Nutritional Sciences 7(4), November, 2004, pp.201-207

Inhibitory Effects of Mulberry Fruit on Intestinal Disaccharidase Activity and Hyperglycemia in Streptozotocin-Induced Diabetic Rats*

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The current study examined the effects of freeze-dried mulberry fruit on disaccharidase activity in the small intestine and the lowering of blood glucose in streptozotocin (STZ)-induced diabetic rats. Sprague-Dawley male rats were randomly assigned to one normal and three streptozotocin (STZ)-induced diabetic groups. The diabetic groups were fed a mulberry fruit-free diet (DM-group), 0.3% mulberry fruit diet (DM-F group) or 0.6% mulberry fruit diet (DM-2F group). After they were fed the experimental diets for three weeks, diabetes was induced with an intraperitoneal injection of streptozotocin 50 mg/kg b.w before sacrificing 9 days later using the same experimental treatments. Analyses of anthocyanins, flavonoid and 1-deoxynojirimycin (DNJ) of lyophilized mulberry fruit were carried out and the major anthocyanins were rutin (142.5 mg), isoquercitrin (10.3 mg), quercetin (5.8 mg), morin (1.6 mg) dihydroquercetin (3.83 mg), cy-3-*O*-glucopyranoside (230.45 mg) and cy-3-*O*-rutinoside (131.5 mg) on the basis of 100 g dry weight. Total DNJ content was 2.39 mg/g dry weight of lyophilized mulberry fruit. Blood glucose level decreased in the diabetic rats fed the mulberry fruit supplement. The content of the liver glycogen increased in the diabetic rats fed the mulberry fruit supplement. Disaccharidase activity in the proximal part of the intestine, such as that of maltase, sucrase and lactase in the mulberry fruit supplementation groups, were lower than that of the DM group. These results suggest that mulberry fruit possess a suppressive effect on hyperglycemia, possibly by inhibiting the activity of disaccharidase in the small intestine of rats.

Key words: Diabetes, Mulberry fruit, Blood glucose, Disaccharidase activity

INTRODUCTION

The number of people suffering from diabetes has rapidly increased, especially those with chronic diseases such as hyperlipidemia, atherosclerosis and hypertension, due to unsound dietary habits and insufficient exercise. Diabetes mellitus causes impaired metabolism of the carbohydrates, proteins, lipids and electrolytes with increased blood glucose. Moreover, if hyperglycemia is not controlled, several complications may arise including cardiovascular diseases such as hyperlipemia, hypertension and arteriosclerosis. There is also induced insulin-dependent diabetes.[1-3]

But blood glucose can be controlled to prevent diabetes and other complications. Recently, many investigators have conducted anti-diabetic studies and have worked on anti-diabetic functional food material development using natural resources from silkworms,[1] mulberry leaves[4-6] and green tea.[7-9] Attention has been drawn to the study of mulberry fruit.[10,11]

Fruit of the mulberry, which belongs to the *Morus alba* L. is used in Oriental medicine. It is picked when its color is black or claret, which can occur between May and June.[12] In particular, the crude drug "Sangsimja", the fruit of *M. alba* (Moraceae), has been used in traditional Chinese medicine to cure and prevent diabetes, anemia, hypertension and arthritis.[12,13] Recently, mulberry fruit has been reported to initiate several biological actions and to be anti-diabetic,[14] anti-oxidative,[15-18] anti-inflammatory[17] and anti-hyperlipidemic.[19] An *in vitro* study of antioxidants in mulberry fruit found that it contained various ingredients such as flavonoids, silbenes, prenylflavonoids, coumarin and deoxynoijirimycine (DNJ). Nonetheless, very few studies have focused on blood glucose control in relation to mulberry fruit. Yoo *et al.*[4] reported that mulberry leaves could lower blood glucose by controlling disaccharidase activity in the small intestine. Therefore, the current study observed the effects of mulberry fruit on disaccharidase activity in the small intestine and on lowering blood glucose in streptozotocin (STZ) induced diabetic rats.

