Inhibitory effect of *Saccharomyces cerevisiae* extract obtained through ultrasound-assisted extraction on melanoma cells

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**Abstract**

Although the immune enhancing effect of yeast has been widely reported, studies specifically investigating its effects on skin cancer are lacking. Therefore, this study aimed to develop a yeast extract capable of inhibiting melanoma cells using ultrasound technology, which can lyse the cell walls allowing subsequent rapid yeast extraction. To compare the extraction efficiency across different extraction methods, the total yield, as well as total glucan, α-glucan, and β-glucan yields were measured. Ultrasound-assisted extract of yeast (UAey) was found to effectively inhibit melanoma cell growth and proliferation as well as the expression of cyclin D1 and c-myc, in vitro. Additionally, the extract reduced melanoma tumor volume and cyclin D1 levels in BALB/c nu/nu mice. The optimal extraction conditions were 0.2 M NaOH, 3 h, 70 °C, 20 kHz, and 800 W, resulting in an increased total extraction and β-glucan yields of 73.6% and 7.1%, respectively, compared with that achieved using a conventional chemical (0.5 M NaOH) extraction method. Taken together, the results of this study suggest that UAey may represent an effective anti-skin cancer agent.

1. Introduction

The yeast biotechnology field is highly diverse, including fundamental biological, biomedical, biocontrol, environmental biotechnology, traditional fermentation, feed ingredients, biocatalysis, and protein production research [1]. Among the principal yeast species, *Saccharomyces cerevisiae* has traditionally been used in the brewing and baking industries offering the advantage of being well-characterized and genetically tractable [2]. Yeast extract, which is the water-soluble portion of autolyzed yeast, contains glucose polysaccharide, mannose polysaccharide, proteins, phospholipids, and sphingolipids [3,4] and is widely used as a nutritional resource, food flavoring agent, additive, and vitamin supplement [5].

β-Glucan, one of the major cell wall components of yeast, is multifunctional. In fact, its market is projected to grow at a compound annual growth rate of 7.57% from 2017 reaching USD 476.5 million by 2022 [6]. β-glucan extraction first requires the extraction of all cell wall components following cell lysis. Yeast lysis methods involve the use of chemical treatment (NaOH, HCl, acetic acid, etc.) with high-temperature, physical treatment (ultrasound and homogenization), or high-pressure or enzyme treatment (self-digestion and specific lysis enzyme) [7]. However, the water solubility of β-glucan depends on its structure, with large amounts of alkali and acid required to extract insoluble β-glucan [8].

Since the development of sustainable green technology in 1991, research has been conducted to reduce or eliminate the use of chemicals and solvents that are harmful to human health and the environment [9]. Examples of these green technologies include ultrasound-assisted extraction (UAE), microwave-assisted extraction, accelerated solvent extraction, and supercritical liquid extraction; among which, UAE is useful for obtaining high-quality extracts at increased yields [10].

The ultrasound frequency is above the audible zone (16–20 kHz) and is divided into a power ultrasound zone (20–2 MHz, low frequency and high power) and diagnostic ultrasound zone (2–10 MHz, high frequency and low power) [11]. UAE corresponds to the power ultrasound zone and is suitable for extracting bioactive compounds tightly bound to the
yeast cell wall.

Numerous studies have assessed the functions of yeast and have reported that yeast culture provides intracellular protection from oxidative damage, inhibits neutrophil reactive oxygen species formation, and increases the natural killer cytotoxic response and B cell activation in vitro [12]. Moreover, *S. cerevisiae* and its glycan can reportedly modulate intestinal inflammation in mice [13]. Through various in vivo experiments on terrestrial animals, rodents, and aquatic animals, the use of yeast as feed has been shown to induce immunostimulatory effects [14]. Although the immune enhancement effects of yeast have been widely examined, studies related to anticancer activity have focused primarily on drug transporters rather than on functionality [15,16]. Therefore, the objectives of this study were: 1) to optimize the yeast extraction process using an ultrasound system and 2) to verify the growth suppression of melanoma by using yeast ultrasonic extracts.

2. Material and methods

2.1. Extraction condition of commercial yeast extract

Freeze-dried yeast powder from *S. cerevisiae* was obtained from Jainbio Co., Ltd. (Gyeonggido, Korea). The extraction conditions of the control (conventional method) included 0.5 M NaOH, extraction time of 5 h, sample and extraction solution ratio of 1:10, and extraction temperature of 90 °C. Ultrasound extraction conditions included ultrasonic treatment (VX 750; Sonics & Materials, Newton, CT, USA) with step horn (probe 1/2”, 13 mm) at a frequency of 20 kHz and 80% amplitude (800 W) under the above conditions with the NaOH concentration adjusted to 0.1, 0.25, and 0.5 M, and the extract neutralized by HCl followed by desalting and freeze-drying. The extraction yield was calculated according to Equation 1 [17].

