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Differential Expression of Kidney Proteins in Streptozotocin-induced Diabetic Rats in Response to Hypoglycemic Fungal Polysaccharides

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Abstract Diabetic nephropathy remains a major cause of morbidity and mortality in the diabetic population and is the leading cause of end-stage renal failure. Despite current therapeutics including intensified glycemic control and blood pressure lowering agents, renal disease continues to progress relentlessly in diabetic patients, albeit at a lower rate. Since synthetic drugs for diabetes are known to have side effects, fungal mushrooms as a natural product come into preventing the development of diabetes. Our previous report showed the hypoglycemic effect of extracellular fungal polysaccharides (EPS) in streptozotocin (STZ)-induced diabetic rats. In this study, we analyzed the differential expression patterns of rat kidney proteins from normal, STZ-induced diabetic, and EPS-treated diabetic rats, to discover diabetes-associated proteins in rat kidney. The results of proteomic analysis revealed that up to 500 protein spots were visualized, of which 291 spots were differentially expressed in the three experimental groups. Eventually, 51 spots were statistically significant and were identified by peptide mass fingerprinting. Among the differentially expressed renal proteins, 10 were increased and 16 were decreased significantly in diabetic rat kidney. The levels of different proteins, altered after diabetes induction, were returned to approximately those of the healthy rats by EPS treatment. A histopathological examination showed that EPS administration restored the impaired kidney to almost normal architecture. The study of protein expression in the normal and diabetic kidney tissues enabled us to find several diabetic nephropathy-specific proteins, such as phospholipids scramblase 3 and tropomyosin 3, which have not been mentioned yet in connection with diabetes.

Keywords: Diabetes mellitus, fungal polysaccharides, kidney proteins, proteomics, renal proteins, streptozotocin

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During the past few years, proteomics has been extensively applied to various biomedical fields. Most applications deal mainly with expression proteomics to determine global expression profiles of proteins in cells, tissues, and organs during normal and disease states and to approach to new drug development [29, 36, 37]. Current applications of renal proteomics are to better understand renal physiology, to explore the complexity of disease mechanisms, and to identify novel biomarkers and new therapeutic targets [17, 42, 54].

The kidney is an organ that eliminates waste products from the plasma, and maintains homeostasis of essential cellular biomolecules. Because 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 the physiology and pathophysiology of many diseases [63–65].

Diabetes mellitus is a common metabolic disorder in which the hyperglycemic state seems to adversely affect the homeostasis of various organ systems, frequent lesions including diabetic angiopathy, neuropathy, retinopathy, and nephropathy [2, 8, 28, 48].

Diabetic nephropathy is the most important cause of death in insulin-dependent diabetes mellitus [40], as 30%–45% of the patients eventually develop end-stage renal failure [5, 44]. Although current therapies successfully reduce proteinuria and slow the rate of progression of diabetic renal injury, renal failure remains a major diabetic complication [40]. 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 [11, 14, 21, 33, 47].

Multiple biochemical mechanisms for diabetic nephropathy have been proposed: inappropriate activation of the protein kinase C-mitogen-activated protein kinase pathway; activation of the polyol pathway; increased accumulation of advanced

glycation end products; and influence of oxidative stress [3, 12, 19, 24, 31, 58, 60].

Recently, the search for appropriate hypoglycemic agents has been focused on various traditional medicinal sources, owing to the various side effects and toxicity raised by current synthetic antidiabetic drugs [66, 74]. In this regard, mushrooms are potent exemplary sources of natural medicines with antidiabetic activity. Many investigators have endeavored to study the hypoglycemic effect of either the fruiting body or the mycelia of various edible and medicinal fungi [22, 23, 25, 75–77].

In our previous consecutive studies [20, 26, 27], we have found that the fungal extracellular polysaccharides (EPS) produced by mycelial culture of a medicinal mushroom *Phellinus baumii* has a strong antidiabetic activity against streptozotocin (STZ)-induced diabetic rats. In addition, it was also observed that the EPS significantly altered and particularly normalized dysregulated plasma proteins in STZ-induced diabetic rats.

