Isolation, Characterization and α-glucosidase Inhibitory Activity of Crude Beta Glucan from Silver Ear Mushroom (Tremella fuciformis)

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Abstract: β-glucan is a polysaccharide derived from mushrooms’ cell wall mainly used for increase the immune system and it is also used to reduce the blood glucose level. This research was aimed to isolate and characterize crude β-glucan from Tremella fuciformis, as well as assess its bioactivity by doing α-glucosidase inhibition test. Hexane and ethyl acetate were used to remove impurities prior to hot water extraction to get the isolated β-glucan, resulting in 2.58±0.33%. 13C-NMR, 2H-NMR and FT-IR were used to confirm the characteristic of crude β-glucan. C-H and CH2 groups were detected within δH 3.5-4.2 ppm, shown by 2H-NMR. Meanwhile anomeric proton that indicated β-glycosidic linkage was shown at 4.8-5.2 ppm which was also confirmed by FT-IR in 1028 cm-1 region. The most abundant spectra shown by 13C-NMR in chemical shift δC 71.58; 71.87; 72.90; 73.68; 73.47; 76.53; followed by δC 60.41; 60.95; indicated CH and CH2. Anomeric carbon that indicated glycosidic carbon was shown at 93.13-102.01 ppm. FT-IR showed the OH group at 3500-3000 cm-1, followed by 1250-1122 cm-1 for ν-C-O and 1500-1400 cm-1 for ν-C-H absorption. In addition, methyl group which belong to furanose was shown by δH at 1.07 and 1.21 ppm which also conformed by the result of GC-MS showed the crude β-glucan contain glucose, fucose, mannose, melibiose and D-galactose. Antidiabetic activity was measured by performing α-glucosidase inhibitory evaluation. Isolated crude β-glucan was found to have a weak activity on α-glucosidase since at 50 ppm, it has lower inhibitory activity than quercetin.

Key words: β-glucan, Tremella fuciformis, α-glucosidase inhibitory activity

INTRODUCTION

Mushrooms are not only functioned as functional food, but also natural food, processed food, pharmaceuticals and cosmetics. In last few years, polysaccharides as a major component in food has been recently attracted much attention (Cao et al., 2006). The biological importance of mushroom arises from their chemical components, particularly active polysaccharides like β-glucan. According to Carbonero et al. (2006), β-glucan itself may act as biological response modifier that can increase the immune system. Another general activities of β-glucan are anti-inflammatory effect, wound healing, anti-tumor activity and lowering cholesterol and blood glucose level.

Tremella fuciformis (silver ear mushroom or jamur kuping putih) are categorized as edible mushroom. Besides, mushroom also has shown its activity in controlling high blood sugar (Halpern, 2007). This is due to the role of α-glucosidase inhibitor in limiting the digestion of starch and sugar absorption in the body. The length and branches of β-glucans from various mushrooms are different. This research was aimed to isolate and characterize crude β-glucan from Tremella fuciformis, as well as assess its β-glucosidase inhibitor activity.

MATERIALS AND METHODS

Materials: Main material used in this research was silver ear mushroom purchased from local supermarket in Puit, confirmed as Tremella fuciformis from Indonesian Institute of Sciences-Research Center for Biology. The materials used for isolation method and analysis were hexane, ethyl acetate, distilled water, ethanol, dimethyl sulfoxide (DMSO), KBr, α-glucosidase, buffer phosphate, p-nitrophenyl-α-D-glucopyranoside (PNP-Glu) (Sigma Co. Ltd.) and Na2CO3 (pro-analysis).

The equipment used in this experiment were analytical balance, oven vacuum, rotary evaporator, macerator, thermometer, spatula, nylon cloth, Whatman
paper No. 1, Buchner pump, refrigerator, beaker glass, centrifuge tube, centrifuge (Beckman J2-21M/E Centrifuge), test tube and pH meter. Meanwhile, the equipments used for analysis were micropipettes, vortex, waterbath, spectrophotometer Hitachi spectrophotometer U-2001, Fourier transform-infrared spectroscopy (FT-IR) Shimadzu prestige-21, Nuclear Magnetic Resonance (NMR) JEOL JM ECA-500 and Gas Chromatography-mass Spectrometry (GCMS) Agilent Technologies 6890 Gas Chromatograph and 5973 Mass Selective Detector.

