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Research article

β-Glucan fragmentation by microfluidization and TNF-α-immunostimulating activity of fragmented β-glucans

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ABSTRACT

Fragmentation of β-glucans secreted by the fungus *Ophiocordyceps dipterigena* BCC 2073 achieved by microfluidization was investigated. The degree of β-glucan fragmentation was evaluated based on the average number of chain scissions (α). The effects on the α value of experimental variables like solid concentration of the β-glucan suspension, interaction chamber pressure, and number of passes through the microfluidizer were examined. Kinetic studies were conducted using the relationships of the α and suspension viscosity values with the number of passes. Evidence indicated that α increases with the interaction chamber pressure and the number of passes, whereas the solid concentration shows the inverted effect. Kinetic data indicated that the fragmentation rate increases with β-glucan solid concentration and interaction chamber pressure. Furthermore, since β-glucan molecular weight is a key factor determining its biological activity, the effect of β-glucans of different molecular weights produced by fragmentation on tumor necrosis factor (TNF)-α-stimulating activity in THP-1 human macrophage cells was investigated. Evidence suggested that β-glucans have an immunostimulating effect on macrophage function, in the absence of cytotoxic effects. Indeed, β-glucans characterized by a range of molecular weights produced via microfluidization exhibited promise as immunostimulatory agents.

1. Introduction

Natural substances have become the focus of significant attention for their use in healthcare or medical therapeutics. The natural substances β-glucans are a family of carbohydrates found in the cell walls of yeasts, fungi, lichens, bacteria, and some plants, such as oats and barley. β-Glucans are derived from various sources, so they are characterized by different structures, molecular weights,

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physicochemical properties, and biological activities [1]. While plant-derived β-glucan are typically unbranched, those from yeast and fungi exhibit branching structure [2,3]. As a consequence of the presence of β-(1–6) side-chains branches found in yeast and fungi β-glucans, these carbohydrates effectively bind to receptors found on the surface of immune cells, thus exhibiting immunostimulating and immunomodulating activities [4,5]. The biological effects of β-glucans depend on their structure, size, branching frequency, conformation, solubility, and, especially, molecular weight [6]. Naturally, the molecular weight of β-glucans exhibits variation based on their source, extraction technique, and fungal species, especially evident in fungi-derived β-glucans. Several research studies explore the immunostimulatory potential of β-glucans across various molecular weights. Each molecular weight range offers distinct advantages and may be preferred for different applications [5,7,8]. However, the specific effects can vary depending on factors like the source and structure of the β-glucans and the receptors they interact with on immune cells. Therefore, investigating the impact of molecular weight variation on the immunomodulatory activity of β-glucan is crucial for gaining insights into their biological properties and optimizing their potential applications in various fields.

Microfluidization has been recognized as an effective method for the fragmentation of polysaccharides while preserving their chemical structure. This technique achieves significant reduction in particle size by subjecting suspensions or solutions containing polysaccharides to high shear forces in interaction chamber, resulting in a more uniform and fine dispersion [9–12]. This is advantageous in applications where smaller particle sizes are desired, such as in the development of drug delivery systems, nano-emulsions, or enhanced functional properties of polysaccharides. Microfluidization has been implemented to depolymerize natural polymer solution prepared in dilute acid or basis. Kasaai et al. investigated the degradation of chitosan solutions by microfluidization, finding that factors such as pressure, exposure time, and hydrodynamic parameters like molecular weight and polymer concentration in solution affected the extent and efficacy of depolymerization [11]. Similarly, Hee Cho et al. studied the effects of high-pressure homogenization on β-glucan solution, observing a decrease in viscosity and average molecular weight of the β-glucans with increasing pressure and the number of passes [12]. The findings suggest microfluidization emerges as a promising technique for precisely controlling the molecular weight of β-glucans. By adjusting the parameters of the microfluidization process, it is possible to obtain β-glucans with varying molecular weights and physicochemical properties.

In the present study, microfluidization was employed to reduce the molecular weight of β-glucans secreted by *Ophiocordyceps dipterigena* BCC 2073, a fungus known for predominantly producing (1–3, 1–6)-β-glucans. The objective was to evaluate the effects that microfluidization had on the fragmentation of β-glucans fragmentation. Notably, we evaluated the impact of various experimental variables, including the solid concentration of the β-glucan suspension, interaction chamber pressure, and number of passes through the microfluidizer, on the average number of β-glucan chain scissions. Additionally, the effect that β-glucans of different molecular weights produced after the fragmentation process had on TNF-α activity in human macrophages was also investigated.

2. Materials and methods

2.1. Materials

β-Glucans secreted by the fungus *O. dipterigena* BCC 2073 were provided by the Biocontrol Technology Research Team, Biorefinery and Biochemical products Group, National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency, Thailand. $NaNO_3$, NaN_3, and DMSO-d_6 were obtained from Sigma-Aldrich (USA). THP-1 human monocytic cell line (ATCC® TIB-202TM) was purchased from ATCC. The cells were maintained in RPMI 1640 medium (Gibco BRL, NY, USA) containing 10% fetal bovine serum (Gibco, Germany), 1% penicillin/streptomycin (Gibco), and they were cultured in an incubator at 37 °C in 5% CO_2 and a 95% humidified atmosphere. The cells were provided with fresh medium two to three times per week.

A β-1,3-1,6-D-glucan produced by O. *dipterigena* BCC 2073 was optimized and characterized [7]. O. *dipterigena* BCC 2073 was first grown on potato dextrose agar (PDA) (Difco, Becton, Dickinson and company, MD, USA) at 25 °C for 5–7 days. An agar block (1 cm3) containing the growing culture was cut into small pieces and transferred to 25 mL of potato dextrose broth (PDB) (Difco, Becton, Dickinson and company, MD, USA) in a 250-mL Erlenmeyer flask. This liquid seed culture was incubated for 5–7 days at 25 °C on a rotary shaker at a shaking speed of 200 rpm (New Brunswick, NJ, USA). The medium used in a 10-L bioreactor consisted of 2.5 g/L yeast extract, 68.98 g/L glucose, 0.5 g/L KH_2PO_4, 0.2 g/L K_2HPO_4, 0.2 g/L $MgSO_4{\cdot}7H_2O$, 0.14 g/L $MnSO_4{\cdot}H_2O$, 1 mL/L trace element solution (trace elements consisted of 14.3 g/L $ZnSO_4{\cdot}H2O$, 2.5 g/L $CuSO_4{\cdot}5H_2O$, 0.5 g/L $NiCl_2{\cdot}6H_2O$ and 13.8 g/L $FeSO_4{\cdot}H_2O$) and 1 mL/L vitamin solution (Blackmores, NSW, Australia). The culture was agitated at 300–400 rpm and aerated at 1 vvm, but pH was not controlled. The cultivation was carried out for 10 days. The culture filtrate was then mixed with four volumes of 95% ethanol, stirred vigorously for 10–15 min and stored at 20 °C for at least 12 h. β-glucan precipitate was redissolved in distilled water, and any insoluble material was removed by centrifugation at 10,000 g for 20 min. The β-glucan solution was then dialyzed against 4 L of distilled water with 2 kDa molecular weight cut off (Spectrum Laboratories, USA) for 24 h and freeze dried to yield high molecular weight β-glucan of 900–950 kDa.

