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Rhodobacter sphaeroides Extract Improves Glucose Homeostasis in Streptozotocin-Induced Diabetic Mice

Chih-Chiang Wang[1,2], Wen-Sheng Liu[1,3], Fu-Hsin Chang[3], Pei-Yi Tsai[4], Ming-Kai Tsai[2], Jeng-Chuan Shiang[2] and Zhi-Hong Wen[1]*

[1]*Department of Marine Biotechnology and Resources, National Sun Yat-sen University, Kaohsiung, Taiwan*
[2]*Department of Medicine, Kaohsiung Armed Forces General Hospital, Kaohsiung, Taiwan*
[3]*Asia-Pacific Biotech Developing, Inc. Kaohsiung, Taiwan*
[4]*Department of Pharmacology, Institute for Drug Evaluation Platform, Development Center for Biotechnology, Taiwan*

Abstract

Insulin resistance is one of the most important mechanisms of diabetes mellitus. Anti-oxidants had been proved to improve insulin resistance. However, the direct relation between anti-oxidants and glucose homeostasis is still elusive. Recently, we found that Lycogen™ (extracts of *Rhodobacter sphaeroides* WL-APD911) has lycopene-like activity. Furthermore, Lycogen™ showed more potent anti-oxidative effect and less cytotoxicity than lycopene. To evaluate the effect of Lycogen™ on blood glucose levels, STZ-induced diabetic mice were randomly divided into four groups: (1) diabetes control group; (2) diabetes+Lycogen™ 50 mg/kg; (3) diabetes+Lycogen™ 100 mg/kg; and (4) diabetes+Lycogen™ 200 mg/kg. After 7 days of treatment, the blood glucose level in the Lycogen™ 200 mg/kg group was significantly lower than the disease control group ($p<0.01$). In the oral glucose tolerance test, the blood glucose level was significantly lower in the Lycogen™ 200 mg/kg group than the disease control group ($p<0.05$). Our results confirmed that Lycogen™, a potent anti-oxidant, can significantly lower blood glucose levels in a diabetic mice model.

Keywords: *Rhodobacter sphaeroides*; Lycogentm; Anti-Oxidants; Diabetes mellitus

Introduction

Diabetes mellitus (DM) continues to be a major burden on society globally. The International Diabetes Federation has predicted that the global burden of diabetes will increase to 552 million by 2030. In spite of newer and effective treatment, glucose control remains relatively poor in a subset of subjects diagnosed and treated with diabetes. Therefore, extensive research is still being performed to develop potential anti-diabetic agents [1]. Reduction of action of insulin to activate the glucose transport system in skeletal muscle (referred to as "insulin resistance") is the most important mechanism of DM. Oxidative stress has been increasingly recognized as a common underlying mechanism of insulin resistance [2-6]. Recent studies support this connection that the direct exposure of mammalian skeletal muscle to an oxidant stress results in the stimulation of mitogen-activated protein kinase (MAPK) and that MAPK signaling is mechanistically associated with the diminished insulin-dependent stimulation of insulin signaling and glucose transport [7-10]. Thus, strategies to prevent and ameliorate oxidative stress have become important in the treatment of diabetes. However, the association between anti-oxidants and glycemic control was still elusive in previous observational studies [11-16].

Bacteria can produce some compounds in response to their environment. These compounds are widely used in pharmaceutical applications. Carotenoids, responsible for pigments in plants, deep green vegetables and yellow fruits, possess antioxidant properties. Carotenoids are also biosynthesized in photosynthetic bacteria such as *Rhodobacter sphaeroides* [17] with the function of photoprotection [18]. Recently, we extract a novel fractionate (Lycogen™) from transformant *Rhodobacter sphaeroides* WL-APD911 [19,20]. Lycogen™, which contained ζ-carotene, neurosporene, spheroidenone and methoxyneurosporene according to nuclear magnetic resonance spectroscopy analysis, has anti-oxidative activity. ζ-Carotene is the precursor of neurosporene, which is the precursor of lycopene. Furthermore, Lycogen™ showed more potent anti-oxidative effects and less cytotoxicity than lycopene in our study. Therefore, we aimed to study the effect of Lycogen™ on blood glucose levels.

