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Modern Biotechnology of *Phellinus baumii* – From Fermentation to Proteomics

*Hye Jin Hwang, Sang Woo Kim and Jong Won Yun**

Department of Biotechnology, Daegu University, Kyungsan, Kyungbuk 712–714, Korea

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Summary

Phellinus baumii is a mushroom used as a folk medicine for a variety of human diseases in several Asian countries. Recently we have reported for the first time about the antidiabetic effect of the crude exopolysaccharides (EPS) produced from submerged mycelial culture of *P. baumii* in streptozotocin (STZ)-induced diabetic rats. The diabetic rats study revealed that orally administrated *P. baumii* EPS lowered the blood glucose levels and stimulated insulin excretion in diabetic rats, and consequently restored the functions of pancreas, liver, and kidney, suggesting that the EPS might be useful for the management of human diabetes mellitus. We undertook proteomic analyses for plasma, pancreas, liver, and kidney of the rats to search for novel biomarkers for monitoring diabetes before and after EPS treatments. In this article, we describe the production of EPS in submerged culture of *P. baumii* and studies of their hypoglycemic activity. We also explore the issue of proteomic analyses for mining biomarkers of diabetes.

Key words: diabetes mellitus, exopolysaccharides, hypoglycemic effect, *Phellinus baumii*, proteome

Introduction

Microbial exopolysaccharides (EPS) are a class of high value biopolymers with a wide variety of industrial applications (*1,2*). In particular, many kinds of EPS have been produced from submerged cultures of basidiomycetes or entomopathogenic fungi (*3–22*) because solid culture does not guarantee a standardized product, with product composition varying from batch to batch (*23–27*).

Phellinus spp., basidiomycetes belonging to the family *Polyporaceae*, have diverse physiological effects, especially antitumor activity (*25,28–31*). The fruiting bodies of *P. baumii* and *P. linteus* are used as a folk medicine for a variety of human diseases in several Asian countries. Pharmacological activities ascribed to polysaccharide-containing extracts of these fruiting bodies include antioxidant activity and prevention of acute pulmonary inflammation (*32,33*). However, although there are a great number of reports about the pharmacological activities of EPS from fruiting bodies of *Phellinus* species (*34,35*), there are few published reports about the production of EPS in submerged culture and characterization of their biological activities (*8,10,12*). Recently, we have found that EPS produced in submerged cultures of *P. baumii* showed strong antidiabetic activity against streptozotocin (STZ)-induced diabetic rats (*16*).

Though different types of oral hypoglycemic agents are available, along with insulin, for the treatment of diabetes mellitus, there is increasing demand by patients to use natural products. Insulin cannot be used orally and continuous use of the synthetic antidiabetic drugs causes side effects and toxicity (*36,37*). Due to their effectiveness, limited side effects, and relatively low cost, herbal drugs are widely prescribed even when their biologically active compounds are unknown. In many countries, much effort has been put into the discovery of na-

*Corresponding author; Phone: ++82 53 850 6556; Fax: ++82 53 850 6559; E-mail: jwyun@daegu.ac.kr

tural antidiabetic drugs from various medicinal plants (*38–54*). Mushrooms and entomopathogenic fungi are also potential sources of natural medicines with antidiabetic activity. Many investigators have tested the fruiting bodies or mycelia of various edible/medicinal fungi for hypoglycemic effects (*41,55–62*), but little attention has been given to EPS obtained by mycelial culture (*63–68*).

There are numerous marker proteins for the diagnosis of diabetes mellitus (*69–73*), but there has been no proteomic study of diabetes before and after the treatment with natural antidiabetic remedies, such as mushrooms and other herbal medicines. It is interesting to link proteomics technology to the discovery of diabetes-associated proteins (biomarkers), which is possible by detailed analysis of those proteins after treatment with fungal polysaccharides (see Fig. 1). In this paper, we describe the production of EPS of *P. baumii* by submerged culture and characterization of their hypoglycemic activity. We also explore possible applications to proteomic analyses for mining biomarkers of diabetes.

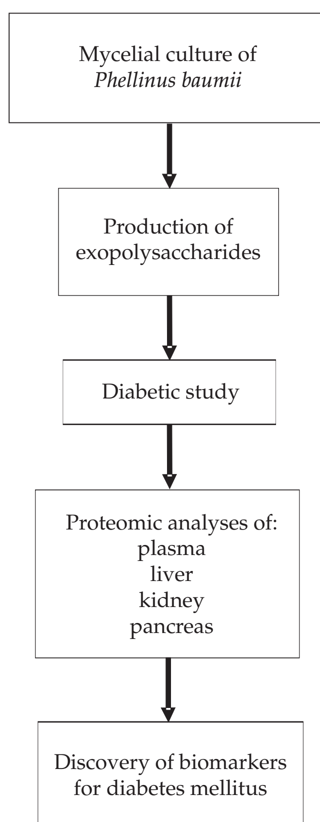

Fig. 1. A strategy for the application of fungal polysaccharides to diabetic proteomics

Materials and Methods

Microorganism and culture procedure

Phellinus baumii DG-07 was used throughout the experiments. The stock culture was inoculated on a potato dextrose agar (PDA) slant, incubated at 28 °C for 6 days and then stored at 4 °C. It was subcultured monthly. For inoculum preparation, *P. baumii* was grown on a PDA medium (2.4 % potato dextrose broth and 2 % agar) in a Petri dish, and then transferred to the seed culture medium by punching out a portion (5 mm diameter) of the agar plate with a sterilized homemade cutter. The seed culture was grown in a 250-mL flask containing 50 mL of PMP medium (2.4 % potato dextrose broth, 1 % malt extract, 0.1 % peptone) at 28 °C with shaking at 150 rpm for 4 days. Submerged culture of *P. baumii* for the production of EPS was performed in a 5-litre stirred-tank fermentor under the following culture conditions: fructose 20 g/L, yeast extract 20 g/L, $CaCl_2$ 0.55 g/L, temperature 30 °C, aeration rate 2 vvm, agitation speed 150 rpm, initial pH=5.0, and working volume 3 L.