2.2. Fragmentation

β-Glucan suspensions characterized by various solid contents in the 0.5%–3% (w/w) range were prepared in distilled water at 25 °C. Before each microfluidization treatment, the original *O. dipterigena* BCC 2073-derived β-glucan was mixed with distilled water using a high-speed homogenizer (T25 Digital Ultra-turrax model, IKA, Germany) at 10,000 rpm speed for 10 min. Fragmentation was performed on a microfluidizer (LV1 Low volume model, G10Z chamber type, Microfluidics, USA) at 25 °C by varying the chamber

pressure from 10,000 to 20,000 psi and the number of passes from 3 to 20. The fragmented β-glucans were subjected to freeze-drying for analysis.

2.3. Sample characterization

2.3.1. Molecular weight

The weight-average molecular weight (M_w) for each sample was determined by gel permeation chromatography (GPC) performed using a WATERS instrument (Waters Technologies, USA) equipped with a refractive index detector. The samples were prepared at a concentration of 1 mg/mL in 0.05 M $NaNO_3$, and they were subjected to filtration through a 0.45 μm nylon syringe filter before being injected into the GPC device. Analyses were performed at 30 °C on a Shodex SB-806 M HQ column. The mobile phase was deionized water containing 0.02 % w/w NaN_3. Pullulan (M_w:23,000–736,000 kDa) was used as standard. The average number of chain scissions (α) was calculated from the M_w value of the control β-glucan ($M_{w,0}$) and that of fragments ($M_{w,f}$) as follows:

$$\alpha = M_{w,0}/M_{w,f} - 1 \quad (1)$$

Analysis of variance (ANOVA) was employed to determine statistically significant differences between the M_w of the control β-glucan and that of fragments obtained after microfluidization. After performing the ANOVA, a Fisher's multiple comparison test was conducted for the comparison of differences between the means of each factor level. The significance level chosen for both the ANOVA and Fisher's multiple comparison test was 0.05.

2.3.2. Apparent viscosity

The apparent viscosity was measured for each sample employing a modular rheometer (MAR60 model, Thermo Fisher Scientific, USA) fitted with a 1° angle cone (plate geometry: 60 mm diameter and 0.053 mm gap). Measurements were performed in duplicate utilizing the fragmented β-glucans in suspension form at a shear rate of 20 s^{-1} and temperature of 20 °C.

2.3.3. Structural characteristics

The chemical and molecular structures of the β-glucan before and after fragmentation were investigated by attenuated total reflectance–Fourier-transform infrared (ATR–FTIR) spectroscopy and by ^{13}C nuclear magnetic resonance spectroscopy (^{13}C NMR), respectively. The FTIR spectra were recorded on a NICOLET 6700 model (Thermo Scientific Fisher) in transmission mode in the 4000–400 cm^{-1} wavenumber range. The ^{13}C NMR measurements were conducted using a Bruker ADVANCE 500 MHz model (Bruker Biospin, AV-500, Switzerland) spectrometer. The spectrometer operates at a frequency of 500 MHz. The number of scans performed for each measurement was set to 40,000, indicating that the data acquisition process was repeated 40,000 times to improve the signal-to-noise ratio. To prepare the sample solutions for NMR analysis, aliquots of the β-glucans were dissolved in deuterated dimethyl sulfoxide (DMSO-d_6). The final concentration of the samples in the DMSO-d_6 solution was adjusted to 5 mg/mL before the measurement. This concentration ensures a sufficient amount of the sample for accurate NMR analysis and allows for clear spectral signals to be obtained.

2.4. Cytotoxicity and immune system stimulation activity

2.4.1. Cytotoxicity assay

The cytotoxic effects of β-glucans on THP-1 cells were determined implementing a colorimetric MTT assay. Briefly, the cells were plated in 96-well plates at densities of 5×10^4 cells/well. Subsequently, the cells were treated with various concentrations (0–50 μg/ml) of β-glucans of different molecular weight. The highest concentration of dimethyl sulfoxide (DMSO; 0.1%) was used as the vehicle control. Afterwards, the plates were incubated for 24 h at 37 °C. Subsequently, the medium was removed by centrifugation conducted at 3000 rpm for 5 min, and 0.5 mg/ml MTT solution was added to each well. The plates were further incubated for 4 h at 37 °C, and the supernatants were discarded. The formazan crystals that had formed in each well were then dissolved in 100 μl of DMSO. The amount of purple formazan was determined using a multimode microplate reader (Synergy; BioTek Instruments, Inc.) at 595 nm. All the measurements were carried out in triplicate. Cell viability is presented as the percentage of the control.

2.4.2. Differentiation of THP-1 monocytes to macrophages

In order to induce the differentiation of THP-1 monocytes to macrophages, THP-1 monocytes were incubated in the presence of 60 ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma) in a 24-well cell culture plate for 48 h as previously described [13]. After 48 h of incubation, PMA and non-attached cells were removed from the plate, and the adherent cells were washed twice with RPMI 1640 medium. The adherent cells were then further incubated in the culture medium for 24 h before conducting any other experiment.

2.4.3. Cytokine quantification

To determine whether β-glucans could stimulate TNF-α secretion in human immune cells, after the monocyte-to-macrophage induction (see paragraph 2.4.2), the cells were treated with or without 5 and 50 μg/ml β-glucans for 3, 6, 12, and 24 h. The supernatants were then collected and subjected to centrifugation aimed at conducting an enzyme-linked immunosorbent assay (ELISA). The concentration of TNF-α was evaluated using the Human TNF-alpha Quantikine ELISA Kit (R&D Systems, USA). Briefly, an antibody specific for human TNF-α was used to coat the wells of the plate prior to adding the standards and protein samples, according to the

manufacturer's instructions. Subsequently, the plate was incubated at 4 °C overnight. The solution was then discarded, and the plate was washed four times with the wash buffer included in the kit. Human TNF-α conjugate was then added to each well, and the resulting mixtures were incubated for 2 h at room temperature under gentle shaking. Subsequently, the unbound antibody was removed by washing, and the substrate solution was added to each well; the resulting mixtures were then incubated for 30 min at room temperature under gentle shaking and protected from light. In each well, a blue color developed whose intensity was proportional to the amount of bound TNF-α. The stop solution included in the kit was then added to each well, and the color was observed to change from blue to yellow. The intensity of the yellow color was measured within 30 min using a multimode microplate reader (Synergy; BioTek Instruments, Inc.) at 450 nm.