Materials and Methods

Lycogen™

Rhodobacter sphaeroides WL-APD911 (DSM 25056) was a new strain, isolated from mutants using chemical mutagenesis (*Rhodobacter sphaeroides*; Bioresource Collection and Research (BCRC), Hsinchu, Taiwan). The *R. sphaeroides* WL-APD911 was cultured in broth. After harvesting, the bacterial broth was centrifuged and washed with ethanol. The bacterial residue is extracted with acetone and then centrifuged by 7500 rpm for 5 min. The supernatant is filtered through filter paper and a 0.2 μm filter into a round-bottomed flask. The color of the final supernatant is dark red. Acetone is removed completely in an oven at 55°C. The extract of *R. sphaeroides* WL-APD911 was named Lycogen™. The Lycogen™ is available from Asia-Pacific Biotech Developing, Inc. (Kaohsiung, Taiwan).

DPPH radical-scavenging activity

The free radical-scavenging activity was determined using the method described by Braca et al. [21]. Freshly prepared DPPH solution, lycopene and Lycogen™ extract at various concentrations (25 μM, 50 μM and 100 μM) were mixed and incubated at 37°C for 30 min. The absorbance at 517 nm was determined, and the percentage of

***Corresponding author:** Dr. Zhi-Hong Wen, Department of Marine Biotechnology and Resources, National Sun Yat-sen University, 70 Lienhai Rd., Kaohsiung 80424, Taiwan, Tel: +886-7-5252000; E-mail: ndmcndmcndmc@gmail.com

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inhibition activity was calculated as [(A0–A1)/A0] x100, where A0 is the absorbance of the control, and A1 is the absorbance of the extract/standard. The inhibition curves were constructed, and IC50 values were obtained.

Cytotoxicity assessment

The effects of Lycogen™ on the cell viability of Hs68 cells (human fibroblast cells, purchased from Bioresource Collection and Research Center) were evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. For the MTT assay, various concentrations of Lycogen™ and lycopene were separately added to the medium (Dulbecco's Modified Eagle's Medium) 24 h before the cell viability assay. In brief, MTT solution was added to each well, and the plates were incubated at 37°C for 4 h. The formazan product was then dissolved in 100 µl DMSO at 37°C for 30 min, and the absorbance at 570 nm was measured with a microplate reader. Cell cytotoxicity was determined and expressed as the percentage of viable cells of the total number of cells counted. The values are the means ± SD (n ≥ 3) for each treatment.

Experimental animals

Seven-week-old C57BL/6 male mice were obtained from a supply company (BioLASCO Co., Ltd., Taipei, Taiwan). The animals were housed in stainless steel cages at 25 ± 2°C with a relative humidity range of 40%-70% and an alternating 12-hour light-dark cycle, and they were fed standard laboratory chow and water. The experimental protocol was approved by the Laboratory Animal Care and Use Committee (IACUC) of the Development Center for Biotechnology.

Establishment of experimental diabetes model

Diabetes was induced by the intraperitoneal injection of STZ (50 mg/kg; Sigma, USA) in freshly prepared citrate buffer (0.1 M, pH 4.5) for four consecutive days. The blank control group was intraperitoneally injected with an equivalent amount of buffer. Diabetic mice were confirmed by measuring the 4 h fasting blood glucose levels from the tail vein at one week after injection with STZ. Animals with a blood glucose level above 250 mg/dl were considered to be diabetic and included in the experiment. The serum insulin level was also detected to verify the diagnosis of diabetes.

Oral glucose tolerance test (OGTT)

After 4 h of fasting, the basal blood glucose level was measured in test mice. Lycogen™ was orally administered to each group, and 30 min later, the mice were fed glucose (1.5 g/kg) orally. The blood glucose level was detected at 15, 30, 60, 90, and 120 min after feeding.

Statistical analysis

The results were expressed as the mean+standard error (mean ± SE.). A one-way ANOVA was used to test the difference between groups. If $P<0.05$, Dunnett's multiple-range t-test was used to identify the difference between the diabetes group and control group.