In the present study, in an attempt to investigate differential expression of renal proteins, we launched a proteomic study of diabetic rat kidney in response to EPS therapy.

Materials and Methods

Preparation of the EPS

The basidiomycetous fungus *Phellinus baumii* was isolated by the authors from a mountainous district in Korea. The submerged culture of *P. baumii* was performed as described previously [18]. Briefly, the mycelial culture was carried out in the medium containing 20 g/l fructose, 20 g/l yeast extract, and 0.55 g/l $CaCl_2$ at 30°C for 14 days in a stirred-tank bioreactor. The carbohydrate and protein contents in the crude EPS were 71.0% and 29.0%, respectively. The crude EPS consisted of mainly arginine (14.1%) and glycine (12.0%) in the protein moiety and mainly mannose (87.5%) and galactose (7.0%) in the carbohydrate moiety.

Animal Experiments

Male Sprague-Dawley rats (Daehan Experimental Animals, Seoul, Korea) weighing 130–150 g at 5 weeks of age were used for diabetic studies. The animals were housed in individual stainless steel cages in an air-conditioned room (23±2°C with 55±5% humidity) under a 12:12-h light-dark cycle. A commercial pellet diet (Sam Yang Co., Seoul, Korea) and water were provided throughout the experiments. After one week of acclimatization, the rats were subjected to a 16-h fast. Diabetes was induced by intravenous injection of streptozotocin (50 mg/kg body weight, dissolved in 0.01 M sodium citrate buffer, pH 4.5). Control rats were injected with vehicle alone. Diabetes was verified 48 h later by evaluating blood glucose levels with the use of glucose oxidase reagent strips (Lifescan, Milpitas, CA, U.S.A.). Rats with a blood glucose level ≥300 mg/dl (16.7 mmol/l) were considered to be diabetic. All the animals were randomly divided into three groups with six animals in each group: normal healthy control group (N group), where normal rats received 0.9% NaCl solution; STZ-induced diabetic group (STZ group), where diabetic rats were treated with 0.9% NaCl solution; EPS-treated diabetic group (EPS group), where diabetic rats were treated with EPS at the level of 200 mg/kg body weight daily for 14 days. These experiments were approved by the Committee for Laboratory Animal Care and Use, Daegu University. All procedures were conducted in accordance with the "Guide for the Care and Use of Laboratory Animals" published by the National Institutes of Health.

Oral Glucose Tolerance Test

After overnight fasting, blood samples (0.2 ml) were taken from the rats by orbital sinus puncture. Glucose solutions were administered orally (2 g/kg body weight), and blood samples were taken at −30 min to +180 min before and after glucose administration for analysis of the glucose levels using glucose oxidase reagent strips (Lifescan). Plasma insulin levels were measured using a rat insulin kit (SHIBAYAGI, Gunma, Japan) with rat insulin as a standard, for quantitation of insulin by the sandwich technique of enzyme immunoassay.

Preparation of the Kidney Protein Sample

Kidney tissues were removed immediately after sacrifice, and then excessive blood contents of kidney tissues were eliminated using a cold NaCl solution. Kidney tissues were 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% CHAPS, 1 mM PMSF, 20 mM DTT, 2% 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 the methanol/chloroform method prior to electrophoretic separation. The precipitate was resuspended in rehydration buffer, and then kept at −80°C until use.

Two-dimensional Gel Electrophoresis (2-DE)

2-DE was performed on individual samples from the six animals in each group and run three times per sample to minimize gel-to-gel variation. IPG IEF of samples was carried out on nonlinear pH 3–10, 18-cm IPG DryStrips (Amersham Biosciences, Little Chalfont, Buckinghamshire, England) in the PROTEIN IEF cell (Bio-Rad, Hercules, CA, U.S.A.) using the protocol suggested by the manufacturers. For a short time, 50 µg (~8 µl) of solubilized kidney protein was mixed into 342 µl of rehydration solution. After focusing,

gel strips were equilibrated in a solution containing 6 M urea, 2% SDS, 1% DTT, 30% glycerol, and 50 mM Tris (pH 6.8) for 15 min, followed by incubation in the same solution, but replacing DTT with 2.5% iodoacetamide, for an additional 15 min. The equilibrated IPG strips were then gently rinsed with electrophoresis buffer and then placed on a 20×20 cm 12% polyacrylamide gel for resolution in the second dimension. The 2-D SDS-PAGE was performed sequentially at a constant voltage of 20 mA per gel for 10 h. After SDS-PAGE, the separated gels were visualized using silver staining.