* This study was supported by the special Grants Research Program (No. 102004-3) of the Problem-Oriented Technology Development Project for Agriculture and Forestry, Ministry of Agriculture and Forestry, Republic of Korea.
Accepted : November 6, 2004
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MATERIALS AND METHODS

1. Preparation of Plant Materials

The Cheongil mulberry fruit that was used in this experiment was grown in the fields of the Youngcheon Silkworm Culture Agricultural Co-operative Association, Youngcheon, Korea and was harvested in early June 2003. All of the mulberry fruit that was used underwent abrasion to 100 mesh size after being freeze-dried for the experiment.

2. Analysis of Anthocyanin Contents

Anthocyanin content of the mulberry fruit was determinated using the method of Lee *et al.* as previously reported (20). Freeze-dried mulberry fruit (1.0 g) was extracted twice with 50 mL of 0.5% trifluoroacetic acid (TFA) in 80% aqueous EtOH under ultrasonic cleaner, filtered and evaporated under reduced pressure. The EtOH extract was redissolved in 10 mL of 80% aq. EtOH and left to stand overnight in a refrigerator. The souble extract (2 mL) was passed through 0.45 μm membrane filter (Gelman, USA) and injected in HPLC for quantification of mulberry fruit anthocyanins. HPLC analysis was performed using a Gilson 506B HPLC system equipped with 170 UV-vis detector, Gilson Unipoint™ 3.0 software and 231XL autosampler with a 10 μl loop, using a YMC-Pack Pro C_{18} column (5 μm, 4.6 I.D.×250 mm, YMC Inc., USA) with a mobile phase of 1.0% H_3PO_4 in CH_3CN-H_2O-HOAc (7.5:85.5:7, v/v) at a flow rate of 1.0 mL/min with a UV detector at 520 nm. Individual anthocyanins were identified by a comparison of their retention time with those of the two standard anthocyanins [cyanidin-3-*O*-β-D-glucoside (anthocyanin 1), cyanidin-3-*O*-β-D-rutinoside (anthocyanin 2)] isolated from a previous report (21). Duplicate analyses were conducted on duplicate samples. Linear correlation coefficients were superior to 0.999 for each anthocyanin. The calibration lines ($y=10433\times-30.296$ for anthocyanin 1, $y=5346.7\times-0.4025$ for anthocyanin 2) used were determined using a least squares regression method. The concentration of anthocyanins were determined using the calibration curves of two standard anthocyanins, and expressed as mg% of dried weight. Recovery rates of anthocyanin 1 and 2 were 82 and 80%, respectively.

3. Analysis of Flavonoid Contents

Flavonoids of the mulberry fruit were determinated using the method of Lee *et al.* as previously reported.[20] Freeze dried mulberry fruit (1.0 g) was extracted twice with 50 mL of 80% aqueous MeOH under ultrasonic cleaner, followed by filtration and evaporation. The MeOH extract was redissolved in 10 mL of 80% aq. MeOH and left to stand overnight. The extract (5 mL) was passed through polyamide (polyamide C-200, 75~150 μm, Wako Pure Ind. Ltd. Osaka, Japan) cartridges that were preconditioned with 80% aq. MeOH (4 mL) and water (10 mL). The first 3 mL was discarded and the next 1 mL was used for HPLC analysis for quantification of flavonoids of mulberry fruit. HPLC analysis was performed on the same HPLC equipment and conditions for anthocyanin analysis, with the exception of a mobile phase [gradient elution from solvent A (4.5% formic acid in H_2O) to solvent B (90% aq. CH_3CN) for 60 min] and UV detection at 280 (dihydroquercetin) and 360 nm (four flavonoids except for dihydroquercetin). Quantification of flavonoids was performed similarly in the case of anthocyanins. Recovery rates of all flavonoids were above 90%.