Measurements of rheology and morphology

Morphological details of the samples were evaluated using an image analyzer with software coupled to a light microscope through a charge-coupled device (CCD) camera according to the method outlined by Tucker and Thomas (*74*). Rheological measurements were performed on samples collected from the bioreactor at regular intervals using a digital viscometer (Brookfield Engineering Laboratories, Inc., Stoughton, MA, USA) fitted with a small sample adapter. Culture broths were centrifuged at 10 000×*g* for 20 min, and the supernatant was filtered through a Whatman filter paper No. 2 (Whatman International Ltd., Maidstone, UK). The resulting culture filtrate was mixed with four volumes of absolute ethanol, stirred vigorously and left overnight at 4 °C. The precipitated EPS was centrifuged at 10 000×*g* for 20 min and the supernatant was discarded. The precipitates of crude EPS were lyophilized and the mass was estimated. Dry mass of the mycelium was measured after repeated washing of the mycelial pellet with distilled water and drying at 90 °C overnight to constant mass.

Purification of the EPS

The ethanol precipitates of the crude EPS components were dissolved in 0.2 M NaCl buffer to the concentration of 10 g/L, and loaded onto a Sepharose CL–4B column (2.4 cm×100 cm, Sigma Chemical Co., Louis, MO, USA). The column was eluted with the same buffer at a flow rate of 0.6 mL/min. The total carbohydrate content of the EPS produced from *P. baumii* was determined by the phenol sulfuric acid method (*75*) using glucose as the standard. Total protein was determined by the method of Lowry *et al.* (*76*) with bovine serum albumin as the standard. The protein moiety in the EPS was monitored by absorbance at 280 nm, whilst the carbohydrate moiety was monitored at 480 nm.

Diabetic rats study

Male Sprague-Dawley rats (Daehan Experimental Animals, Seoul, Korea), weighing 130–150 g, were housed in individual stainless steel cages and acclimatized with free access to food and water for at least one week in an air conditioned room ((23±2) °C with (55±5) % humidity) under a 12:12-hour light-dark cycle. The rats were fed with a commercial pellet diet (Sam Yang Co., Seoul, Korea) throughout the experimental period.

After one week of acclimatization, the rats were subjected to a 16-hour fast. Diabetes was induced by in-

tramuscular injection of streptozotocin (STZ; Sigma Chemical Co., Louis, MO, USA) dissolved in 0.01 M sodium citrate buffer (pH=4.5) at a dose of 50 mg/kg body mass (*77*). Two days after the injection of the STZ, fasting blood glucose was determined and the rats with blood glucose >300 mg/dL were included in the group of diabetics. All the animals were randomly divided into four groups with six animals in each group: Group N: normal control rat group was treated with 0.9 % NaCl solution; Group NP: normal control rat group was treated with *Phellinus baumii* EPS at the level of 200 mg/kg body mass; Group S: STZ-induced diabetic rat group, the so-called diabetic control rats, which were treated with 0.9 % NaCl solution; Group SP: STZ-induced diabetic rats treated with *Phellinus baumii* EPS at the level of 200 mg/kg body mass daily for 14 days.

Blood samples for the proteomics study were obtained without anesthesia by resection of the terminal 1–2 mm of the rats' tails after 14 days; a total of 0.5–0.6 mL of blood was drawn into tubes containing sodium EDTA. Plasma was separated by centrifugation (3000×*g*, 10 min) and then stored at –30 °C until analysis. The protein content of plasma samples was determined using the Bradford method (*78*) with protein assay dye reagent concentrate (Bio-Rad, Hercules, CA, USA).

Two-dimensional electrophoresis for the proteomic study

Rat tissues (*e.g.* liver, kidney, and pancreas) were removed immediately after sacrifice. Excess blood was eliminated from the tissues using a cold NaCl solution. Each tissue was pulverized into a powder under liquid nitrogen and stored at –80 °C until use. Frozen tissues (40 mg) were solubilized in 200 μL of rehydration buffer containing 7 M urea, 2 M thiourea, 4 % 3-((3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid (CHAPS), 1 mM phenylmethylsulfonylfluoride (PMSF), 20 mM dithiothreitol (DTT), 2 % immobilized pH gradient (IPG) buffer and a trace of bromophenol blue. An ultrasonic generator was used for 2×30 s with 1 min on ice between each round to aid solubilization. Samples were centrifuged at 13 000×*g* for 15 min and then the supernatant was transferred into new tubes. Protein from the supernatant was precipitated by methanol/chloroform prior to electrophoretic separation. The precipitate was resuspended in rehydration buffer and then kept at –80 °C until use.

Two-dimensional electrophoresis images were made in triplicate for the three rats in normal (Group N), diabetic control (Group S), and EPS-treated diabetic (Group SP) groups (*i.e.* a total of 9 gel images for each group) and normalized prior to statistical analysis. IPG isoelectric focusing (IEF) of samples was carried out on pH=4–7 or pH=3–10, 17 cm IPG DryStrips (Bio-Rad) in the Protean IEF cell (Bio-Rad) using the protocol recommended by the manufacturer. IPG strips were rehydrated passively overnight in strip holders in 350 μL of rehydration solution containing 3 μL of plasma sample. IEF was carried out as follows: 15 min at 250 V, 3 h at 250–10 000 V, 6 h at 10 000 V, and then held at 500 V until ready to run the second dimension. Briefly, 200 μg (~3 μL) of the plasma sample were mixed with 347 μL of rehydration solution containing 7 M urea, 2 M thiourea, 4 % CHAPS, 1 mM PMSF, 20 mM DTT and 2 % IPG buffer. After focusing, the gel strips were equilibrated in a solution containing 6 M urea, 2 % SDS, 1 % DTT, 30 % glycerol, and 50 mM Tris-HCl (pH=6.8) for 15 min, followed by further incubation in the same solution (except that DTT was replaced with 2.5 % iodoacetamide) for additional 15 min. The equilibrated IPG strips were gently rinsed with electrophoretic buffer and then placed on a 20×20 cm 12 % polyacrylamide gel for resolution in the second dimension. The fractionation was performed with the Laemmli-SDS-discontinuous system, at a constant voltage of 20 mA per gel for 10 h, after which the gels were visualized by silver staining.