2.4.4. Statistical analysis

In the study of cytotoxicity and immune system stimulation activity, all measurements were performed conducting at least three separate experiments to ensure reliability and reproducibility. The data are presented as mean ± standard error of the mean and were analyzed by GraphPad Prism version 5.01 software (GraphPad Software, Inc). To determine the statistical significance of the data, a two-way analysis of variance (ANOVA) was conducted. Following the ANOVA, a Bonferroni post-test was performed to compare the means between different groups and identify statistically significant differences. A P value below 0.05 ($P < 0.05$) was considered statistically significant.

3. Results and discussion

3.1. Changes in the number of chain scissions

Microfluidization was implemented as an effective process to achieve the fragmentation of β-glucans in the absence of chemical reagents. The β-glucan suspensions were subsequently passed through the microfluidizer at different pressures and implementing different numbers of passes. To achieve a qualitative estimate of β-glucan fragmentation, the M_w of each sample was determined and calculated in terms of the average number of chain scissions (α). The calculated values for the α parameter of β-glucans exposed to microfluidization conducted at various values for the solid concentrations, interaction chamber pressures, and number of passes through the microfluidizer are reported in Figs. 1–3, respectively. In detail, in Fig. 1 are reported data reflecting the solid concentration dependence of the α value of β-glucan samples after the fragmentation conducted at 20,000 psi over three cycles. The highest α value was measured at a β-glucan suspension concentration of 0.5% (w/w). Notably, a decrease in α value was observed to be associated with increases in solid concentration of the β-glucan suspension up to a value of 2% (w/w); as the said concentration increased further, the α value was observed to undergo almost no change. This observation indicates that β-glucans were more susceptible to fragmentation at low concentration. According to the theoretical reaction rate, increasing the β-glucan suspension concentration should increase the reaction rate due to the increase frequency of collisions between the two β-glucan chains in a specific period. But despite those collisions occurring, they do not always result in the β-glucan chains scission. The fact that the α value did not change at high β-glucan suspension concentrations may be caused by the viscosity of the said suspension. In a system with a higher concentration, the polymer chains are more closely packed together. The proximity of polymer chains promotes stronger intermolecular interactions, such as van der Waals forces, hydrogen bonding, or electrostatic interactions. These interactions contribute to the stabilization of the polymer chains and make them viscose [14]. To verify this presumption, the viscosity of the β-glucan suspension was measured. A plot of the apparent viscosity of the β-glucan suspension as a function of the suspension's solid concentration is reported in Fig. 4, and it reveals an exponential increase of the viscosity with the value of the solid concentration. The decreased collision frequency in highly viscous

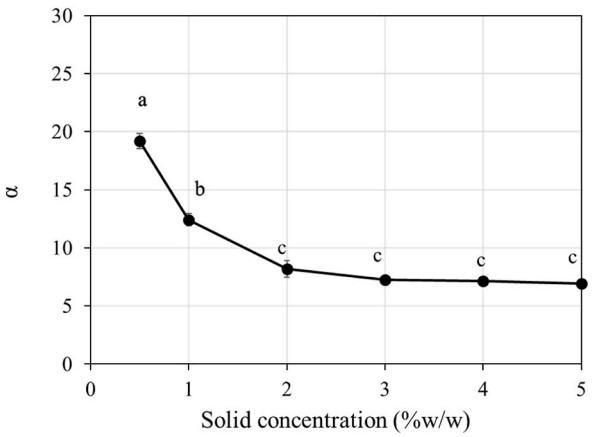

Fig. 1. Solid concentration dependence of the average number of chain scissions (α) in β-glucans secreted by *Ophiocordyceps dipterigena* BCC 2073 after fragmentation conducted at 20,000 psi over three cycles. Different lowercase letters indicate a significant difference among the treatments ($p < 0.05$).

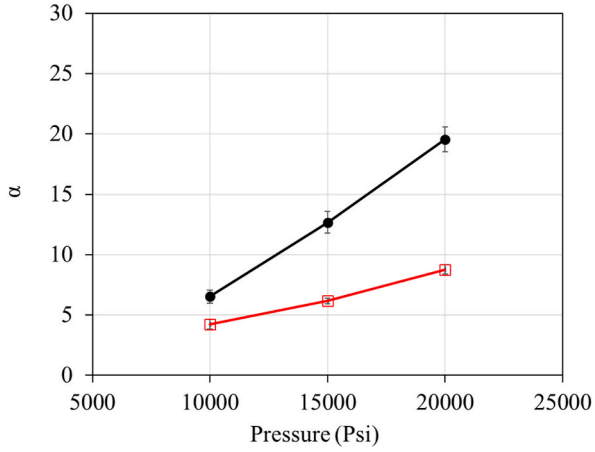

Fig. 2. Pressure dependence of the average number of chain scissions (α) in 0.5% w/w (●) and 2% w/w (□) suspensions (solid concentrations) of β-glucans secreted by *Ophiocordyceps dipterigena* BCC 2073 after being subjected to fragmentation over three cycles. Different lowercase letters indicate a significant difference among the treatments ($p < 0.05$).