Isolation of Crude β-Glucan: Isolation of crude β-glucan was conducted according to Wasterlund et al. (1993) with modification. The flowchart of β-glucan isolation is shown in Fig. 1, done in duplicate. Successive extraction were

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Fig. 1: Flow chart of isolation of crude β-glucan (modified from Wasterlund et al., 1993)
done in order to remove the impurities. Extraction was done for 24 h by using hexane pro analysis and ethyl acetate pro analysis. Hot water extraction was done for 2 h at 96°C for getting the water soluble β-glucan. Initially, 452 g dried silver ear mushroom was ground into powder by using dry blends and then extracted by using non-polar solvent (n-hexane), semi polar solvent (ethyl acetate) and polar solvent (distilled water). Extraction was done by using macerator to increase the extraction rate. Ethanol was used to precipitate the β-glucan, with ratio 1:4 (v/v). The isolated, crude β-glucan was then analyzed by using NMR, FT-IR, GC-MS and assessed the α-glucosidase inhibitory activity by performing α-glucosidase inhibitory evaluation.

Chemical characterization of crude β-glucan: NMR was used for determining the structure of β-glucan. It provides information on chemical structure of polysaccharides, based on its NMR resonance. It was used to obtain structural information on high-molar mass molecules and their building blocks (Bacic et al., 2009). About 20 mg sample was diluted in deuterated DMSO at 80°C and put into NMR tube. Its chemical shift resonances were analyzed to identify the β-glucan structure according to the established method (Mursito et al., 2010).

Fourier Transform-Infrared spectroscopy (FT-IR) was used to identify the functional groups such as OH-, C=H and C=O of the crude β-glucan. About 5 mg sample was ground with 300 mg dried KBr and scanned using FT-IR by 500-4000 cm⁻¹ wavelength (Mursito et al., 2010).

Sugar types of crude β-glucan were determined by using Gas Chromatography-Mass Spectrometry (GC-MS). About 6.15 mg crude β-glucan was hydrolyzed with 0.5 mL HCl 3M and boiled at 100°C for 1 h. After cooling down to room temperature, it was evaporated at 75°C with vacuum evaporator and eventually dried in the vacuum oven. Derivatization was done by removing water with P₂O₅, for one hour and added with 25 μL Pyridine and 75 μL solution of MSTFA (N-methyl-trimethylsilyltrifluoroacetamide)/NH₃/Ethanol (100:0.2:0.6, v:v:v). After incubated at 60°C for 20 min, 5 μL sample was injected into GC-MS. The system used for Gas Chromatograph with auto sampler and 5973 Mass Selective detector and chemstation data system with electron impact and it used column HP 5 MS, Capillary Column. Helium was used as the carrier gas at a flow rate 1 μL min⁻¹.

α-Glucosidase inhibitory evaluation: Assay of α-glucosidase was conducted according to Artanti et al. (2011) with slight modification. Enzyme solution was made by dissolving 1.0 mg α-glucosidase in 100 mL phosphate buffer (pH 7.0) that contains 200 mg bovine albumin serum. Before it was used, 1 mL of the enzyme solution was diluted with 9 mL phosphate buffer. The mixture contains 250 μL 5 mM p-nitrofenil α-D-glucopyranoside as a substrate, 490 μL 100 mM phosphate buffer and 10 μL solution sample in DMSO. After the mixture incubated at 37°C for 5 min, 250 μL enzyme solution was added and incubated for 15 min. Enzymatic reaction was stopped by adding 1 mL 200 mM sodium bicarbonate. The absorbance of p-nitrophenol was obtained by using 400 nm.

RESULTS AND DISCUSSION

Material preparation: Dried silver mushroom (452 g) was firstly ground in order to get a finely powder of dried silver mushroom. Besides, moisture content of dried silver ear mushroom was measured and found to be 11.89%.

Isolation of crude β-glucan: Successive extraction was conducted with different polarity of the solvent. Three stages extraction was used with the following solvent such as hexane, ethyl acetate and water, respectively. Hexane and ethyl acetate solvent were used for extraction to remove the non-and semi-polar compounds prior to isolation of β-glucan. Non polar compounds such as oil, waxes and semi polar compounds like sterols and flavonoid were expected to be removed in Tremella fuciformis sample after the first two extraction steps (Bhat et al., 2006). In addition, the presence of lipids might hinder the water extraction.