Gels were imaged on a UMAX PowerLook 1120 (Maxium Technologies, Inc., Taipei, Taiwan) and the resulting 16-bit images were converted to TIF format prior to exporting and analysis. Intensity calibration was carried out using an intensity stepwedge before gel image capture. Images were compared using a modified version of ImageMaster 2D software ver. 4.95 (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK). A reference gel was selected at random from the gels of the control group for each experiment and detected spots from the other gels in the control data set were matched to those in the selected reference gel. The relative absorbance and relative volume were also calculated in order to correct for differences in gel staining. Each spot intensity volume was processed by background subtraction and total spot volume normalization; the resulting spot volume percentage was used for comparison.

Protein identification

Enzymatic in-gel digestion of protein spots was similar to that described by Shevchenko *et al.* (*79*), using modified porcine trypsin. Gel pieces were washed with 50 % acetonitrile to remove SDS, salts, and stains. The gel was then dried to remove solvent, rehydrated with trypsin (8–10 ng/μL), and incubated for 8–10 h at 37 °C. The proteolytic reaction was terminated by adding 5 μL of 0.5 % trifluoroacetic acid. Tryptic peptides were recovered by combining the aqueous phase from several extractions of gel pieces with 50 % acetonitrile. After concentration, the peptide mixture was redissolved in the buffer and desalted using C_{18}ZipTips (Millipore), and the peptides were eluted with 1–5 μL of acetonitrile. An aliquot of this solution was mixed with an equal volume of a saturated solution of α-cyano-4-hydroxycinnamic acid in 50 % acetonitrile, and 1 μL of the mixture spotted onto a target plate.

Protein analysis was done using an Ettan MALDI-ToF mass spectrometer (Amersham Biosciences). The peptides were evaporated with an N_2 laser at 337 nm using a delayed extraction mode. They were accelerated with a 20 kV injection pulse for a time of flight analysis. Each spectrum was the cumulative average of 300 laser shots. The search program ProFound, developed by the Rockefeller University (*80*), was used for protein identification by peptide mass fingerprinting. Spectra were calibrated with trypsin auto-digestion ion peak *m/z* (842.510, 2211.1046) as internal standards.

Statistical analysis

All experimental results were compared by one-way analysis of variance (ANOVA) using the Statistical Package of Social Science (SPSS) program and the data were expressed as means ± standard error (S.E.). Group mean values were considered to be significantly different at $p<0.05$, as determined by the technique of protective least-significant difference (LSD) when ANOVA indicated an overall significant treatment effect, $p<0.05$.

Results and Discussion

Submerged culture of P. baumii

P. baumii grows well under the following conditions: fructose 20 g/L, yeast extract 20 g/L, $CaCl_2$ 0.55 g/L, pH=5.0, and 30 °C (*12*). Under these conditions, the maximum mycelial biomass and EPS obtained in a 5-liter stirred-tank bioreactor were 17.6 and 3.8 g/L, respectively (Fig. 2A).

During fermentation, the morphology of *P. baumii* changed from small pellets with radial growth of filamentous mycelia to small feather-like mycelial clumps. Pellets fragmented rapidly after day 12 due to prolonged agitation, depletion of substrate, changing pH, and oxygen limitation inside the pellet. Subsequently, the mean equivalent diameter dropped from 5.2 mm to 2.6 mm (Fig. 2B). The pellet circularity of *P. baumii* remained unchanged throughout the entire culture period.

The broth rheology in fungal fermentations depends on the morphology and on the yields of mycelial biomass and EPS. The apparent viscosity of the fermentation broth increased rapidly during the early culture period, and then a rapid drop was observed at a later stage of the culture period (Fig. 2C). This drop in viscosity was related to the fragmentation into small feather-like mycelial clumps. The apparent viscosity was influenced more by the mycelial biomass concentration than by the EPS concentration. The culture broth of *P. baumii* had extremely high apparent viscosities. Fig. 2D shows the variations in the consistency index (K) and the flow behavior index (n) of the fermentation broth. The consistency index (K) rose from the beginning of the fermentation for 14 days and then declined. This behavior is quite common for liquid fermentations of mushrooms (*81,82*).

The morphology of fungi during submerged culture is related to their metabolic activities (*81,83,84*). Many investigators claim that the different morphology of fungal mycelia under different initial pH values is one of the most important factors affecting biomass accumulation and metabolite formation (*85,86*). The mycelial morphology of *P. baumii* was very sensitive to the culture pH and culture time (Fig. 3).