Image Capture and Analysis

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 export and analysis. Comparison of the images was performed using a modified version of ImageMaster 2D software V4.95 (Amersham Biosciences). A reference gel was selected at random from the gels of the control group set. Detected spots from the other gels in the data set were then matched to those in the selected reference gel. The relative optical density and relative volume were also calculated in order to correct for differences in gel staining. Differentially expressed proteins were found by differential analysis and Student's *t*-test. A $p<0.05$ was considered significant. Each spot intensity volume was processed by background subtraction and total spot volume normalization; the resulting spot volume percentage was used for comparison.

Enzymatic Digestion of Proteins in Gels

Protein spots were enzymatically digested in-gel in a manner similar to that previously described by Shevchenko *et al.* [56] using modified porcine trypsin. Briefly, gel pieces were washed with 50% acetonitrile to remove SDS, salt, and stain; 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 the addition of 5 μl of 0.5% trifluoroacetic acid. Tryptic peptides were recovered by combining the aqueous phase from several extractions of gel pieces with 50% aqueous acetonitrile. After concentration, the peptide mixture was desalted using C_{18} ZipTips (Millipore Co., Bedford, MA, U.S.A.), 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% aqueous acetonitrile and 1 μl of mixture spotted onto a target plate.

Protein Identification

Protein analysis was performed using an Ettan MALDI-TOF (Amersham Biosciences). The peptides were evaporated with a 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 (http://129.85.19.192/profound_bin/WebProFound.exe), was used for protein identification by peptide mass fingerprinting (PMF). Spectra were calibrated with the trypsin autodigestion ion peak *m/z* (842.510, 2211.1046) as internal standards.

Histochemistry for Light Microscopy

Formalin-fixed, paraffin wax-embedded renal tissue samples were prepared from control and experimental groups using routine procedures [62]. Briefly, tissue samples were fixed in 10% formalin solution. They were dehydrated through a graded ethanol series, cleared in xylene, and embedded in paraffin wax. Sections were cut at 4 μm thickness, deparaffinized, and rehydrated. Tissue blocks were chosen carefully after histological assessment of the sections stained with hematoxylin and eosin.

Statistical Analysis

All experimental groups were compared by one-way analysis of variance (ANOVA) using the Statistical Package of the Social Science (SPSS) program. All data were expressed as means±SE. Group means were considered to be significantly different at $p<0.05$, as determined by the technique of protective least-significant difference (LSD) when the ANOVA indicated an overall significant treatment effect, $p<0.05$. In 2-DE gel analysis, differential analysis and Student's *t* test ($p<0.05$) using the related volume of each spot (>0.04%) allowed for the detection of significantly up- and downregulated polypeptides with a minimum ratio of two.

RESULTS

Hypoglycemic Effects of the EPS

Prior to the proteomics study, the effect of the fungal polysaccharides on the plasma glucose level was investigated in the STZ-induced diabetic rats. The results revealed that orally administrated EPS, when given 48 h after STZ treatment, exhibited an excellent hypoglycemic effect, lowering the average plasma glucose level of the EPS-fed diabetic rats to about 55% of the control levels (Fig. 1A). Figs. 1B and 1C show the changes in the levels of blood glucose and insulin, respectively, during oral glucose tolerance test (2 g/kg body weight). The diabetic control rats showed significant increase in the blood glucose at 60 min, whereas blood glucose concentration in EPS-treated rats was remarkably lowered at 60 min after glucose administration ($p<0.05$). As shown in Fig. 1C, the rise of plasma insulin observed in control rats was abolished in STZ-treated rats, explaining the hypoglycemic effect of the EPS. These results prompted us to conduct further proteomic studies.

