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**Effects of silkworm powder on glucose absorption
by human intestinal epithelial cell line Caco-2**

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1 **Abstract**

2 Inhibitors of glucose absorption play an important role in the treatment of diabetes. In
3 this research, we report that silkworm powder has inhibitory effects on glucose
4 absorption in human intestinal epithelial cells. Silkworm powder inhibited α
5 -glucosidase activation and glucose transporter (SGLT1) expression. These results
6 suggest that silkworm powder can be used as a natural functional food for the
7 prevention and alleviation of type-2 diabetes.

8

9 Key words: Silkworm powder, Caco-2 cells, Glucose absorption, α -glucosidase,
10 Sodium-dependant glucose transporter, Type-2 diabetes

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13 **Introduction**

14 Diabetes is complex in nature, and changing lifestyles, food habits and stress have
15 further complicated it. In particular, type-2 diabetes is an increasingly common disease,
16 which causes a number of life-threatening complications. The number of diabetics is
17 increasing by 4-5% yearly with an estimated 40-45% of individuals over 65 years
18 having either type-2 diabetes or impaired glucose tolerance [1]. Various approaches are

1 used to control type-2 diabetes via reduction of blood glucose level. Promising
2 approaches are the use of (1) insulin sensitizers (such as glycogen synthase kinase 3 and
3 protein tyrosine phosphatase-1B inhibitor), (2) inhibitors of gluconeogenesis (such as
4 pyruvate dehydrogenase kinase inhibitor), and (3) inhibitors of disaccharide hydrolysis
5 (such as acarbose, voglibose, and deoxynojirimycin).

6 The increase in the capacity of the intestine to absorb glucose through
7 human intestinal epithelial cells is attributable to a combination of specific enzymes
8 (such as α -glucosidase) and glucose transporters. In type-2 diabetes
9 (noninsulin-dependent diabetes mellitus; NIDDM), there is an increase in the capacity
10 of the intestine to absorb glucose. Moreover, sodium-dependent glucose transporters
11 (SGLT1s) were found to be more abundant in human intestinal epithelium isolated from
12 duodenal biopsies of type-2 diabetes patients than from healthy controls. This was
13 determined from the threefold increases in SGLT1 mRNA measured [2].

14 In Asian countries including Korea, silkworm powder has long been
15 favored as an antidiabetic agent, but its efficacy has not been tested using modern
16 scientific methods [3]. In this study, we investigated the effects of silkworm powder on
17 inhibition of α -glucosidase activity and on the expression of SGLT1 in human intestinal
18 epithelial cells.

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3 **Materials and methods**

4 *Cell culture*

5 Caco-2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM)
6 supplemented with 10% fetal calf serum (Sigma), 1% penicillin-streptomycin, and 1%
7 nonessential amino acids (Cosmo Bio). They were incubated in an atmosphere of 5%
8 CO₂ at 37 °C. The cells were passaged at a split ratio of 4 to 8 every 3 or 4 days. For the
9 extraction of total RNA, cells were seeded onto Petri plates at a density of 1×10⁶ cells
10 per plate.

11

12 *Preparation of silkworm powder solution*

13 Silkworm powder (10g) were extracted at 105 °C with distilled water (100 ml) for 15
14 min. Extracted solution (10% (w/v)) were filterated with the filter unit (Millipore, pore
15 size; 0.22 μm). Silkworm powder was obtained from CB Japan Co.

16

17 *TER measurement*

18 The condition of the Caco-2 cell monolayer was evaluated by measuring the

1 transepithelial electrical resistance (TER). TER is considered to be correlated with the
2 change in paracellular permeability of the cell monolayer [4]. The cell monolayers on
3 Millicell-HA were rinsed with DMEM and then set in a 12-well plate containing
4 DMEM. DMEM was added to the apical side, and the TER was measured with a
5 Millicell-ERS instrument (Millipore). TER, which can be used to evaluate the
6 confluency of cells, become constant ($600-700 \Omega \times \text{cm}^2$) 3 weeks after seeding. After
7 confirming that the Caco-2 cell monolayer has formed completely, we determined the
8 α -glucosidase activity.