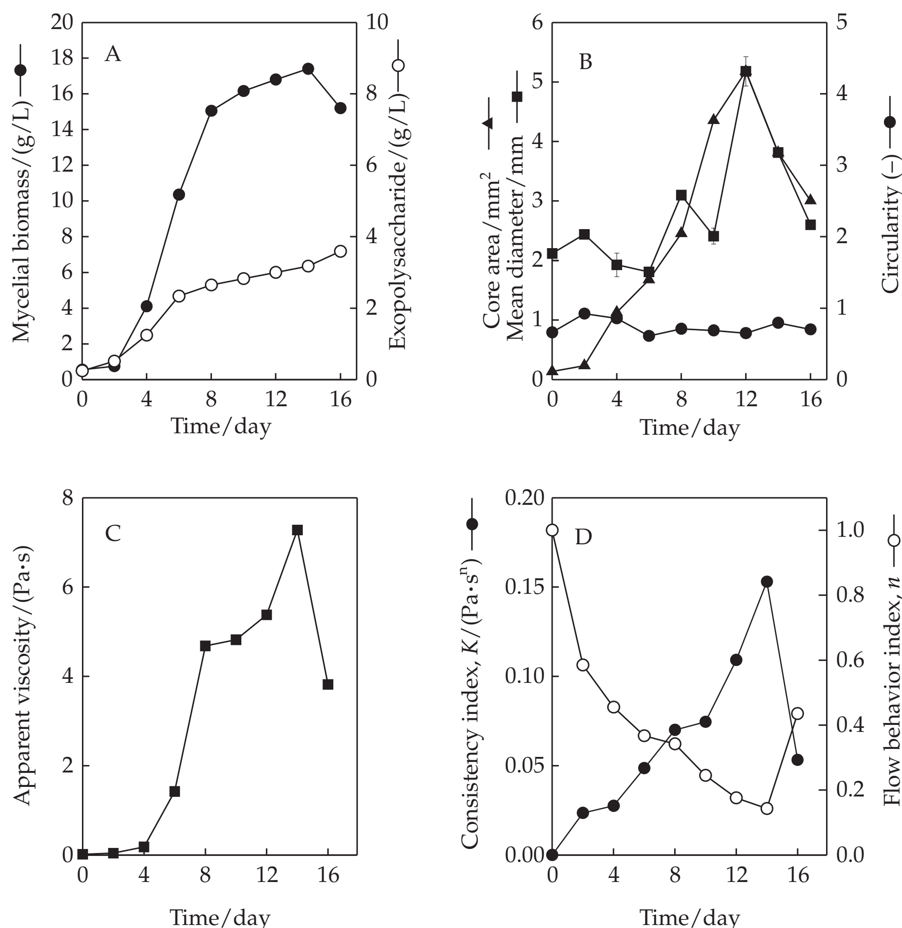

Fig. 2. (A) Typical time profiles of mycelial biomass and exopolysaccharide concentration, (B) mycelial pellet sizes, (C) apparent viscosity, and (D) consistency index and flow behavior index in submerged mycelial culture of *Phellinus baumii*

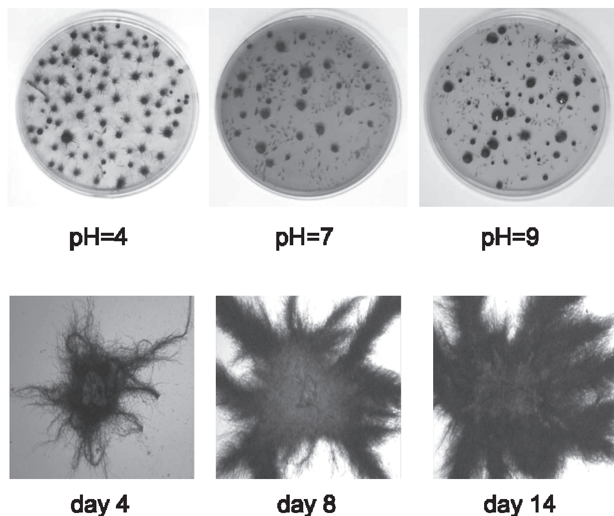

Fig. 3. Morphological changes in mycelia of *Phellinus baumii* in different culture pH (upper panels) and culture time (lower panels) in a stirred-tank bioreactor

Chemical compositions of the EPS

The crude EPS was purified by gel filtration in a Sepharose CL–4B column. Two different heteropolysaccharides and two proteoglycans were eluted. The carbohydrate and protein contents in the crude EPS were 71.0 and 29.0 %, respectively. The protein moiety of the crude EPS consisted mainly of arginine (14.1 %) and glycine (12.0 %), while the carbohydrate moiety consisted mainly of mannose (48.7 %) and arabinose (38.4 %).

Hypoglycemic effect of the EPS

The food intake of the diabetic control rats (Group S) increased by 28.1 %, whereas body mass gain was reduced by about 44.1 % as compared to the normal control rats (Group N). Moreover, STZ-treated rats (Groups S and SP) also had a markedly lower food efficiency ratio than those in the N and NP groups (*16*). Fig. 4 shows the plasma glucose level in the various rat groups over a

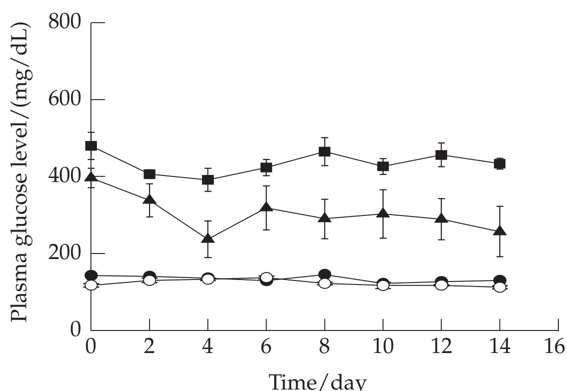

Fig. 4. Plasma glucose levels in the various rat groups during two weeks. Group N (●), Group NP (○), Group S (■), Group SP (▲). EPS and buffer were administrated 48 h after diabetes induction. All data are expressed as mean ± S.E. ($p<0.05$). Data points for Group N and Group NP are incorporated together because they showed no significant difference

14-day period. In the two diabetic groups (S and SP), the initial concentrations of plasma glucose were almost the same, with both groups within the error range. In the diabetic control group (Group S), the plasma glucose level continuously increased during the experimental period, reaching a final level of 610 mg/L. In contrast, the administration of *P. baumii* EPS lowered the plasma glucose level of the Group SP rats by 52 % on day 14, when compared to that of the diabetic control group (Group S). Also, a remarkable reduction of aspartate aminotransferse (AST) and alanine aminotransferse (ALT) levels upon oral administration of *P. baumii* EPS indicates a remedial effect on the liver function. There were no significant differences in the mass of the heart and pancreas, whereas those of the liver, spleen, kidney, and lung were considerably increased in the diabetic groups (Groups S and SP), as compared to Group N (*16*).

The hypoglycemic activity of *P. baumii* EPS produced in submerged culture is better than that of polysaccharides from fruiting bodies or mycelial extracts. Furthermore, the EPS from submerged culture is more practical for use in medical practice because a complicated extraction process is required in order to obtain polysaccharides from the fruiting body and it is difficult to obtain a uniform chemical composition.

The chemical structure and viscosity of mushroom polysaccharides are related to the hypoglycemic activity (*55,56,59,87,88*). Administration of EPS from *P. baumii* counteracted the diabetogenic effect of STZ, which involves the induction of the generation of reactive oxygen species (ROS) in rat islets. EPS treatment reduces the degree of diabetes by reducing STZ-induced destruction of β-cells. The oral administration of *P. baumii* EPS therefore has potential in preventing diabetes, since pancreatic damage induced by environmental chemicals and other factors is a cause of diabetes (*61*).

