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Purified Polysaccharides Extracted from Grey Oyster Mushroom [*Pleurotus sajor-caju* (Fr.) Sing.] Stimulate Glucose Uptake in C2C12 Myotubes through the activation of AMP-Activated Protein Kinase (AMPK) and Glucose Transporter 1 (GLUT1) Proteins

(Polisakarida Dimurnikan Diekstrak daripada Cendawan Tiram Kelabu [*Pleurotus sajor-caju* (Fr.) Sing.] Merangsang Pengambilan Glukosa dalam Miotiub C2C12 melalui Pengaktifan Protein Kinase Diaktifkan AMP (AMPK) dan Protein Pengangkut Glukosa 1 (GLUT1))

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ABSTRACT

The grey oyster mushroom [*Pleurotus sajor-caju* (Fr.) Sing.], an edible mushroom, has been known as a source of bioactive compounds, including polysaccharides. Polysaccharides from this mushroom have been shown to possess antidiabetic activity both *in vivo* and *in vitro*. However, antidiabetic mechanism of partially purified or purified polysaccharides from the gray oyster mushroom has not been characterized. In this study, we extracted and purified polysaccharides from gray oyster mushrooms and used them to investigate the antidiabetic mechanism in the context of C2C12 myotubes. Using Fourier Transform Infrared spectroscopy (FTIR) analysis and enzymatic assay, we showed that the polysaccharide sample, namely 9S1-1, contains β-glucose, α-glucose, and mannose as the monosaccharide composition, and β-glucan is the major type of polysaccharide in the sample. This 9S1-1 sample dose-dependently stimulated glucose uptake in C2C12 myotubes. Further analysis showed that the sample activated the AMP-activated protein kinase (AMPK) but not Akt serine/threonine kinase (AKT) phosphorylation, suggesting that the stimulation is AMPK-dependent. Moreover, we showed that compound c, an inhibitor of AMPK, inhibited glucose uptake in 9S1-1-stimulated cells, confirming the requirement of AMPK in the glucose uptake activated by the 9S1-1 sample. In addition, it promoted glucose transporter protein type 1 (GLUT1) but not GLUT4 protein expression. These results suggest that GLUT1 may be responsible for the stimulation of glucose uptake in 9S1-1-activated cells. Together, these data illustrate the antidiabetic mechanism of polysaccharides isolated from the gray oyster mushroom and the potential use of the polysaccharide as an antidiabetic agent.

Keywords: AMPK; antidiabetic; GLUT1; Gray oyster mushroom; *Pleurotus sajor-caju*

ABSTRACT

Cendawan tiram kelabu [*Pleurotus sajor-caju* (Fr.) Sing.], cendawan yang boleh dimakan, telah dikenali sebagai sumber sebatian bioaktif, termasuk polisakarida. Polisakarida daripada cendawan ini telah terbukti mempunyai aktiviti antidiabetis secara *in vivo* dan *in vitro*. Walau bagaimanapun, mekanisme antidiabetis bagi polisakarida yang telah dimurnikan sebahagian atau dimurnikan daripada cendawan tiram kelabu belum dicirikan. Dalam kajian ini, kami mengekstrak dan memurnikan polisakarida daripada cendawan tiram kelabu dan menggunakannya untuk mengkaji mekanisme antidiabetis dalam konteks miotiub C2C12. Dengan menggunakan analisis spektroskopi Transformasi Fourier Inframerah (FTIR) dan asai enzim, kami menunjukkan bahawa sampel polisakarida, iaitu 9S1-1, mengandungi β-glukosa, α-glukosa dan manosa sebagai komposisi monosakarida dan β-glukan ialah jenis utama polisakarida

dalam sampel. Sampel 9S1-1 ini merangsang pengambilan glukosa dos berkeperluan dalam miotiub C2C12. Analisis lanjut menunjukkan bahawa sampel mengaktifkan protein kinase diaktifkan AMP (AMPK) tetapi bukan fosforilasi Akt serin/threonina kinase (AKT), mencadangkan bahawa rangsangan adalah sandaran AMPK. Selain itu, kami menunjukkan bahawa sebatian c, iaitu perencat AMPK, merencat pengambilan glukosa dalam sel yang dirangsang 9S1-1, mengesahkan keperluan AMPK dalam pengambilan glukosa yang diaktifkan oleh sampel 9S1-1. Di samping itu, ia menggalakkan protein pengangkut glukosa jenis 1 (GLUT1) tetapi bukan ekspresi protein GLUT4. Keputusan ini menunjukkan bahawa GLUT1 mungkin bertanggungjawab untuk rangsangan pengambilan glukosa dalam sel yang diaktifkan 9S1-1. Data ini menggambarkan mekanisme antidiabetis polisakarida yang dipencilkan daripada cendawan tiram kelabu dan potensi penggunaan polisakarida sebagai agen antidiabetis.

Kata kunci: AMPK; antidiabetis; Cendawan tiram kelabu; GLUT1; *Pleurotus sajor-caju*

Introduction

Diabetes mellitus (DM), a global health problem, is a non-communicable chronic metabolic disease caused by the body's inability to regulate blood glucose. This results in high blood glucose levels and damage to tissues and organs. The trend of DM is increasing. It is estimated that the number of adults living with DM will reach 643 million by 2030 and 783 million by 2045 (International Diabetes Federation 2021). The disease places a significant burden on both patients and the global healthcare system, as it caused at least USD 966 billion in healthcare expenditures (International Diabetes Federation 2021).

Clinically, DM can be classified into four types: type 1 diabetes mellitus (T1DM) in which the pancreas can produce little or no insulin; type 2 diabetes mellitus (T2DM), which is caused by the inability of the body to respond to insulin; gestational diabetes (GDM), which happens during pregnancy if the body cannot produce sufficient insulin to control blood glucose; and specific types due to other causes such as drugs, genes, or diseases (Association 2021). DM could potentially lead to heart attack, kidney failure, blindness, stroke, and limb amputation. While medications for DM are available, they may have side effects such as weight gain and hypoglycemia (Chaudhury et al. 2017). Therefore, it is essential to explore alternative medicines that are safe and cost-effective.