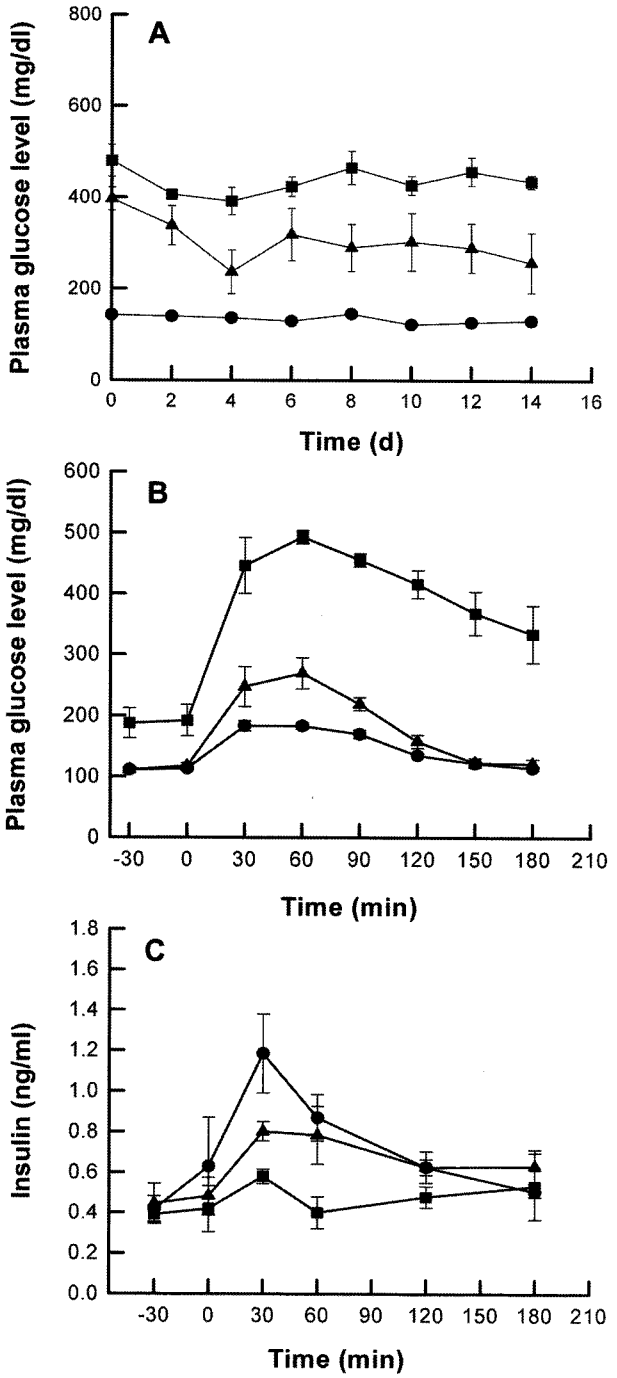

Fig. 1. Effect of fungal exopolysaccharides (EPS) on (**A**) the plasma glucose level in STZ-induced diabetic rats during two weeks. Effect of the EPS on (**B**) the plasma level and (**C**) the insulin level on oral glucose tolerance test in STZ-induced diabetic rats.

N Group (●): normal healthy control rat group received 0.9% NaCl solution; STZ Group (■): STZ-induced diabetic rat group treated with 0.9% NaCl solution; EPS Group (▲): EPS-treated diabetic rat group treated with EPS at the level of 200 mg/kg body weight daily for 14 days. EPS and buffer were administrated 48 h later. All data were expressed as mean±SE ($p<0.05$). Data points for N Group in Fig. 1A are incorporated together with no significant difference.