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\text{Extraction yield } (\%) = \frac{\text{Dry weight of ultrasound-assisted extract (g)}}{\text{dry sample used for extraction (g) } \times 100(1)}
\]

2.2. Chemical analysis

The proximate characteristics (moisture, crude fat, crude protein, ash, and carbohydrate content) of yeast were analyzed using the AOAC method [18]. The carbohydrate content was measured by the phenol–sulfuric acid method using a total carbohydrate colorimetric assay kit from BioVision, Inc. (Milpitas, CA, USA). The β-glucan content was measured in accordance with the protocol of the mushroom and yeast β-glucan assay kit from Megazyme International Ltd. (Bray, Co. Wicklow, Ireland) [19].

2.3. Anti-proliferation assay

The murine melanoma cell line, B16F10 (4 × 10^3 cells per well), purchased from the Korean Cell Line Bank (Seoul, Korea), were seeded into 96-well plates and incubated in Dulbecco’s modified eagle medium supplemented with 10% fetal bovine serum (v/v) and 1% (v/v) penicillin-streptomycin at 37 °C in a humidified atmosphere containing 5% CO₂ for 12 h. The cells were treated with different concentrations of yeast extract. After incubation for 72 h, cell proliferation was measured using Cell Titer96 Aqueous One Solution (Promega, Madison, WI, USA). Briefly, 20 µL of Cell Titer96 Aqueous One Solution was added to each well and the cells were incubated for 1 h at 37 °C in a 5% CO₂ incubator. The absorbance was estimated at 492 nm.

2.4. Western blot analysis

Protein was extracted from B16F10 cells using 1 × Cell Lysis Buffer (Cell Signaling Technology, Danvers, MA, USA). The protein concentration was estimated using a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). The proteins were separated based on molecular weight by electrophoresis using Any KD™ Mini-PROTEAN® TGX™ Precast Protein gels (Bio-Rad, Hercules, CA, USA) and subsequently transferred to Immobilon P membranes (Millipore, Billerica, MA, USA). The membranes were blocked with 5% fat-free milk for 1 h and incubated with specific primary antibodies at 4 °C overnight. After hybridization with a horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology), the protein bands were visualized using ECL reagents (Bio-Rad) with a Chemiluminescence Imaging System (Uvitec, Cambridge, UK) [20].

2.5. Animals and xenograft

The animal protocol used in this study was reviewed and approved by the Institutional Animal Care and Use Committee of Korea Food Research Institute (Approval Number KFRI-M—19020). Male six-week-old BALB/c nu/nu mice were obtained from Orient Bio (Seongnam, Korea) and acclimatized to the laboratory conditions for 1 week with their health status carefully monitored before tumor implantation. Animal rooms were maintained on a 12-h light–dark cycle with 10 times/h ventilation, 23 ± 2 °C, and 50 ± 10% relative humidity. All mice were housed in individually ventilated cages (DGM70 and Gm500, Tecniplast, Buguggiate, Italy) and had ad libitum access to sterilized standard laboratory diet (Teklad Irradiated Global 18% Protein Extruded Rodent Diet, Envigo, Indianapolis, IN, USA) and water. Mice were anesthetized using isoflurane with oxygen and subsequently subcutaneously injected on the back with 5 × 10^6 B16F10 cells in 200 µL of phosphate-buffered saline. Tumor size and body weight were measured three times per week and mice were euthanized when the tumor volume reached > 1,000 mm³. Six days after B16F10 cell inoculation, all mice were separated into control (0.2 mL saline) and sample-treated (500 mg/kg) groups according to the size of the tumor on the day of therapy commencement. Saline or the sample was injected intraperitoneally every 2 days (n = 10 for each group). At the end of the experiment, the all mice were sacrificed, and tumors were surgically extracted. The largest and smallest diameters of the tumor were measured with calipers and the tumor volume (mm³) was calculated as the largest diameter (mm) × smallest diameter² (mm²)/2.

2.6. Statistical analysis

The functional results are presented as the mean ± standard deviation. GraphPad Prism 8 software (GraphPad, Inc., La Jolla, CA, USA) was used for analysis using Pearson correlation coefficient (two-tailed, confidence interval: 95%) and one-way analysis of variance. P values < 0.05 were considered statistically significant.