Edible mushrooms, nutritious foods grown for consumption worldwide, may offer an alternative medicine for DM as well as other metabolic-related diseases. Mushrooms contain various bioactive molecules such as phenolics, terpenoids, polysaccharides, glucans, and lectins (Kumar et al. 2021). Among these compounds, the antidiabetic activity of mushroom polysaccharides is well documented (Aramabašić Jovanović et al. 2021). To date, many types of edible mushrooms have been illustrated for their antidiabetic activity such as *Lentinus edodes*, *Agaricus bisporus*, *Phellinus linteus*, *Ganoderma lucidum*, and *Pleurotus* spp. (Aramabašić Jovanović et al. 2021; Kumar et al. 2021). In addition to the antidiabetic activity, mushrooms were reported to possess other biological activities, such as anticancer (Park 2022), anti-inflammatory (Elsayed et al. 2014), antioxidant (Kosanić, Ranković & Dašić 2012), anti-microbial (Gebreyohannes et al. 2019), anti-obesity (Tung et al. 2020), and the potential anti-COVID-19 activity (Chun, Gopal & Muthu 2021). Therefore, edible mushrooms are functional foods that offer health benefits beyond basic nutrition for the body.

Among edible mushrooms, the gray oyster mushroom [*Pleurotus sajor-caju* (Fr.) Sing.] is of great interest. Extracts from the gray oyster mushroom were shown to possess many biological activities, including antidiabetic activity (Kanagasabapathy et al. 2012; Sermwittayawong et al. 2018). One of the key antidiabetic compounds in mushrooms is polysaccharides, particularly β-glucan, which is found in the cell wall of mushrooms. β-glucan is a polymer of β-glucoses connected typically through β(1→3) glycosidic bonds, exhibiting diverse activities including antidiabetic properties (Chakraborty & Devi Rajeswari 2022; Wan et al. 2022). It was shown that a β-glucan-containing crude polysaccharide extract from gray oyster mushrooms prevented insulin resistance, inflammation, and hyperglycemia in mice fed with a high-fat diet (Kanagasabapathy et al. 2012). Similarly, the oral administration of an aqueous extract from the gray oyster mushroom reduced fasting blood glucose, body weight, and urine sugar in streptozotocin-induced diabetic rats (Ng et al. 2015). In addition, work from our group showed that partially purified polysaccharides from

gray oyster mushrooms stimulated glucose uptake in L6 myotubes in a dose-dependent manner (Sermwittayawong et al. 2018).

Although the antidiabetic activity of the extract from the gray oyster mushroom has been studied, it remains unclear how the polysaccharides mechanistically confer the activity. In this present work, we purified, characterized, and analyze the antidiabetic activity of the polysaccharides using C2C12 myotube, a mouse cell line which has been used as a model for skeletal muscles (Abdelmoez et al. 2019; Casadei et al. 2009). We demonstrated that the polysaccharides stimulated AMP-activated protein kinase (AMPK) pathway, which promotes GLUT1 protein expression to activate glucose uptake in the cells. Thus, this study advances our understanding of how mushroom polysaccharides mechanistically promote glucose uptake in muscle cells and illustrates the application of polysaccharides as an antidiabetic compound.

Materials and Methods

Antibodies and Reagents

The source for each antibody and dilution factor used in this study are specified as follows: anti-beta actin antibodies (MABT523, Merck) were used at 1:10,000 dilution; anti-Akt antibodies (4691, Cell Signaling) were used at 1:2,500 dilution; anti-phospho-Akt antibodies (4060, Cell Signaling) were used at 1:2,500 dilution; anti-AMPK α-subunit antibodies (2532, Cell Signaling) were used at 1:1,000 dilution; anti-phospho AMPK α-subunit antibodies (Thr172) (2535, Cell Signaling) were used at 1:2,000 dilution; anti-GLUT1/SLC2A1 antibodies (A11208, ABclonal) were used at 1:1,000 dilution; anti-GLUT4 antibodies (A7637, ABclonal) were used at 1:1,000 dilution; and anti-rabbit-IgG (H+L) HRP conjugated antibodies (AP307P, Merck) were used at 1:10,000 dilution. C2C12 myoblast (CRL-1772) was purchased from the American Type Culture Collection (ATCC), USA. Insulin (I9278), metformin (PHR1084), and a glucose assay kit (GAGO20) were purchased from Sigma-Aldrich.

Extraction and Purification of Mushroom Polysaccharides

The gray oyster mushroom was a gift from Duangdao Mushroom Farm (Banpru, Hat Yai, Songkhla, Thailand). The farm obtained the mushroom spores from the Department of Agriculture, Ministry of Agriculture and Cooperatives, Thailand. We followed the same extraction and purification protocol used to create mushroom polysaccharides from gray oyster mushroom used in our previous study (Sermwittayawong et al. 2020).

The extraction of polysaccharides was performed using a hot-water extraction method. In brief, two kilograms of fresh mushrooms were washed, air-dried, sliced, and heated in an oven at 70 °C for 48 h or until dried. The mushrooms were then ground into powder. Polysaccharides were extracted with hot water. Distilled water was added to the mushroom powder at a ratio of 8:1 water (mL) to mushroom (g). Boiling was performed at 80-90 °C for 4 h with constant stirring. After cooling, the sample was subjected to centrifugation to separate the supernatant from the residues. The boiling was repeated once for 2 h, and the supernatant from the first and second extractions were combined. Polysaccharides in the supernatant were then precipitated by adding ethanol to the supernatant at a 5:1 v/v ratio, and the mixture was incubated for 12-16 h at 4 °C. After incubation, the sample was centrifuged to collect the polysaccharide precipitate, and the supernatant was discarded. The polysaccharides were then resuspended in water and subsequently dialyzed to eliminate ethanol.

