Slow Acting Protein Extract from Fruit Pulp of *Momordica charantia* with Insulin Secretagogue and Insulinomimetic Activities

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The protein from Thai bitter gourd (*Momordica charantia*) fruit pulp was extracted and studied for its hypoglycemic effect. Subcutaneous administration of the protein extract (5, 10 mg/kg) significantly and markedly decreased plasma glucose concentrations in both normal and streptozotocin-induced diabetic rats in a dose-dependent manner. The onset of the protein extract-induced antihyperglycemia/hypoglycemia was observed at 4 and 6 h in diabetic and normal rats, respectively. This protein extract also raised plasma insulin concentrations by 2 fold 4 h following subcutaneous administration. In perfused rat pancreas, the protein extract (10 μg/ml) increased insulin secretion, but not glucagon secretion. The increase in insulin secretion was apparent within 5 min of administration and was persistent during 30 min of administration. Furthermore, the protein extract enhanced glucose uptake into C2C12 myocytes and 3T3-L1 adipocytes. Time course experiments performed in rat adipocytes revealed that *M. charantia* protein extract significantly increased glucose uptake after 4 and 6 h of incubation. Thus, the *M. charantia* protein extract, a slow acting chemical, exerted both insulin secretagogue and insulinomimetic activities to lower blood glucose concentrations *in vivo*.

Key words *Momordica charantia*; antihyperglycemia; protein extract; pancreatic perfusion; insulin secretagogue; insulinomimetic

Diabetes mellitus is as a metabolic disease caused by an insufficient of insulin secretion or insulin resistance. There are two types of diabetes: type 1 (insulin-dependent diabetes mellitus or IDDM) and type 2 (non-insulin-dependent diabetes mellitus). Type 2 diabetes is continuously growing to be a major health problem in modern societies. There are many classes of pharmacological agents for type 2 diabetes such as sulfonylureas, biguanides, thiazolidinediones and alpha glucosidase inhibitors. However, these drugs have also shown adverse effects, including lactic acidosis and diarrhea.1) Numerous chemical compounds from traditional plants are considered for diabetic control in many countries. *Momordica charantia* Linn. (MC) is commonly known as karela or bitter gourd. The extracts from fruit pulp, seed, leaves and whole plants of MC have shown antihyperglycemic effect in animal models such as alloxan-induced diabetes,2) streptozotocin (STZ)-induced diabetes,3) and glucose-loaded rats.4) MC also displays antitumor,5) anti-human immunodeficiency virus (HIV),6) anti-ulcerogenic7) and hypotriglyceremic activities.8)

Khanna et al.9) reported the first method for isolation of an active protein from the seeds, tissue, and tissue culture of seedlings of MC by acid–ethanol extraction. The active protein extract compound isolated by Khanna et al. was called protein extract-p or p-insulin, and consisted of a methionine-containing protein with a minimum size of 11 kDa. Subcutaneous administration of protein extract-p exerted the hypoglycemic activity in gerbils, langurs and diabetic patients.9) Thai bitter gourd is commonly used as a traditional remedy for diabetes in Thailand. The extract also causes a decrease in blood glucose concentrations in STZ-induced diabetic rats and also increases glycolytic enzymes activity.10) Zinc has been found to have insulin mimetic activity by stimulating tyrosine phosphorylation of insulin receptor. It also potentiates insulin-mediated activation of insulin receptor substrate-1 (IRS-1).11) In our MC protein extract preparation, we followed Khanna’s method, but did not perform the step utilizing a zinc solution to crystallize the protein extract. To date, there have been no reports regarding the direct stimulatory effect of the protein extract on insulin and glucagon secretions using the pancreatic perfusion technique.

The aim of this study was to investigate the effects of zinc-free protein extract from bitter gourd fruit pulp on plasma glucose concentration and insulin responses in normal and STZ-induced diabetic rats. Furthermore, we investigated the direct effect of the MC protein extract on insulin and glucagon secretions from the perfused rat pancreas as well as glucose uptake in C2C12 rat myocytes and 3T3-L1 rat adipocytes.