4. Analysis of DNJ Content

Lyophilized mulberry fruit (0.1 g) was extracted with 10 mL of distilled water at 60 ℃ for 1hr, followed by centrifugation and these steps were repeated three times. The supernatants were pooled, diluted to 100 mL with distilled water and reacted with 9-fluorenylmethyl chloroformate (Fluka, Switzerland) by means of Cole *et al's.* modified method.[21] The reaction mixture was syringe-filtered (nylon, 0.45 μm) and injected into HPLC. Mobile phase was acetonitrile-0.1% of aqueous acetic acid (1:1, v/v) and flowed isocratically for 13 min through Phenomenex Luna C18 (2) column (5 μm ODS; 4.60×250 mm I.D.). DNJ was detected as an FMOC derivative by fluorescence detector (excitation 264 nm and emission 314 nm).

5. Experimental Animals and Diets

Male Sprague-Dawley rats weighing between 90 and 110 g were purchased from Bio genomics (Seoul, Korea). The rats were individually housed in stainless steel cages in a room with controlled temperature (20-23 ℃) and lighting (alternating 12 h period of light and dark). They were fed a pellet commercial non-purified diet. (Samyang, Seoul, Korea) for 7 days after arrival. They were randomly divided into one normal group and three diabetic groups of 10 experimental rats each. The four groups were fed experimental diets for 3 weeks and the diabetic groups were given a mulberry fruit-free diet (DM group), 0.3% mulberry fruit diet (DM-F group) or 0.6% mulberry fruit diet (DM-2F group), according to the level of mulberry fruit supplemented (Table 1). The experimental design was approved by the committee for the care and use of laboratory animals at the Catholic University of Daegu.

6. Experimental Diabetes

Diabetes was induced by an intravenous injection of STZ (50 mg/kg body weight) in a citrate buffer (pH 4.3) via the tail vein. Rats with a blood glucose concentration of 16.7 mmoL/L after 9 days were used for the experiment.

Table 1. Composition of experimental diet (g/kg diet)

Ingredients \ Groups	N	DM	DM-F	DM-2F
Corn starch[1]	698	698	695	692
Casein[2]	150	150	150	150
DL-methionine[3]	2	2	2	2
Mineral mix[4]	40	40	40	40
Vitamin mix[5]	10	10	10	10
Corn oil[6]	50	50	50	50
Cellulose[7]	50	50	50	50
Mulberry fruit[8]	-	-	3	6
Total(g)	1000	1000	1000	1000

1) Sam Yang Co., Seoul, Korea
2) Lactic Casein, 30 mesh, New Zealand Dairy Board, Welligton, N. Z.
3) Sigma Chem. Co., St. Louis, Missouri, U.S.A
4) Mineral mix, AIN-76 (g/kg mixture) : Calcium Phosphate, dibasic ($CaHPO_4 \cdot 2H_2O$) 500, Sodium chloride (NaCl) 74, Potassium citrate, monohydrate ($K_3C_6H_5O_7 \cdot H_2O$) 220, Potassium sulfate (K_2SO_4) 52, Magnesium oxide (MgO) 24, Manganous carbonate (45-48% Mn) 3.5, Ferric citrate (16-17% Fe) 6, Zinc carbonate (70% ZnO) 1.6, Cupric carbonate (53-55% Cu) 0.3, Potasium iodate (KIO_3) 0.01, Sodium selenite ($Na_2SeO_3 \cdot 5H_2O$) 0.01, Chromium potassium sulfate [$CrK(SO_4)_2 \cdot 12H_20$] 0.55, filled up to 1,000 with sucrose, Harlan TEKLAD Co.
5) Vitamin mix, AIN-76A (g/kg mixture) : p-Aminobenzoic Acid 11.0132, Ascorbic Acid, coated (97.5%) 101.6604, Biotin 0.0441, Vitamin B_{12} (0.1% trituration in mannitol) 2.9736, Calcium Pantothenate 6.6079, Choline Dihydrogen Citrate 349.6916, Folic Acid 0.1982, Inositol 11.0132, Menadione 4.9559, Niacin 9.9119, Pyridoxine HCl 2.2026, Riboflavin 2.2026, Thiamin HCl 2.2026, Dry Vitamin A Palmitate (500,000 U/g) 3.9648, Dry Vitamin D3 (500,000 U/g) 0.4405, Dry Vitamin E Acetate (500 U/g) 24.2291, Corn Starch, Harlan TEKLAD Co.
6) Dong Bang oil Co., Seoul, Korea
7) Sigma Chem. Co. CMC (Sodium carboxyl methyl cellulose, non-nutritivefiber), St. Louis, Missouri, U.S.A
8) Mulberry fruit powder