Next, the sample was subjected to a sequential enzymatic digestion with α-amylase, followed by digestions with proteinase, and subsequently proteinase k. The remaining protein in the sample was subsequently removed by extracting using the Sevag reagent (chloroform/butanol 4:1, v/v) twice. The digested sample was dialyzed to remove buffer and small molecules. To purify polysaccharides, the sample was fractionated with Diethylethanolamine (DEAE) Sepharose Fast Flow column chromatography. Each fraction was subjected to the phenol-sulfuric assay and the Bradford assay to measure total carbohydrate and total protein, respectively. The column fractionated the sample into 3 peaks from low salt to high salt concentrations, namely 9S1-1, 9S1-2, and 9S1-3, respectively (Figure 1). Each peak was pooled separately, dialyzed against water, lyophilized, and stored in a desiccator until needed.

Measuring the Concentration of β-Glucan

The β-glucan content in the sample was measured via enzymatic assays using a β-glucan Assay Kit (yeast and mushroom from Megazyme, Ireland, following the manufacturer's instruction. In brief, the digestion of control β-glucan (contains 49% β-glucan) and the sample

were performed in parallel. Glycosidic bonds in the samples were hydrolyzed to near completion with H_2SO_4, and the remaining glucan fragments were subsequently hydrolyzed with specific exo-1,3-β-glucanase and β-glucosidase. The hydrolysates and digested products were used to measure the amount of glucose using glucose oxidase. The results reflect the amount of total glucan, which consists of both α- and β-glucans. In a separate experiment, the samples were digested with amyloglucosidase and invertase. The resulting D-glucose from the digestion was quantified using glucose oxidase. This gave the amount of α-glucan. Thus, the total β-glucan in the sample can be simply calculated by subtracting the amount of total glucan from the amount of α-glucan. The resulting amount of total β-glucan from the subtraction was then normalized against the control β-glucan, which contains 49% β-glucan, as clearly specified by the company.

DETERMINATION OF TOTAL CARBOHYDRATE

Total carbohydrate concentration in the sample was analyzed using the phenol-sulfuric acid method (Dubois et al. 1951). Briefly, 200 µL of sample was mixed with 200 µL of 5% phenol. Following that, 1 mL of concentrated sulfuric acid was added to the mixture, which was incubated at room temperature for 30 min. The absorbance at 470 nm was measured, and the total carbohydrate concentration was calculated based on a standard curve of glucose.

DETERMINATION OF TOTAL PROTEIN

The concentration of total protein in each sample was quantified using the Bradford assay (Bradford 1976). In a nutshell, 2.5 mL of Bradford reagent was added to 50 µL of sample and incubated for 20 min at room temperature. After incubation, the absorbance at 595 nm was measured, and the amount of protein was determined from a standard curve of bovine serum albumin (BSA).

CELL CULTURE AND DIFFERENTIATION

The mouse C2C12 skeletal muscle cell line was maintained in a low-glucose complete Dulbecco's modified Eagle's medium (cDMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin solution (100 U/mL penicillin and 100 µg/mL streptomycin) at 37 °C in a humidified atmosphere with 5% CO_2. C2C12 were seeded at a density of 24,000 cells/mL in a multi-well plate. Two days after plating, the process of differentiation was initiated by introducing cDMEM containing 2% horse serum (HS), which was changed every 2 days for a total of 3 changes or until the cells fused to form myotubes.

STIMULATION OF GLUCOSE UPTAKE AND CELL VIABILITY ASSAYS

To access whether the sample could stimulate glucose uptake in the cells, C2C12 myotubes were treated with the 9S1-1 sample at a desired concentration (62.5, 250, and 1000 µg/mL). Insulin and metformin (an antidiabetic drug) were employed as positive controls and applied at 100 nM and 1 mM, respectively. Following 24 h of treatment, the culture medium was collected for the glucose measurement assay, while the cells were utilized for a cell viability measurement. To analyze the viability, the cells were incubated with 0.25 mg/mL of MTT solution at 37 °C for 2 h. Then, the MTT was removed, and dimethyl sulfoxide (DMSO) was added to solubilize the purple formazan crystals in the cells. The absorbance at 550 nm was measured, and the viability of the cells was determined by comparing it with the untreated cells.

EXPERIMENT WITH COMPOUND C

C2C12 myotubes were pretreated with 20 µM compound c, an AMPK inhibitor purchased from Sigma-Aldrich (P5499), for 30 min prior to the co-incubation between a stimulator (9S1-1) and compound c at the same concentration. Insulin and metformin were positive controls. After 24 h of treatment, the culture medium was collected for the glucose measurement assay as described below.

GLUCOSE MEASUREMENT ASSAY

The glucose measurement assay was performed using a glucose assay kit, as recommended by the manufacturer. Briefly, the collected medium was diluted 1:2 with water and then mixed with the glucose assay reagent. Subsequently, the mixtures were incubated for 30 min at 37 °C. After the incubation, the reaction was halted by adding 6 M H_2SO_4, and the absorbance at 540 nm was measured. The amount of glucose in the medium was calculated based on a glucose standard curve. The greater the amount of glucose left in the medium, the lower the stimulation of glucose uptake was. The percentage of glucose uptake stimulation by the purified mushroom polysaccharides was calculated using the following equation:

$$\%\ stimulation = \frac{\left(A_{untreated\ control} - A_{sample}\right) \times 100}{A_{untreated\ control}}$$

WESTERN BLOTTING ANALYSIS

C2C12 myotubes in 6-well plates treated with 9S1-1 sample at 1 mg/mL concentration for different length of time (0, 30, 60, and 120 min) were collected for Western blotting. To collect the cells, the plate was placed on ice, and the cells were washed twice with ice-cold 1XPBS. After removing 1XPBS, 200 µL of cold lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM NaF, 1 mM sodium orthovanadate (Na_3VO_4), 1% nonidet P-40 (NP-40), 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), phosphatase inhibitor tablet (Roche), and protease inhibitor tablet) was added into each well and incubated on ice for 5 min. Cells were resuspended, and the lysates were transferred to a new 1.5 mL Eppendorf tube. After chilling on ice and vortexing 3 times for 10 min each, the sample was centrifuged at 12,000 rpm for 30 min at 4 °C. Subsequently, the supernatant was transferred to a new tube and mixed with 2X protein gel loading buffer in a ratio of 1:1. Proteins in the sample were fractionated with a 10% sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Fractionated protein in the gel was subsequently transferred to a polyvinylidene difluoride (PVDF) membrane. Western blotting was carried out as previously described (Jakkawanpitak, Hutadilok-Towatana & Sermwittayawong 2020), with the exception that the chemiluminescence signal was visualized using the Alliance Q9-ATOM Advance Chemiluminescence Imager, UVITEC.