Effect of EPS on the plasma proteome of diabetic rats

We obtained good resolution of plasma proteins by using a system in which the first dimension separation was carried out in a pH=4–7 IEF strip. The plasma maps obtained from our gels showed a pattern similar to those of The Rat Serum Protein Study Group (*89*). As shown in Fig. 5, less than 500 individual spots were detected, with masses ranging from 6 to 200 kDa and isoelectric points ranging between pH=4–7. Nineteen proteins from these spots were found to be differentially regulated, of which 17 spots were identified as principal diabetes-associated proteins.

The distinct effect of diabetes induction on the pattern of rat plasma proteins includes the downregulation of albumin, Apo E, α_1-inhibitor-3, fetuin β, Gc-globulin, hemopexin, vitronectin, and transthyretin monomer, and the upregulation of Apo A-I, Apo A-IV, ceruloplasmin, α_1-antitrypsin, serine protease inhibitor III, and transferrin. Interestingly, levels of these proteins were restored to those of healthy rats by EPS treatment, although the order of magnitude of the changes differed widely. Two proteins showed distinct differential expression with opposite trends: transthyretin tetramer was significantly downregulated and Ig kappa light chain was significantly upregulated upon diabetes induction; both of

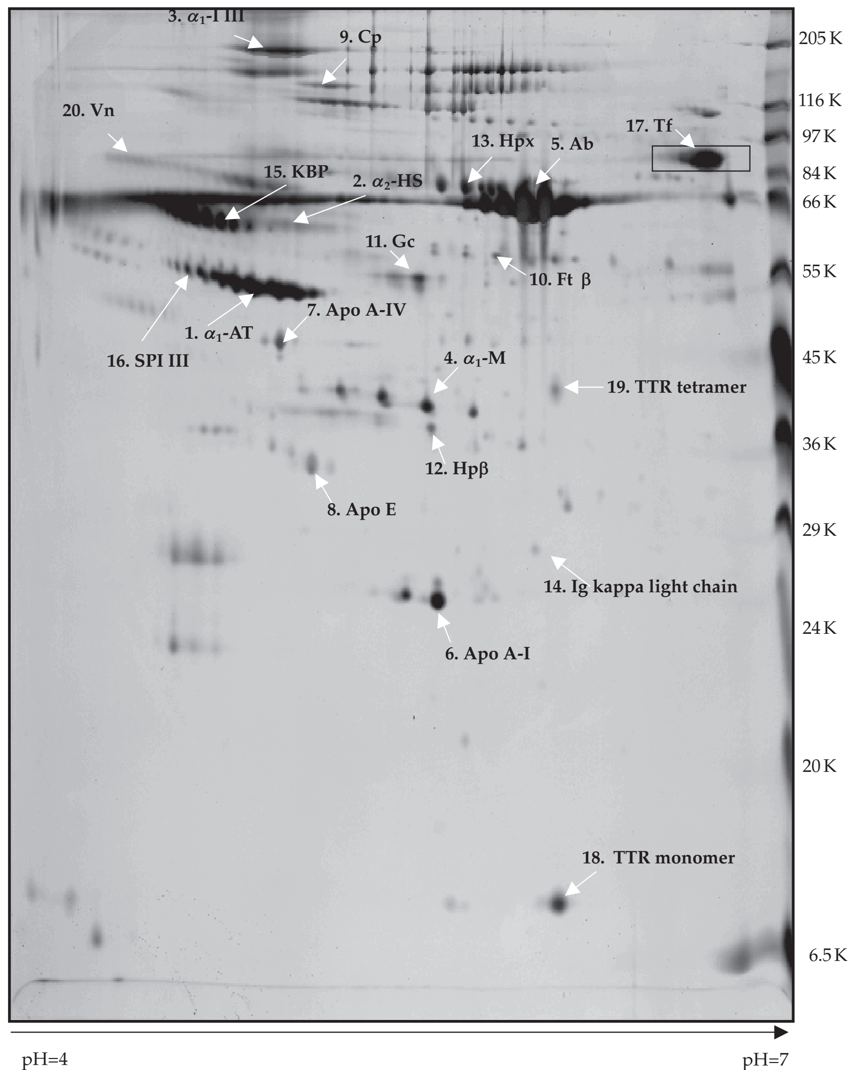

Fig. 5. Silver-stained rat plasma two-dimensional electrophoresis image. Twenty differentially regulated proteins were marked with arrows together with identified major rat plasma proteins. Abbreviations: α_1-I III: α_1-inhibitor III; α_1-AT: α_1-antitrypsin; α_1-M: α_1-macroglobulin; Ab: albumin; Apo: apolipoprotein; Ce: carboxylesterase; Cp: ceruloplasmin; Ct: clusterin; Fn: fibronectin; Ft β: fetuin beta; Gc: Gc-globulin; Hp: haptoglobin; Hpx: hemopexin; Ht: histamine; Ig: immunoglobulin; KBP: kallikrein-binding protein; RBP: retinol-binding protein; SPI: serine protease inhibitor; Tf: transferrin; TTR: transthyretin; TTRP: transthyretin precursor; Vn: vitronectin

these proteins were also normalized to levels typical of those of healthy groups after EPS treatment.

The oxidative stress proteins (Cp and Tf) and proteins related to lipid metabolism (Apo A-I, Apo A-IV, Apo E) were expressed differently in the STZ–induced diabetic rats (Fig. 6). Many investigators suggest that diabetes is connected with marked alterations in the levels and distributions of the major apolipoproteins associated with circulating lipoproteins (*90–93*).

So far, rat plasma has not been commonly used in pharmacological and toxicological research. Our study on rat plasma may stimulate a search for important biomarkers. The model established in our experiment may reflect human insulin-requiring diabetes, which will help us develop biomarkers for human diabetes (*94*). However, it is possible that biomarkers that are discovered using STZ-induced diabetes may be specific for this condition without relevance to natural type 1 diabetes mellitus (T1DM) or other types of human diabetes. Further studies are needed before any conclusions can be made as to which proteins are important for monitoring the progression of diabetes or the relevance of our results to human diabetes.

Our proteomic analysis of rat plasma has enabled us to be the first to report that a fungal polysaccharide contributes to normalization of the levels of transthyretin tetramer, Ig kappa light chain, apo A-I, apo E, haptoglobin, and Gc-globulin, even though the molecular mechanisms for such changes are not clarified yet.

