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β-glucan-containing polysaccharide extract from the grey oyster mushroom [*Pleurotus sajor-caju* (Fr.) Sing.] stimulates glucose uptake by the L6 myotubes

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Abstract

Mushroom β-glucan has the ability to affect many cellular functions, including cellular glucose uptake. Although cumulative evidence in literature suggests a connection between β-glucan and reduction of blood glucose concentration, a mechanism of how β-glucan affects cellular glucose uptake has not been demonstrated. In this study, we analyzed the effect of β-glucan containing polysaccharide extract from mushrooms on glucose uptake by the L6 myotubes. We extracted crude polysaccharide from fruiting bodies of the grey oyster mushroom (*Pleurotus sajor-caju*), using hot water and protein removed by the Sevag reagent. The presence of β-glucan in the extract was confirmed by FT-IR analysis. We found that the partially purified polysaccharide extract from the grey oyster mushroom stimulated glucose uptake by the rat L6 muscle cells. In addition, laminarin, a small soluble β-glucan, enhanced the cellular glucose uptake. Results from this study suggest that β-glucan in the extract might promote the effect to the cells. The effect of purified mushroom β-glucan on the L6 cells requires additional analyses, and mechanistically how the mushroom β-glucan affects blood glucose concentration becomes our future goal.

Introduction

β-glucans are polysaccharides found in the cell wall of fungi, plants and some bacteria (Gawronski *et al*., 1999). They consist of glucose molecules that link through β(1→3), β(1→4) and β(1→6) glycosidic bonds. As part of the pathogen associated molecular patterns (PAMPs), β-glucan affects several pathways in the immune and non-immune systems. For example, it can induce cytokines and nitric oxide production in macrophages and promote monocyte adhesion (Byeon *et al*., 2009; Carbonero *et al*., 2012; Satitmanwiwat *et al*., 2012) and activate neutrophils and natural killer cells combat against cancer cells (Yoon *et al*., 2008). Furthermore, β-glucan was shown to reduce total and LDL cholesterol level of hypercholesterolemic in adult individuals (Braaten *et al.*, 1994; Kerckhoffs *et al.*, 2003) and blood glucose in both animals and humans (Lo *et al.*, 2006). In addition, it also has the ability to prevent occurrence of glucose intolerance in mice high-fat diet (Kanagasabapathy *et al*., 2012). Thus, β-glucan possesses several activities, which depend on structure, size, solubility, and the degree of branching (Graham *et al.*, 2006). For example, highly branched β-glucan was shown to be a better immune stimulator than one with less frequent branches (Kubala *et al*., 2003).

One of the best sources for β(1→3) with β(1→6) glucan is mushroom. β-glucan isolated from mushrooms has been subjects for many intense research investigations. One of the mushrooms: the grey oyster mushroom [*Pleurotus sajor-caju* (Fr.) Sing.], which is easily found in Thailand, has been used for β-glucan isolation. It was found that β-glucan from this mushroom was highly branched - it contained a single β(1→6) glucose side chains on every second and third glucose residues (Carbonero *et al.*, 2012). This β-glucan was shown to stimulate nitric oxide production in macrophages (Carbonero *et al*., 2012; Satitmanwiwat *et al.*, 2012). In addition to the immunomodulatory activity, β-glucan from this mushroom was shown to reduce blood glucose concentration in mice fed with high-fat diets (Kanagasabapathy *et al*., 2012).

Glucose, the major metabolic fuel in animals, enters cells through glucose transporter proteins especially, through the isoform 4 (GLUT-4), which is expressed in skeletal muscles, heart muscles, and fat tissue (Shi and Kandror, 2008). The L6 myotube cell line is the best-characterized cellular model of skeletal muscle origin for studying glucose uptake (Klip

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2009). Recently, it was shown that the polysaccharide extract from *Astragalus membranaceus* Bunge stimulated glucose uptake in the L6 muscle cells (Liu *et al*., 2013). However, this study failed to show the presence of β-glucan in the polysaccharide extract. In addition, the effect of β-glucan on glucose uptake by the L6 muscle cells has yet to be illustrated. Although the recent study showed the ability of β-glucan-rich polysaccharide extract from the grey oyster mushroom to reduce blood glucose (Kanagasabapathy *et al*., 2012), the direct effect of the polysaccharide on the L6 myotube has not been shown. Therefore, we carried out experiments to test the effect of carbohydrate extract from the grey oyster mushroom on the L6 cells. We found that the β-glucan-containing polysaccharide extract from this mushroom stimulated glucose uptake in the L6 myotube in time- and dose-dependent fashions. Likewise, laminarin, a small soluble β-glucan from plant, showed the same effect.