7. Sample Collection and Preparation

After blood was drawn from the artery of the abdominal region, serum was obtained by centrifuging at 3000×g for 10 minutes. The serum was stored at -80 ℃ after being fast frozen. The livers and small intestine were excised, and washed in 9 g/L of NaCl . The liver was frozen rapidly in lipid nitrogen and stored at -80 ℃ until use.

8. Measurement of Blood Glucose

Blood glucose contents of the rats were measured after a 12-hr fast using the enzymatic kit AM 201 K (Asan Co., Korea).

9. Measurement of Glycogen Content in the Liver

The glycogen contents of liver were determined according to the method of Lo *et al.*[22] About 70~80 mg of the liver abrasion was filled with liquid nitrogen in a poison cup and added to the 1 mL of KOH (30%). After mixing it was incubated at 100 ℃ for 30 min. It was then left for more than 12 hours at 0~5 ℃ after mixed with 1.5 mL ethanol (95%). After being centrifuged at 3000 rpm for 25~30 min, the resultant was put into 350 μl of distilled water, 0.5 mL of 5% phenol, 2.5 mL of Na_2SO4 after being mixed with 3 mL of distilled water and measured at 490~492 nm.

10. Measurement of Maltase, Sucrase and Lactase Activity in Intestinal Mucosa

Maltase, sucrase and lactase activity were measured according to Dahlqvist's method.[23] The small intestine was excised and washed in 9 g/L of NaCl on ice in order to remove the duodenum. After being washed with 9 g/L of NaCl, the organs were kept in cold storage after being cut open. They were divided into proximal, middle and distal section as three equal parts. Water was removed using cheesecloth. The mucosa was scraped off with a microscopic slide glass and weighed. The mucosa was then homogenized with four times the quantity of cold distilled water. Diluted enzymes in the amount of 0.1 mL were mixed with 0.056 M disaccharide solution/0.1 M sodium malate buffer (pH 6.0) 0.1 mL and incubated at 37 ℃ for 60 min. They were cooled in tap water after being added to 0.8 mL of distilled water and incubated in boiling water for 2 min, 0.5 mL of the test sample was added to 3 mL of glucose oxidase. It was vortexed and incubated at 37 ℃ for 60 min and absorbance was read at 420 nm.

11. Statistical Analysis

All data were assessed by analysis of variance (ANOVA). If significance was found by ANOVA, comparisons among group means were made using Tukey's Honestly Significant Difference test.[24]

RESULTS

1. Quantification of Anthocyanins, Flavonoids and DNJ

Table 2 shows the quantification of flavonoids, anthocyanins and DNJ. The contents of rutin, isoquercitrin, quercetin, morin and dihydroquercetin were 142.5, 10.3, 5.8, 1.6 and 3.83 mg per 100 g dry weight, respectively, of the cheongil mulberry fruit. The contents of cy-3-*O*-glucopyranoside and cy-3-*O*-rutinoside were 230.45 and 131.5 mg per 100 g dry weight, respectively. The content of DNJ was 2.39 mg/g dry weight.