9

10 *Measurement of α -glucosidase activation*

11 To determine the α -glucosidase activity, the cells were plated at 2×10^5 cells/cm² onto a
12 12-mm polycarbonate transwell filter with 0.4 μm pores and a collagen-coated surface.
13 The medium was replaced every three days until use. The cells were grown for 21 days
14 before conducting the experiments. The culture medium was removed and both the
15 apical and basal chambers were washed 3 times with 1 ml of phosphate-buffered saline
16 (PBS). The culture medium in the apical chamber was replaced with 250 μl of PBS, 100
17 μl of 1% (w/v) soluble starch, 50 μl of porcine pancreatic α -amylase, and 100 μl of
18 silkworm powder solution (0.1, 1% (w/v)). In the basal chamber, 500 μl of PBS was

1 added instead of culture medium. The assay plate was incubated at 37°C in a humidified
2 atmosphere of 5% CO₂ for 2 h. After incubation, the monosaccharide (glucose)
3 converted from disaccharide in the apical chambers was determined by the glucose
4 oxidase (GOD) method [5, 6]. This α -amylase-added Caco-2 system was established as
5 a useful model to evaluate the effects of α -glucosidase inhibitors on starch digestion [5,
6 7].

7

8 *RNA extraction and reverse transcription (RT)*

9 The silkworm powder solution treated plates were incubated at 37°C in a humidified
10 atmosphere of 5% CO₂ for 2 h. After incubation, the total RNA of Caco-2 cells was
11 isolated using ISOGEN (NIPPON GENE Co LTD, Tokyo). The total RNA was then
12 quantified by measuring its absorbance at 260 nm using a UV spectrophotometer. Only
13 RNA samples having a 260/280 ratio higher than 1.8 was used for the RT reaction.
14 Template cDNA was obtained from total RNA by the SuperScript II Reverse
15 Transcriptase system (Invitrogen). Briefly, RNA was denatured at 65°C for 5 min,
16 incubated with 1 μ l oligo(dT)₁₂₋₁₅ primers, and chilled at 4°C. After adding 200 units of
17 SuperScript II Reverse Transcriptase, the reaction mix was incubated at 42°C for 60
18 min, then at 70°C for 10 min.

1

2 *Real-time Qualitative PCR*

3 For the quantification of mRNA, nested primers were designed using the 'Primer3
4 input' software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi/primer3_www.cgi).

5 Quantitative PCR reactions were performed using a MiniOpticon instrument (BioRad)

6 following the protocol of the iQ SYBR-Green Supermix system (BioRad). Briefly, the

7 RT mix (2 μ l) was used as template for the real-time PCR mix containing 0.5 μ M

8 forward (5'-CTTCACCATGGACATCTACGCCAA-3') and reverse

9 (5'-CTGGTGATGGACTGGATGTAATCG-3') nested primers (2 μ l each) and 2 \times

10 SYBR-Green Supermix (10 μ l). The amplification conditions were as follows: 10 s at

11 95°C, 30 s at 62°C and 30 s at 72°C for 34 cycles. At the end of the reaction, a melting

12 curve analysis was carried out to check for the presence of primer-dimers [8].

13

14 *Statistical analysis*

15 All experiments were conducted at least in quadruplicate (n=4) and results are

16 expressed as mean \pm S.D. Data were analyzed by one-way ANOVA followed by

17 Dunnett test (Stat-100, BIOSOFT, U.K) to determine significant differences among the

18 means.