Materials and Methods

Extraction of polysaccharide from mushroom

The polysaccharide was extracted using the method from Yap and NG with some modifications (Yap and Ng, 2001). First, 400 g of fresh fruiting bodies of grey oster mushroom (*P. sajor-caju*) were washed and diced into small pieces. Then, they were baked in a hot air oven at 60°C for 3 days or until completely dry, and subsequently grinded into powder. The dried, powdered mushroom weighted approximately 35 g. They were boiled for 3 hours on a heating plate stirrer at 80-90°C. After cooling down at room temperature, the polysaccharide was precipitated by adding one volume of 95% ethanol and incubated for 14-18 h at 4°C. The whole mixture was centrifuged at 3,024 x g for 20 minutes at 4°C, and the supernatant was discarded. The pellets were combined and flash frozen with liquid nitrogen prior to lyophilization. The sample was dissolved in one volume of water and boiled for 8 hours on a heating plate stirrer at 80-90°C. After cooling down at room temperature, the sample was incubated at 4°C for 14-18 hours to allow precipitation of impurities. The precipitates were removed by centrifugation at the same speed, and the soluble material was mixed with one volume of 95% ethanol to precipitate the polysaccharides. After 14-18 hours of incubation at 4°C, the precipitated polysaccharides were collected by centrifugation at respectively 3,024 x g and 5,927 x g for 20 minutes each at 4°C. They were then flashed frozen and lyophilized. The trace moisture in the lyophilized sample was completely removed by baking at 70°C hot air oven for 1 day.

To remove proteins from crude polysaccharide, the Sevag reagent (chloroform:butanol ratio of 4:1) was employed. Briefly, the crude polysaccharide was dissolved with water to 10 mg/ml. The Sevag reagent was added to the sample with a ratio of 1:1, and subsequently mixed and votexed. The mixture was centrifuged at 12,096 x g for 10 minutes at room temperature. After centrifugation, the sample was separated into two layers. The top layer is the aqueous solution, which contains the polysaccharides and the bottom layer is the Sevag reagent. The top layer was carefully pipetted without picking up the interface, which contains some proteins, and transferred to a clean tube. The solution was pooled and the extraction was repeated twice. Then, the aqueous layer was dialysed against 2L distilled water. Water used for dialysis was changed everyday until the volume of sample did not change. Finally, the dialysed sample was frozen at -80°C, lyophilized and subsequently dried at 70°C. The weight of the sample was recorded, and the sample was kept at -20°C until needed. The components of the extract were analyzed with fourier transformed infared spectroscopy (FT-IR).

Determination of protein

Proteins were determined by the Lowry assay (Lowry *et al.*, 1951). Briefly, 100 µl 1 mg/ml sample was mixed with 3 ml alkaline copper solution (2% Na_2CO_3 in 0.1 M NaOH, 1% sodium potassium tartrate, 1% $CuSO_4.7H_2O$). After incubation at room temperature for 10 minutes, 300 µl of Folin and Ciocalteu's phenol reagent was added, and the incubation was continued for another 30 minutes. The optical density (OD) at 500 and 650 nm of the sample was measured, and the amount of protein was calculated by comparing with a standard curve from bovine serum albumin (BSA).

Determination of carbohydrate

Total carbohydrate in the sample was determined using the phenol-sulfuric acid method (DuBois *et al*., 1956). Briefly, 500 µl 0.25 mg/ml sample was mixed with 500 µl 5% phenol. Then, 2 ml sulfuric acid was added to the mixture and incubated for 20 minutes at room temperature. The OD at 470 nm was measured, and the total sugar was calculated by comparing with a standard curve from glucose.