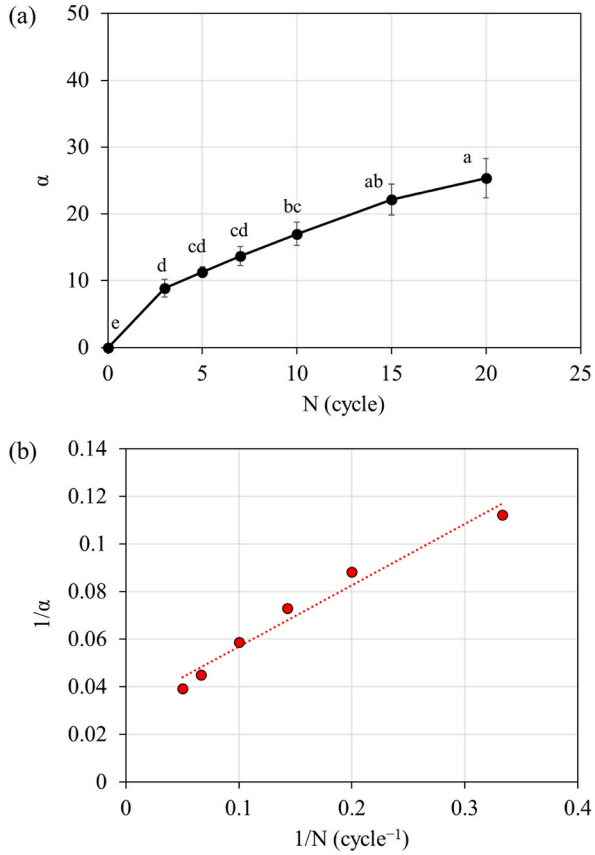

Fig. 3. (a) Number of passes (N) dependence of the average number of β-glucan chain scissions (α) and (b) plot of the reciprocal of the α value *versus* the reciprocal of the number of passes through the microfluidizer (1/N) of a 2% w/w suspension (solid concentration) of β-glucans secreted by *Ophiocordyceps dipterigena* BCC 2073 β-glucan after fragmentation conducted at 20,000 psi. Different lowercase letters indicate a significant difference among the treatments ($p < 0.05$).

suspensions can result in less chain scission. Chain scission often occurs through mechanical forces generated during collisions or interactions with reactive species. When the diffusion of β-glucans is hindered, their chances of encountering the necessary mechanical forces or reactive species for chain scission are reduced. Consequently, the overall chain scission process is less likely to occur. Indeed,

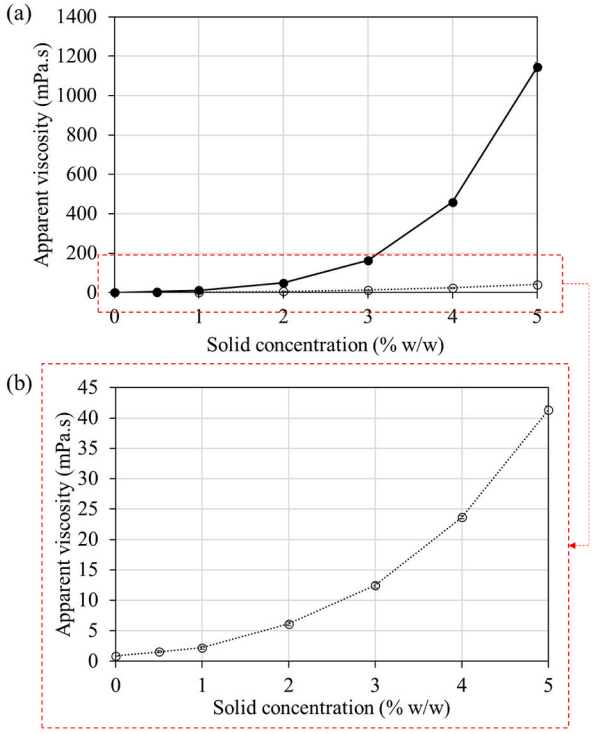

Fig. 4. Dependence of the apparent viscosity of a suspension of β-glucans secreted by *Ophiocordyceps dipterigena* BCC 2073 on the β-glucan solid concentration determined before (solid line) and after (dotted line) the suspension underwent fragmentation conducted at 20,000 psi over three cycles.

sufficient inlet pressures or input forces are required to assist molecular weight reduction in the case of a highly viscous fluid.

In Fig. 2 is reported a plot reflecting the interaction chamber pressure dependence of the α value of β-glucan suspensions at the concentration of 0.5 % and 2% (w/w), after being subjected to fragmentation over three cycles. Evidence indicates a significant increase in the α value associated with an increase in the interaction chamber pressure for both concentrations. Increasing the pressure can enhance the α value due to several reasons. Higher pressure can exert greater shear forces on the β-glucan molecules, leading to increased mechanical stress and breaking of the glycosidic bonds within the polysaccharide chains [15]. This can result in higher fragmentation and a greater number of chain scissions. Furthermore, the differing change in the α value per unit pressure between the two concentrations of β-glucan suspension suggests that the concentration of the suspension has an influence on the fragmentation process. This also corroborates the result described above that a β-glucan suspension of 0.5% (w/w) concentration was more susceptible to fragmentation than a 2% (w/w) suspension.

The number of passes dependence of the α value of β-glucan samples subjected to fragmentation at 20,000 psi is reflected by the hyperbolic curve reported in Fig. 3(a). As expected, the α value increased with the number of passes through the microfluidizer. This observation is attributable to the shear-induced mechanical degradation of the polymer taking place through a mechanism. As the number of passes through the microfluidizer increases, the polymer experiences repeated exposure to shear forces, leading to a higher likelihood of chain scission. However, we observed that the β-glucan fragmentation rate declined after three consecutive passes through the microfluidizer. This result is similar to the study of chain scission of chitosan by microfluidization reported by Kasaai et al. These researchers also found that the degradation rate of chitosan was higher at the beginning of the process than later on during it and that the change in rate was observed after two passes through the microfluidizer [11]. The decline in the fragmentation rate after multiple consecutive passes through the microfluidizer may be explained by a random scission model. The random scission model assumes that chain scission occurs randomly along the polymer chain, and the probability of scission is proportional to the number of bonds available for breakage within the chain [16]. According to this model, shorter β-glucan chains or lower molecular weight β-glucans produced after multiple consecutive passes through the microfluidizer were less likely to experience scission compared to longer chains or higher molecular weight β-glucan. Consequently, the rate of fragmentation declined. That is to say, the number of chain scissions is limited by the number of bonds available for breakage or the threshold value of the molecular weight of β-glucan. The relationship between the α value and the number of passes through the microfluidizer considering the threshold value of the molecular weight of β-glucan could be defined as equation (2):

$$\alpha = \alpha_{max} \times N/(K + N) \tag{2}$$

where $\alpha_{max} = M_{w,0}/M_{w,l}$ - 1 is the maximum number of chain scissions, $M_{w,l}$ is the threshold value of the molecular weight of β-glucan

representing a critical molecular weight in which significant chain scission occurs, and K is the number of passes at which the value of α equals $\alpha_{max}/2$. The data reported in Fig. 3(b) illustrate the linearizing relationship between the reciprocal of the α value and the reciprocal of the number of passes to simplify the α_{max} and K values analysis, a relationship described by the following equation (3):

$$\frac{1}{\alpha} = \left(\frac{1}{\alpha_{max}}\right) + \left(\frac{K}{\alpha_{max}}\right) \times \left(\frac{1}{N}\right) \quad (3)$$

The values of α_{max} and K were 32.05 and 8.25, respectively, for the fragmentation of a 2% w/w β-glucan suspension at 20,000 psi. Furthermore, α_{max} and K were observed to have values of 49.50 and 4.60, respectively, in the fragmentation of 0.5% w/w β-glucan at 20,000 psi (data not shown). The calculated values of $M_{w,l}$ were 42.49 and 64.93 kDa for the 0.5% w/w and 2% w/w β-glucan suspensions undergoing fragmentation at 20,000 psi, respectively. As the fragmentation process advances, the polymer chains undergo scission until they reach the threshold value of the molecular weight or the limit molecular weight, after which the likelihood of further fragmentation decreases. Understanding the limit molecular weight is valuable for optimizing the fragmentation process. By carefully controlling reaction conditions such as time, temperature, and reaction environment, it becomes feasible to attain desired fragment sizes and customize the properties of the resulting fragments.