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2

3 **Results and Discussion**

4 The inhibitory effect of silkworm powder on α -glucosidase activity in Caco-2 cells is
5 shown in Fig. 1. Silkworm powder solution at 1% (w/v) concentration has a higher
6 inhibitory effect on glucose conversion than acarbose (10 μ M), a commercial
7 α -glucosidase inhibitor. However, silkworm powder not showed porcine pancreatic
8 α -amylase (data not shown). It has been reported that deoxynojirimycin (DNJ), a potent
9 α -glucosidase inhibitor, is 2.7-fold more concentrated in silkworms than in mulberry
10 leaves which are used to feed them [9]. Mulberry trees (*Morus alba L.*) are cultivated in
11 China, Korea, and Japan, and their leaves are used to feed silkworms (*Bombyx mori L.*)
12 [10]. The silkworm has special enzymes that can concentrate DNJ from mulberry leaves
13 [11]. The structure of DNJ from silkworm powder was determined by variety of 1D and
14 2D NMR spectral data and HRFABMS, and the yield was 0.3% [3].

15 From this result, it can be hypothesized that DNJ components, which are
16 concentrated by silkworms, inhibit glucose production from starch. Consequently, the
17 converted glucose from disaccharide was decreased by silkworm powder in human
18 intestinal epithelial cells. Next, we investigated the effects of silkworm powder on

1 SGLT1 mRNA expression by human intestinal epithelial cells. Results showed that
2 silkworm powder solution (1%, w/v) inhibited the expression of SGLT1 mRNA (Fig. 2).
3 Most of the SGLT1 inhibitors such as phlorizin inhibit the binding of glucose to SGLT1
4 [12]. However, silkworm powder inhibited the expression of the SGLT1 mRNA.
5 Moreover, phlorizin reduces the barrier function of intestinal epithelial cells, such as by
6 causing the diffusion of tight junctional protein (ZO-1) and decreasing the TER [13, 14].

7 From this result, it can be concluded that silkworm powder can be used as
8 an inhibitor of glucose absorption in human intestinal epithelial cells. The inhibition
9 mechanism by silkworm powder is composed of 2 stages. The first stage is the
10 inhibition of glucose production from disaccharide through the inhibition of
11 α -glucosidase activity, and the second stage is the inhibition of glucose transporter
12 mRNA expression.

13 In conclusion, silkworm powder inhibited glucose absorption by human
14 intestinal epithelial cells through the inhibition of α -glucosidase activity and SGLT1
15 expression.

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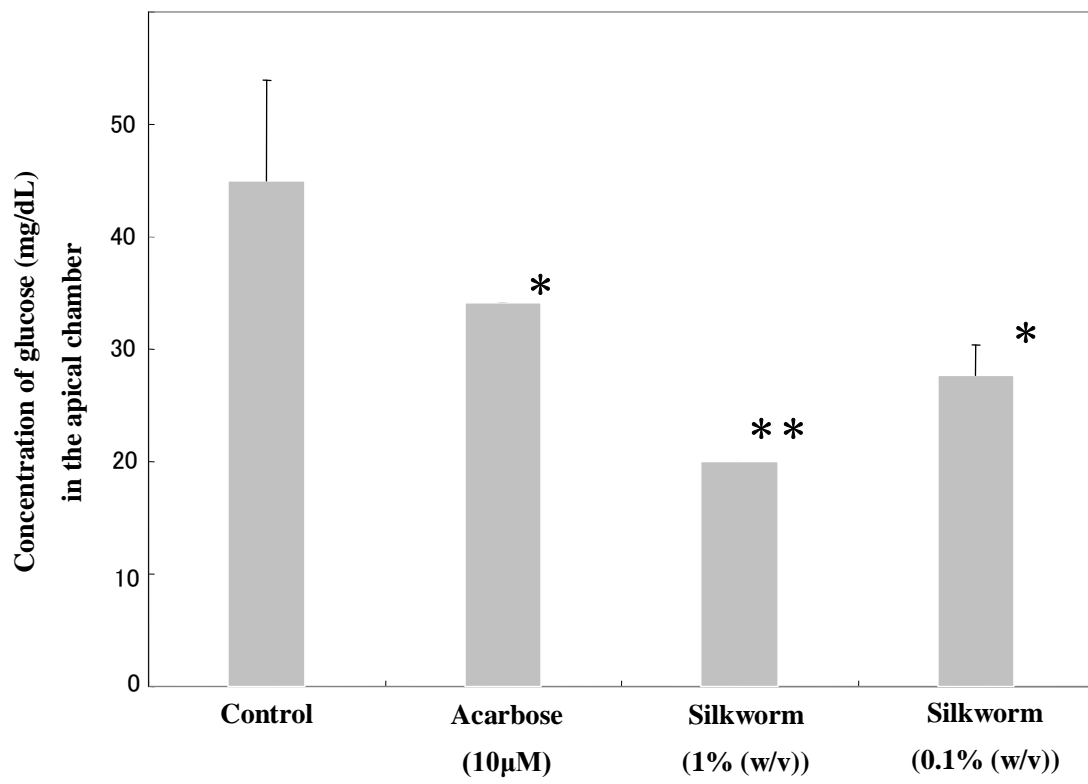
1 Figure 1