MATERIALS AND METHODS

**Materials** Streptozotocin and fetal bovine serum were purchased from Sigma Chemical (St. Louis, MO, U.S.A.). Rat and bovine insulin and glucagon were donated by Eli Lilly Laboratories (Indianapolis, IN, U.S.A.). Insulin antibody was donated by Dr. V. Leclerq-Meyer of Free University of Brussels, Belgium. Glucagon antibody was donated by Dr. Joseph Dunbar of Wayne State University (Detroit, MI, U.S.A.).12) [3H]-labeled glucagon was purchased from Linco Research (St Charles, MO, U.S.A.). 2-Deoxy-[3H]-glucose was purchased from Perkin-Elmer (Boston, MA, U.S.A.).

**Animals** Male Wistar rats procured from the National Laboratory Animal Center (Salaya, Thailand) weighing 250—300 g were used in these experiments. The animals were acclimatized for 1—2 weeks before being used in the experiment. They were maintained in the laboratory animal facility and given food pellets (CP, Bangkok, Thailand) and water *ad libitum*, following the Animal Care and Use Committee Guide of the Faculty of Veterinary Science, Chula-
longkorn University.

**Extraction of Proteins from Thai Bitter Gourd Fruit**  The fruit of Thai bitter gourd was purchased from a local market in Ang Thong province, Thailand and authenticated by the Department of Botany, Chulalongkorn University. The method of Khanna was followed with modification for extraction. Briefly, the fresh pulp was sliced and extracted at 4°C with ice-cold acid-ethanol (0.05 M H$_2$SO$_4$, 60% ethanol). The mixture was filtered through a muslin cloth and centrifuged at 8000×g for 10 min. The supernatant was collected and the pH adjusted to 3.0 using an ammonia solution. Then, 4 volumes of aceton was added to 1 volume of supernatant for precipitation, and the mixture was kept at 4°C for 24 h. The precipitate was dialyzed with a dialysis membrane (molecular weight cut-off: 10 kDa). After dialysis, the MC protein extract was lyophilized and kept at −20°C until use. It was dissolved in 0.9% NaCl and then centrifuged at 2000×g to remove the insoluble part of the extract before using in the following experiments.

**Induction of Diabetes in Rats**  The rats were fasted overnight and received STZ (50 mg/kg, intraperitoneally). STZ was freshly prepared by dissolving it in citrate buffer (0.01 M, pH 4.5) and maintained on ice prior to use. One week following STZ administration, diabetes was confirmed by measuring the fasting blood glucose concentration. The diabetic rats with a blood glucose concentration of >200 mg/dl were used in the experiments. The experiments were performed 8—10 d after STZ administration.

**Effect of MC Extract on Normal and Diabetic Rats**  Normal and diabetic rats were fasted for 6 h and divided into 4 groups with 8 rats in each group. Group 1 received a 0.2 ml of subcutaneous administration of 0.9% NaCl. Groups 2, 3, and 4 received 3 different doses of the MC protein extract (1, 5, 10 mg/kg), respectively. Blood samples were collected in chilled heparinized tubes from the tail vein before and 1, 2, 4, 6, and 8 h after administration, and centrifuged (2000×g) at 4°C for 5 min. The plasma was frozen for subsequent analysis of glucose and insulin. The plasma glucose concentrations were determined by the glucose oxidase method (Sigma Chemical, St. Louis, MO, U.S.A.) and the absorbance was measured with a spectrophotometer at the wavelength 450 nm. Plasma insulin concentrations were determined by using radioimmunoassay (RIA) kits (Diagnostic Products Corporation, Los Angeles, CA, U.S.A.).

**In Situ Pancreatic Perfusion**  The rats were fasted for 12 h before experiments with 3 rats in each group. The rats were anesthetized with pentobarbital sodium (60 mg/kg, intraperitoneally) and were maintained at 37°C on a hot plate during the experiment. The celiac artery and the hepatic portal vein were cannulated with polyvinyl tubing. Then, the pancreas was immediately perfused with Krebs-Ringer bicarbonate buffer (KRB) supplemented with 20 mmol/l HEPES, 5.5 mmol/l glucose, 1% dextran, and 0.2% BSA as a basal medium. The KRB was continuously aerated with 95% O$_2$—5% CO$_2$ at pH 7.4. The perfusion rate was set at 1 ml/min. The rats were euthanized immediately after the placement of cannulas and the beginning of the flow. The perfusion was equilibrated for 20 min. Following the baseline period of 10 min, the perfusate containing 10 μg/ml MC protein extract was administered for 30 min, followed by a washout period of 10 min. The perfusate containing arginine (1 mM) was administered as a positive control for 5 min at the end of each experiment. The effluent fractions were kept at 4°C and subsequently assayed for insulin and glucagon using RIA as previously described.