Materials and Instruments

The materials and instruments included 15 ml centrifuge tubes (Corning, USA), a centrifuge (ALC PK131R, Italy), a blood glucose meter (glucometer ACCU-CHEK advantage II, Roche Diagnostics, USA), blood glucose test paper (glucose test Comic books, strips ACCU-CHEK advantage II, Roche Diagnostics, USA), microcentrifuge tubes (SSI and USA), streptozotocin (Sigma, USA), sodium citrate (Sigma, USA), citric acid (Sigma, USA), 1-ml syringes (Terumo, the Philippines), a mouse insulin ELISA kit (cat. # 10-1247-01, Mercodia, Sweden), DPPH (Sigma, USA), and an MTT assay kit (Tosoh, Tokyo, Japan).

Results

Anti-oxidative activity and cytotoxicity assessment

In the DPPH test, Lycogen™ showed more potent anti-oxidative activity than Lycopene, especially at higher concentrations (Figure 1). The human fibroblast (Hs68) toxicity assessment of Lycogen™ showed no cytotoxic effects on human fibroblasts at concentrations below 5 µM. In contrast to Lycogen™, 0.1 µM lycopene showed cell toxicity in human fibroblasts. More significant cell toxicity was found at higher concentrations of lycopene (Figure 2).

Blood glucose and insulin levels

Diabetic mice were randomly divided into four groups, (1) diabetes control group; (2) diabetes+Lycogen™ 50 mg/kg; (3) diabetes+Lycogen™ 100 mg/kg; and (4) diabetes+Lycogen™ 200 mg/kg. Lycogen was prepared with pumpkin seed oil once a day for 14 consecutive days. The diabetes control group and blank control group, which was only administered pumpkin seed oil, was treated once a day for 14 consecutive days. After the end of the 14-day course, the oral glucose tolerance test (OGTT) was performed on the next day. The experimental design is shown in Table 1.

After injection with STZ, the blood glucose level was significantly increased ($p<0.005$) in STZ-induced diabetic mice (Figure 3). The success rate of diabetes induction was 92% (23/25). The serum insulin concentration of STZ-induced diabetic mice was significantly lower than the control group ($P<0.01$), which confirmed the successful induction of the diabetic model (Figure 4). Changes in the body weight of the mice during the treatment period are shown in Figure 5. The results showed that Lycogen™ treatment did not cause weight loss in any group of mice. The blood glucose level after 7 days of Lycogen™ treatment is shown in Figure 6. Treatment with Lycogen™ 200 mg/kg significantly reduced the blood glucose concentration compared with the disease control group ($P<0.01$). No significant difference was found between Lycogen™ 50 mg/kg, 100 mg/kg and disease control groups. The oral glucose tolerance test was performed after 14 days of Lycogen™ administration (Figure 7). At 60 min after oral glucose loading, the blood glucose level in the Lycogen™ 200 mg/kg group

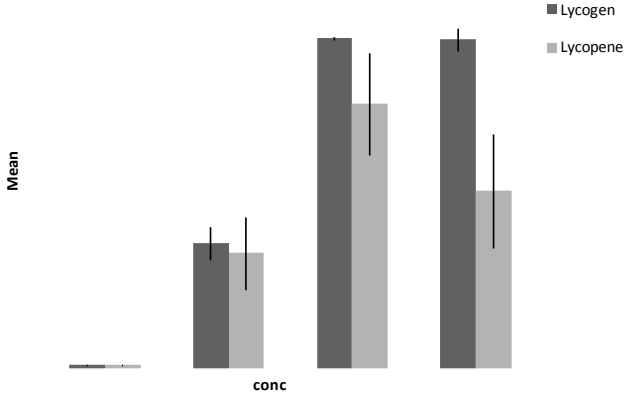

Figure 1: DPPH antioxidant test: Lycogen compared with Lycopene.

