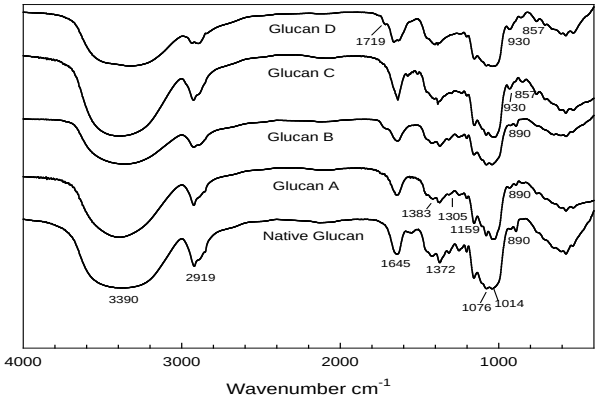

Figure 2. FT-IR spectra of different glucan samples.

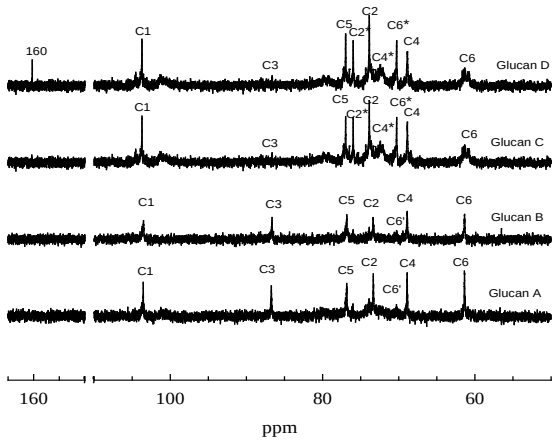

Figure 3. ^{13}C NMR spectra of different glucan samples.

Compared with glucan A and B, three major new signals at 76.0 (C2*), 72.4 (C4*) and 70.2 (C6*) ppm were observed from glucan C and D, showing that the substitution positions of glucan C and glucan D were C2, C4 and C6. In addition, the signal at 72.4 ppm (C4*) was weaker than the signal at 76.0 (C2*) and 70.2 (C6*) ppm, indicating that the phosphate group concentration at the C4 position was significantly lower than those at C2 and C6. It can therefore be concluded that phosphorylation at C4 is hindered, possibly due to steric hindrance by the neighboring oxygen in the glucose ring. A new peak appeared at 160.3 ppm for glucan D, which could be attributed to COOH, suggesting that COOH moieties were present in glucan D.

3.4. Helix-Coil Transition Analysis of Different Glucan Samples

Figure 4 shows the change of maximum absorbance (λ_{max}) of Congo red-polysaccharide complex solution with alkaline concentration. Laminarin served as the β-1,3-linked triple helical control. Dextran T-500 served as the random coil control. Congo red in NaOH served as the negative control. In the presence of NaOH (0.025-0.05 M), the Congo red-laminarin or Congo red-glucan (glucan A, B, C and D) solution exhibited a red shift from low to high. And the λ_{max} keep stable when the concentration of NaOH from 0.05-0.25 M. The red shift of λ_{max} arising from the laminarin or glucan with Congo red is suggestive of the existence of triple helical in all the glucan sample solution. It is well established that triple helical existing in glucan solutions tend to form a complex with Congo red, resulting in a red shift of the maximum absorbance λ_{max} of Congo red.

3.5. Effect of Different Glucan Samples on the Survival of Mice Challenged with E. coli

Experimental peritonitis induced by Escherichia coli could be modified by the addition of glucan and its derivatives, which have immunostimulatory activities. The bioactivity of glucans was therefore studied using the survival rate of mice with E.coli induced peritonitis as an assay. ICR/HSD mice were injected intraperitoneally with glucans (200 mg/kg) 24 hours prior to intraperitoneal challenge with 1 × 109 viable E. coli. Compared

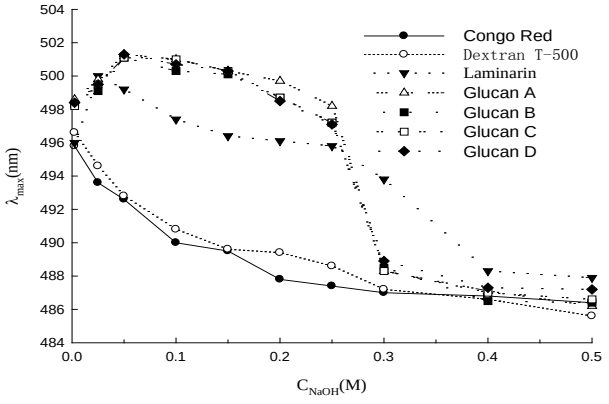

Figure 4. Helix-coil transition analysis of glucans according to the absorption maximum of the Congo red-polysaccharide complex at various concentrations of NaOH。

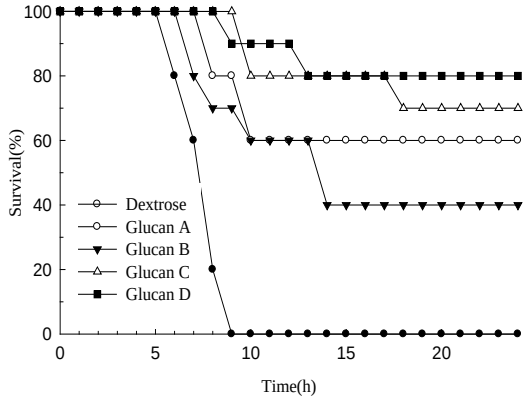

Figure 5. Effects of different glucan samples on survival rate of mice challenged with *E. coli.*

to the control group (dextrose), all glucans had a potent immunoprophylaxis efficacy that significantly ($p < 0.05$) enhanced the survival rate and the survival time of ICR/HSD mice challenged with E. coli (**Figure 5**). The survival rate of mice was 60 %, 40 %, 70 % and 80 % respectively, for glucan A, glucan B, glucan C and glucan D. In contrast, the control group mice showed 0 % survival after 9 hours. This indicated that introduction of phosphate group not only enhanced the solubility, but also improved the immunoprophylaxis efficacy.

4. Conclusion

Four kinds of soluble glucans were successfully prepared by four processes. DMSO and urea was used as denaturant could facilitate the breakdown of the intermolecular hydrogen bonds between the glucan chains. As a result, more hydroxyl group exposed, which was beneficial to the solubility and substitution reaction of glucan. Meanwhile, urea has a significant effect on yield, and DMSO has a significant effect on solubility. From FT-IR and ^{13}C NMR spectra we can see that there was no difference between native and glucan A, B. Glucan C and D was successfully phosphorylated. And the substitution positions were C2, C4 and C6. However the hydroxyl group of glucan D at C6 was partially oxidized into a carboxyl group, which was caused by urea and H_3PO_4 together. All the glucans had the triple helical structure and the immunoprophylaxis efficacy. However the immunoprophylaxis efficacy of phosphorylated glucan was better. The introduction of phosphate group not only enhanced the solubility of glucan, but also improved the survival rate of mice challenged with *E. coli*.

5. Acknowledgements

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