Table 2. Quantification of flavonoids, anthocyanins and 1-deoxynojirimycin (DNJ) from Cheongil mulberry fruit

Mulberry fruit	Flavonoids (mg/100g, dry weight)					Anthocyanins (mg/100g, dry weight)		DNJ (mg/g dry weight)
	Rutin	Isoquercitrin	Quercetin	Morin	Dihydroquercetin	Cy-3-O-glu[1]	Cy-3-O-rut[2]	
Cheongil	142.5	10.3	5.8	1.6	3.83	230.45	131.5	2.39

1) *Cyandidin 3-O-β-glucopyranoside.*
2) *Cyandidin 3-O-β-rutinoside.*

2. Small Intestine Weight and Length Changes

Body weight, intestine weight, intestine length and intestine index are shown in Table 3. Body weights significantly decreased in the diabetic groups as compared to the normal group, but the difference among the diabetic groups was not significant Intestine weight and length were not significantly different among the diabetic groups. The small intestine index in the diabetic groups was significantly higher than that of the normal group but the effect of the mulberry fruits was not observed.

Table 3. Effects of mulberry fruit on body weight, intestine weight, length and intestine index in STZ-induced diabetic rats

Group	Body weight(g)	Intestine weight (g)	Intestine length (cm)	Intestine index* (g/100g body wt)
Normal	304.00±9.90[a]	6.51±0.26[NS]	98.50±6.36[NS]	2.14±0.08[b]
DM	229.00±31.11[b]	7.82±1.27	119.00±14.42	3.42±0.55[a]
DM-F	265.50±4.94[b]	7.74±1.16	112.33±4.73	2.93±0.16[a]
DM-2F	258.33±17.00[b]	6.52±1.08	104.67±15.53	2.79±0.32[a]

*Intestine index : intestine weight (g)/100 g body weight
All values are the mean±SE (n=10). Values with different superscript letters are significantly different at p<0.05 by Tukey's-HSD test. The experimental conditions are the same as Table 1.

3. Blood Glucose Levels

The levels of blood glucose (Fig. 1) in the DM group increased more than 187% to that of the normal group, but the figures for the DM-F and DM-2F groups decreased 36% and 38%, respectively, compared to the DM group.

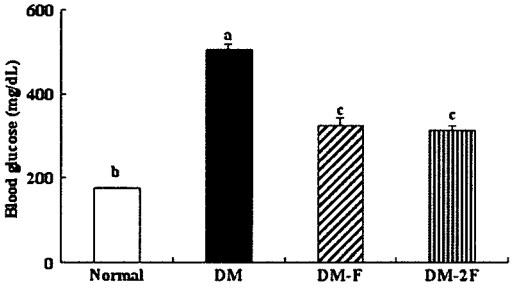

Fig. 1 Effects of mulberry fruit on blood glucose levels in STZ-induced diabetic rats.
All values are the mean±SE (n=10).
Values with different superscript letters are significantly different at p<0.05 by Tukey's-HSD test.
The experimental conditions are the same as Table 1.

4. Glycogen Content in the Liver

The content of glycogen (Fig. 2) in the DM group decreased 27% compared to that of the normal group, but those of the DM-F and DM-2F groups increased 21% and 18%, respectively, compared to that of the DM group.

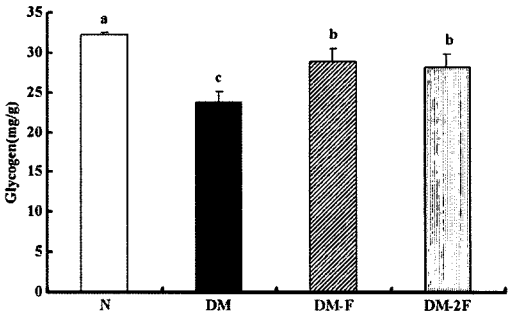

Fig. 2 Effects of mulberry fruit on glycogen contents of liver in STZ-induced diabetic rats.
All values are the mean ± SE (n=10).
Values with different superscript letters are significantly different at p<0.05 by Tukey's-HSD test.
The experimental conditions are the same as Table 1.

