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Systematic extraction and characterization of β-glucan from the industrial brewing yeast (*Saccharomyces cerevisiae*) waste material and the immunomodulatory studies on tumor necrosis factor-alpha and interleukin-6 cytokine balance of the mouse macrophage cell lines

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ABSTRACT

Objectives: The aim of this investigation was to create value to the industrial waste production by systematic extraction and purification of β-glucan (BBG) from the industrial brewing yeast (*Saccharomyces cerevisiae*) waste material. In addition, the immunological activity of the obtained BBG was also evaluated. **Materials and Methods:** The BBG from the yeast waste material of the brewing production was isolated by cell autolysis fractioning, solvent extraction, and drying. The obtained BBG was characterized by High-performance liquid chromatography, nuclear magnetic resonance and comparison with information in literature. The characterized BBG was then examined for its immunomodulatory activity on RAW 264.7 cells. **Results:** The optimal extraction method of BBG from the brewing waste material showed high extraction yield (13.14% w/w). In addition, the immunomodulatory study on RAW 264.7 cell lines showed that the isolated BBG could suppress the tumor necrosis factor-alpha and interleukin-6 production in the cell lines at 6 and 24 h, compared to the lipopolysaccharide control.

INTRODUCTION

Thailand has been known as one of the most beer production in the Southeast Asia. This production has generated a large amount of it's by process waste of yeast which results in high chemical oxygen demand and biological oxygen demand values of the waste water, and thus high treatment cost.

Saccharomyces cerevisiae is one of the most common species of baker's and brewer's yeast. The yeast is used during the production of bread, beer and ethanol and ends up as by

process waste. Research has shown that this by process yeast is safe and rich in protein, Vitamin B, chitin, and most importantly β-glucan (BBG) [1]. BBG contains beneficial properties that can be applied in skin care especially for sensitive skin [2]. It is a polysaccharide molecule comprising repeating glucose units. It can be found in microorganisms, such as yeast, mushroom, and plant such as oats and barley [3]. It is also a powerful stimulator of the immune system against infection from viral, bacteria and fungi, which lead to cancer and stress related immune suppression [4]. Moreover, it contains wound healing, anticoagulant, anti-cytotoxic, antioxidant, and free radical scavenging properties that increase antibiotic effectiveness and reduces the low-density lipoprotein cholesterol level in the body [5]. In addition, in the food industry, yeast BBG is utilized as fat substitutes, dietary fiber additives and thickening agents to improve the texture of food [6].

In the current market, most BBG skin care formulas claim the benefit on moisture retention capability with secondary benefit as stabilizers in emulsion or gel based products [7]. Several scientific studies claimed that BBG promotes hair regeneration by activation of hair follicles [8,9]. It also contains wound healing properties that lower skin infection and enhance the tensile strength of skin tissue [10,11].

Therefore, BBG can act as an immunomodulator that enhances macrophage activity resulting in positive collagen biosynthesis in the wound healing both in animal and human.

The aim of this study was to isolate BBG from the yeast waste material of the brewing production by cell autolysis fractioning, solvent extraction and drying. The structure and composition of BBG from brewing yeast industry was studied by high-performance liquid chromatography (HPLC), gas chromatography–mass spectrometry, and nuclear magnetic resonance (NMR). Furthermore, the effects of BBG were examined on the production of interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) by the Abelson leukemia virus transformed monocyte/macrophage cell line (RAW 264.7) that was treated with lipopolysaccharide (LPS).

MATERIALS AND METHODS

Materials

LPSs from *Escherichia coli* 055:B5 (purified by phenol extraction) were purchased from Sigma-Aldrich, MO 63103, USA. Standard mouse TNF-α recombinant protein (1 μg/ml), standard Mouse IL-6 recombinant protein (1 μg/ml), mouse TNF-α enzyme-linked immunosorbent assay (ELISA) Ready-SET-Go! and mouse IL-6 ELISA Ready-SET-Go! were obtained from eBioscience, Inc., CA 92121, USA.

Preparation of Yeast Waste and Extraction of BBG

Yeast material collection and storage

The solvent extractions of yeast BBG were modified from Freimund's method [12] to find the most optimal conditions. The 300 g of 15% w/w dry solid yeast raw material was washed with 900 g deionized water twice and adjusted to 15% dry matter using vacuum evaporator and adjust pH to 5 prior the extraction. The autolysis process was performed in a closed container with continues stirring. The process was varied under four conditions by varying the time (16 or 24 h) and temperature (55°C or 80°C).