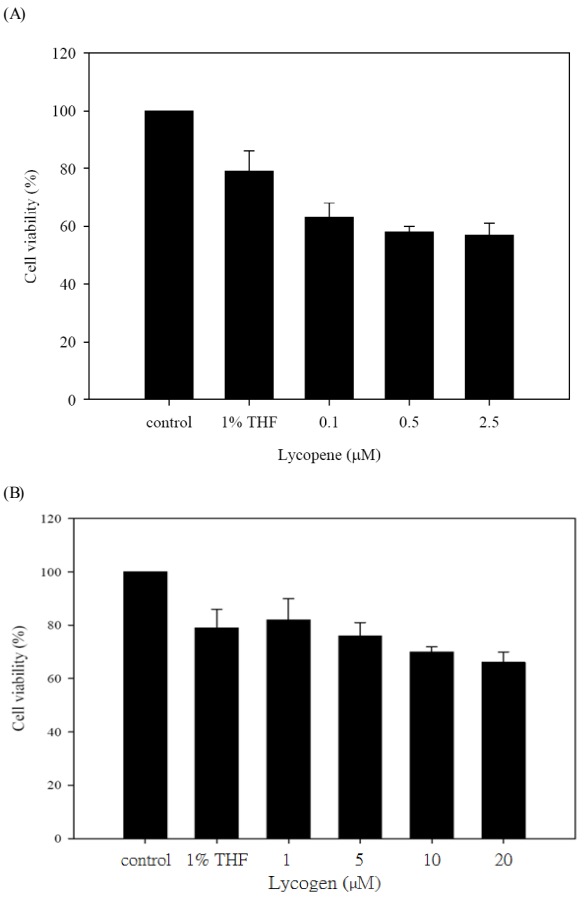

Figure 2: Effects of lycopene and Lycogen on cell cytotoxicity in Hs68 cells (human fibroblast cells). Cells were treated with different concentrations of lycopene(A) or Lycogen (B) for 24 h. THF (tetrahydrofuran; 1%) served as solvent control for lycopene and Lycogen. Cell cytotoxicity was determined by MTT and expressed as a percentage of viable cells in the total number of cells counted. Values are means ± SD (n ≥ 3) for each treatment.

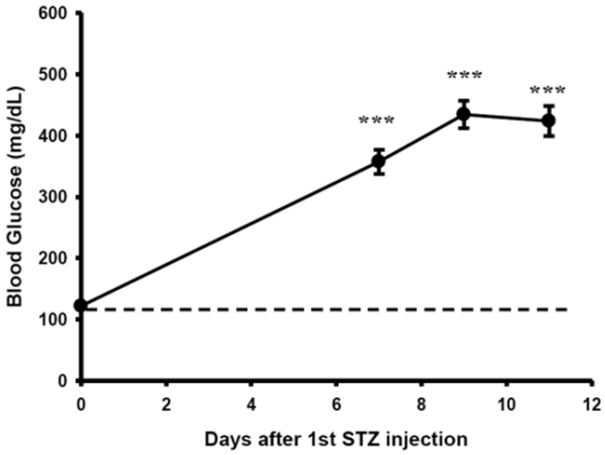

Figure 3: Blood glucose change after STZ injection in mice. The dashed line is the average blood glucose level of the blank control group. *** P <0.005 compared with the blank control group.

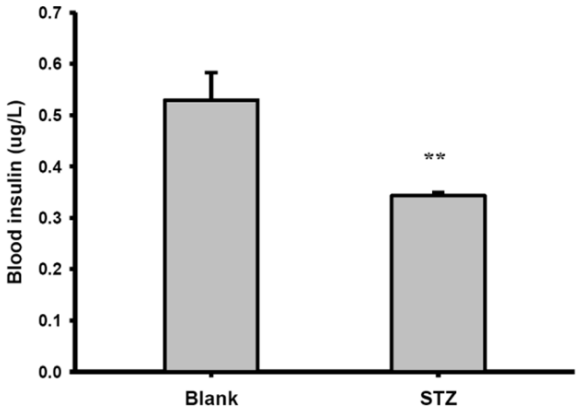

Figure 4: Insulin levels in STZ-induced diabetes mice. ** P <0.01 compared with the blank control group.