2-DE Gel Separation and Protein Identification

To investigate the effect of EPS on the alterations of rat kidney proteins, the kidney proteomes of the three experimental groups (*e.g.*, N, STZ, and EPS groups) were arrayed on a nonlinear pH 3–10 2-DE gel system, and the proteins were identified by PMF MALDI-TOF. Up to 500 protein spots were visualized on each 2-DE gel by sliver staining (Fig. 2). The 2-DE image analysis of kidney proteome showed that 291 spots were differentially expressed among the three experimental groups, where 142 spots were increased and 149 spots were decreased after diabetes induction. We applied 59 spots, excluding statistically nonsignificant spots, for identification by MALDI-TOF analysis (see the marked proteins in Fig. 2). Consequently, 51 proteins out of 59 spots were identified by PMF. Positions for all of these identified proteins on 2-D gels were in the expected range of their theoretical isoelectric points (p*I*) and molecular weights (M_w). All identified proteins in the proteome map are summarized in Table 1.

Differential Expression of the Kidney Proteins

A silver stained 2-DE map in Fig. 2 shows the positions of putative markers identified by differential expression. A total of 51 protein spots were differentially expressed in diabetic rats when compared with the normal healthy rats.

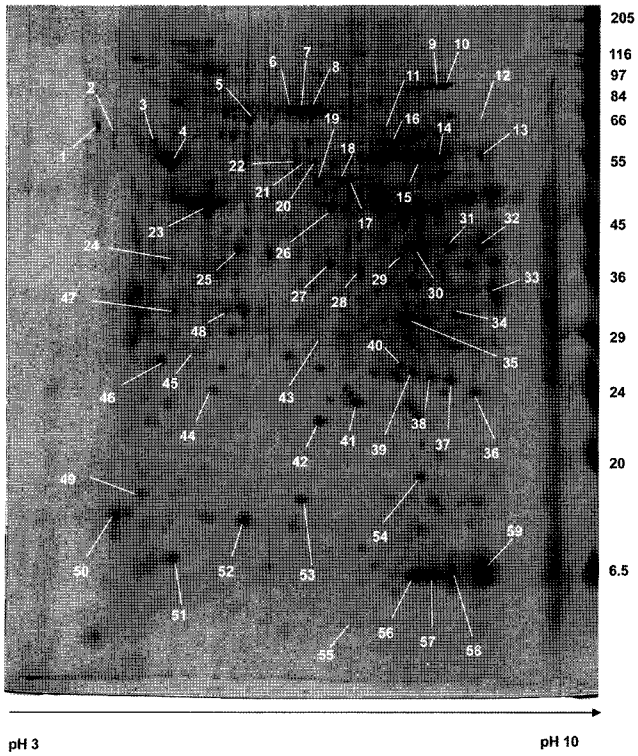

Fig. 2. A silver stained 2-DE map showing positions of putative markers identified by differential expression.

Details of these proteins are listed in Table 1.

Table 1. List of identified proteins that were differentially expressed upon diabetes induction and treatment of fungal polysaccharides.