STATISTICAL ANALYSIS

All data were expressed as mean ± SD in triplicate for at least two separate experiments. Statistical significance was analyzed using one-way analysis of variance (ANOVA), with Duncan's multiple comparison tests. Two datasets with different letters are significantly different with $p < 0.05$.

RESULTS AND DISCUSSION

PURIFICATION AND CHARACTERIZATION OF THE POLYSACCHARIDES

Three polysaccharide peaks: 9S1-1, 9S1-2, and 9S1-3 resulted from DEAE column chromatography (Figure 1) were subjected to total protein and carbohydrate analyses (Table 1). In general, fractions from the 9S preparation exhibited higher concentrations across all three fractions compared to the polysaccharide samples from the 7S preparation (7S1-1, 7S1-2, and 7S1-3), which were used in our previous report (Sermwittayawong et al. 2020). Among the 3 peaks, the 9S1-1 sample contains the highest concentration of carbohydrate (94.00%) and the lowest concentration of protein (1.79%). The carbohydrate content of the 9S1-1 sample was comparable to that of the 7S1-1, which was at 94.36% (Sermwittayawong et al. 2020). However, the protein concentration of the 9S1-1 sample was slightly higher than that of the 7S1-1 sample, which was recorded as 0.08% (Sermwittayawong et al. 2020). Due to the highest concentration of carbohydrate and the lowest concentration of protein, we selected the 9S1-1 sample for subsequent analysis and experiments.

TABLE 1. Yield, percent carbohydrate, and percent protein

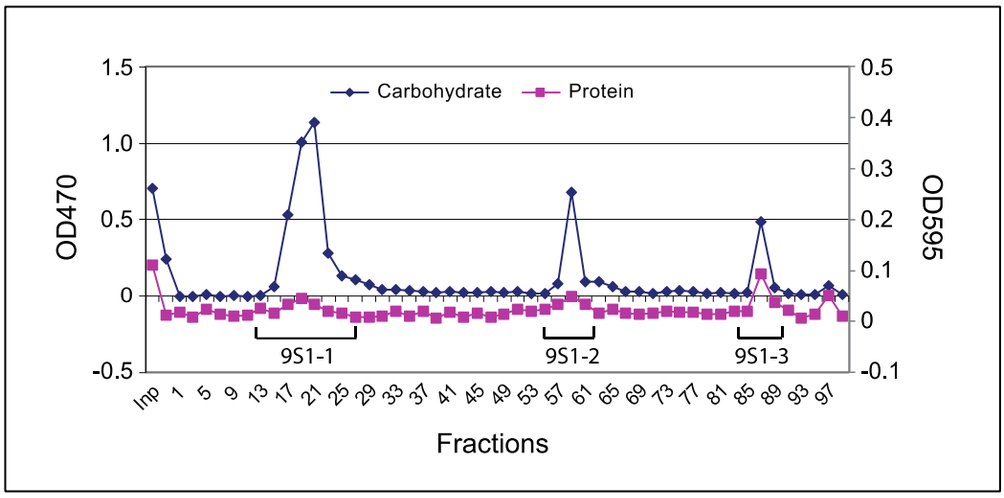

FIGURE 1. Purification of polysaccharides. The extracted polysaccharides from the mushroom were fractionated with DEAE Sepharose Fast Flow. Each fraction was subjected to the phenol-sulfuric assay to measure carbohydrate and the Bradford assay to measure protein

of the 3 polysaccharide fractions obtained from DEAE column chromatography. The percent carbohydrate and protein are reported as the average ± S.D. (n =3)

Fractions	Amount obtained (g)	Percent carbohydrate	Percent protein
9S1-1	3.28	94 ± 7.49	1.79 ± 0.15
9S1-2	0.40	64.67 ± 4.21	3.91 ± 0.13
9S1-3	0.39	44.44 ± 1.13	6.44 ± 0.21

9S1-1 sample underwent Fourier Transform Infrared spectroscopy (FTIR) analysis. Results in Figure 2 showed that the FTIR spectrum from the 9S1-1 sample bears similarity to the FTIR spectra of the 7S1-1 fractions, as reported previously (Sermwittayawong et al. 2020). For example, the vibrations at 1045 cm^{-1} and 1077 cm^{-1} represent β-(1→3) glucans (Galichet 2001). In addition, the vibration at 911.6 cm^{-1} indicates the presence of mannan (Galichet 2001). However, the peak at 1022 cm^{-1}, which indicates the α-(1→4) glycosidic bonds (Synytsya & Novak 2014), was shown in the 9S1-1 sample but not present in the S1-1 and 7S1-1 polysaccharides. Therefore, the FTIR results suggest that the 9S1-1 sample contains at least α-glucan, mannan, and β-glucan.

In addition, we utilized a β-glucan test kit to quantify the amount of β-glucan in the 9S1-1 sample. Results in Table 2 show that the 9S1-1 sample contains α-glucan at a concentration above 1% and β-glucans at approximately 56% (after adjusting the calculated concentration with the control, which has 49% β-glucan). Thus, the 9S1-1 sample contains mostly β-glucan. The presence of both α- and β-glucans based on the test kit is consistent with the observation from FTIR analysis. The presence of α-glucan most likely reflects the incomplete digestion using α-amylase during the purification process (Sermwittayawong et al. 2018).