5. Intestinal Mucosa Maltase, Sucrase and Lactase Activity

Intestinal mucosa disaccharidase activity is shown in Fig. 3. The maltase activity of the proximal and middle parts of the DM group significantly increased compared to that of the normal group, but the figures for the DM-F and DM-2F groups decreased compared to that of the DM group. Activity in the distal part of the DM group significantly increased compared to that of the normal group but the figures for the DM-F and DM-2F groups decreased 29% and 32%, respectively, compared to that of the DM group (Fig. 3, A). Sucrase activity in the proximal part of the intestine in the DM group significantly increased compared to that of the normal group, but the figures for the mulberry fruit supplementation groups significantly decreased compared to that of the DM group. Sucrase activity in the middle and distal parts in the diabetic groups significantly increased compared to that of the normal group (Fig. 3, B). Lactase activity in the proximal part in the DM group significantly increased compared to that of the normal group, but the figures for the DM-F and DM-2F groups decreased compared to that of the DM group. There was no significant difference between the normal group and diabetic groups in terms of lactase activity of the middle and distal (Fig. 3, C). Therefore, we found that mulberry fruit supplementation lowered maltase, sucrase and lactase activity in the proximal part of the small intestine and had a hypoglycemic effect in diabetic rats.

DISCUSSION

The purpose of this study was to investigate the effects of mulberry fruit on disaccharidase activity in the small intestine and the lowering of blood glucose in diabetes-induced experimental rats.

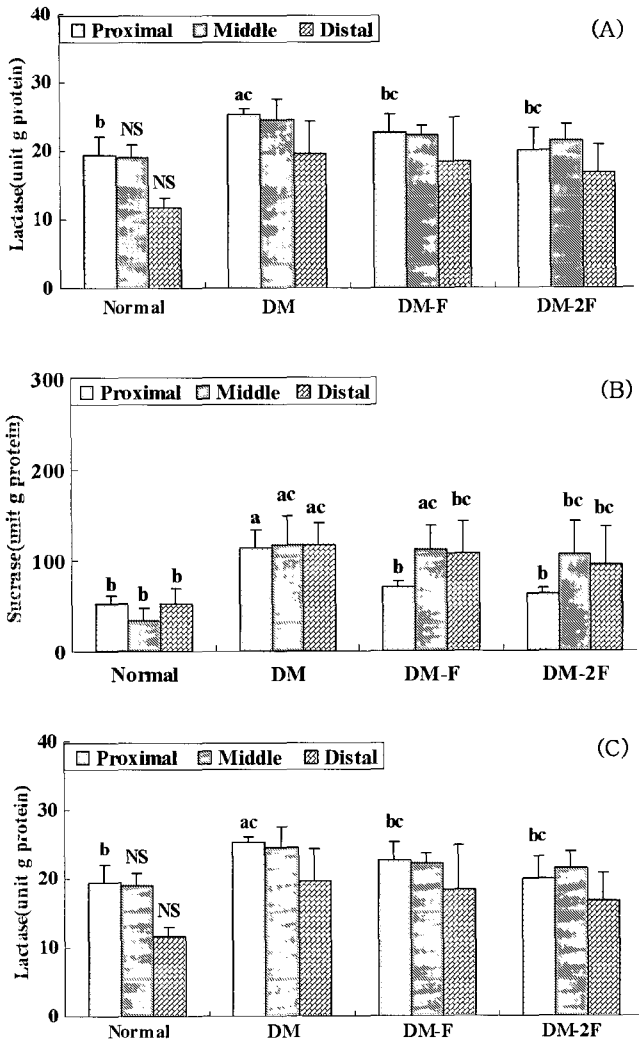

Fig. 3 Effects of mulberry fruit on intestinal mucosa maltase (A), sucrase (B) and lactase (C) activities in STZ-induced diabetic rats.