3.2. Changes in viscosity

The plots in Fig. 4 reflect the dependence of the apparent viscosity of β-glucan suspensions on the said suspensions' solid concentrations, before (η_0) and after (η) the fragmentation process. Notably, in Fig. 4(b) is reported a blown-up section of the plot reported in Fig. 4(a) that allows to focus on the apparent viscosity of the β-glucan suspension after the fragmentation process. Evidence indicates that viscosity increases exponentially with the solid concentration of the β-glucan suspension, both before and after fragmentation. A significant reduction in the apparent viscosity of the β-glucan suspension was also observed after the fragmentation process. The reduction in apparent viscosity was normalized in terms of η/η_0 to remove data redundancy before analysis. In Fig. 5 are reported data reflecting the dependence of the normalized apparent viscosity (η/η_0) on the number of passes through the microfluidizer (N). Evidence indicates that fragmentation resulted in a reduction in the apparent viscosity of the sample. Specifically, the apparent viscosity of the β-glucan suspension dropped sharply at the beginning of the fragmentation process; after three passes, however, the value of this parameter began to decrease quite slowly. The reason for this observation is that polymer–polymer interactions between larger molecules are stronger than between smaller molecules, so that suspension viscosity is higher in the presence of the former types of interactions than in the presence of the latter [10]. The observed change in viscosity can be described by a double exponential decay function represented by the following equation (4):

$$\eta_N/\eta_0 = A.e^{-k_1 N} + (1\text{-}A).e^{-k_2 N}$$

where A is the amplitude of the first exponential function, (1−A) is the amplitude of the second exponential decay, and k_1 and k_2 are the relevant rate constants. The values of A, k_1, and k_2 for various solid concentrations of the β-glucan suspensions and various interaction chamber pressures are listed in Table 1. For each solid concentration of the β-glucan suspension, the value of k_1 was larger than that k_2, which means that the viscosity reduction rate at the beginning of the fragmentation process is faster than at the end of it. By comparing k_1 values determined for β-glucan suspensions of different solid concentration, we surprisingly found that suspensions with higher solid concentrations need a lower number of passes to reach the same viscosity reduction or the same η/η_0 value as suspensions with lower solid concentrations. This finding is probably caused by an increase in the frequency of collisions between the two β-glucan chains when increasing the solid concentration. Higher concentrations mean more collisions and more opportunities for fragmentation. In addition, by comparing k_1 values determined for β-glucan suspensions treated at different interaction chamber

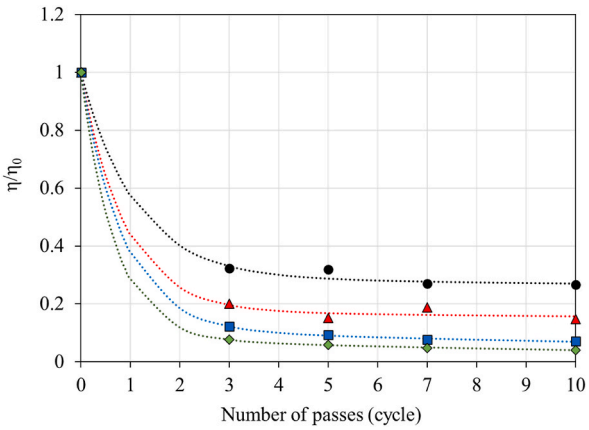

Fig. 5. Plot of the normalized apparent viscosity (η/η_0) as a function of number of passes through the microfluidizer at 20,000 psi in the case of β-glucan suspensions of 0.5% w/w (●), 1% w/w (▲), 2% w/w (■), and 3% w/w (◆) solid concentrations.

Table 1
Values for the kinetic constants of the double exponential decay function describing the fragmentation of a β-glucan suspension at various values for the suspension's solid concentration and the interaction chamber pressure.

Solid concentration (% w/w)	Pressure (Psi)	A	k_1	k_2
0.5	20,000	0.7100	0.9050	0.0070
1	20,000	0.8254	1.1267	0.0101
2	10,000	0.7596	1.1385	0.0562
2	15,000	0.8341	1.2041	0.0598
2	20,000	0.8875	1.1827	0.0474
3	20,000	0.9192	1.4755	0.0707

pressures, we found that suspensions treated at higher pressures need a lower number of passes to reach the same viscosity reduction as suspensions treated at lower pressures. To discuss the relationship between Mw and viscosity after fragmentation at various solid concentrations, Fig. S1 illustrates the dependence of the apparent viscosity of β-glucan suspensions on the weight-average molecular weight (M_w). The results indicate that the reduction in viscosity of β-glucan suspensions is significantly associated with the reduction in their Mw, except at concentrations equal to or lower than 1% w/w. This suggests that at concentrations below 1% w/w, the viscosity of each Mw value showed no significant difference.