3. Results

3.1. Extract condition of commercial yeast using ultrasound-assisted extraction

Results of the proximate analysis of commercial yeast are shown in Table 1. The commercial yeast had low water and fat content, high crude protein and carbohydrate content, and considering the presence of ash,
it was expected to contain various minerals.

To compare the efficiency of conventional and ultrasonic extraction methods, extract total yield and glucan content were measured (Table 2). The experimental conditions were adjusted to NaOH concentration of 0.1, 0.25, and 0.5 M with extraction times of 1, 3, and 5 h. The total yield of extracts obtained using the conventional method was lower than that obtained using ultrasound-assisted extracts of yeast (UAEY). Although the β-glucan content of the extract from ultrasound in 0.1 M NaOH was very low at 2–3%, it increased significantly when the NaOH concentration was increased to 0.5 M.

To optimize laboratory scale ultrasonic extraction, the ratios of solvents and solids, as well as ultrasonic conditions (output and frequency) were fixed, and the ultrasonic processing time (2 and 3 h), temperature (50 °C, 70 °C, and 90 °C), and solvent concentration (0.15 and 0.2 M) were changed. The total yield and β-glucan content of UAEY extracted in 0.2 M NaOH at 70 °C were highest at 73.6% and 71.1%, respectively, after ultrasonic treatment for 3 h (Table 3).

3.2. Anti-proliferative effect of UAEY in B16F10 melanoma cells

Based on the conditions shown in Table 3, the relative degree of B16F10 melanoma cell proliferation was found to decrease in all samples compared with that in the untreated group (Fig. 1A). Moreover, UAEY-treated cells, following extraction with 0.2 M NaOH at 50 °C for 3 h exhibited the greatest reduction in proliferation.

Abundance of the cancer proliferation biomarkers cyclin D1 [21,22] and c-myc [23] was detected by western blot analysis. The highest suppression of biomarkers was observed in UAEY-treated cells following extraction with 0.2 M NaOH at 50 °C for 3 h.

3.3. Inhibitory effect of UAEY on melanoma cells in BALB/c nu/nu mice

For xenograft experiments, samples extracted under conditions showing high B16F10 melanoma cell suppression were used. Notably, the increase in tumor volume was attenuated in the UAEY-treated group compared to the control group (Fig. 2A). This reduction trend started two days after sample treatment, with significant results (p < 0.05) achieved six days after treatment (Fig. 2B). Body weight loss in UAEY-treated mice was not detected during the test, suggesting that UAEY was not overtly toxic to mice at this concentration range. In addition, the expression of cyclin D1 in the melanoma tumor was significantly decreased in the UAEY-treated group (Fig. 2C).

3.4. Industrial ultrasound extraction system

The layout of the industrial extraction system for mass production of yeast ultrasound extracts is shown in Fig. 3. The ultrasound extraction system consists of a raw material tank, ultrasonic treatment unit, ultrasonic oscillator, steam generator, and main controller. This circulatory system begins with the raw material tank, followed by repeated passage through the ultrasonic treatment unit. The raw material tank was designed at a size that can accommodate up to 1 ton of samples, with a stirrer and temperature sensor installed inside. The ultrasound processing unit comprises three cells connected to each other, and three ultrasonic oscillators with a maximum output of 1,500 W per cell connected to enable a maximum output of 4,500 W, and an ultrasonic oscillator with frequency fixed at 20 KHz. In addition, a steam generator is connected to control the temperature.

4. Discussion

During mass production of yeast, standardization of the yeast product is difficult. Since there is no notification standard for carbohydrate, protein, and functional component of yeast, generally recognized as safe strains must be used, and the detection criteria for heavy metals and pathogenic bacteria should be met [24]. During production and processing of yeast as a food additive, the final product is affected by variable growth and/or culture and harvesting conditions [25,26]. Additionally, yeast extraction studies have demonstrated that the physiological and chemical activities of extracts vary depending on the extraction method or extraction factor [19,27]. Therefore, proximate analysis was conducted to determine the general characteristics of yeast used in this study. Compared to other commercial brewers and bread yeast of the same species (S. cerevisiae), the proximate characteristics of the yeast in this study were similar, as most proteins and carbohydrates were detected [28,29]. During the yeast extraction process, the extract and glucan content yields were higher when UAE was used compared to the conventional extraction method. This improved yield is likely due to the mechanism of ultrasound technology. That is, when ultrasound waves disperse into solvent, the cavitation bubble continues to expand and contract by external water; as these bubbles bursts, due to their inability to withstand expansion, strong micro-jets are generated toward the cell wall, breaking the cell wall and allowing extraction of glucan, the main component of the yeast cell wall [30,31]. Although ultrasound treatment can cause sonochemical degradation and altered molecular structure, FT-IR spectroscopy analysis has shown that the structure of β-glucan and polysaccharides are not impacted by UAE [32–35].
Meanwhile, Chen et al. [36] reported that ultrasonic treatment of insoluble (1 → 3)-β-D-glucan increases water solubility, as well as antitumor activity, without altering its primary chemical structure. In addition, ultrasound and alkali treatment of yeast β-glucan may increase its exposure to solution by loosening the aggregated structure and enlarging its surface area [37].