Spot No.[a]	Acc. No.[b]	Protein identity[c]	Sequence coverage[d] (%)	*pI*[e]	M_w[f] (kDa)
1	gi|33340011	Beta-galactosidase-like protein isoform 2	12	9.4	72.82
2	gi|41054820	Neurogenesin 1	13	9.5	60.82
3	gi|30582091	Gonadotropin-regulated testicular RNA helicase; GRTH	8	5.7	55.29
4	gi|6729935	Chain B, rat liver F1-ATPase	46	4.9	51.33
5	gi|56385	Hsc70-ps1	26	5.4	71.14
6, 7, 8	gi|113580	Serum albumin precursor [contains: neurotensin-related peptide (NRP)]	14, 14, 16	6.1	70.70
9, 10	gi|38541404	Mitochondrial aconitase (nuclear *aco2* gene)	25, 32	8.2	86.16
11, 12	gi|34867525	Similar to CCTtheta, theta subunit of the chaperonin containing TCP-1 (CCT)	14	5.4	60.14
13	gi|6729934	Chain A, rat liver F1-ATPase	15	8.4	55.38
14	gi|34867320	Hypothetical protein XP_346778	19	8.8	58.24
15	gi|56200	Unnamed protein product	32	8.3	61.75
16	gi|550418	Carboxylesterase ES-4	31	6.3	62.66
17, 18	gi|50926833	Eno1 protein	36, 47	6.2	47.45
19	gi|22096350	Alpha enolase (2-phospho-D-glycerate hydrolyase) (nonneural enolase) (NNE) (enolase 1)	18	5.8	47.53
20	gi|34857203	Similar to hypothetical protein FLJ12572	15	9.2	46.09
21	gi|266684	Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex, mitochondrial precursor (dihydrolipoamide succinyltransferase component of 2-oxoglutarate dehydrogenase complex) (E2) (E2K)	9	8.4	47.68
22	gi|34867155	Similar to ubiquitin-associated protein	10	5.0	50.19
23	gi|57574	Unnamed protein product	25	5.3	42.12
24	gi|37361884	LRRGT00099	29	9.2	43.17
25	gi|55575	Beta-actin	18	5.3	42.06
26	gi|34865868	Aminoacylase 1	37	6.0	46.07
27	gi|37590235	Malate dehydrogenase 1	29	5.9	36.64
28	gi|34861584	Similar to rod outer segment membrane protein 1	8	5.5	37.73
29	gi|1806278	Glycoprotein 56	21	9.0	44.26
30	gi|37590783	Aldo-keto reductase family 1, member A1	34	6.8	36.72
31, 32	gi|238482	Long-chain alpha-hydroxy acid oxidase=FMN-dependent alpha-hydroxy acid-oxidizing enzyme {E.C. 1.1.3.15} [rats, kidney, peptide, 352 aa]	26, 30	8.3	39.51
33	gi|48734887	Vdac1 protein	21	8.5	32.29
34	gi|413911	Cyclin E	19	5.7	46.03
35	gi|41350889	Pgam1 protein	43	6.7	28.93
36	gi|56691	Unnamed protein product	20	9.3	24.89
37	gi|34849845	Glutathione S-transferase, pi 2	24	6.9	23.65
38	gi|136708	Ubiquinol-cytochrome *c* reductase iron-sulfur subunit, mitochondrial precursor (Rieske iron-sulfur protein) (RISP)	15	9.2	27.96
39	gi|57029	H^+-transporting ATP synthase	29	7.0	25.64
40	gi|27720723	Similar to glutathione S-transferase 8 (GST 8-8) (CHAIN 8) (GST CLASS-ALPHA)	41	6.8	25.55
41	gi|34849851	Peroxiredoxin 1	45	8.7	22.32
42	gi|37590239	Unknown (protein for MGC:72861)	41	6.2	18.80
43	gi|27676450	Similar to HSCO protein	24	6.6	28.17
44	gi|34932471	Similar to KIAA0437	25	9.1	24.43
45	gi|128867	NADH-ubiquinone oxidoreductase 24 kDa subunit, mitochondrial precursor	35	6.0	26.85
46	gi|34876811	Similar to golli-interacting protein	20	5.9	33.64
47	gi|34862189	ATP synthase, H^+ transporting, mitochondrial F1 complex, beta polypeptide	19	5.1	56.33
48	gi|112444	Tropomyosin 3, fibroblast - rat	25	4.7	32.83
49	gi|45154722	Phospholipid scramblase 3	11	6.2	32.43
50	gi|34857503	Similar to carbohydrate (chondroitin) synthase 1	12	9.5	91.82

Table 1. Continued.

Spot No.[a]	Acc. No.[b]	Protein identity[c]	Sequence coverage[d] (%)	*pI*[e]	M_w[f] (kDa)
51	gi|24233541	Cytochrome *c* oxidase, subunit Va	22	6.1	16.34
52	gi|204264	α_{2u}-Globulin	48	5.4	17.34
53	gi|818029	Dismutase	26	5.9	15.73
54	gi|127984	Nucleoside diphosphate kinase B (NDK B) (NDP kinase B) (P18)	47	6.9	17.38
55	gi|34852500	6-Pyruvoyl-tetrahydropterin synthase/dimerization cofactor of hepatocyte nuclear factor 1 alpha	30	6.3	12.04
56, 57	gi|47168637	Chain B, crystal structure of perchloric acid soluble protein-A translational inhibitor	40	7.8	14.21
58, 59	gi|34849618	Hemoglobin beta-chain complex	65, 73	8.0	16.08

[a]Spot No. was defined according to spot positions in 2-DE gel indicated as in Fig. 2.
[b]Acc. No.: NCBInr database accession number.
[c]Protein identity: name of each matched protein from the NCBInr database.
[d]Sequence coverage: percent of identified sequence to the complete sequence of the known protein.
[e]p*I*: theoretical isoelectric point of the matching protein.
[f]M_w: theoretical molecular weight of the matching protein in kDa.