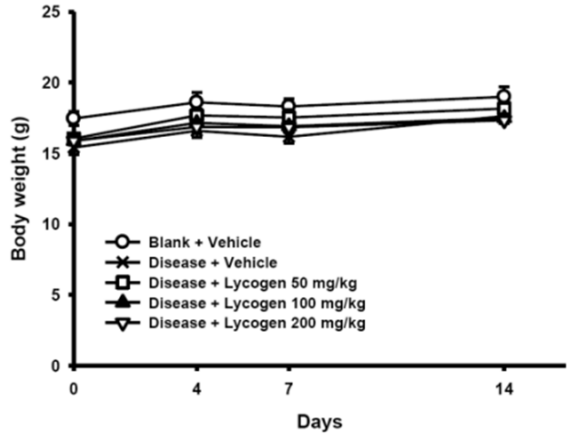

Figure 5: The change of the body weight of the mice in each treatment group during the drug treatment.

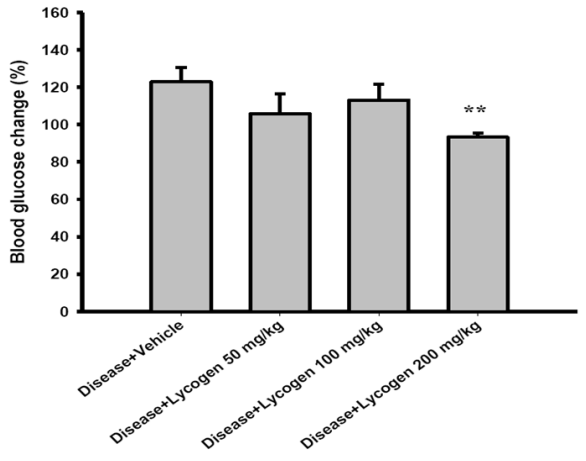

Figure 6: Blood glucose changes in diabetic mice 7 days after treatment. Blood glucose change (%) is calculated as: (7th day glucose level / basal glucose level before treatment) x 100%. ** P <0.01 compared with disease control group.

was significantly lower than the disease control group ($p<0.05$). The insulin level of mice was measured at the same time, and no significant difference was found between any groups (Figure 8).

Homeostatic model assessment (HOMA) is a method used for assessing insulin resistance (IR) using basal (fasting) glucose and insulin concentrations. The HOMA-IR (insulin resistance) index of each group was calculated and shown in Table 2. There was no significant difference in insulin resistance between disease groups (day 0, $p=0.142$ and day 14, $p=0.239$). When Lycogen™ 50 and 200 mg/kg were added, there were significant differences in the insulin resistance index between day 0 and day 14.

Discussion

Oxidative stress plays an important role in insulin resistance. Moreover, anti-oxidants may be a potential treatment of DM. *Rhodobacter sphaeroides* extract, Lycogen™ showed more potent anti-oxidative effects and less cytotoxicity than lycopene. Our data confirmed that Lycogen™ significantly lowered the blood glucose level in STZ-induced diabetic mice (Figures 6 and 7).

In our study, there was no significant difference in blood insulin levels between the disease control group and Lycogen™ treatment groups (Figure 8). Thus, the possibility of Lycogen™ lowering the blood glucose level by increasing insulin release was excluded. In the OGTT, there was no significant difference in the basal glucose level (0 min) between the study groups (Figure 7). Combined with no significant body weight difference between the study groups, this result suggests that it is less likely that Lycogen™ lowered the blood glucose level through decreasing GI tract absorption. There was a significant difference in the insulin resistance index between day 0 and day 14 in the Lycogen™ 50 and 200 mg/kg groups (Table 2). Our data was consistent with previous studies, reduced oxidative stress improved insulin resistance in animal models [2-6].

Lycopene, a carotenoid, has been shown to have more potent antioxidant properties than other carotenoids *in vitro* [22]. In a cross-sectional study, greater dietary lycopene intake was associated with reduced fasting plasma glucose concentrations [23]. Plasma concentrations of lycopene had been shown to have an inverse association with fasting blood glucose [11-15]. However, no significant glucose-lowering effect of lycopene was found in other observational study [16]. The association between lycopene and glycemic control was still elusive in these observational studies.

