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Purification and Characterization of Moran 20K from *Morus alba*

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A new glycoprotein was purified from the aqueous methanolic extract of the root bark of *Morus alba* which has been used as a component of antidiabetic remedy in Oriental Medicine. SDS-PAGE result shows that the molecular weight of the glycoprotein was approximately 20 kDa. This new glycoprotein was named as Moran 20K. The protein lowered blood glucose level in streptozotocin-induced hyperglycemic mice model and it also increased the glucose transport in cultured epididymis fat cells. The amino acid composition of the protein was analyzed, and the protein contained above 20% serine and cysteine such as insulin. The actual molecular weight of the protein was determined as 21,858 Da by MALDI-TOF mass spectroscopy.

Key words : Moran 20K, Glycoprotein, MALDI-TOF Mass

INTRODUCTION

Diabetes had been recognized for about 2,000 years, but the treatment only became available following the discovery of insulin. Since diabetes is a heterogeneous syndrome, it is difficult to give a general definition of the disease other than persistent and inappropriate hyperglycemia. Diabetes is classified into two types, described as type I and type II. A more clinical definition, which accurately describes the major feature of the disease, is the classification of diabetes into insulin-dependent diabetes mellitus (IDDM) and non-insulin-dependent diabetes mellitus (NIDDM) (Kahn *et al.*, 1995).

Insulin-dependent diabetes occurs with autoimmune phenomena, which result from high-diabetes sensitive person affected by environmental factor such as diets, toxin and virus (Garchon *et al.*, 1991). This disease is the disorder of organization specific autoimmune system which is brought about by the specific breakdown of β-cell. This causes the abnormal activation of autoimmune system on β-cell secreted from islets of pancreas (Miller, 1995). Non-insulin dependent diabetes may result from complex defects of adipose tissues and muscle cells, genetic defect of mitochondria, deficiency of insulin receptor and so on. In this case, patients have the insulin resistance and the deficiency of insulin secretion. As the genes causing non-insulin dependent diabetes are not yet discovered, its diagnosis, prevention and treatment are still difficult. Any attempt at understanding the etiology and pathophysiology of diabetes, particularly type II, and at evaluating therapeutic strategies to treat the disease must start with the knowledge of the structure of insulin and its related chemistry. Insulin has been used for delaying chronic complex diseases by relaxing the symptom of insulin-dependent diabetes. Insulin is composed of two chains, A and B. These chains are linked each other by disulfide bridge bond. About 20% out of 51 amino acid residues are cysteine and serine. In type II diabetes, the ability of the pancreas to synthesize and secrete insulin is maintained, but the response to glucose is considerably decreased. 85~95% of diabetes patients fall into non-insulin dependent diabetes.

The root barks of *Morus alba* have been used as oriental medicine for the treatment of diabetes in China and Korea. An aqueous methanolic extract of the root barks of *Morus alba* was known to exhibit a significant hypoglycemic effect and the glycoprotein purified from the extract was named as Moran A (Hikino *et al.*, 1984). The molecular weight of Moran A is 7,500 and its amino acid components (mol%) were glycine (13.7), glutamic acid (11.4), aspartic acid (10.5), alanine (8.9), proline (7.8), threonine (7.1), serine (6.9), hydroxyproline (6.7), cystine (5.0) and other minor amino acids (Hikino *et al.*, 1984).

In an effort to purify hypoglycemic material from *Morus alba*, we purified two glycoproteins from *Morus alba*: 7,500 Da protein supposed as Moran A and approximately 20 kDa glycoprotein. We named this 20 kDa glycoprotein as Moran 20K. The present study shows the biochemical characteristics and biological

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activities of the protein.

MATERIALS AND METHODS

Materials

Root barks of *Morus alba* were purchased from Kyung-Dong herb market in Seoul, Korea. Cellulose membrane, thymol, bovine serum albumin (BSA), pyruvate and triton X-100 were purchased from Sigma Chemical Co. (St. Louis, USA). 2-Deoxy-[1-^{3}H]-D-glucose was purchased from Amersham, UK. All other materials were analytical or biotechnological grade. Male SD rats (180~200 g) and mouse (C57BL/6, 25~30 g) were supplied by Experimental Animal Center of Seoul National University in Seoul, Korea.