Effect of EPS on the liver proteome of diabetic rats

The liver is one of the central metabolic organs in the body, playing a key role in regulating and maintaining homeostasis. It is therefore not surprising that many investigators have found disease-associated target pro-

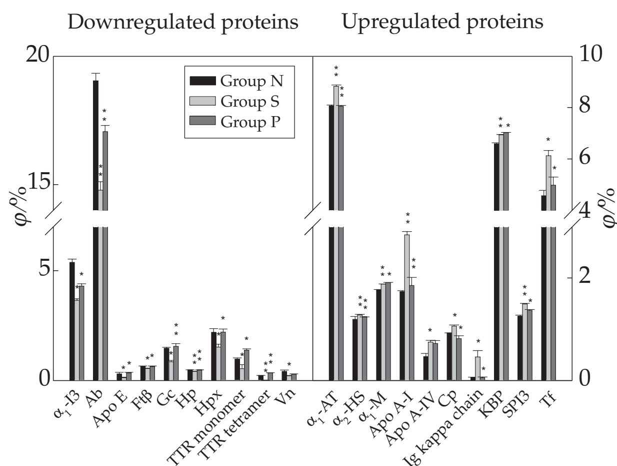

Fig. 6. Alterations in the levels of the significantly regulated rat plasma proteins, where down- and upregulated proteins after diabetes induction are illustrated separately. For each spot, the relative intensity was averaged and expressed as a mean ± S.E. of the three separate experiments
$^*p<0.05$ *vs.* Group N or S, $^{**}p<0.001$ *vs.* Group N or S. Notations for each group are the same as in Fig. 4

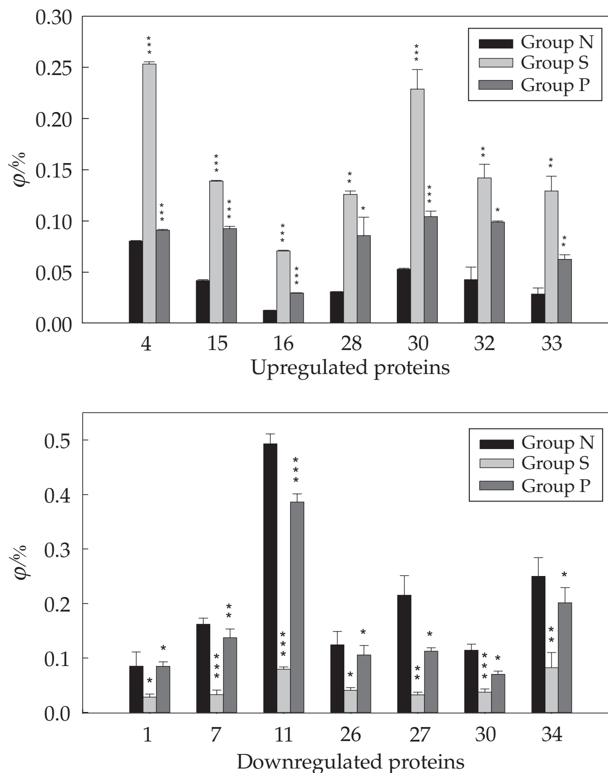

Fig. 7. Proteins up- (U) and down- (D) regulated upon STZ treatment and ameliorated by EPS administration. Each spot intensity volume was processed by background subtraction and total spot volume normalization; the resulting spot volume percentage was used for comparison. All data are expressed as mean ± S.E. ($^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$). Notations for each group are the same as in Fig. 4. Numbers for protein names: U4: carbamoyl phosphate synthetase I (CPS I) precursor; U15: dihydrolipoamide dehydrogenase; U16: methylmalonate semialdehyde dehydrogenase (MMSDH); U28: aldo-keto reductase family 7, member A2 (aflatoxin aldehyde reductase); U30: chain D, structure of glycine *N*-methyltransferase complexed with *S*-adenosylmethionine and acetate; U32: cyclin G-associated kinase (GAK); U33: mitogen-activated protein kinase activator with WD repeats binding protein (MAWDBP); D1: glucose regulated protein, 58 kDa; D7: catalase; D11: cytoplasmic β-actin; D26: mitochondrial H^+-ATP synthase α subunit; D27: peroxiredoxin I (Prx I); D30: nucleoside diphosphate kinase B (NDK B); D34: chain A, solution structure of cellular retinol binding protein (RBP) type-I in complex with all-*trans*-retinol

teins in liver tissues (*95–97*). Diabetes is closely linked to liver dysfunction, as well as to impairment of the pancreas. It is also associated with various structural and functional liver abnormalities, including changes in glycogen metabolism (*98*), lipid metabolism (*99*) and antioxidant status (*100,101*).

In the present proteomic study of diabetes with rat liver, we identified 69 significantly changed proteins (34 upregulated and 35 downregulated). Of those, 12 were unidentified proteins that have not been reported to be related with diabetes mellitus. These numerous diabetes-related changes in protein expression could be associated with the appearance of diabetes-related pathologies. Interestingly, all 69 altered proteins in the diabetic rats were partially or fully restored to levels of those for non-diabetic control rats upon EPS treatment (for details, see Fig. 7). Many of the alterations of protein levels in this study are in accordance with observations noted previously by other investigators, while 16 proteins were associated with diabetes mellitus for the first time.

The relationship between the antidiabetic effect of EPS and the changes in liver protein profiles are still not clear. Therefore, the molecular basis of protein modulation by EPS in diabetic rats should be explored further. The results of the proteomic analysis provide impetus for further molecular and mechanistic studies on the therapeutic action of EPS, and must be explored before EPS can be administered as a possible insulin replacement in the management of diabetes mellitus.

Effect of EPS on the kidney proteome of diabetic rats

The kidney eliminates waste products from the plasma and maintains homeostasis of essential cellular biomolecules. Since nephrology deals mainly with physiology, application of proteomics to renal research is considered to be a good model to demonstrate that proteomic data can contribute to physiology and pathophysiology of many diseases (*102–104*). Diabetic nephropathy is the most important cause of death in insulin-dependent diabetes mellitus (IDDM), as 30–45 % of the patients eventually develop end-stage renal failure (*105–107*). Although current therapies successfully reduce proteinuria and slow the rate of progression of diabetic renal injury, renal failure remains a major complication (*108*). Thus, earlier diagnosis and better understanding of the pathophysiology of diabetic nephropathy are needed to achieve better therapeutic outcomes. Therefore, extensive efforts have been made to delineate the pathogenetic mechanisms involved in diabetic nephropathy (*109–114*).

