

## Anti-Diabetic Mechanism of Maitake (*Grifola frondosa*)

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**ABSTRACT:** Glucose tolerance tests were conducted on KK-A<sup>y</sup> mice, model animals of NIDDM (non-insulin dependent diabetes mellitus). The elevated blood glucose levels after 15 minutes and 30 minutes of Maitake-fed group were 0.64 times and 0.76 times those of the control group respectively, indicating inhibition of a significant blood glucose increase. Insulin receptor capability of liver cells then was examined using the liver perfusion method. Downward regulation was observed among the Maitake-fed group, while a state of tolerance was seen among the control group. Next, glucose absorption activity at the enteron and sucrase activity at the mucosa of the small intestine were examined. Neither inhibition of glucose at the small intestine nor inhibition of sucrase activation was observed when Maitake powder or X-fraction was administered. These results suggested that Maitake's anti-diabetic activity is not related to the inhibition of glucose absorption at the enteron, but with the process of metabolism of absorbed glucose.

### 1 INTRODUCTION

In general, fruiting bodies of some mushrooms in the Basidiomycotina have various biological activities (Maeda *et al.* 1973). The authors have previously reported that high molecular polysaccharides obtained from Maitake (*Grifola frondosa*) are effective against NIDDM (non-insulin dependent diabetes mellitus) using the KK-A<sup>y</sup> mouse which is genetically diabetic (Kubo *et al.* 1994). The objective of this study was to identify the active material and examine its mechanism.

## 2 MATERIALS AND METHOD

### 2.1 Preparation of solid-feed with powdered Maitake

Fruit bodies of Maitake were dried at 65C for 4 hours and pulverized ( $\phi 200\mu$ ). This Maitake powder was mixed with commercial food CE-2 (Clea Japan Inc.) in a ratio of 1:4 and 800 ml/kg of distilled water was added. After thoroughly kneading, 3 x 3 cm squares were cut which were dehydrated at 80C for 20 hours and 20% Maitake solid-feed (20% M-feed) was prepared.

### 2.2 Preparation of X-fraction

Diethylether-ethylalcohol soluble-fraction (ES-fraction) was obtained after powdered Maitake (300 g) was mixed with 1500 ml of diethylether and ethylalcohol (1:1) mixture extracted at 70C for 6 hours. The residue was extracted with 3000 ml of distilled water at 121C for 30 minutes by autoclaving to obtain hot water soluble material which was added with ethylalcohol to make a final concentration of 50%. The material then was allowed to stand at 4 C for 12 hour to collect the floating material (X-fraction).

### 2.3 Preparation of solid feed with X-fraction

The X-fraction obtained from Maitake powder (equal amount of 20% M-feed) was added to 1500 g of CE-2 (control-feed), and the solid-feed was prepared in the same way as stated in 2.1.

### 2.4 Animals

Seven week old female spontaneously diabetic mice (KK- $A^y$ ) or seven week old male Spragu-Dawley (SD) rats were raised on laboratory chow and water *ad libitum* in a temperature-controlled room,  $24 \pm 1C$  and 55% humidity under specific pathogen-free conditions for one week in our laboratory before use in this experiment.

### 2.5 Glucose tolerance test by oral administration

Eight-week-old female KK- $A^y$  mice were fed 20% M-feed for a certain period, but no feed was given for 18 hours before collection of the blood. Glucose (2g per kg body weight) was orally administered and the blood glucose value and plasma insulin value were measured at 0, 15, 30, 60 and 120 minutes after administration.

### 2.6 Effect on hepatic glycogen

Maitake-fed mice and control mice (fed with normal-feed) were given 0.6 mg per kg body weight of epinephrine intraperitoneally and the blood was collected after 1 hour. Then the liver was extirpated and the amount of glycogen was measured.

### 2.7 Treatment and measurement of hepatic glycogen

Extirpated liver (1 g) was treated with 2 ml of 30% KOH at 100C for 30 minutes. Then saturated  $Na_2SO_4$  (0.2 ml) and 95% ethylalcohol (5 ml) were added. The precipitated glycogen was centrifuged at 3000 rpm at 20 C for 10 minutes and 10 ml of distilled water was added to obtain the glycogen solution. After adding 1 ml of 1.2N HCl to 1 ml of glycogen solution, the mixture was heated at 100C for 2 hours. Then it was cooled and neutralized by 0.5% NaOH. The value of glucose was measured by the Anthrone method. The value of glycogen was calculated by multiplying 0.93 times the obtained glucose.