After breaking, the yeast cell wall with autolysis process and separation of yeast sludge through centrifugation at the speed of 7500 rpm for 15 min. The 115.00 ± 7.88 g sediment was then extracted with ethanol at 1:3 ratios for 30 min and centrifuged at 10°C for 15 min. The collected sediment was adjusted pH to 7 to perform enzyme extraction with 5 g papain extract (Specialty Natural Products Co., Ltd., activity 1025 TU) in a total volume of 280 g at 50°C for 5 h and the solution mixture was centrifuged at 7500 rpm 10°C for 15 min. The sediment was then washed with deionized water and acetone by stirring followed by centrifugation under the same condition before drying process to collect the BBG. The extraction was repeated in triplicate.

Analysis of BBG Extracted from the Yeast Waste Materials

NMR

Identification of BBG was carried out on Jeol Resonance Delta T2 NMR spectrophotometer resonating at 600 MHz for ^{1}H NMR. The 5 mg of BBG samples were dissolved in D_2O 600 μl and kept in a refrigerator at −76°C for 15 min prior 1 day lyophilize. The sample was then added with 600 μl D_2O before the NMR measurement.

HPLC

The quantification of BBG was be performed by hydrolysis before HPLC measurement. 1 ml of 2.5 M trifluoroacetic acid in deionized water was added into 1 mg sample and purge with N_2 gas. The solution was then heated at 100°C for 4 h then evaporated with N_2 gas at 40°C. The obtained sample was then dissolved with 100 ml H_2O for HPLC analysis (Shimadzu LC-2030). TSK gel Sugar AXI (4.6 mm I.D. × 150 mm) was used as column under 70°C. The eluent was 0.5 M boric acid-NaOH buffer (pH 8.5) was set at 0.4 ml/min flow rate. Reagents 1 and 2 were 0.5% 2-cyanoacetamide (0.25 ml/min) and 0.25 M NaOH (0.25 ml/min) with reaction temperate of 110°C. Fluorescence (Ex 331 nm; Em 383 nm) is used as the detector.

Cell Culture Preparation

The RAW 264.7 cell lines were collected from Dr. Atsushi Ichikawa, Chiba University. The cell lines were incubated in DMEM cell culture medium (Nacalai Tesque, Inc., Kyoto Japan) with 10% fetal bovine serum (Life Technology, Tokyo Japan) and 1% penicillin-streptomycin mixed solution, lot L5B3459 (Nacalai Tesque, Inc., Kyoto, Japan) at 36.5°C in 5% CO_2 humidify incubator. RAW 264.7 cells (5×10^5) were treated in poly-D-lysine Cellware 6 well-plate with 2 ml cell suspension in each well for 24 h before the experiment (Figure 1a and b). The plate was prepared in triplicate. During the sample treatment, the medium was replaced with fresh medium containing LPS (1 μg/ml), obtained the brewer (BBG) BBG sample (100 μg/ml), and LPS (1 μg/ml) with the obtained BBG sample (100 μg/ml). One well was marked as a negative control. After 6 and 24 h, the cell solution was harvested and remove dead cell for further experiment.

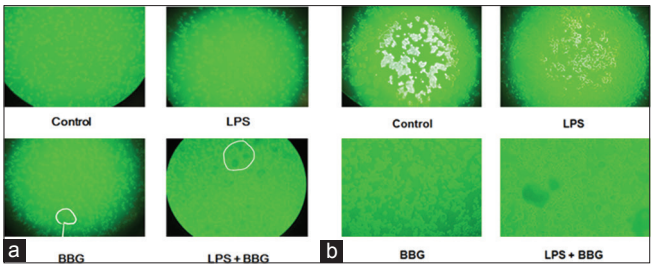

Figure 1: Cell observation after the sample treatment on poly-D-lysine Cellware 6 well-plate at 0 h (a) and at 24 h (b). Control is phosphate-buffered saline (PBS): dimethelsulfooxide (DMSO) solvent. Lipopolysaccharide (LPS) is 1 μg/ml LPS in diluted in PBS. β-glucan (BBG) is 100 μg/ml β-glucan sample diluted in BPS: DMSO solvent. LPS + BBG is 1 μg/ml LPS in diluted in PBS and 100 μg/ml β-glucan sample diluted in PBS: DMSO solvent