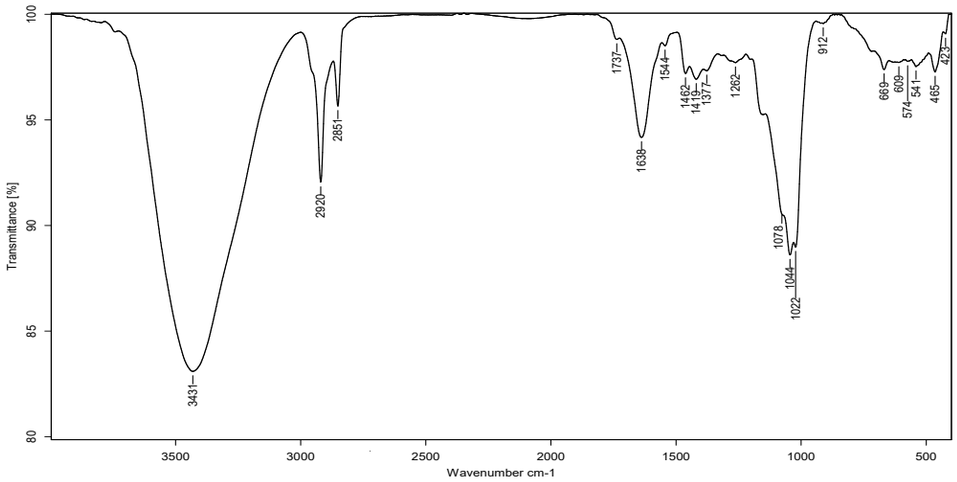

FIGURE 2. FTIR spectrum of 9S1-1 sample

TABLE 2. Quantification of the amount of β-glucan using the β-glucan test kit

Samples	Average % w/w			
	Total glucan	alpha-glucan	beta-glucan	Adjusted % beta-glucan according to the control (49%)
Control	62.13	0.65	61.48	49.00
9S1-1	71.81	1.68	70.13	55.89

THE 9S1-1 SAMPLE STIMULATES GLUCOSE UPTAKE IN C2C12 MYOTUBES IN A DOSE-DEPENDENT FASHION

Previously, we showed that polysaccharides from the gray oyster mushroom dose-dependently stimulated glucose uptake in L6 myotubes (Sermwittayawong et al. 2018). In addition, previous work showed that inulin, a type of polysaccharides that consists mainly of β-(2→1) fructosyl-fructose, stimulated the uptake of 2-deoxyglucose in C2C12 myotubes in a dose-dependent manner (Yun et al. 2009). Given these findings, we hypothesized that these 9S1-1 sample could stimulate glucose uptake in the C2C12 myotubes. To test this hypothesis, we first analyzed whether the 9S1-1 sample would affect the viability of the cells. As depicted in Figure 3(A), the 9S1-1 sample concentration ranging from 62.5 to 1000 μg/mL exhibited the ability to sustain C2C12 myotube viability at nearly 100%, suggesting that the polysaccharides are not toxic to the cells. Then, we tested whether the polysaccharides could stimulate glucose uptake in the cells. The results show that the 9S1-1 sample stimulated glucose uptake proportionally to the increasing amount of the polysaccharides (Figure 3(B)), suggesting that the polysaccharides exert a dose-dependent promotion of glucose uptake in muscle cells.

THE 9S1-1 SAMPLE PROMOTES GLUCOSE UPTAKE IN C2C12 MYOTUBES THROUGH AMPK BUT NOT AKT PHOSPHORYLATION

AMP-activated protein kinase (AMPK) and Akt serine/threonine kinase (AKT) have been identified to play pivotal roles in stimulating glucose uptake within myotubes. AMPK, a protein complex that consists of α, β, and γ subunits, functions as the primary sensor of

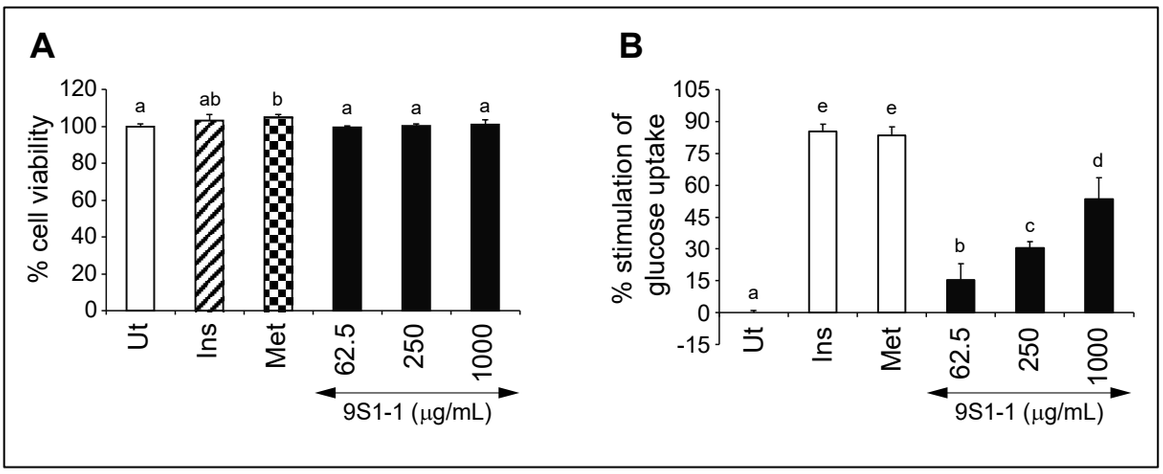

FIGURE 3. 9S1-1 sample is not toxic to C2C12 myotubes and dose-dependently stimulates glucose uptake in the cells. (A) shows the average percent cell viability of C2C12 myotubes treated with 9S1-1 sample in the increasing dose. The abbreviations 'UT', 'Ins', and 'Met' stand for untreated, insulin, and metformin, respectively. (B) shows the average percent stimulation of glucose uptake in C2C12 myotubes. Error bars represent standard deviations (n=3)