Table 2 shows the results of alterations in rat kidney proteins, both upon diabetes induction and EPS treatment. Among the differentially expressed proteins, the levels of 10 proteins were increased and 16 were decreased after diabetes induction. Of these, three of the significantly increased proteins after diabetes induction included E2, phospholipid scramblase 3, and 6-pyruvoyl-tetrahydropterin synthase/dimerization cofactor of hepatocyte nuclear factor 1 alpha (Fig. 3). The expression levels of E2 and phospholipid scramblase 3 were significantly increased by 3.45-fold and 1.67-fold, respectively in the STZ-induced diabetic group, and their levels were considerably restored to those of normal healthy rats after EPS treatment (Table 2). In addition, the expression level of 6-pyruvoyl-tetrahydropterin synthase/dimerization cofactor of hepatocyte nuclear factor 1 alpha was increased by 1.75-fold after diabetes induction, and its level was slightly decreased to 1.45-fold by EPS administration (Table 2).

Among the 16 proteins decreased by STZ-induced diabetes, α_{2u}-globulin, glycoprotein 56, Hsc70-ps1, long-chain alpha-hydroxy acid oxidase, neurogenesin 1, and tropomyosin 3 were considerably decreased. In turn, after EPS administration, they increased in concentration to a different magnitude (Fig. 4).

The levels of α_{2u}-globulin, glycoprotein 56, long chain alpha-hydroxy acid oxidase, and neurogenesin 1 were also decreased after diabetes induction, and these levels were partially restored to the levels of normal healthy rats by EPS therapy (Table 2). Hsc70-ps1 and tropomyosin 3 were also decreased after diabetes induction, and were increased to 1.27-fold and 1.31-fold, respectively, by EPS administration (Table 2).

Histological Study

We examined histopathologically stained sections of rat kidney cortices obtained from the three experimental groups, using light microscopy. The kidney of normal healthy rat showed obviously normal morphologies of glomeruli and tubules (Fig. 5A). It was observed that the kidney of STZ-induced diabetic rat showed glomerular thickening (see the arrow a in Fig. 5B), and the glomeruli displayed some cellular proliferation with fibrosis (see the arrow b in Fig. 5B). The glomerulus of the STZ-induced diabetic rats showed slight glomerular hypertrophy and hyaline degeneration as compared with normal healthy and EPS-treated rats (see the arrow c in Fig. 5B). In contrast, the impaired glomeruli in the diabetic rats were restored to that of normal healthy rats after EPS therapy (Fig. 5C).

Discussion

Many researchers have investigated proteomic analysis using not only whole kidney tissue but also glomerular podocyte, post-mitochondrial fraction, and urine sample [1, 28, 32, 36, 49, 73]. Recently, Thongboonkerd [63] suggested that alterations in the renal elastin-elastase system in type 1 diabetic nephropathy, identified by proteomic analysis, indicate the potential value of proteomic analysis in defining pathophysiology. More importantly, a variety of markers of diabetic nephropathy were found with urinary sample from many diabetic subjects, which included albumin, transferrin, fibronectin, and so on [16]. Meanwhile, efforts to find markers of renal failure in diabetes using whole kidney tissue have not been extensively made so far.