To optimize the ultrasound extraction of yeast, the total yield, as well as total glucan, α-glucan, and β-glucan content were measured under different conditions. Pearson correlation analysis for the four variables (Table 2) revealed a strong positive correlation between total glucan and β-glucan content ($r = 0.934; p < 0.005$, confidence interval (CI) of $r = 0.7397–0.9847$); no correlation was detected between other variables. The correlation results are indicated in Table 3 (total glucan vs. β-glucan content: $r = 0.864$, $p = 0.026$, and CI of $r = 0.1762–0.9850$), which were not obtained for the other variables. Considering that a high yield does not necessarily indicate a high glucan content, both aspects should be considered when optimizing the extraction conditions.

The Wnt/β-catenin pathway can regulate cell proliferation and embryonic development of melanoma cells and promote the activity of bioactive compounds with anti-melanoma properties [38]. Several signaling molecules, including p53, MMP7, c-myc, c-Jun, cyclin D1, COX-2, and FRA1 participate in this pathway [39]. Specifically, inhibition of c-myc induces cellular crisis via telomerase dysfunction and oxidative stress [40], while cyclin D1 has a central role in the pathogenesis of most cancers [41]. Results of the current study indicate that UAEY treatment inhibited melanoma cell proliferation, while reducing the abundance of cyclin D1 and c-myc in vitro, and inhibited melanoma tumor growth and cyclin D1 levels in vivo. A similar study was performed with polysaccharides isolated from Zizyphus jujuba and reported antiproliferative effects toward melanoma cells [42], while pectic polysaccharides isolated from Angelica gigas Nakai inhibit melanoma cell metastasis [43]. These findings indicate that β-glucan in yeast, as well as other polysaccharides inhibit melanoma cell growth. Therefore, further studies are needed to identify the specific substances capable of inhibiting melanoma while also quantifying the expression of other cytokines.

Although extraction at the laboratory scale has been optimized, extraction optimization at the industrial scale is necessary to increase industrial utilization. A commercialized static ultrasound system is available for lab-scale extraction; however, the use of circular ultrasound systems is essential for mass production as the area affected by ultrasonic waves is limited, thus, large quantities of samples must be circulated using a pump to increase ultrasonic efficiency [17]. This was considered when developing the industrial ultrasound extraction system; hence, the ultrasonic flow cell was designed to be cylindrical and was divided into four sections. The first cylindrical tube, located in the innermost portion, is the passage through which the specimen is transferred, where functional components are extracted through ultrasonic waves. The cooling water was then allowed to circulate between the innermost and second cylinders, however, the temperature can be adjusted from 0 °C to 90 °C. The outer wall of the second cylinder is

![Fig. 1. Inhibition of melanoma cell proliferation (A) and cyclin D1 and c-myc expression (B) by ultrasound-assisted extract of yeast (UAEY).](image-url)
equipped with an ultrasonic element. Moreover, the outermost cylinder functions as a protective membrane to protect the ultrasonic elements.

5. Conclusion

Taken together, polysaccharides including β-glucan were extracted from S. cerevisiae using an ultrasound system. The inhibitory effect of this extract was then confirmed using skin cancer (melanoma) cells both in vivo and in vitro. Hence, the development of an ultrasonic extraction method is possible for materials that are structurally rigid or have poor solubility, thereby contributing to green technology that is economically feasible.

Fig. 2. Inhibition of skin cancer growth by ultrasound-assisted extract of yeast (UAEY) in BALB/c nu/nu mice (A), tumor size of BALB/c nu/nu mice at 6 days after B16F10 cell inoculation (B), and cyclin D1 expression in control and UAEY-treated groups (C). b.w, body weight.

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CRediT authorship contribution statement

Su Jin Eom: Validation, Formal analysis, Writing - original draft. Tae-Gyu Lim: Methodology, Investigation, Visualization. Hyunjhung Jhun: Methodology, Investigation, Visualization. Nam Hyouck Lee: Conceptualization, Resources. Min-Cheol Kang: Investigation. Kyung-Mo Song: Project administration.
Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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