Quantitative Determination of TNF-α and IL-6 Production by ELISA

Two reactions were performed in triplicate by ELISA using Mouse ELISA Ready-SET-Go! (eBioscience, Inc., CA 92121, USA) to quantify the cytokines production of TNF-α and IL-6. The two coat corning costar 9018 ELISA plates were coated with 100 μl/well of 4 μg/ml TNF-α (lot# E12409-1634), 4 μg/ml IL-6 (lot# E05328-1633) capture anti body in coating buffer and incubated overnight at 4°C. The plates were then aspirate and wash by ×1 phosphate buffered saline, 0.05% Tween-20 and ELISA diluent to remove excess antibody. Standard mouse TNF-α recombinant protein 1 μg/ml (lot# E08248-1643) and standard IL-6 (lot# 4280337) were prepared in 2-fold serial dilution to make 8 points standard curve. Samples were prepared to 1/20 dilution, direct injection accordingly before adding at 100 μl/well. After aspirate and wash, 100 μl/well of 4 μl/ml detection antibody anti-mouse TNF-α (250X) (lot# E12411-1634), 4 μl/ml detection antibody anti-mouse IL-6 (250X) (lot# E12615-102) were added into each plate and incubate at room temperature for 1 h. After aspirate and wash, 100 μl/well of 4 μl/ml enzyme Avidin-HRP (250X) (lot# E00005-1636) was added to each well and incubate at room temperature for 30 min. The plates were washed 14 times and soak for 2 min with wash buffer to increase the wash effectiveness before adding 100 μl/well substrate ×1 3,3',5,5'-tetramethylbenzidine solution (lot# E00007-1642). After 15 min incubation, 1 M H_3PO_4 solution was added to each well and read plate at 450 nm on a Sunrise Thermo A-5082 (Tecan, Tokyo, Japan). Results were calculated into pg/ml.

RESULTS AND DISCUSSION

The Extraction Yield of BBG

Modified BBG extraction from Freimund's method [12] was compared and evaluated to find the most optimal extraction conditions on the brewing yeast waste material. The results expressed as extraction yield and the protein content of each extraction process are shown in Table 1. The autolysis process (Step 1) was aimed to break the yeast cell wall to separate in inner protein liquid from the insoluble cell wall. During the process, yeast cells went through 24 h under pH 5 at 50°C with papain enzyme. The separation resulted in the obtained insoluble sediment cell wall of 115.0 g (34.9% w/w) with a protein content of 21.6%. The obtained cell wall went through a series of extraction to purify the BBG by breaking layers of chitin and separating fatty acid, vitamins and amino acids. The process includes the ethanol extract and papain extraction. Protein content was used as the rough indicator of the overall purification process. The total yield of the obtained BBG was calculated to be 43.4 g (13.14% w/w). The content and the structure of the BBG were to be further analyzed.

Table 1: The extraction yields and protein contents of BBG obtained from repeating 3 times of autolysis processes

Brewer yeast	Weight (g)	Weight (%)	Protein content (%)
Yeast sludge	330	100.0	25.2
Cell wall 1	115.0±7.88	34.9	21.6
Cell wall 3	46.7±2.74	14.2	12.5
BBG	43.4±4.54	13.1	Not available

BBG: β-glucan

Chemical Analysis of BBG from the Brewing Yeast Waste Material

NMR

^{1}H NMR spectra were recorded on a Jeol Resonance Delta T2 NMR spectrophotometer resonating at 600 MHz, and the chemical shifts were reported in parts per million (ppm). Ultraviolet absorbance values were measured on ultraviolet-spectrophotometer (Tecan, Tokyo, Japan). The obtained BBG sample from the brewer yeast waste material was carried out on NMR spectrophotometer resonating at 600 MHz for their ^{1}H. The ^{1}H-NMR spectrum of extracted glucan from brewer waste materials (Figure 2) showed the anomeric protons resonate at the region 4.10-4.5 ppm. The α anomeric configuration showed $^3J_{1,2}$ coupling constants of <4 Hz whereas β configuration presents coupling constants >7.0 Hz [13]. Based on the above results and the published data in literature [13], it was suggested that the isolated glucan from the brewing yeast waste material was BBG.

HPLC

The hydrolysis of the isolated BBG prior the HPLC analysis allowed us to identify its monomeric sugar molecules and to quantify the concentration of BBG present in the samples. It was found that BBG from the brewing yeast waste material contained mainly glucose up to 99.30%, with minor proportions of mannose and rhamnose (Figure 3).