cellular energy (Trefts & Shaw 2021). This complex is activated by the rise in the amount of cellular AMP nucleotide, leading to the phosphorylation of many residues in AMPK, especially the Thr172 residue of the α-subunit, which is the hallmark of AMPK activation (Trefts & Shaw 2021). Activated AMPK phosphorylates many target proteins, including the AKT substrate of 160 kDa (AS160), resulting in translocation of GLUT4 to the plasma membrane (Eickelschulte et al. 2021). In addition, AKT functions in many cellular activities, such as cell cycle progression, glucose metabolism, protein synthesis, and cell viability (Nitulescu Mihai et al. 2018). AKT can be phosphorylated at many positions, especially at Ser473, which is required for the maximal activation (Wei et al. 2019). AKT is activated by a series of protein kinases that receive the signal from insulin receptors, including phosphatidylinositol 3-kinase (PI3K) and 3-Phosphoinositide-dependent kinase 1 (PDK1) (Manning & Toker 2017). Like AMPK, AKT can phosphorylate AS160, which subsequently causes translocation of GLUT4 to the plasma membrane and activation of glucose uptake into the cells.

To this end, we performed Western blotting to analyze the phosphorylation of Thr172 of the α-subunit of AMPK and Ser473 of the AKT proteins. The results in Figure 4 show that phosphorylation of AMPK was significantly increased from 30-120 min (Figure 4(A) and 4(B)), suggesting the role of AMPK in the stimulation of glucose uptake. In contrast, the phosphorylation of AKT remained unchanged over time (Figure 4(A) and 4(C)), suggesting that the polysaccharides do not

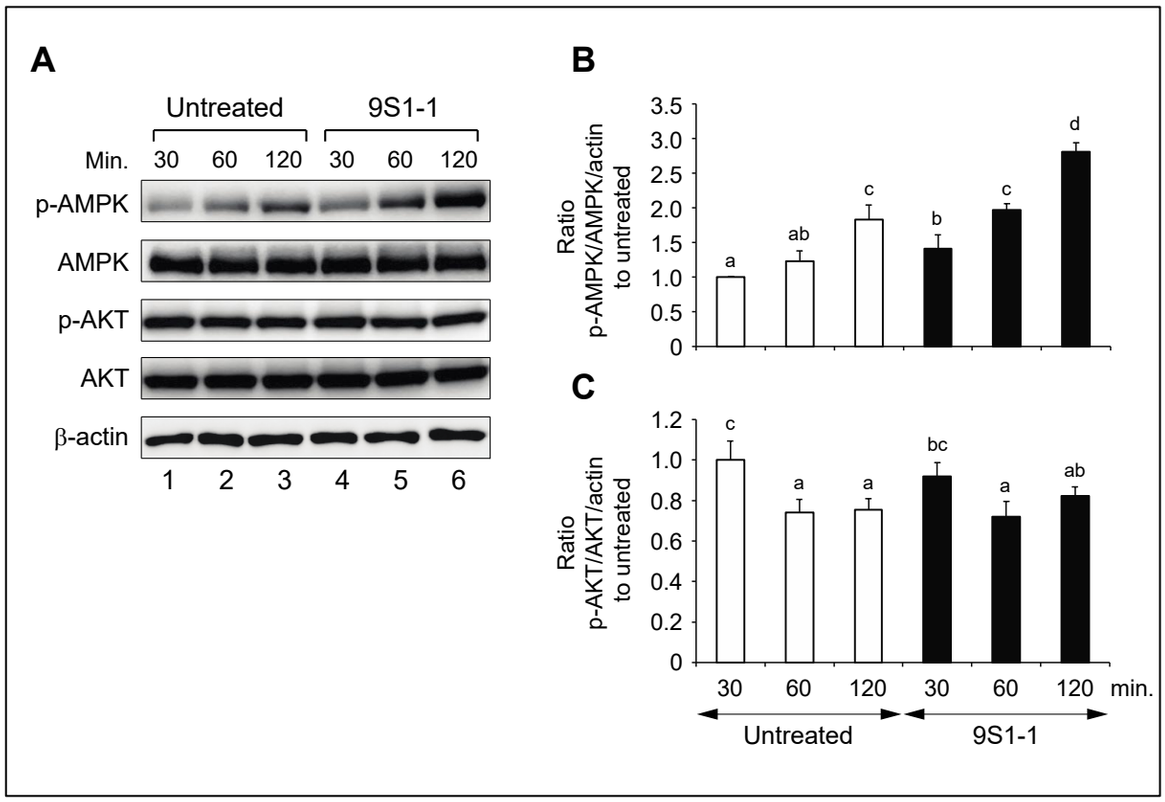

FIGURE 4. 9S1-1 sample promotes AMPK but not AKT activation in C2C12 myotubes. (A) shows Western blotting experiments for time-dependent protein expression and phosphorylation of AMPK and AKT in the cells treated with 1 mg/mL of 9S1-1 sample for 30, 60, and 120 min. (B) shows the histogram of the ratio between the Western blotting signals of phosphorylated protein to total protein in (A), normalized with β-actin in respect to the untreated set. Error bars represent standard deviations (n=3)

stimulate glucose uptake in C2C12 myotubes through the AKT kinase.

To further delve into the role of AMPK in polysaccharide-induced glucose uptake in C2C12 myotubes, we used the compound c inhibitor. If AMPK is involved in the signaling, then compound c should inhibit glucose uptake. Therefore, we performed the experiment by stimulating the cells with insulin, metformin, or the 9S1-1 sample, in the presence or absence of compound c. The results show that compound c completely inhibited glucose uptake in C2C12 myotubes induced by the 9S1-1 sample, and partially inhibited glucose uptake stimulated by insulin and metformin, which served as positive control sets (Figure 5). Therefore, we concluded that the mushroom polysaccharides stimulate glucose uptake in C2C12 myotubes via AMPK but not AKT pathways.