### 2.8 Glucose absorption test at the enteron

The small intestines were extirpated from 8 week old SD rats after they were anesthetized with sodium pentobarbital. These rats were not given any food for 24 hours after 20% M-feed had been fed for a certain period. A section from 20 cm to 10 cm distance from the ligament of Treitz was isolated and removed from the stomach side; 10 cm as sample 1 and the tail side 10 cm as sample 2, respectively. Then the everted small intestine was quickly prepared trying not to damage the mucosa.

The everted small intestine was connected to Teflon tubes and 5 cm of sac was made by dismembering the bottom. The prepared everted small intestine was soaked in 10 mM glucose solution saturated with mixed gas of 95% oxygen and 5% carbon dioxide. The sac was filled with glucose-free solution and the temperature was set at 37C. The solution in the sac was collected (10  $\mu$ l each time) for every other 12 minutes until 180 minutes elapsed from a start time of zero.

### 2.9 Small intestine perfusion test (*in situ*) (Deren *et al.* 1967)

Rats in each group had not been fed for 24 hours before the operation. The orifice of the duodenocholedochus was ligated and the lower part was cut off to insert a silicon tube (for in-flow). Then the connecting part of ileocecum was also cut off to insert a silicon tube (for out-flow) in the

same manner. In this test, whole small intestine was used from the pylorus to ileocecum and the perfusion treatment was performed for 30 minutes. This procedure removed the content in the intestine with phosphate buffered saline (PBS, pH 7.4) at 37C saturated with mixed gas of 95% oxygen and 5% carbon dioxide. When the perfusion was in the normal state, the PBS with 300 mg/dl glucose was distributed at 1 ml/min for 60 minutes by a Perista pump. Samplings were made by collecting 4 ml of the fluids at the ileocecal connection part.

#### 2.10 Measurement of disaccharidase activity (Dahlqvist 1968)

After the completion of enteron perfusion *in situ*, the whole small intestine was extirpated. From about 20 cm of jejunum (about 10 cm lower than the ligament of Treitz) where sucrase activity is most highly concentrated. The mucosa was collected and 1 ml of PBS was added to 100 mg of mucosa and the mixture was homogenized with a Teflon-homogenizer. After centrifugation, the supernatant was collected and used as crude enzyme. Substrate of 700  $\mu$ l of PBS containing 10 mM sucrose was added to this crude enzyme solution (300  $\mu$ l) and incubated at 37C for 30 minutes. The liberated glucose was measured by the glucose oxidase method after treatment for 5 minutes at 100 C.

#### 2.11 Preparation of liver cells as insulin targets (Tanaka *et al.* 1978)

Mice livers were perfused in collagenase solution. The perfused livers were extirpated and the cells were filtered by 150 mesh nylon filter after thorough dispersion in the medium. The filtrate was centrifuged at 500 rpm for 1 minute and hepatocytes were obtained. After treatment with a cell suspension ( $5 \times 10^5$  cells/ml), the hepatocytes were divided into 35 mm Petri plates coated with collagen,  $1 \times 10^5$  cells per dish, and cultured at 37C with 5% CO<sub>2</sub> gas. After 4 hours, the medium was replaced and culturing for 20 hours to obtain monolayer primary cultured hepatocytes which were used for the function test of insulin receptors.

#### 2.12 Measurement of insulin binding ability of insulin receptors

After formation of a monolayer of hepatocytes, a certain amount of hot insulin and various concentrations of cold insulin were added. Culturing then was continued at 4C for 18 hours. At the end of the incubation period, the rate of specific binding of insulin labeled <sup>125</sup>I to the cells was determined.

### 3 RESULTS

The mechanism of anti-diabetic action was examined. The oral glucose tolerance test indicated that the ability to recognize glucose was lowered substantially by Maitake. Also, the same anti-diabetic activity was observed when the X-fraction, a high molecule polysaccharide, having  $\beta$ -1,6 main chain with  $\alpha$ -1,4 branches, was administered. The increase of blood glucose of the experimental group was 0.64 times compared to that of the control group after 15 minutes of glucose administration and 0.76 times after 30 minutes, respectively. Also, the value of plasma insulin was lowered.

Blood glucose of KK-A<sup>y</sup> mice was measured at 1 hour after 0.6 mg/kg body weight of epinephrine was intraperitoneally injected to both the 20% M-feed group and normal-feed group. Blood glucose in the M-feed group almost doubled while the control group increased only 1.4 times. The value of hepatic glycogen was reduced significantly.

Since several factors are known to be associated with the metabolism of chronic diabetes as seen in KK-A<sup>y</sup> mice, the variation of blood glucose and hepatic glycogen was examined using normal mice after inducing hyperglycemia by epinephrine to exclude these factors. The glucose level was elevated, but the hepatic glycogen was decreased even with Maitake administration. This is the same results as when KK-A<sup>y</sup> mice were tested.