The two-dimensional electrophoresis image analysis of kidney proteome showed that 291 spots were differentially expressed among the three experimental groups:

142 spots were increased and 149 spots were decreased after induction of diabetes. We excluded those spots that had a relatively low difference in expression level. This left 59 spots, which were applied for identification by MALDI-ToF analysis (data not shown). Consequently, 51 proteins out of 59 spots were identified by peptide mass fingerprinting (PMF). These proteins showed significant differences in expression between normal and diabetic control rats.

Among these 51 proteins, the expression of 26 proteins responded to EPS treatments; the levels of 10 proteins increased after diabetes induction and then decreased again to normal values, while the levels of 16 proteins decreased after diabetes induction and then increased again in response to EPS treatment. In particular, the proteins that were significantly increased after diabetes induction included E2, phospholipid scramblase 3, and 6-pyruvoyl-tetrahydropterin synthase/dimerization cofactor of hepatocyte nuclear factor 1 alpha (Fig. 8). The expression levels of E2 and phospholipid scramblase 3 increased 3.45-fold and 1.67-fold, respectively, in the STZ-induced diabetic group, and their levels were almost restored to those of normal healthy rats after EPS treatment (Fig. 8). The expression level of 6-pyruvoyl-tetrahydropterin synthase/dimerization cofactor of hepatocyte nuclear factor 1 alpha was increased 1.75-fold after diabetes induction, and administration of EPS decreased its level slightly, to 1.45 times the normal value (Fig. 8).

Among the 16 proteins whose expression was decreased by STZ-induced diabetes, α_{2u}-globulin, glycoprotein 56, Hsc70-ps1, long chain alpha-hydroxy acid oxidase, neurogenesin 1, and tropomyosin 3 decreased considerably, but increased again after EPS administration, although to different degrees (Fig. 8). The levels of α_{2u}-globulin, glycoprotein 56, long chain alpha hydroxy acid oxidase, and neurogenesin 1 were only partially restored to the levels of normal healthy rats by EPS therapy. On the other hand, EPS administration restored the levels of Hsc70-ps1 and tropomyosin 3 to values 1.27-fold and 1.31-fold higher than those of normal healthy rats.

Effect of EPS on the pancreas proteome of diabetic rats

Two-dimensional electrophoresis was used to investigate differential protein expression in rat pancreas before and after diabetes induction and after EPS treatment. The first studies using two-dimensional electrophoresis for protein separation in relation to islets, β-cell lines and T1DM were made in the 1980s, and they described changes in the protein expression pattern in pancreatic islets from mice with virus-induced hyperglycemia. In 1987, Nepom *et al.* (*115*) used two-dimensional electrophoresis and immunoprecipitation to analyze HLA (human leukocyte antigen) molecules from T1DM patients and demonstrated that hybrid HLA molecules are associated with heterozygosity. Other investigators have used two-dimensional electrophoresis to describe insulin secretory granule biogenesis, exocytosis and the effect of glucose on β-cells (*116*). More recent studies have analyzed the proteome of pancreatic islets, but have not directly addressed the pathogenesis of T1DM (*117–122*). Furthermore, pancreatic islets have been studied by proteome analysis to investigate the effect of the insulin-sensitizer drug Rosiglitazone on protein expression (*72,73*).

The pancreatic proteome resulted in more than 2000 individual spots. Thirty-four proteins from a total of about 500 matched spots were differentially expressed, of which twenty-six spots were identified as proteins whose expression had previously been associated with

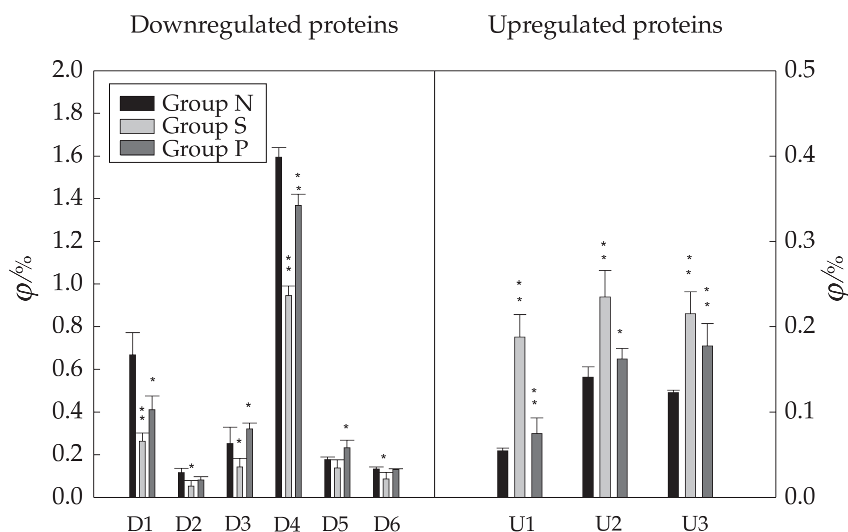

Fig. 8. Alterations in the levels of the significantly regulated rat kidney proteins, where down- and upregulated proteins after diabetes induction are separately illustrated. For each spot, the relative intensity was averaged and expressed as a mean ± S.E. of the three separate experiments. *p<0.05 *vs.* Group N or S, **p<0.001 *vs.* Group N or S. Group notations are the same as in Fig. 4. Abbreviations: D1: α_{2u}-globulin; D2: glycoprotein 56; D3: Hsc70-ps1; D4: long chain alpha hydroxy acid oxidase=FMN-dependent alpha hydroxy acid-oxidizing enzyme; D5: neurogenesin 1; D6: tropomyosin 3; U1: dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex, mitochondrial precursor (dihydrolipoamide succinyltransferase component of 2-oxoglutarate dehydrogenase complex) (E2) (E2K); U2: phospholipid scramblase 3; U3: 6-pyruvoyl-tetrahydropterin synthase/dimerization cofactor of hepatocyte nuclear factor 1 alpha

diabetes (Fig. 9). Of these proteins, ten were upregulated and sixteen were downregulated ($p<0.05$) after diabetes induction, and the altered proteins were restored ($p<0.05$) upon EPS treatment. We have found for the first time that carbonyl reductase (18.6-fold, $p<0.001$) and MAW-DBP (31.4-fold, $p<0.01$) were upregulated upon diabetes induction, and that the levels of these two proteins were completely restored by EPS treatment. Moreover, we obtained eight unidentified proteins that had not been reported to be related with diabetes mellitus (data not shown). These results show that EPS treatment after induction of diabetes can be a useful tool in the search for potential markers for diagnosis and therapeutic manipulation of diabetes mellitus.