Silver ear mushroom powder that had been extracted with hexane and ethyl acetate was then extracted with hot water at 96°C for 2 h to extract the β-glucan compound. High temperature was needed to enhance the extraction. Hot water extraction was the main step in isolating β-glucan due to its solubility in water as polysaccharide (Wasterlund et al., 1993). Silver ear mushroom powder was boiled at 96°C for 2 h with a continuous stirred agitator and followed by filtration using nylon cloth to get the filtrate. Residue was removed and eventually a yellow-viscous filtrate solution was collected. Filtrate was centrifuged at 9000 rpm at 4°C to remove the impurities. Isolation of polysaccharide like the β-glucan was done by applying centrifugation for separation (Biliaderis and Lysdcorczyk, 2006). Centrifugation was carried out at low temperature to avoid the disruption of β-glucan due to high forces resulted from high speed. However, instead of filtration by using filter paper, centrifugation was carried out to get the β-glucan due to its viscosity (Mursito et al., 2010). Ethanol precipitation which is a common method...
to purify polysaccharides was used to precipitate the β-glucan (Biladeris and Izydorczyk, 2006). Temperature was kept low in order to enhance the precipitation with a white flocculate precipitate resulting after being centrifuged with 9000 rpm at 4°C. The precipitated was considered as isolated β-glucan which has light brown in color and yielded in 11.647 g (2.58%).

Characterization of β-glucan by nuclear magnetic resonance: Carbon (13C-NMR) and Proton NMR (1H-NMR) spectra of isolated compound were analyzed by comparison with previously published spectra (Mursito et al., 2010). 1H-NMR is used to analyze organic molecules containing hydrogen atom. 1H-NMR absorption occur in the range 0 to 10 δ. Unlike 1H-NMR, 13C-NMR absorption occur in the range 0 to 230 δ. It allows the identification of carbon atom in an organic molecule.

Crude β-glucan sample was analyzed by 1H-NMR by dissolving sample in deuterated DMSO and examined at 80°C. Result of 1H-NMR profile (Fig. 2) indicated the polysaccharide profile at 3-4 ppm. In particular, the presence of chemical shift (δH) around 3.5-4.2 ppm showed the existing of -CH-OH, -CH2OH group and around 4.8-5.2 ppm showed the anomeric protons in the isolated compound which revealing the β-glycosidic linkage (Silverstein et al., 2005). Besides, there was traces of other sugar compound signaled as doublet at 1.21 ppm which was assigned to be fucose. Fucose is normally contained in Tremellaceae (Khondkar, 2009). Above all, the chemical shift obtained was accordance to literature data; chemical shifts around 3.2-4.6 ppm are confirmed the characteristic of the β-glucan (Mursito et al., 2010).

Crude β-glucan sample was analyzed by 13C-NMR by dissolving sample in deuterated DMSO and examined at 80°C. The chemical shifts (Fig. 3) were showed of δC 71.58; 71.87; 72.90; 73.08; 73.47; 76.53; followed by δC 60.41; 60.95. These chemical shifts indicated the presence of CH and CH2 group, respectively (Mursito et al., 2010; Silverstein et al., 2005). The characteristic of β-glucan was shown by the presence of glycosidic carbon detected at δC 93.13-102.01 (Bhat et al., 2006; Stephen et al., 2006). There were also minor compound detected at chemical shift δC 172.37-172.40 indicates the presence of carboxyl derivative group (Silverstein et al., 2005).

Characterization of β-glucan by fourier-transform infrared spectroscopy: The FT-IR spectrum showed a typical carbohydrate pattern. The characteristic of very strong broad band between 3000 and 3500 cm⁻¹ region indicates the presence of OH stretching in hydrogen bonds which also means there was a strong interaction of the polysaccharide chains. The characteristic of -C-O-stretching was shown on the band between 1250-1122 cm⁻¹ region while C-H stretching at 1500-1400 cm⁻¹ region. The presence of β-glucons absorption band was shown between 1000 and 1100 cm⁻¹, particularly 1028 cm⁻¹ (Kozarski et al., 2011; Mursito et al., 2010; Stephen et al., 2006).