Next, Maitake's effect on glucose absorption from the enteron was examined. Absorption tests *in vitro* by everted small intestine were conducted. No significant difference was observed on the stomach side (sample 1) between the Maitake group and control group. However, glucose absorption was higher in the Maitake group on the tail side (sample 2), indicating that the ability of glucose absorption varies depending on the part of the small intestine.

Next, a small intestine perfusion test *in situ* was conducted to investigate the ability of Maitake X-fraction in absorbing glucose. It was found that the X-fraction could not inhibit glucose absorption. Also, there was no difference between the groups in the activity of sucrase.

These results suggest that the anti-diabetic mechanism of Maitake or the X-fraction is directly associated with insulin receptors. Therefore, hepatocytes were isolated as insulin target cells and the capability of receptors to insulin was measured. There was a reversal relationship between <sup>125</sup>I-insulin capability and cold insulin concentrations and so-called down regulation was observed among the Maitake group, while the control group showed the state of tolerance and no reaction was seen against the variation of insulin concentrations.

#### 4 DISCUSSION

Recently, biological activities of mushrooms have been studied (Maeda *et al.* 1974, Nanba and Kuroda 1987, Hikino *et al.* 1985). Authors have reported these activities from tests with Maitake (Adachi *et al.* 1988, Hishida *et al.* 1988, Nanba *et al.* 1987). Tomoda *et al.* (1986) reported that Mannentake (*Ganoderma lucidum*) lowers blood glucose level. Authors have also examined anti-diabetic activity of Maitake and reported its effectiveness against NIDDM in 1994 (Kubo *et al.*).

In this study, oral glucose tolerance tests using Maitake powder and its extract, X-fraction ( $\beta$ -1,6 main chain having (-1,4 branches) were conducted against KK-A<sup>y</sup> mice, the model of NIDDM. In the experimental group, which were given Maitake-feed (20% M-feed), significant improvement in glucose recognition was observed compared to the control group given normal-feed. The level of plasma insulin also was lowered in the Maitake group. These results indicate that Maitake could reduce the insulin resistance or could increase insulin sensitivity. Similar results were obtained with the X-fraction, indicating that the X-fraction may be the active material to exhibit anti-diabetic activity. The mechanism to explain such results of glucose tolerance test is yet to be elucidated, but it is believed to be closely related with glucose absorption from the small intestine and with glucose release or absorption from the liver and peripheral tissues. Therefore, the glycometabolism in liver was further examined.

One of the factors for elevating blood glucose may be the acceleration of glycogen disassimilation in the liver. Highly concentrated epinephrine was administered to KK-A<sup>y</sup> mice to accelerate disassimilation of hepatic glycogen and elevate the blood glucose level. Blood glucose was elevated, however, even with Maitake administration. Epinephrine was injected into normal mice in the same way, and it was found that blood glucose was elevated while the load of hepatic glycogen was reduced. This result indicates that Maitake is not involved in the activities to inhibit disassimilation of glycogen and, thus, inhibit elevation of blood glucose.

Glucose absorption at the enteron was examined *in vitro* using the everted small intestine. Glucose absorption varies depending on the region of the small intestine. It is found that Maitake has no ability to inhibit glucose absorption at the upper part of small intestine where glucose absorption is active. Also, the X-fraction did not inhibit glucose absorption (in fact, it even slightly expedited the absorption) at the enteron *in situ* where normal biological conditions are maintained and where all of the digestive tract from the stomach to the rectum is used.

Carbohydrates are absorbed at epidemal cells in the mucosa of the small intestine. Sucrase activity was examined to determine the influence

of Maitake and the X-fraction on digestion of saccharides. There was no significant difference in sucrase activity between the control and Maitake/X-fraction groups. This would indicate that the Maitake/X-fraction are not associated with the inhibition of glucose absorption and disaccharidase activity at the enteron.

These results suggested that Maitake's anti-diabetic action could be associated with insulin receptors themselves, and functional tests of insulin receptors were conducted. A reverse relationship between insulin concentration and number of receptors often are observed to show adaptability, i.e., where the receptor number decreases under high insulin concentrations while the number increases under insulin shortage. Downward regulation was confirmed from the Maitake group, while the control group was in the state of tolerance, i.e., the state of diabetes. This result indicates that insulin resistance as seen in the state of diabetes is reduced to enhance insulin sensitivity; this confirms the fact that Maitake and the X-fraction are closely associated with insulin receptors. The activity of the X-fraction to insulin receptors will be further elucidated in future studies.

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