The involvement of AMPK in glucose uptake in polysaccharide-stimulated muscle cells has been illustrated. For example, a previous study showed that inulin dose-dependently stimulated 2-deoxyglucose uptake in C2C12 myotubes and promoted both α-AMPK phosphorylation at Thr172 and AKT phosphorylation at Ser473 (Yun et al. 2009). The authors further showed that compound c (an inhibitor of AMPK) and LY294002 (an inhibitor of PI3K/AKT) blocked inulin-induced 2-deoxyglucose uptake, suggesting that inulin promoted glucose uptake in C2C12 myotubes via AMPK and AKT pathways. Another study showed that laminarin from *Salicornia herbacea* stimulated glucose uptake in L6 myotubes in a dose-dependent manner, and compound c inhibited this stimulation (Kim et al. 2020). Similarly, another report showed that *Astragalus* polysaccharides (APS) activated the AMPK complex to promote glucose uptake in myotubes, and compound c blocked AMPK and AS160 in the cells stimulated by APS (Liu et al. 2013). Therefore, different types of polysaccharides stimulate glucose uptake in muscle cells, commonly through the activation of the AMPK complex.

The precise mechanism by which the AMPK complex in polysaccharide-stimulated C2C12 myotubes undergoes phosphorylation remains unresolved. Previous studies have identified kinases that phosphorylate the AMPK complex, and those kinases include liver kinase B1 (LKB1) and calcium/calmodulin-dependent protein kinase kinase 2 (CAMKK2) (Steinberg & Carling 2019). Both kinases belong to the serine-threonine kinase family. LKB1 was shown to play a role in myogenic differentiation (Mian et al. 2012), and the deficiency of LKB1 in skeletal muscle was shown to affect AMPK activation and glucose uptake induced by 5-aminoimidazole-4-carboxamide ribonucleoside stimulated (AICAR) or muscle contraction (Sakamoto et al. 2005). Likewise, CAMKK2 or CAMKKβ was also shown to be involved in glucose uptake. For example, it was shown that *Astragalus* polysaccharides promoted glucose uptake in the L6 myotubes through the stimulation of CAMKK2, which subsequently phosphorylated the AMPK complex (Liu et al. 2013). In addition, CAMKK2 was shown to be involved in the stimulation of glucose uptake in C2C12 (Chen et al.

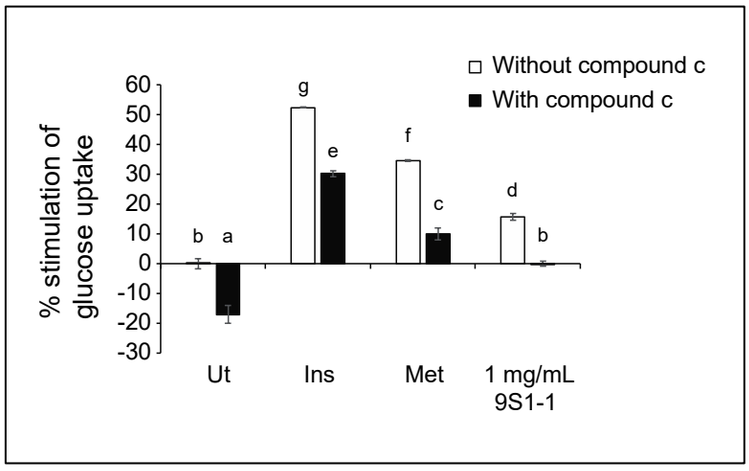

FIGURE 5. Compound c inhibits glucose uptake in 9S1-1-stimulated C2C12 myotubes. The chart shows the average percent stimulation of glucose uptake in the treated cells, in the absence or the presence of 20 μM compound c. The abbreviations 'UT', 'Ins', and 'Met' stand for untreated, insulin, and metformin, respectively. Error bars represent standard deviations (n=3)

2020; Ferdowsi et al. 2022). Therefore, whether LKB1 or CAMKK2 are responsible for AMPK phosphorylation in mushroom polysaccharide stimulated cells, additional research investigation is required.

THE 9S1-1 SAMPLE PROMOTES GLUT1 BUT NOT GLUT4 PROTEIN EXPRESSION

GLUT1 and GLUT4, as key glucose transporters, hold vital roles within C2C12 (Wong, Al-Salami & Dass 2020). GLUT1 contributes to basal glucose uptake within cells (Pragallapati & Manyam 2019), while GLUT4 can be activated through both insulin and non-insulin mechanisms (Klip, McGraw & James 2019). The findings that the 9S1-1 sample stimulated glucose uptake in C2C12 via the activation of AMPK has led us to consider the potential involvement of GLUT4 in this glucose uptake process. Surprisingly, the Western blotting results show an increase in protein expression of GLUT1 in C2C12 myotubes after a 120-min treatment with the 9S1-1 sample. In contrast, the protein expression of GLUT4 remains constant throughout the period of treatment (Figure 6). These results suggest that the 9S1-1 sample stimulated GLUT1 protein expression in the muscle cells and a possibility that GLUT1 but not GLUT4 may be responsible for the stimulation of glucose uptake in the cells.