Effect of crude polysaccharide on glucose-uptake

Glucose uptake was determined using a glucose (GO) assay kit. Briefly, L6 rat skeletal muscle cells were grown in α-MEM (low glucose) supplemented with 10% fetal bovine serum (FBS) at 37°C under 5%

CO_2. L6 cells were differentiated to L6 myotubes by 2% horse serum (HS)-containing medium in 48 well culture plates. The various concentrations of crude extracts were added to the cells and incubated for 24 and 48 hours. Insulin (500 nM) and metformin (2 mM) were used as positive controls. After incubation, medium was collected and used to determine the glucose level. The assay reagent (Glucose Oxidase/ Peroxidase Reagent and o-Dianisidine Reagent) was added to the sample and incubated for 30 minutes at 37 ^oC. The reaction was stopped by adding 50 µl 12 N H_2SO_4. The remaining glucose in the sample was measured using the A_{540} absorbance. This reagent specifically reacts with glucose, which is ultimately converted them into a colored substance.

Results and Discussion

Purification of the crude polysaccharide extract from P. sajor-caju

In this study, the crude polysaccharide was extracted using hot water. Proteins in the extract were removed using the Sevag reagent. The total polysaccharide was estimated by the phenol-sulfuric modified method (DuBois *et al.*, 1956). We found that the polysaccharide content before and after the Sevag extraction was increased from 56% to 93.5%, respectively. A previous study also used the Sevag reagent to remove proteins from the polysaccharide purify β-glucan (Satitmanwiwat *et al*., 2012). In this work, we did not further purify the polysaccharide after the Sevag extraction and did not determine the amount of β-glucan in the partially purified extract. However, a study done by Kanagasabapathy and colleagues also extracted the polysaccharides from the grey oyster mushroom using hot-water extraction method and found approximately 80% β-glucan in their crude extract (Kanagasabapathy *et al*., 2012). Additional studies reported that the branched β(1→3) and β(1→6)-D-glucan is predominated in the hot water polysaccharide extract from *Pleurotus ostreatus* and *Pleurotus eryngii* (Synytsya *et al*., 2009). Collectively, these studies suggest that β-glucan is the major component in our partially purified polysaccharide from the grey oyster mushroom.

FT-IR analysis

To further confirm the presence of β(1→3)-glucan in our partially purified extract from the dried mushroom, the FT-IR spectroscopy technique was employed. Previous FT-IR analysis of the yeast polysaccharide cell wall and purified β-glucan

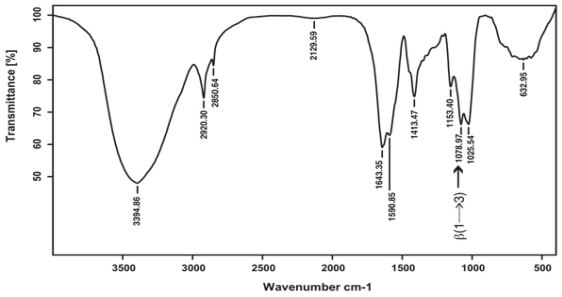

Figure 1. FT-IR spectra of the partially purified polysaccharide extracted from the dried grey oyster mushroom

was used as references (Galichet *et al*., 2001; Satitmanwiwat *et al*., 2012). For example, the peaks in the range of 950 - 1,200 cm^{-1} indicate the presence of polysaccharides. The peaks at 1,150 - 1,160 cm^{-1} define the stretching of glycosidic bonds. Other peaks and shoulders for β-glucan can be found at 1,376, 1,317, 1,162, 1,100, 1,080, 1,040 and 990 cm^{-1} (Synytsya *et al.*, 2009). As shown in Figure 1, we found a peak at 1,078 cm^{-1}, which represents the β(1→3)-glucans. Additional peaks at 1,153 and 1,025 cm^{-1} correspond to the pyranose ring and β(1→4) glucans, respectively (Galichet *et al*., 2001; Satitmanwiwat *et al*., 2012), suggesting cellulose contamination in our sample. No evidence of other sugars was found in the FT-IR analysis. In addition to the sugar, the two peaks at 1,590 cm^{-1} and 1,643 cm^{-1} represent vibration of proteins (Figure 1). Proteins are overlapped with water near 1,640 cm^{-1} (Synytsya *et al.*, 2009). Other peaks at 2,920, 3,394 cm^{-1} are C-H stretching vibration and hydroxyl stretching vibration, respectively (Synytsya *et al.*, 2009; Satitmanwiwat *et al.*, 2012).