Glycoprotein detection in SDS-PAGE

In order to detect glycoprotein, SDS-PAGE electrophoresis (Laemmli, 1970) was performed. The gel was fixed by submerging in 25% 2-propanol/10% acetic acid for 2 hour at room temperature and the same procedure was repeated. The gel was immersed in the above solution containing 0.2% (V/V) thymol for 90 min with gentle rocking and then in 80% sulfuric acid/ 20% (V/V) ethanol. The solution was rocked until the gel clarified and glycoproteins appeared as pink/red bands (Racusen, 1979).

Purification

Five kilograms of Morus Radicis Cortex was extracted with methanol:Water (1:1, 50 l×12) for 4 days at room temperature. The aqueous methanol solution was concentrated under reduced pressure to give the extract. The extract was dialyzed with cellulose membrane against water for 7 days to get the non-dialyzable portion, and it was centrifugated (10,000 g, 30 min, at 4°C) and the supernatant was diluted to three times with column buffer (50 mM potassium phosphate buffer, pH 8.0). The diluted solution was applied to DEAE cellulose (Pharmacia Co., Uppsala, Sweden) column previously equilibrated with the same buffer at 4°C. Moran 20K protein eluted in pass-through, was concentrated to 3 ml by using Centriprep-10 (Amicon Inc., USA). The sample was then applied to Sephacryl S-200 column previously equilibrated with the same buffer at 4°C. Sample of each step were analyzed by using 15% SDS-PAGE (Fig. 1). The fractions containing Moran 20K protein were pooled and dialyzed against distilled water. The experiments followed were performed with this desalted sample. The concentration was measured by Bradford method (Pierce Coomassie protein assay reagent).

Hypoglycemic effects of Moran 20K

To induce hyperglycemia of mouse (C57BL/6), mice were employed in groups of five and had given streptozotocin (50 mg/kg) dissolved in citrate buffer 10 ml by *i.p.* injection for five days. Moran 20K (200 mg/ kg) and CMC (0.5%, 5 ml/kg) were administrated orally to streptozotocin-induced hyperglycemic mice (n=5, 8 days passed after streptozotocin injection), respectively. Blood was drawn from the orbital sinus by micro-hematocrit tubes periodically. The glucose level of plasma obtained by centrifugation of blood was measured with a glucose analyzer by the glucose oxidase method.

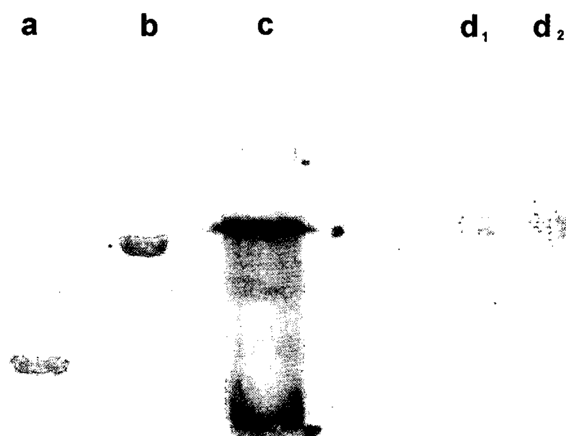

Fig. 1. SDS-PAGE of Moran 20K purified from a gel chromatography using Sephacryl S-200 column. Purified protein was loaded on 15% polyacrylamide gel in the presence of 0.1% SDS. Molecular weight standard; a. Lysozyme (14.4 KDa); b. Cyclic AMP Receptor Protein monomer (CRP, 23.5 KDa); c. Crude Morus alba extract; d_1 and d_2. Purified Moran 20K.

In vitro assay of Moran 20K

Glucose transport assay were performed by Olefsky's method (Olefsky, 1975). Epididymis fat cells separated from SD rat (2-4×10^5 cells/ml) by Rodbell's method (Rodbell, 1964) were preincubated in KRH buffer (pH 7.4) containing 1% BSA, 20 mM pyruvate for 20 minutes. Each sample was added and incubated for 2 hours at 37°C. Subsequently twenty microliters of 2-deoxy-[1-^{3}H]-D-glucose (2-DOG, 2.5 μmol, 4 μCi/μmol) was added, incubated for 3 minutes and the reaction was stopped by oil centrifugation. The radioactivity of the cell layer cut from tube centrifugated was estimated by liquid scintillation counter, using 2:1 mixture of toluene base scintillation cocktail containing 0.5% 2, 5-diphenyl oxazole, 0.01% 1,4 bis[2-(5-phenyl-oxazole)] benzene and triton X-100.