Biological Activities of the Isolated BBG

Immunomodulatory study

The immunomodulatory study on RAW 264.7 cell lines of the obtained BBG was performed on TNF-α and IL-6 cytokine. The treated cell line was observed prior (0 h) the experiment (Figure 1a) which showed particle sizes and overall distribution of the negative control, positive control, and samples. After 24 h of incubation (Figure 1b), more cells were observed in all conditions, but with well spreading distribution for the negative control (control); with completely cell structural change of most

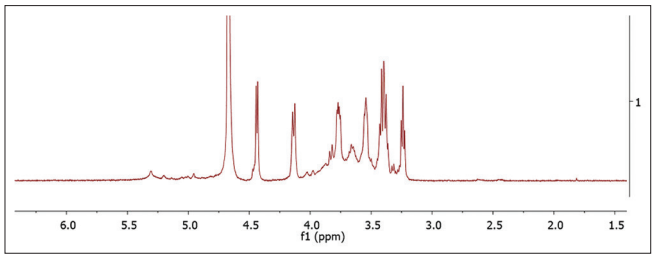

Figure 2: The ^{1}H nuclear magnetic resonance spectra of β-glucan from the brewing yeast waste material in D_2O

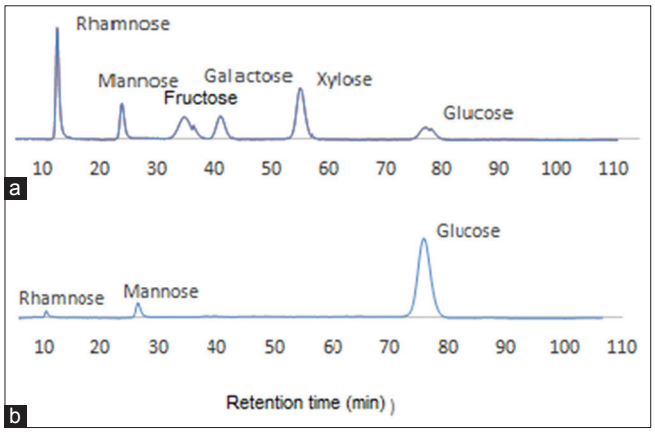

Figure 3: The high-performance liquid chromatography chromatogram of neutral sugars. (a) A mixture of six monosaccharide standards (rhamnose, mannose, fructose, galactose, xylose, and glucose). (b) The composition of monosaccharides in obtained β-glucan from the brewing yeast waste material

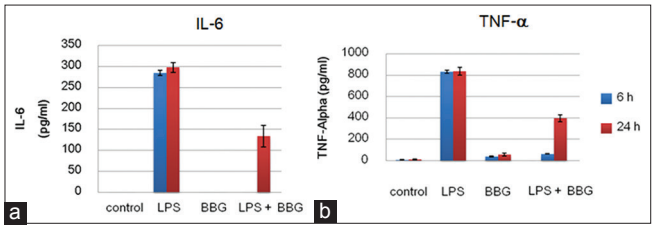

Figure 4: (a) Production of interleukin-6 by the RAW 264.7 cell lines in response to polysaccharides from the brewing yeast β-glucan. Cells were treated with lipopolysaccharide (LPS) (1 μg ml^{-1}) or samples for 6 and 24 h as described in experimental. (b) Production of tumor necrosis factor-alpha by the RAW 264.7 cell line treated for 6 h with LPS (100 ng/ml) or samples as described in experimental. Statistical analyses were performed by one-way analysis of variance (ANOVA) followed by Tukey's test for selected pairs. The results represent the mean ± standard error (n = 3). *P < 0.05; **P < 0.01; ***P < 0.001 versus LPS positive control

cell shape for the positive control (LPS); and with approximately half of cell structural change at sample (LPS + BBG).

The culture medium of the treated cells was measured using sandwich ELISA kits for IL-6 and TNF-α as described in experimental. The results are shown in Figure 4. The results clearly showed the dramatic suppression of BBG sample over LPS at 6 h and observed less suppression of BBG sample at 24 h as compared to the inflammation stimulation control (LPS) (Figure 4).

CONCLUSION

From this work, the industrial brewing yeast waste material as a source of BBG can be found beneficial in food, health and cosmetic applications. The modified purification process under the conditions of 24 h, pH 5 at 50°C with papain enzyme showed the most effective cell breakdown, and the extraction yield of extracted BBG from raw yeast waste material was 13.14% w/w. In this study demonstrated the positive anti-inflammatory property of the obtained BBG on TNF-α and IL-6 cytokine. The ELISA data performed on TNF-α and IL-6 cytokine showed the reduction of cytokine production due to the treatment of BBG sample. According to the results of this study could be suggested that the yeast waste material is an effective natural alternative ingredient to be utilized in the immune modulating agent for ecological food, cosmetic, and health.

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