Our FT-IR result shows that the sample still contains some proteins, which might not completely be removed by the Sevag reagent (Synytsya *et al*., 2009). We also found a significant loss in the total carbohydrate after the Sevag extraction (data not shown). Therefore, this extraction method might not be the best technique for eliminating proteins from the polysaccharide. Consistently, it has been suggested that this extraction technique might not be able to remove polysaccharide-binding proteins (Synytsya *et al*., 2009). Previous studies used specific enzymes to eliminate some unwanted carbohydrates (e.g. cellulose and hemicellulose) in β-glucans (Ookushi *et al.*, 2008; Satitmanwiwat *et al*., 2012). Thus, a similar technique using an enzyme to remove proteins could also be utilized. This enzymatic digestion might be a better technique than the Sevag extraction method because it might confer a better yield and purity to

the polysaccharide.

Effect of the partially purified polysaccharide extract on glucose uptake

The relationship between polysaccharide and reduction of blood glucose has been demonstrated. For example, a previous study found that the effect of polysaccharide extracted from the fruiting bodies of *Tremella aurantia* reduced blood glucose in diabetic rats (Kiho *et al*., 1995). In addition, polysaccharide from *Pleurotus tuber-regium* has many properties such as, antihyperglycemia, antihyperlipidemic, and antioxidant in diabetic rat model (Huang *et al*., 2012). Moreover, extracellular polysaccharide from *Phellinus linteus* prevented high blood glucose in diabetes patients (Kim *et al*., 2001). Together, these data led us to investigate the effect of our partially purified β-glucan from the grey oyster mushroom and the ear mushroom (*Auricularia auricula*) on glucose uptake by the L6 cells. We also included laminarin, a small soluble β-glucan from *Laminaria digitata* in the experiment. Results in figure 2 show that polysaccharide extracted from the grey oyster mushroom and laminarin stimulated glucose uptake in the L6 cells, suggesting that β-glucan in the extract probably caused the effect. Unlike those samples, polysaccharide extracted from the ear mushroom showed no effect on the cells (Figure 2). These polysaccharides did not affect viability of the cells (data not shown). From these data, we continued to investigate the effect of the polysaccharide extract from the grey oyster mushroom on glucose uptake in this cell type. We found that the level of glucose uptake increased as the concentration of the extract increased, and the stimulation of the uptake was clearly evident in 48-hour incubation (Figure 3).

The role of polysaccharide in regulation of glucose uptake has been illustrated. A recent study showed that Astragalus polysaccharide (APS) extracted from *Astragalus membranaceus* stimulated glucose uptake in the L6 myotubes through the AMP-activated protein kinase (AMPK) pathway (Liu *et al*., 2013). It was found that the APS-dependent AMPK pathway stimulation subsequently promoted the phosphorylation of Akt substrate of 160 kDa (AS160), which led to the excotytosis of the glucose transporter 4 (GLUT-4) to promote glucose uptake by this cell type (Liu *et al*., 2013). GLUT-4 has been shown to be a major glucose transporter in the skeletal muscle cells (Sakamoto and Holman 2008). The involvement of the Akt pathway and AS160 in glucose uptake by skeletal muscles has been demonstrated (Treebak *et al*., 2006; Choi *et al*., 2012). This Akt/AMPK pathway might be responsible for β-glucan-induced