Amino acid composition analysis

At first, samples were classified into three parts, each for general amino acid, cysteine and tryptophan

analysis. Each sample was diluted to 1/10, was taken 20 μl and then dried. The samples were hydrolyzed by HCl at 110°C except cysteine and tryptophan. Cysteine was hydrolyzed after peroxidation. Tryptophan was hydrolized by alkali. Amino acids hydrolyzed were converted into phenylisothiocyanate derivatives. After complete dry, they were solubilized by solvent A (1.4 mM NaHAc, 0.1% trifluoroacetic acid, 6% CH_3CN at pH 6.3). After microcentrifugation, supertanant was loaded into HPLC to analyze peak area.

MALDI-TOF measurement

The Hewlett Packard G 2030A machine was used for MALDI-TOF analysis. The samples in distilled solution were mixed with a cinapinic acid as matrix prior to analysis. Desorption and ionization were achieved by the application of a focused burst of ultraviolet light from nitrogen laser (λ=337).

RESULTS AND DISCUSSION

In vivo assay

The hypoglycemic activity of Moran A was reported by Hikino *et al.*, 1984. Although Moran 20K is a glycoprotein like Moran A, it was not certain that Moran 20K had the same activity as Moran A. Therefore, we assayed hypoglycemic effect of Moran 20K. In this assay, Moran 20K lowered blood glucose level of streptozotocin-induced hyperglycemic mice, compared with control but the lowered levels of blood glucose didn't show the significant difference from the control. This result is shown in Fig. 2.

In vitro assay

Glucose, mannose and N-acetylglucosamine cause stimulation of insulin release and biosynthesis, and are also metabolized by the islets. Non-metabolizable sugar such as 3-O-methylglucose or 2-deoxyglucose does not cause the secretion of insulin. In Fig. 3, both crude and purified Moran 20K increased the amounts of 2-DOG uptake in a dose-dependent manner, considerably at 1 hour after administration but at 2 hours after adminstration, it doesn't seem to be increased. While the reason of which is unknown, it may be caused by the death of fat cells for the toxicity of Moran 20K or the degradation of Moran 20K, 2 hours later. The asterisk shows the significant difference from the control. Therefore, Moran 20K seemed to have an effect on the increase of glucose transport, especially at 1 hour after administration.

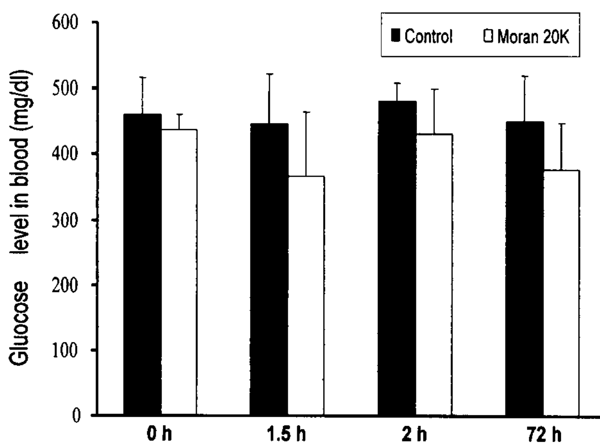

Fig. 2. *In vivo* activity test of Moran 20K: as control, streptozotocin-induced hyperglycemic mice (n=5) was treated with 0.5% CMC (5 ml/kg) and Moran 20K (200 mg/kg) were administrated to streptozotocin-induced hyperglycemic mice (n=5).