**Cell Culture**  3T3-L1 adipocytes were maintained in Dulbecco’s modified high glucose Eagle’s medium (DMEM) and supplemented with 10% FBS at 37°C (5% CO$_2$ in air). Preadipocytes 3T3-L1 were grown in 24-well plates until 2 d postconfluence. Differentiation was induced by addition of 0.5 mmol/l isobutylmethylxanthine (IBMX), 1 μmol/l dexamethasone (DEX), and 167 nmol/l bovine insulin in DMEM with 10% FBS. Two days after induction, the IBMX and DEX were placed with 10% FBS and 167 nmol/l bovine insulin. The medium was subsequently replaced again with fresh medium for 2 more days. C$_6$C$_{12}$ cells were grown in 24-well plates in high glucose DMEM and supplemented with 10% FBS, 1% antibiotic solution (penicillin G and streptomycin) at 37°C (5% CO$_2$ in air) until 70% confluence. To induce differentiation into myocytes, confluent cells were exposed to DMEM supplemented with 2% horse serum for 4 more days.

**Glucose Uptake Assay**  After incubation with the MC protein extract for 14—18 h, C$_6$C$_{12}$ myocytes and 3T3-L1 adipocytes were washed once with KRB containing no glucose or BSA, and then incubated with the same KRB at 37°C for 30 min. Ten μg/ml of the MC protein extract or 10 nmol/l bovine insulin was added to the KRB and incubated for another 15 min. At 15 min, 0.2 μCi of $^3$H-glucose was added to the wells, which were incubated for another 15 min. The reaction was terminated by washing the cells twice with 1 ml of 100 mmol/l ice-cold PBS and solubilized in 0.1 N NaOH in a volume of 400 μl/well. The cell-associated radioactivity was measured with a liquid scintillation counter.

**Data Expression and Statistical Analysis**  Data are expressed as the mean±S.E. The effluent concentrations of insulin and glucagon are expressed as a percentage of the baseline level. Data were analyzed using ANOVA followed by Dunnett’s multiple comparison test. The significance level was set at p<0.05.

**RESULTS**

**Effects on Plasma Glucose in Normal and STZ-Induced Diabetic Rats**  The results in Fig. 1 show the effect of the MC protein extract on plasma glucose concentration in normal and STZ-induced diabetic rats. The MC protein extract (5, 10 mg/kg, subcutaneously) significantly and markedly decreased plasma glucose concentrations in both normal (Fig. 1A) and diabetic rats (Fig. 1B) in a dose-dependent manner. In normal rats, the onset of the MC protein extract-induced hypoglycemia was observed at 6 h after subcutaneous administration. In STZ-induced diabetic rats with overnight fasting, the plasma glucose concentrations were 3 times higher than those of normal rats within 1 week after STZ administration (normal control rats: 95±3 mg/dl; STZ-induced diabetic rats: 263±12 mg/dl). The onset of 5 mg/kg and 10 mg/kg protein extract-induced antihyperglycemia in diabetic rats was observed at 8 h and 4 h after subcutaneous administration, respectively. The highest concentration of the MC protein extract used in this experiment (10 mg/kg) ex-
erted a 43% maximal reduction in plasma glucose from 261±8 mg/dl to 148±14 mg/dl at 8 h. In contrast, the MC protein extract at 1 mg/kg failed to decrease the plasma glucose concentration in both normal and diabetic rats.

Effects on Plasma Insulin in Normal and STZ-Induced Diabetic Rats  As shown in Table 1, 10 mg/kg MC protein extract increased plasma insulin concentrations in both normal and diabetic rats. In normal rats, the protein extract significantly increased the plasma insulin concentration at 4 and 6 h after subcutaneous administration, when compared with the basal concentration at time 0; the plasma insulin concentrations were 1.7 and 1.5 times of the concentration at time 0, respectively.

One week after diabetes induction, the STZ-induced diabetic rats had insulin insufficiency. The action of STZ in pancreatic \( \beta \)-cells is accompanied by alteration in blood insulin and glucose concentrations. It is taken up by pancreatic \( \beta \)-cells via GLUT2,\(^{14}\) leading to impaired glucose oxidation\(^{15}\) and decreased insulin biosynthesis and secretion.\(^{16,17}\) Our results showed that the basal plasma insulin concentrations in these diabetic rats were about 7-fold lower than those of normal rats (normal rats: 837±62 pg/ml; diabetic rats: 112±14 pg/ml). Subcutaneous administration of 10 mg/kg MC protein extract significantly increased plasma insulin concentrations, which reached 200±14 pg/ml at 4 h, and 313±32 pg/ml at 6 h after subcutaneous administration; the plasma insulin concentrations were 1.7 and 2.6 times of the concentration at time 0, respectively.