All values are the mean±SE (n=10).
Values with different superscript letters are significantly different at $p<0.05$ by Tukey's-HSD test.
The experimental conditions are the same as Table 1.

Rutin, isoquercitrin quercetin, morin, dihydroquercetin, cy-3-*O*-glucopyranoside and cy-3-*O*-rutinoside contents were 142.5, 10.3, 5.8, 1.6, 3.83, 230.45 and 131.5 mg per 100 g dry weight, respectively. DNJ (1-deoxynojirimycin), which is an alkaloid belonging to the polyhydroxylated piperidine family, exists abundantly in mulberry leaves and its roots.[25] This substance has been known to be one of the major blood glucose lowering substances among a diversity of mulberry extracts and a powerful competitive inhibitor for α-glucosidase.[4] The content of DNJ was 2.39 mg/g of dry base. So we could found that DNJ content in mulberry fruit had 1.5 times more than that of the mulberry leaves.[4]

Body weights significantly decreased in the diabetic groups compared to those of the normal group and these results were similar to those of Junod A *et al.*[26] But there was no difference among the diabetic groups.

The body weight gains in the diabetic groups decreased significantly compared to that in the normal group because the starvation state by the inhibition of cellular glucose is characteristic of diabetes. There was no difference intestine length among the diabetic groups. The small intestine index in the diabetic groups was significantly higher than that of the normal group, but the effect of the mulberry fruit was not observed. The levels of blood glucose in the DM group increased more than those of the normal group, but those of the mulberry fruit supplementation groups significantly decreased compared to those of the DM group. This result was similar to the findings of Kim *et al.*[14] that a hypoglycemic effect was brought about by mulberry fruit in STZ-induced diabetic mice. The effect causes a lack of insulin because peroxinitrite breakdown in the pancreatic β-cell by nitric oxide, which is product *in vivo* induced by STZ, reacts with the superoxide anion and creates peroxinitrite that induces hyperglycemia and drops the β-cell degree of sensitivity for glucose. The depression of insulin brought about by the peroxinitrite reduces the glucose utilization ratio in cells and promotes glyconeogenesis and causes hyperglycemia.

Glycogen synthase of the liver promotes glycogenesis because it is activated by irritated protein phosphatase insulin. Protein phosphatase activity of the liver declines in STZ-induced diabetic rats as glycogenesis declines. The glycogen contents in the diabetic groups were lower than those in the normal group. This results correspond to those in a study that found glycogenolysis was enlarged because glycogen phophorylase was activated owing to a lack of insulin in the breakdown of the β-cell. The glycogen contents in the mulberry fruit supplementation groups were significantly higher than those of the DM group.

The lowering of blood glucose and the secretion of insulin are thought to delay the digestion and absorption of glucose in intestine owing to disaccharidase activity combining competitively, which means polysaccharide is dissolved into the monosaccharide in the wool of the small intestine.[27] From the current results of observing the effect of mulberry fruit on the activity of the intestinal mucosa disaccharidase of STZ-induced diabetic rats, the disaccharidase activity in the diabetic groups increased compared to that of the normal group.

These results are similar to those of Lee *et al.*[28] Also, we determine that mulberry fruit supplementation reduced maltase, sucrase and lactase activity in the proximal part of the small intestine and had a hypoglycemic effect in diabetic rats.

This result was similar to that previously reported by

Lee *et al.*[29] in that the activity was controlled in the proximal part and prevented hypoglycemia. When carbohydrate are ingested, hyperglycemia is assimilated with glucose by the digestive process of the dissacharidase of the mucosa.

In conclusion mulberry fruit was found to decrease the blood glucose thanks to decreased disaccharidase activity in diabetes-induced experimental rats. Accordingly, the glycogen content in the DM group was lower than that in the normal group, but the glycogen content in the mulberry fruit supplementated diabetic groups were significantly higher than that of the DM group.

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