3.3. Chemical and molecular structures

The chemical and molecular structural changes of β-glucans resulting from the fragmentation process were monitored via the FTIR and ^{13}C NMR techniques, respectively. In Fig. 6 (a) and (b) are reported the FTIR spectra of β-glucan samples recorded before and after undergoing fragmentation, respectively. The spectra exhibit similar vibration signals, indicating that no significant changes in the chemical structure of β-glucan occurred as a result of the fragmentation process. The broad bands appearing in the 3600–3200 cm^{-1} wavenumber range are assigned to the stretching vibration of the OH bond commonly found in polysaccharides. The bands at 2919 and 2850 cm^{-1} are assigned to the absorption vibration of asymmetric and symmetric CH_2 stretching, respectively [17–19]. The bands at 1417 and 1357 cm^{-1} are attributed to the absorption associated with the vibration of CH_2 bending and CH_2 twisting, respectively [7]. Additionally, the characteristic absorption vibration for (1,3)-(1,6)-linked β-glucan was found at 1,149, 1,078, 1,038, and 930 cm^{-1} [19]. The band at 930 cm^{-1} is attributed to the C_1H bending of the β- linkage [7] while the bands located at 1148, 1016, 931, 849, and 754 cm^{-1} are characteristic for a (1,4)-(1,6)-linked β-glucan found in fungal β-glucan [19]. The bending of a C_1H bond of an α linkage is associated with a band observed at 849 cm^{-1}. In Fig. 7 are reported the ^{13}C NMR spectra of the β-glucan samples before and after the fragmentation process. Notably, the β-glucan samples isolated before and after fragmentation were dissolved in DMSO-d_6 before the measurement, and the spectra obtained for the two samples are reported in Fig. 7(a), (b), respectively. Both spectra exhibit the C-1, C-3, C-5, C-2, C-4, and C-6 resonance signals due to the (1–3)-β-linked D-glucosyl unit at 101.09, 81.27, 73.43, 71.13, 67.43, and 60.69 ppm, respectively. The resonances at 101.84, 73.8, 73.75, 72.09, 70.49, and 61.29 ppm are attributed to the C-1, C-5, C-3, C-2, C-4, and C-6, respectively, of the (1–6)-β-linked D-glucosyl unit [20–22]. The resonance signals due to C′-3 and C′-5 in substituted C-6 D-glucosyl are seen at 80.45 and 72.75 ppm [22]. In addition, the two spectra include a resonance signal at 99.18 ppm attributed to the α-linked configuration of anomeric carbon [7]. The FTIR and ^{13}C NMR data thus collected demonstrate that the microfluidization process is harmless to the functional groups of β-glucans as well as to the polymers' molecular structure. Indeed, even after undergoing the fragmentation process, the *O. dipterigena* BCC 2073–secreted β-glucans still comprised only (1–3)- and (1–6)-β-linkages. However, after

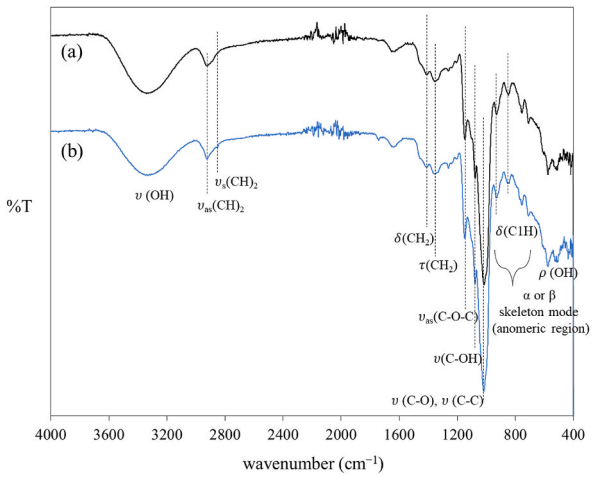

Fig. 6. Fourier-transform infrared spectra of suspensions of β-glucans secreted by *Ophiocordyceps dipterigena* BCC 2073 recorded (a) before and (b) after fragmentation.

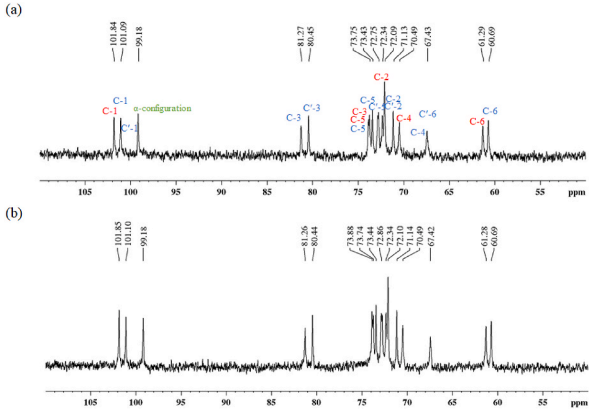

Fig. 7. ^{13}C NMR spectra of β-glucan secreted by *Ophiocordyceps dipterigena* BCC 2073 recorded (a) before and (b) after fragmentation.

the fragmentation process, we found a significant loss in β-glucan content in the sample, as can be evinced from Table 2.

3.4. Cytotoxic and TNF-α-stimulating effects

The cytotoxicity and immunostimulating activity of β-glucans characterized by different M_w values, which had been produced as a result of the fragmentation process, including fragments of 50, 100, 150, 200, 250, 300, 350, and 400 kDa M_w, were investigated, and the results thus obtained were compared with those obtained using β-glucans that had not undergone fragmentation. However, due to the limitations of our experimental design and resources, we did not specifically examine the effects of low-molecular-weight β-glucans in this study. The actual M_w value and β-glucan content of each sample are reported in Table 2. The cytotoxic effects of β-glucans characterized by different M_w values on the THP-1 human monocytic cell line were investigated implementing the MTT assay. The results thus obtained indicated that none of the different β-glucans exhibited any toxicity toward monocytic cells. In fact, no significant differences in cell viability were measured between the β-glucan-treated cells and the vehicle control (data not shown). Therefore, all the different β-glucans were used at concentrations of 5 and 50 μg/ml to investigate their effect on the production of TNF-α by human macrophage cells.

The immunostimulating activities of β-glucans are usually investigated by focusing on macrophage activation. We wanted to demonstrate the effects of β-glucans in a human model. The THP-1 human monocytic cell line was thus chosen to construct a human model, because of the existing literature on these cells' cytokine response and due to the fact that this cell line is the most widely used for *in vitro* studies investigating the function of human macrophages [23,24]. This study thus examined the effect of β-glucans on TNF-α secretion in PMA-induced THP-1 macrophages. Notably, TNF-α is a major inflammatory mediator produced by activated macrophages. In the current study, the THP-1 human monocytic cell line was utilized and induced into a macrophage-like phenotype by exposing the cells to PMA. This treatment was employed to mimic the characteristics and functions of human macrophages in the experimental setting. Typically, THP-1 cells possess a round shape, grow in suspension, and do not adhere to culture plate surfaces as shown in Fig. 8 (a). However, upon induction of differentiation with PMA, these cells undergo changes in morphology and exhibit macrophage-like characteristics. Specifically, the differentiated THP-1 cells adhere to the bottom of the culture plate, spread out, and display an increase in cytoplasmic volume. This is clearly demonstrated in the micrographs presented in Fig. 8 (b) of the study.