Effects on Insulin and Glucagon Secretions from Perfused Rat Pancreas  Figure 2 shows the profile of insulin secretion remained constant during 50 min in the basal control group receiving KRB alone. The perfusate containing 10 \( \mu \)g/ml MC protein extract was administered for 30 min and it increased insulin secretion, which was 2.4-fold that of the basal control group. In contrast, it did not change glucagon secretion (data not shown). The effluent concentration of insulin returned to baseline during the 10-min

Table 1  Plasma Insulin Concentrations of Normal and Streptozotocin (STZ)-Induced Diabetic Rats Receiving Subcutaneous Administration of 10 mg/kg MC Protein Extract

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Plasma insulin (pg/ml)</th>
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<tr>
<td></td>
<td>0 h</td>
</tr>
<tr>
<td>Normal rats</td>
<td></td>
</tr>
<tr>
<td>Control (NS)</td>
<td>837±62</td>
</tr>
<tr>
<td>Extract (10 mg/kg)</td>
<td>811±199</td>
</tr>
<tr>
<td>STZ-induced diabetic rats</td>
<td></td>
</tr>
<tr>
<td>Control (NS)</td>
<td>112±14</td>
</tr>
<tr>
<td>Extract (10 mg/kg)</td>
<td>120±8</td>
</tr>
</tbody>
</table>

The control rats received 0.9% NaCl. Results are expressed as mean±S.E. (n=8). *p<0.05 vs. control group.
washout period in the treated group. Administration of 1 mmol/l arginine increased insulin secretion to 7-fold that of the baseline level at the end of the experiment.

Effects on Glucose Uptake in Rat Myocytes and Adipocytes

The effects of the MC protein extract (10 mg/ml) and insulin (10 nmol/l) on a 2-deoxyglucose uptake assay in transformed C2C12 (Fig. 3A) and 3T3-L1 (Fig. 3B) cells were determined. The 2-deoxyglucose uptakes in both kinds of cells were significantly increased by exposing them to the MC protein extract for 14—18 h and/or insulin for 30 min. Data are expressed as the mean±S.E. (n=3—5). *p<0.05 vs. control group. 2DG, 2-deoxyglucose.

DISCUSSION

The results of the present study showed that the MC protein extract had definite hypoglycemic/antihyperglycemic effect in both normal and STZ-induced diabetic rats via both insulin secretagogue and insulinomimetic pathways. After extraction and dialysis, three different lots of crude protein extracts from MC were assessed for their protein content by an SDS-PAGE method. The result indicated that there were at least two different major proteins and some small amount of low molecular weight compounds in the extracts. The molecular weights of these two major proteins were estimated to be 10 and 20 kDa, respectively (unpublished data). It is possible that one or more of these proteins may play the role as the active principle(s) in lowering blood glucose levels in rats. However, further work in characterizing the protein fractions is warranted to prove this statement.

The dose of STZ that was used in this study to induce diabetes was similar to the one used by many other researchers for studying the antidiabetic effects of various compounds.18—23) STZ selectively destroys insulin-producing beta cells of the pancreas by the induction of high levels of DNA strand breaks in beta cells, causing activation of poly (ADP-ribose) polymerase (PARP), a resultant reduction in cellular NAD+/H11001, and cell death.24) After 1 week of intraperitoneal administration of STZ, rats displayed hyperglycemia, glycosuria, polydipsia, polyphagia and body weight loss. Subcutaneous administration of the MC protein extract into both normal and STZ-induced diabetic rats produced a marked plasma glucose lowering effect in a dose-dependent manner. However, the onset of MC protein extract-induced antihyperglycemia/hypoglycemia was observed at 4 and 6 h in diabetic and normal rats, respectively. In addition, this protein extract also raised plasma insulin concentrations about 2 fold 4 h following subcutaneous administration. This finding is novel since it is the first report of MC protein extract-induced hypoglycemia with a delayed onset. Although, Khanna et al.9) also reported an antihyperglycemic effect of the MC protein, which reached a maximum between 4—8 h after subcutaneous protein extract-p administration, the onset of action was observed within 30 min. This discrepancy may be explained by the fact that our protein extract preparation was not extracted using zinc acetate solution as was that of Khanna et al.9) Zinc has been shown to increase glucose uptake in adipocytes within 30 min of administration. In addition, it stimulated tyrosine phosphorylation of insulin receptors.11) Therefore, it is possi-
The MC-induced hypoglycemia may be due to a mixture of steroidal saponins called charantins, insulin-like peptides and alkaloids. Day et al. have reported that these chemicals are divided into two categories with different time-dependent effects; one is present in the aqueous solution and the residue after alkaline chloroform extraction exerted a rapid hypoglycemic effect; the other is present in the acidic wash of chloroform extraction, which exerted a slow antihyperglycemic effect. However, they only studied the hypoglycemic effect of orally administered extracts of MC. Although our extract also generated a slow hypoglycemic effect, it may be different from the one mentioned by Day et al.4 since our extract is protein in nature, which would be destroyed by enzymes in the gastrointestinal tract.