Lycogen™ showed more potent anti-oxidative activity and less cytotoxicity than lycopene in this study (Figures 1 and 2). Our results showed that the blood glucose level was significantly lower in the high-dose group (Lycogen™ 200 mg/kg). However, no significant difference was found in the glucose level between the lower dose group and control group (Figures 6 and 7). Glucose-lowering effect of Lycogen™ was significant in higher dose but not in lower dose. Lycogen™ improved glycemic control in dose-dependent manner was suspected. This dose-dependent relation can explain the inconsistent findings in previous observational studies. In consideration of the dose-dependent effect, the more potent and less toxic Lycogen™ may be a potential candidate for the development of a new anti-diabetic agent.

Our results confirmed that Lycogen™, a potent anti-oxidant, can significantly improve insulin resistance and lower blood glucose levels in a diabetic mice model. Further investigation is warranted to clarify its possibility of use in diabetic patients.

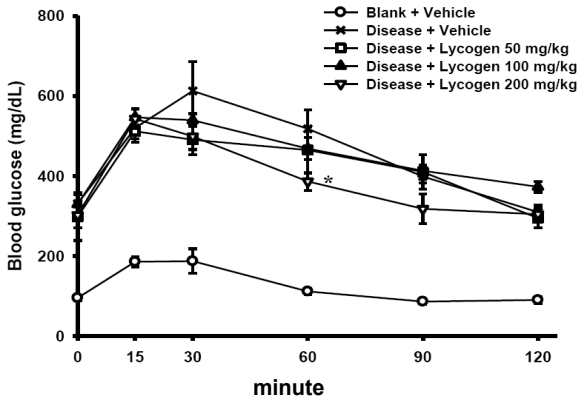

Figure 7: OGTT results of each treatment group. * P <0.05 compared with the disease control group.

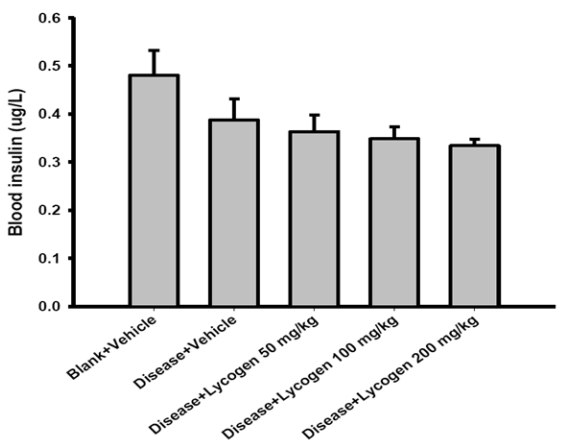

Figure 8: The blood insulin concentration 14 days after treatment.

Group	Lycogen Dose	Frequency	Mice number
Blank+vehicle	-	once daily for 14 days	5
Disease+vehicle	-	once daily for 14 days	5
Disease+Lycogen-1	50 mg/kg	once daily for 14 days	5
Disease+Lycogen-2	100 mg/kg	once daily for 14 days	5
Disease+Lycogen-3	200 mg/kg	once daily for 14 days	5

Table 1: Study design.

Treatment	Insulin resistance index		p value
	Day 0	Day 14	
Blank+Vehicle	3.9±1.8	2.8±0.8	0.115
Disease+Vehicle	7.8±1.0	7.7±1.1	0.814
Disease+Lycogen 50 mg/kg	8.0±2.7	6.1±1.8	0.021
Disease+Lycogen 100 mg/kg	7.9±1.1	7.0±1.2	0.389
Disease+Lycogen 200 mg/kg	10.2±1.8	5.9±1.4	0.003

p=0.142, p=0.239
HOMA-IR (insulin resistance) index= insulin（μU/mL×glucose（mmol/L）/22.5

Table 2: Insulin resistance index in day 0 and day 14 of Lycogen treatment. There was no significant difference between each disease group at day 0 (p=0.142) or day 14 (p=0.239).

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