The ability of β-glucans to stimulate TNF-α production in THP-1 human macrophage cells was investigated. The human macrophage cells were treated with a blank solution or with β-glucans solutions of 5 or 50 μg/ml concentrations for 3, 6, 12, and 24 h; afterwards, an ELISA assay was conducted to determine the effect of β-glucans of different molecular weights on TNF-α production in THP-1 human macrophage cells. Fig. 9(a–d) illustrates TNF-α production in THP-1 human macrophage cells at various time points stimulated by different concentrations and M_w of β-glucans. The results suggest that both the concentration and M_w of β-glucans play a

Table 2
Actual weight-average molecular weight (Mw) and β-glucan contents of fragmented β-glucans.

Sample	M_w (kDa)	β-glucan content (%)
β-glucans before fragmentation	2350.8 ± 908.08	71.6 ± 2.04
50 kDa	61.4 ± 3.52	32.7 ± 0.82
100 kDa	104.5 ± 5.26	44.2 ± 1.81
150 kDa	157.2 ± 10.23	52.5 ± 1.53
200 kDa	200.6 ± 7.13	55.3 ± 0.58
250 kDa	237.2 ± 5.00	56.4 ± 1.96
300 kDa	285.9 ± 20.78	58.1 ± 1.24
350 kDa	349.3 ± 15.89	65.8 ± 0.96
400 kDa	412.8 ± 32.51	59.1 ± 0.89

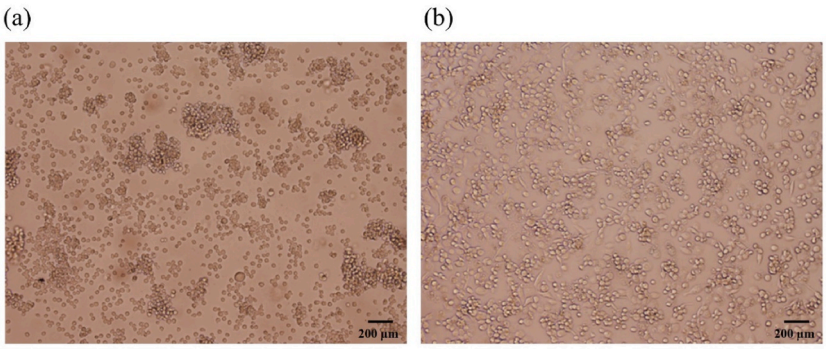

Fig. 8. Micrographs affording evidence of the phorbol 12-myristate 13-acetate (PMA)-induced differentiation of THP-1 monocytic cells to macrophages. (a) Micrograph of THP-1 monocytic cells and (b) micrograph of THP-1 cells after they were induced by the addition of PMA at a concentration of 60 ng/ml for 48 h. The cells were photographed under an inverted microscope at × 10 magnification.

role in stimulating TNF-α secretion. Concentration of β-glucans exhibited a statistically significant effect on TNF-α production, with a concentration of 50 μg/ml leading to greater TNF-α production compared to 5 μg/ml and the vehicle control. When comparing different M_w of β-glucans, the 250 kDa β-glucan significantly increased TNF-α production compared to other M_w of β-glucans. However, no significant differences were observed among the other M_w values (including unfragmented β-glucan and β-glucans of 50, 100, 150, 200, 300, 350, and 400 kDa Mw) at all time-points tested in their ability to stimulate TNF-α secretion. The interaction effect suggests that at a concentration of 5 μg/ml, the influence of M_w of β-glucans on TNF-α production may not be as prominent for most M_w of β-glucans. However, an exception is observed with the 250 kDa β-glucan, which exhibits significantly higher TNF-α production compared to other M_w even at this lower concentration. At a concentration of 50 μg/ml, the effect of M_w becomes more pronounced. Specifically, the 250 kDa β-glucan demonstrates significantly higher TNF-α production compared to other molecular weights, and this difference was evident as early as 3 h post-treatment. This suggests that the impact of M_w of β-glucans on TNF-α production is more pronounced at higher concentrations of β-glucans. The results of a study published in 1995 indicated that β-glucans isolated from the polypore mushroom *Grifola frandosa* characterized by a molecular weight higher than 450 kDa exhibited a more potent induction of TNF-α secretion from macrophages than their counterparts with molecular weights below 450 kDa. Importantly, however, native β-glucans characterized by molecular weights higher than 2000 kDa did not induce cytokine production [8]. Nevertheless, according to a study published in 2003 by Brown and Gordon, immune cells can be directly activated by zymosan' β-glucans with a molecular weight of approximately 504.44 kDa as contrasted with immune-inactive laminarin' β-glucans with a molecular weight lower than 5 kDa [25]. A series of recent studies have implied that the biological activities of β-glucans depend on many factors, not just their molecular weight. Evidence indicates that even β-glucans with a similar structure, molecular weight, and conformation can have significantly different biological activities *in vitro* and *in vivo* [26]. In addition, the transformation of the conformation of β-glucans through a range of a modification methods can directly affect their biological properties [6]. For example, in 2011, Methacanon et al. investigated the relationship between the effects of the γ-irradiation-driven modification of the conformation of β-glucans secreted by *O. dipterigena* BCC2073 and the polymers' IL-8-stimulating activity in normal human dermal fibroblasts. According to the evidence collected, 5 kDa β-glucans exhibited the highest ability to stimulate the production of IL-8. Furthermore, the results of this study suggested that lower molecular weight β-glucans might have a higher chance of binding to more receptors, thus triggering the process that results in the stimulation of IL-8 production [7]. Importantly, however, although both the study by Methacanon and co-workers and our present study investigated β-glucans secreted by *O. dipterigena* BCC2073, the present study investigated the effect that the implementation of the microfluidization method to produce β-glucans of different molecular weight had on the β-glucan samples' ability to stimulate TNF-α secretion in THP-1 human macrophage cells. In fact, the stimulation of cellular responses by β-glucans involves the specific interaction of these polysaccharides with one or more cell surface receptors. Dectin-1 is a major receptor of macrophages, and it has been observed to have the ability to recognize specifically only β-glucans derived from fungal cell walls [27]. The (1–3, 1–6)-β-glucans from the fungal source bind specifically to dectin-1 receptors on the surface of macrophages, resulting in a stimulation of the signaling transduction and an activation of immune cells [28]. However, structurally different β-glucans might interact differently with dectin-1 receptors, contributing to different levels of immune activation [29]. Therefore, *O. dipterigena* BCC2073–secreted β-glucans of different molecular weights, including 50, 100, 150, 200, 250, 300, 350, and 400 kDa β-glucans, as well as unfragmented β-glucans, might interact differently from each other with receptors on macrophages to induce different levels of TNF-α production. Interestingly, the 250 kDa β-glucan exhibited a greater immunostimulating effect than the other β-glucans. Indeed, the 250 kDa β-glucan may be characterized by a higher branching frequency and/or a more appropriate chain length than the other β-glucans. It might thus have a higher chance of binding to receptors, contributing to its observed higher TNF-α-stimulation ability.