In general, after distribution to target tissues, insulin activates its specific receptor, which leads to activation of tyrosine kinase, phosphatidylinositide-3 kinase, and other signals. This event decreases plasma glucose concentrations by promoting glucose transport31) and glycogen synthesis.32) To determine which mechanisms underlie the MC-protein extract-induced hypoglycemia, we investigated its effect on insulin and glucagon secretion from the perfused rat pancreas and on glucose uptake in rat myocytes and adipocytes. To the best of our knowledge, the present study is the first to demonstrate a direct effect of MC protein extract on insulin and glucagon secretions using a pancreatic perfusion technique. These results provide evidence of a direct pancreatic action on beta cells. The persistence of the insulinotropic activity in vivo of the MC protein extract was confirmed by data obtained from perfused rat pancreas. The MC protein extract (10 μg/ml) stimulated insulin secretion in the presence of 5.5 mMol/l glucose. This protein extract induced a transient insulin peak within 5 min of administration, which was 2.4-fold that of the basal level. In contrast, it had no effect on glucagon release at the same concentration. The lack of activity on glucagon release, and the stimulatory effect of MC on insulin secretion would be a benefit to diabetic patients. Interestingly, the MC protein extract increased plasma insulin at 4 and 6 h after subcutaneous administration, but it immediately increased insulin secretion in pancreatic perfusate. The reasons for this discrepancy are not known. It is possible that, in pancreatic perfusion experiments, the much higher dosage of the protein extract (10 μg/ml) was perfused directly into the pancreas, which can stimulate insulin secretion immediately. Based on these results, we hypothesize that 1) 10 mg/kg of the protein extract probably would not be able to yield a concentration close to 10 μg/ml in the pancreas, 2) the absorption of MC from the injection site could be slow, and thus it may have taken a longer time to reach effective concentrations of MC extract to increase insulin secretion, and 3) active metabolites of MC extract, which are much more potent than MC extract itself, might have been responsible for the increase in plasma insulin concentrations at 4 h post-MC. Further studies are warranted to prove or disprove these hypotheses.

The MC protein extract at 10 μg/ml increased glucose uptake in rat adipocytes and myocytes. In addition, the effect of the MC protein extract was not significantly different in the absence or presence of insulin. Furthermore, we investigated the time-course effect of this protein on glucose uptake. We found that this protein increased glucose uptake after incubation for 4 and 6 h (Fig. 4). Therefore, this result confirmed that our MC protein extract indeed exerted a slow hypoglycemic effect by increasing insulin secretion and glucose uptake. Our finding is consistent with the previous report that the protein extracts from bitter gourd fruits and seed as well as the fruit juice display glucose and amino acid uptakes.33,34) However, it is different from the others in terms of the delay in onset of action. Taken together, these results suggested that the MC protein extract exerts both insulin secretagogue and insulin-like activities in lowering blood glucose in vivo. Further study is now in progress to isolate and characterize the MC protein that induces antihyperglycemia/hypoglycemia.

In summary, we have demonstrated that the MC protein extract, a slow acting chemical, was able to decrease plasma glucose levels, and increase plasma insulin secretion in both normal and diabetic rats. This activity was also observed in perfused rat pancreas, which showed a stimulatory effect on insulin but not glucagon secretion. In addition, the MC protein extract stimulated glucose uptake in rat myocytes and adipocytes. Therefore, these results support the MC protein extract as a beneficial therapeutic agent for diabetes mellitus.

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