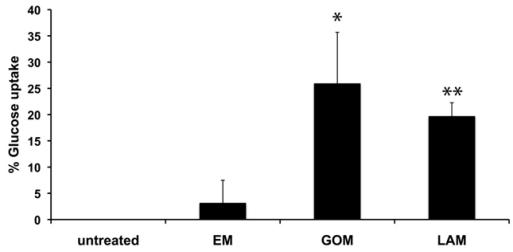

Figure 2. Differential effects of polysaccharides on glucose uptake. L6 myotubes were incubated with different treatments [2 mg/ml crude polysaccharide from the ear mushroom (EM), 2 mg/ml crude polysaccharide from the grey oyster mushroom (GOM), and 2 mg/ml Laminarin] for 48 hours. The histogram shows the average percentages of glucose remaining in the media, calculated from 3 repeats. The error bars represent standard deviations of the data. Statistical differences compare with the control set (untreated) were calculated by two-tailed Student's T-test (* for $P < 0.05$ and ** for $P < 0.001$).

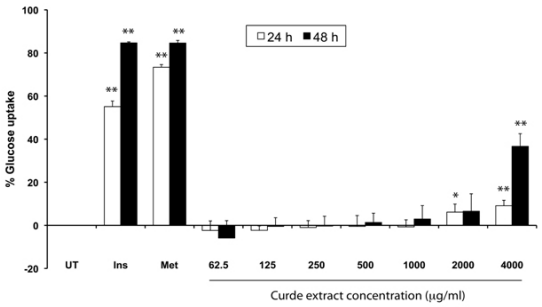

Figure 3. Polysaccharide from fruiting bodies of *P. sajor-caju* stimulated glucose uptake in time- and dose-dependent manners. (A) L6 cells were incubated with the various concentrations of the polysaccharide (62.5-4000 μg/ml). Ins (Insulin) at 500 nM and Met (Metformin) at 2 mM were used as positive controls. UT is abbreviated for untreated. Each represented data point was calculated from 6 repeats, and the glucose uptake experiment was repeated for 3 times. The error bars represent standard deviations of the data. Statistical differences compare with the control set (untreated) were calculated by two-tailed Student's T-test (* for $P < 0.05$ and ** for $P < 0.001$).

glucose uptake by the L6 cells, although the direct connection between β-glucan and glucose uptake has not been confirmed. Another study also showed that β-glucan-rich polysaccharide extract from the grey oyster mushroom decreased the overall blood glucose concentration in mice fed with high-fat diet (Kanagasabapathy *et al.*, 2012). This group showed that their β-glucan-rich extract upregulated GLUT-4 gene expression and downregulated the expression of many proinflammatory cytokines, including interleukine-6 (IL-6) and TNF-α (Kanagasabapathy *et al.*, 2012). However, the experiment was performed in mice, but not in the context of the cell line. Therefore, we hypothesize that β-glucan might activate the expression or exocytosis of GLUT-4 on the L6 cells through the activation of Akt/AMPK

pathway.

The ability of the L6 cell to respond to β-glucan suggests the existent of β-glucan receptor on the cell surface. It has been shown that β-glucan can signal through several receptors on the cell surface. These receptors include dectin-1, complement receptor 3 (CR3), lactosylceramide (LacCer), scavenger receptors (SR) and toll like receptors (TLRs), and the expression of these receptors is cell type specific (Chen and Seviour, 2007). Each of these receptors can signal through different signaling pathway, including PI3K/Akt, Extracellular signal-regulated kinase (Erk), p38 mitogen-activated protein kinase (p38-MAPK) pathways (Chen and Seviour, 2007; Chen and Raymond, 2008). To our knowledge, the specific β-glucan receptor on the L6 myotubes has not been suggested. Thus, identification of the specific β-glucan receptor and characterization of the signaling pathway for the glucose uptake by the L6 myotube await the future investigation.

Conclusion

Partially purified polysaccharide isolated from Pleurotus sajor-caju increased glucose uptake in the L6 muscle cells, possibly due to the presence of β-glucan in the extract. This finding suggests that β-glucan from this mushroom might be used with diabetes patients to help reducing blood glucose concentration. However, additional experiments are required to confirm that this activity is due to β-glucan in the extract. Furthermore, the specific β-glucan receptor and the signaling pathway that lead to the stimulation of glucose uptake by this cell remain our priority tasks in future experimentation.

Acknowledgments

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