![Fig. 2: 1H-NMR result of crude β-glucan](image-url)
A carbohydrate pattern was also shown by the FT-IR result from commercial β-glucan from *Ganoderma lucidum*. The characteristic of β-glucan was specifically shown in 1008 cm⁻¹ (Kozarzki *et al.*, 2011; Mursito *et al.*, 2010; Stephen *et al.*, 2006). The identity of crude β-glucan was coincided with the authentic sample of β-glucan provided by PT Sahabat Lingkungan Hidup.

**Sugar type determination by gas chromatography mass spectrometry (GC-MS):** Table 1 shows the sugar type of crude β-glucan analyzed by GC-MS. Based on the library data of GC-MS, compounds detected were mainly belong to sugar group because the other non polar compounds such as fat had been previously removed in first stage extraction by hexane.

<table>
<thead>
<tr>
<th>Compound</th>
<th>RT</th>
<th>Area (%)</th>
<th>Similarity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fucose</td>
<td>6.55</td>
<td>1.11</td>
<td>91</td>
</tr>
<tr>
<td>Mannose</td>
<td>6.55</td>
<td>1.11</td>
<td>91</td>
</tr>
<tr>
<td>Arabinose</td>
<td>6.99</td>
<td>3.11</td>
<td>81</td>
</tr>
<tr>
<td>Melibiose</td>
<td>17.53</td>
<td>2.99</td>
<td>90</td>
</tr>
<tr>
<td>Glucose</td>
<td>17.82</td>
<td>1.24</td>
<td>87</td>
</tr>
<tr>
<td>Lactose</td>
<td>17.93</td>
<td>1.24</td>
<td>87</td>
</tr>
<tr>
<td>D-Galactose</td>
<td>18.83</td>
<td>1.79</td>
<td>91</td>
</tr>
</tbody>
</table>

Based on Table 1, the crude β-glucan was mainly composed of fucose, mannose, D-galactose, melibiose, glucose, lactose and arabinose. Previous research by Khondkar (2009) has shown polysaccharide of *Tremella* species mainly consisted of sugar such as glucose, mannose, arabinose, glucuronic acid, xylose and fucose which were linked by linked by β-glycosidic linkages. Among *Tremella* species, fucose has been found as particularly in *Tremella fuciformis* (Khondkar, 2009). The presence of fucose was also shown in the presence of methyl group in the 1H-NMR spectra (δH at 1.07 and 1.21 ppm). However, sugar profile of polysaccharide varies from each sources depended on the extraction method and origin of the material (Khondkar, 2009). For comparison, commercial β-glucan which is polysaccharide peptide extract from *Ganoderma lucidum* was consisted of glucose, galactose, arabinose, xylose, mannose and linked by β-glycosidic linkages.

**α-glucosidase inhibitory evaluation:** The crude β-glucan started to show its inhibition to α-glucosidase activity at 50 ppm by resulting yellow transparent color. However, the highest inhibition activity of crude β-glucan was 41.61±2.25% at 50 ppm. On the other hand, 25 ppm quercetin (the known α-glucosidase inhibitor) had already showed 71.41±0.25% inhibition activity. This indicated that crude β-glucan had lower inhibition activity compared to quercetin. The inhibitory activity on α-glucosidase was affected by other impurities containing in crude β-glucan, since stated by Fatmawati *et al.* (2011) that the purity of the active compound may affect the inhibition activity.
CONCLUSION

This study showed the silver ear mushroom yielded 2.58±0.33% of crude β-glucan. The isolated β-glucan was identified based on by the analysis results of FTIR and NMR and comparison with the authentic sample.

The isolated crude β-glucan was found to have β-glucosidase inhibitory activity with its IC₅₀ 41.61±2.25% at 50 ppm. GC-MS results showed crude β-glucan, aside from the β-glucan itself, contained fucose, mannose, D-galactose, melibiose, glucose, lactose and arabinose. Further research on β-glucan including its bioactivity, particularly antidiabetic activity, can be done by in vivo evaluation on animal experiments. The identified β-glucan might also potent as anti-cholesterol and cancer chemoprevention that needs further researches.

REFERENCES