How does EPS act as a hypoglycemic agent?

Although little scientific evidence exists, there are several hypotheses to explain the possible mechanisms for hypoglycemic action of fungal EPS in diabetic animals. Firstly, the viscosity of EPS may delay carbohydrate absorption, thereby improving the control of hyperglycemia, hyperinsulinemia, severe insulin resistance, and impaired glucose tolerance of diabetic mice (*123,124*). Secondly, EPS can, at least in part, restore the immunomodulative imbalance caused by diabetes (*e.g.* enhancing IL-6 production from splenic macrophages). Thirdly, EPS may have a beneficial effect on glucose metabolism in the liver of diabetic animals by increasing the activi-

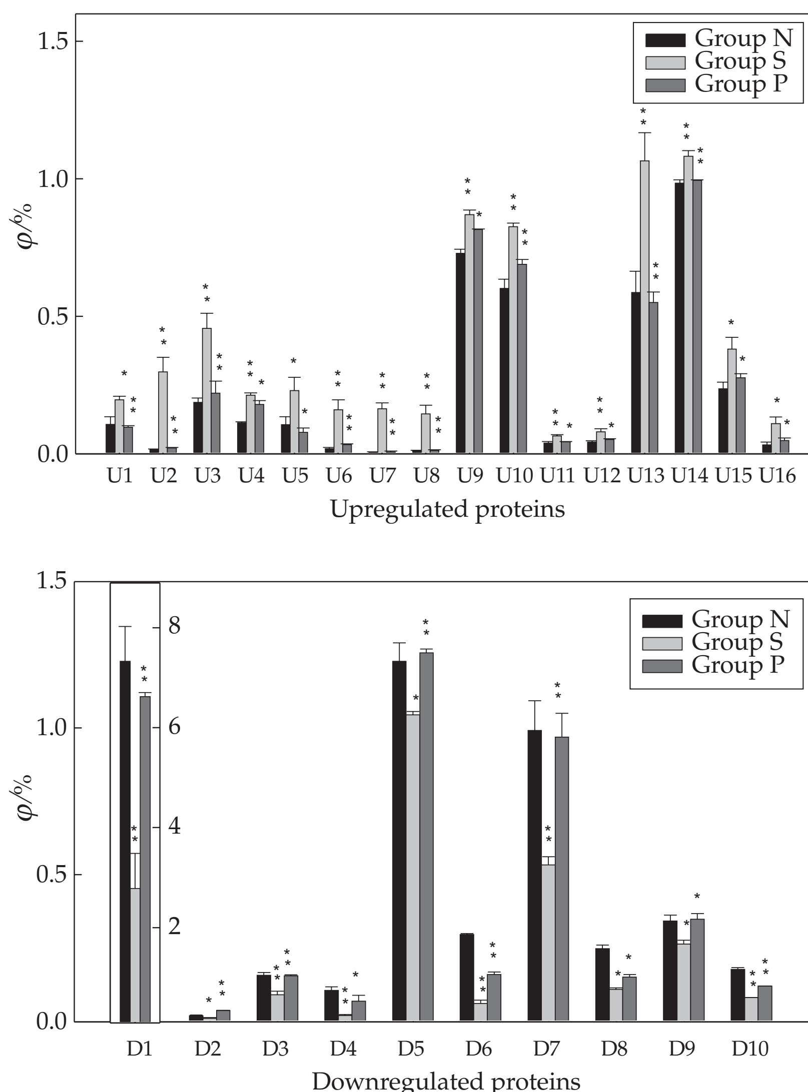

Fig. 9. Alterations in the levels of the significantly regulated rat pancreas proteins, where down- and upregulated proteins after diabetes induction are separately illustrated. For each spot, the relative intensity was averaged and expressed as a mean ± S.E. of the three separate experiments. *$p<0.05$ *vs.* Group N or S, **$p<0.001$ *vs.* Group N or S. Group notations are the same as in Fig. 4. Abbreviations: D1: alpha amylase, pancreatic precursor; D2: alpha amylase precursor; D3: casein kinase 1 alpha; D4: cholesterol esterase preprotein; D5: laminin receptor 1; D6: lithostathine precursor; D7: peroxiredoxin 4; D8: protein disulfide isomerase A3 precursor; D9: similar to WAC; D10: sterol esterase; U1: acetyl-CoA dehydrogenase; U2: carbonyl reductase; U3: destrin; U4: dismutase; U5: glucose regulated protein 58 kDa; U6: homeobox protein Hox-A7; U7: HMG-CoA synthase; U8: MAWD binding protein; U9: myosin light polypeptide 6; U10: nucleotide diphosphate kinase; U11: seven in absentia 1A; U12: similar to alpha glucosidase II, alpha subunit; U13: similar to elastase 3B; U14: similar to eukaryotic translation initiation factor 5A; U15: triacylglycerol lipase; U16: unknown (protein for MGC:91679)

ties of hepatic glucokinase, hexokinase, and glucose-6--phophate dehydrogenase (*125,126*). Finally, EPS may neutralize the ability of STZ to cause β-cell damage. It may even induce regeneration of islet β-cells, given that EPS administered 48 h after STZ treatment significantly restored serum insulin and glucose levels. More detailed biochemical studies of EPS action such as potential antioxidant activity will be necessary to elucidate the mechanism by which EPS alleviates diabetes induced by STZ. These complicated effects of EPS on diabetes can involve the differential expression of many proteins, as described in the present study.

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