Consistent with the protein expression data, we found the upregulation of *Glut1* but not *Glut4* gene expression in the polysaccharide treated cells (data not shown). These data suggest the involvement of GLUT1 in the stimulation of glucose uptake in the cells. To date, only few studies have addressed the role of GLUT1 in glucose uptake stimulation. For instance, it was previously shown that GLUT1 and GLUT4 proteins on the plasma membrane of the soleus muscles treated with 1.0 mM β-phenylpyruvate were increased by 10-fold and 2-fold, respectively (Ben-Abraham et al. 2003), suggesting the role of both glucose transporters in glucose transport into the tissue. Another study showed that AICAR promoted 3-O-methylglucose (OMG) uptake

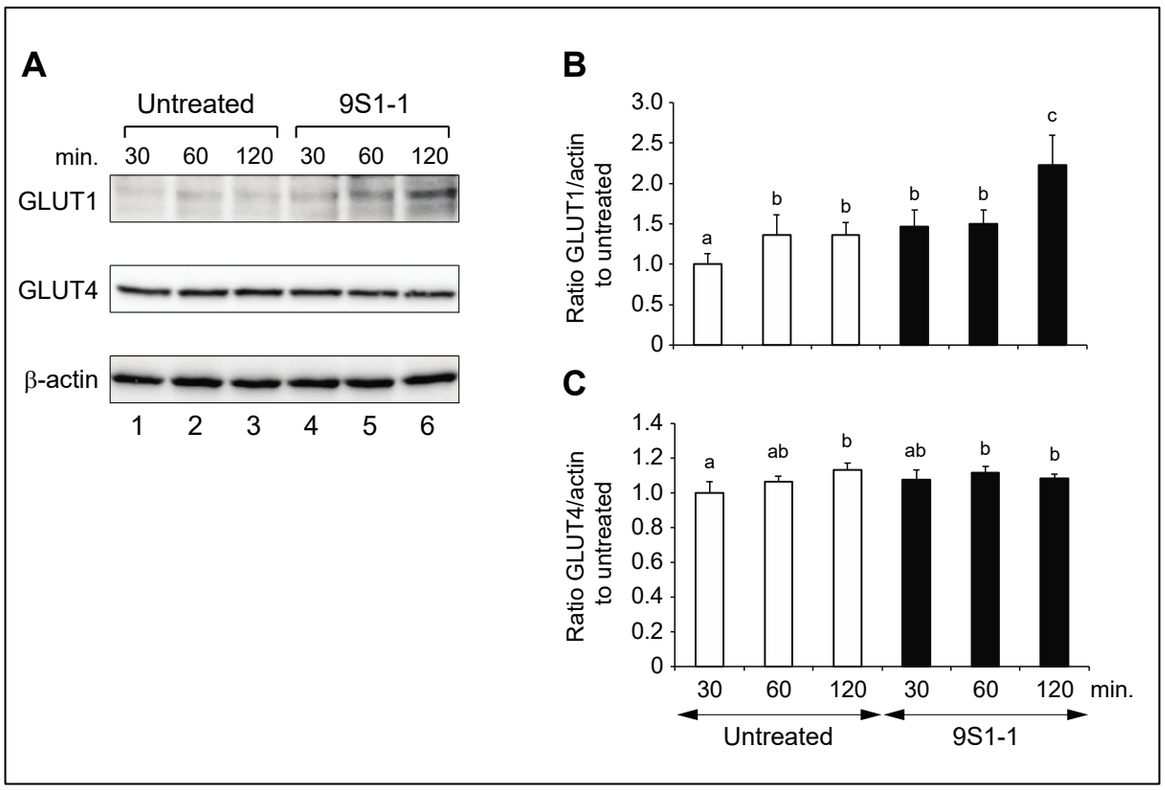

FIGURE 6. GLUT1 but not GLUT4 protein expression is induced in C2C12 myotubes treated with 9S1-1 sample. (A) shows Western blotting experiments for time-dependent protein expression and phosphorylation of GLUT1 and GLUT4 proteins in the untreated cells or cells treated with 1 mg/mL of 9S1-1 sample at 30, 60, and 120 min. (B) shows the histogram of the ratio between the Western blotting signals of GLUT1 to actin from (A). (C) is like (B), except that it is the signal of GLUT4. Error bars represent standard deviations (n=3)

and activated AMPK in 3T3-L1 preadipocytes, C2C12 myoblasts, and Clone 9 cells, all of which expressed only the Glut1 isoform, and translocated GLUT1 to the plasma membrane of Clone 9 cells (Abbud et al. 2000). AICAR also stimulated glucose uptake and the expression of *Glut1* and *Glut4* genes in primary human skeletal myotubes (Al-Khalili et al. 2005).

Despite the findings, the critical question of whether GLUT1 is responsible for the activation of glucose uptake observed in our experiments, remains. It has been known that many glucose transporters, including GLUT1 and GLUT4, are regulated through the translocation to the plasma membrane (Wang et al. 2020). In other words, the produced glucose transporters can uptake glucose into the cells only when they are translocated into the plasma membrane. Consistent with this idea, it was previously shown that although dexamethasone induced Glut4 mRNA expression in C2C12 myocytes by about 10 times, the myocytes did not show an enhancement in glucose uptake stimulated by insulin (Tortorella & Pilch 2002). Thus, despite showing the upregulation in the protein level of GLUT1, we could neither conclude that GLUT1 is responsible for the glucose uptake in the cells, nor rule out a possibility that GLUT4, whose expression remained constant, does not participate in the stimulation of glucose uptake in the cells. The subcellular localization of GLUT1 and/or GLUT4 in the stimulated myotubes is being investigated in our laboratory. Nonetheless, the role of GLUT1 in polysaccharide-stimulated glucose uptake requires future experimentation.

Conclusions

In this study, we have demonstrated that a polysaccharide rich in β-glucan, specifically the 9S1-1 sample, induces a dose-dependent enhancement of glucose uptake in C2C12 myotubes. This effect is mediated through the activation of AMPK, which subsequently leads to an upregulation of GLUT1 protein expression, while maintaining GLUT4 expression unchanged. Consequently, this response results in an increased cellular glucose uptake. This study proposes a potential pivotal role of GLUT1 as the primary mediator of glucose uptake in response to polysaccharide stimulation. Further investigations are imperative to validate the involvement of GLUT1 in the glucose uptake stimulated by mushroom polysaccharides. Overall, this study significantly contributes to our understanding of the antidiabetic mechanism governed by polysaccharides sourced from gray oyster mushrooms.

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