Amino acid composition analysis

Amino acid composition of Moran 20K is summarized in Table I. The amino acid components (mol%) of Moran A were glycine (13.7), glutamic acid (11.4), aspartic acid (10.5), alanine (8.9), proline (7.8), threonine (7.1), serine (6.9), hydroxyproline (6.7), cystine (5.0) and other minor amino acids (Hikino *et al.*, 1984). The result of amino acid analysis of Moran 20K showed that it contained above 20% serine and

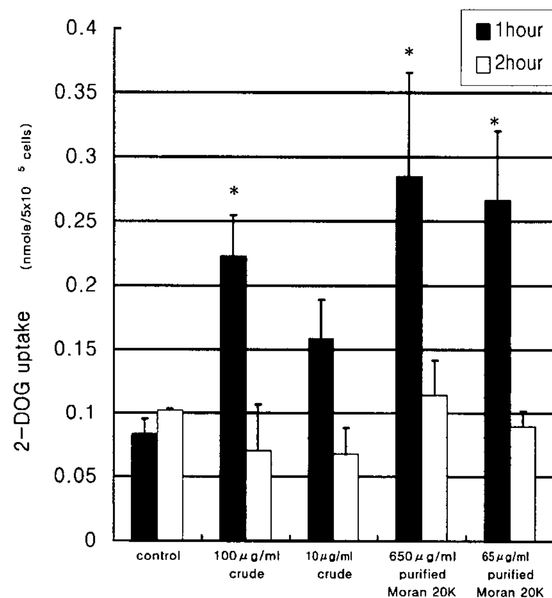

Fig. 3. *In vitro* activity test of Moran 20K: The samples (10 μg/ml and 100 μg/ml crude extracts, 65 μg/ml and 650 μg/ml purified Moran 20K sol'n) was added to the Epididymis fat cells separated from SD rat (2-4×10^5 cells/ml) and incubated for 2 hours at 37°C. Then 2-DOG 20 μl (2.5 μmol, 4 μCi/μmol) was added, incubated for 3 minutes and the reaction was stopped by oil centrfugation. The radioactivity of the cell layer cut from tube centrifugated was estimated by liquid scintillation counter. Results were expressed as means±S.E. of triplicated determinations. Significantly different from the control, *$p<0.05$.

Table I. Amino acid composition analysis

Amino acid	Result	Mol%
Cya*	606.82	13.22
Asx**	411.83	8.97
Glx**	505.52	11.01
Ser	316.43	6.89
Gly	620.67	13.52
His	38.78	0.84
Arg	113.48	2.47
Thr	304.14	6.63
Ala	307.04	6.69
Pro	456.46	9.95
Tyr	112.38	2.45
Val	177.04	3.86
Met	16.27	0.35
Ile	194.08	4.25
Leu	174.31	3.80
Phe	94.31	2.05
Trp	20.18	0.44
Lys	118.77	2.59
Total	4589.41	100.00

*Cya means the sum of cysteic acid & oxidized cystine.
**Asx, Glx mean the sum of asparagine & aspartic acid and glutamine & glutamic acid, respectively.

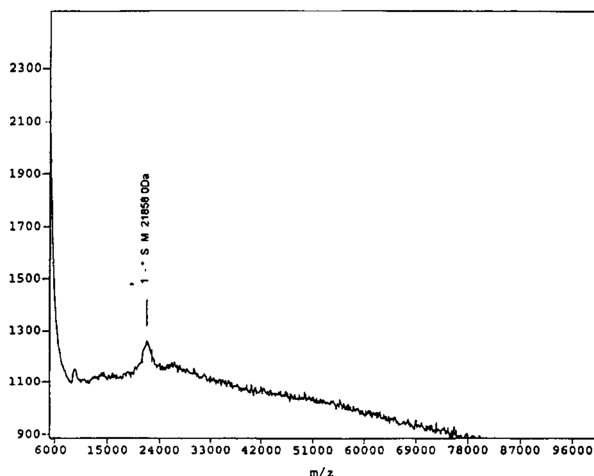

Fig. 4. MALDI-TOF analysis of Moran 20K.

cysteine amino acids such as insulin and was proved to be new material different from Moran A.

Mass measurement

Actual molecular weight determined by MALDI-TOF and its apparent molecular-weight estimated by SDS-PAGE was usually different. In general, the hydrodynamic behavior of glycoprotein-SDS complex cannot meet the real molecular weights of glycoproteins. Quantitatively, it is not known just how much the structural and chemical incongruity of glycoproteins affects their molecular weight estimation. However SDS binding measurements on a number of the glycoproteins suggest that the polypeptide moiety binds the nominal weight ratio of SDS, while the carbohydrate portion exhibit little or no SDS binding (Leach *et al.*, 1980). The actual molecular weight of Moran 20K was determined as 21,858 Da by MALDI-TOF mass spectroscopy (Fig. 4).

ACKNOWLEDGEMENTS

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