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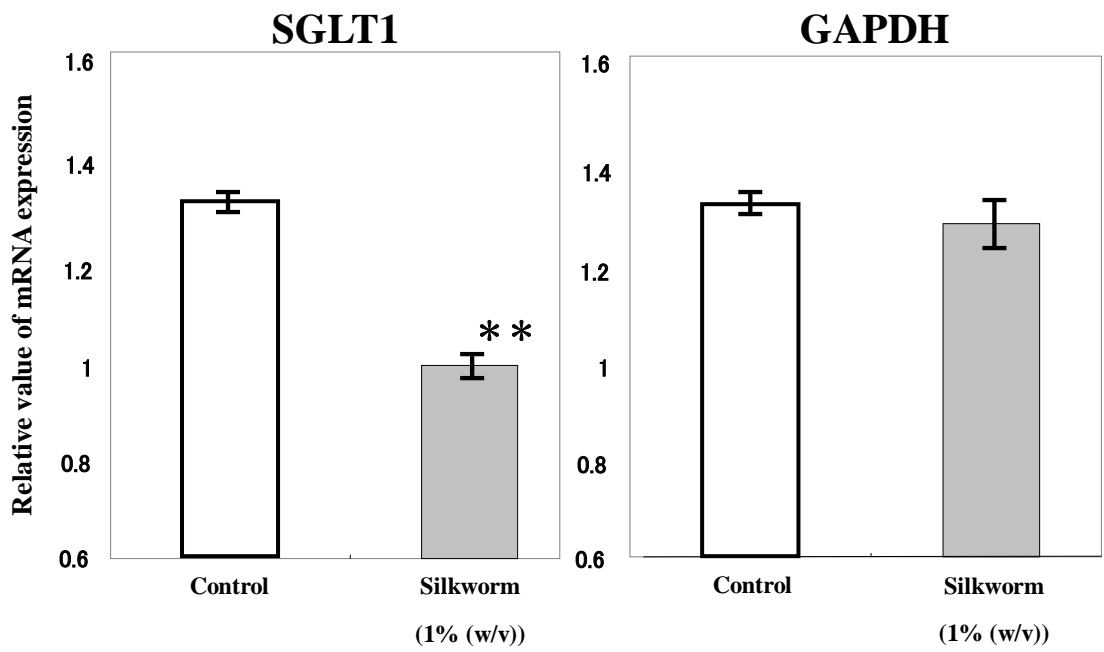
1 Figure 2

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6 Figure 1. Effect of silkworm powder solution on disaccharide hydrolysis by Caco-2

7 cells. Each bar represents the mean with S. D. (n=9). * $p < 0.05$, ** $p < 0.01$

8 vs control.

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11 Figure 2. Effect of silkworm powder solution on the expressions of SGLT1 and GAPDH

12 mRNAs by Caco-2 cells. GAPDH was used as a housekeeping gene

13 Each bar represents the mean with S. D. (n=3). ** $p < 0.01$ vs control.

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