4. Conclusion

The present study demonstrated the implementation of the microfluidization technique to be a potentially effective approach to inducing the fragmentation of the β-glucan secreted by *O. dipterigena* BCC 2073. The fragmentation level increased with the interaction

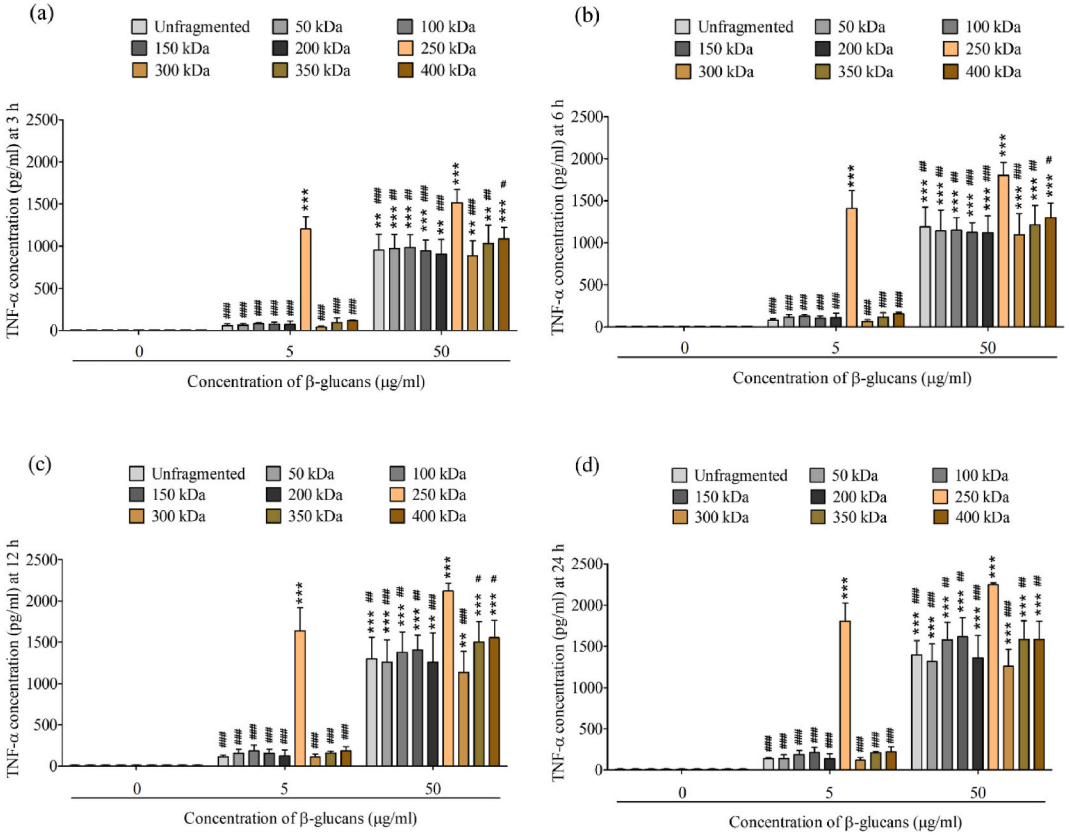

Fig. 9. Data reflecting the induction of TNF-α production in human immune cells afforded by β-glucans of different molecular weights. Notably, the influence of β-glucans on the release of TNF-α by THP-1 macrophages was determined conducting enzyme-linked immunosorbent assays at various time-points after the treatment of the immune cells with β-glucans. Results obtained at the (a) 3 h, (b) 6 h, (c) 12 h, and (d) 24 h time-points. Values are expressed as the mean ± standard error of the mean calculated for three independent experiments. $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with the vehicle control. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ compared with 250 kDa β-glucan.

chamber pressure and with the number of passes through the microfluidizer. Notably, the solid concentration of the β-glucan suspension played a crucial role in influencing its viscosity, resulting in an alteration of β-glucan susceptibility to fragmentation. Fragmentation kinetics could be studied according to the relationship between the number of chain scissions and the number of passes through the microfluidizer as well as according to the relationship between the normalized viscosity and the said number of passes. Evidence indicated that the chain scission can be described by a hyperbolic function considering the threshold value of the molecular weight of β-glucan in term of the maximum number of chain scissions (α_{max}). The normalized viscosity, meanwhile, is best described by a double exponential decay function with various rates of fragmentation that depend on the solid concentration of the β-glucan suspension and the interaction chamber pressure. FTIR and ^{13}C NMR spectroscopy results indicated that the microfluidization process did not destroy the β-glucan structure and functional groups. From a mechanistic perspective, it is recognized that β-glucans possess the capacity to act as immunostimulants. As demonstrated, they can induce the production of TNF-α in THP-1 human macrophage cells in response to antigens. This immunostimulatory activity of β-glucans holds promise for potential applications in immune therapies. Additionally, considering their beneficial effects, β-glucans have the potential to serve as effective nutritional supplements for human consumption. However, it is important to acknowledge the limitations of our study, particularly the lack of investigation into the effects of low-molecular-weight β-glucans. Future research efforts should aim to address this gap and conduct additional experiments to gain deeper insights into the mechanism and diverse effects of β-glucans across a broader range of molecular weights. Such investigations will contribute to a more comprehensive understanding of the therapeutic potential of β-glucans in various applications.

CRediT authorship contribution statement

Phawinee Nanta: Writing – original draft, Investigation, Formal analysis. **Paiwan Buachan:** Writing – original draft, Investigation. **Wichchunee Pinket:** Investigation, Formal analysis. **Wanwisa Srinuanchai:** Investigation, Formal analysis. **Pawinee Pongwan:** Investigation, Formal analysis. **Issara Sramala:** Conceptualization. **Suwatchai Jarussophon:** Validation, Conceptualization. **Wai Prathumpai:** Resources, Conceptualization. **Malai Taweechotipatr:** Conceptualization. **Uracha Rungsardthong Ruktanonchai:** Funding acquisition, Conceptualization. **Kittiwut Kasemwong:** Writing – review & editing, Validation, Supervision,

Methodology, Data curation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:Kittiwut Kasemwong reports financial support was provided by National Science and Technology Development Agency. Kittiwut Kasemwong has patent Reducing molecular weight of beta-glucan and process thereof pending to ipThailand.

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Appendix A. Supplementary data

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