Current therapy aiming to halt the progression of renal damage in established diabetic nephropathy is limited to antihypertensive drugs, especially angiotensin-converting enzyme inhibitors and angiotensin receptor blockers [6]. Although this therapy effectively slows the rate of progression of diabetic renal injury, renal failure remains a common

Table 2. Differently expressed rat kidney proteins upon diabetes induction and ameolioration by EPS treatment.[a]

Protein identity	Alterations (Vol %)[b]		
	N	STZ (STZ/N)	EPS (EPS/N)
Upregulated proteins			
ATP synthase, H^+ transporting, mitochondrial F1 complex, beta polypeptide	0.1918±0.0091	0.2661±0.0323 (1.39)	0.2134±0.0541 (1.11)
Cytochrome *c* oxidase, subunit Va	0.4983±0.0101	0.6602±0.0282 (1.32)	0.5725±0.0201 (1.15)
Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex, mitochondrial precursor (dihydrolipoamide succinyltransferase component of 2-oxoglutarate dehydrogenase complex) (E2) (E2K)	0.0544±0.0036	0.1878±0.0263 (3.45)	0.0749±0.0181 (1.38)
LRRGT00099	0.1126±0.0196	0.1363±0.0137 (1.21)	0.1061±0.0159 (0.94)
Phospholipid scramblase 3	0.1408±0.0120	0.2348±0.0308 (1.67)	0.1621±0.0124 (1.15)
6-Pyruvoyl-tetrahydropterin synthase/dimerization cofactor of hepatocyte nuclear factor 1 alpha	0.1228±0.0029	0.2150±0.0259 (1.75)	0.1775±0.0263 (1.45)
Similar to golli-interacting protein	0.2996±0.0162	0.3806±0.0510 (1.27)	0.3067±0.0227 (1.02)
Similar to HSCO protein	0.1108±0.0065	0.1775±0.0476 (1.60)	0.0961±0.0155 (0.87)
Similar to ubiquitin-associated protein	0.1653±0.0073	0.2323±0.0620 (1.41)	0.1452±0.0211 (0.88)
Vdac1 protein	0.8659±0.2147	1.1071±0.3524 (1.28)	0.8818±0.2005 (1.02)
Downregulated proteins			
α_{2u}-Globulin	0.6680±0.1033	0.2630±0.0384 (0.39)	0.4109±0.0642 (0.62)
Aminoacylase 1	0.4739±0.0617	0.3007±0.0661 (0.64)	0.3651±0.0356 (0.77)
Eno1 protein	0.6491±0.0403	0.4912±0.0288 (0.76)	0.5478±0.0226 (0.84)
Glutathione S-transferase, pi 2	0.3549±0.0412	0.2597±0.0123 (0.73)	0.3198±0.0097 (0.90)
Glycoprotein 56	0.1165±0.0203	0.0526±0.0265 (0.45)	0.0821±0.0157 (0.70)
Hemoglobin beta-chain complex	5.1280±0.6074	3.9112±0.0475 (0.76)	4.1172±0.4685 (0.80)
Hsc70-ps1	0.2528±0.0759	0.1425±0.0408 (0.56)	0.3201±0.0282 (1.27)
Long-chain alpha-hydroxy acid oxidase=FMN-dependent alpha-hydroxy acid-oxidizing enzyme {E.C. 1.1.3.15} [rats, kidney, peptide, 352 aa]	1.5949±0.0441	0.9452±0.0457 (0.59)	1.3682±0.0532 (0.86)
NADH-ubiquinone oxidoreductase 24 kDa subunit, mitochondrial precursor	0.1189±0.0182	0.0946±0.0090 (0.80)	0.1584±0.0221 (1.33)
Neurogenesin 1	0.1329±0.0098	0.0861±0.0314 (0.65)	0.1306±0.0035 (0.98)
Peroxiredoxin 1	0.5008±0.0372	0.3553±0.0672 (0.71)	0.4812±0.0355 (0.96)
Serum albumin precursor [contains: neurotensin-related peptide (NRP)]	1.7480±0.0599	1.2074±0.2085 (0.69)	1.6563±0.0913 (0.95)
Similar to hypothetical protein FLJ12572	0.1542±0.0091	0.1078±0.0287 (0.70)	0.1885±0.0480 (1.22)
Similar to glutathione S-transfer ASE 8 (GST 8-8) (CHAIN 8) (GST CLASS-ALPHA)	0.2561±0.0630	0.1940±0.0350 (0.76)	0.3122±0.1479 (1.22)
Tropomyosin 3, fibroblast - rat	0.1773±0.0126	0.1372±0.0381 (0.77)	0.2324±0.0350 (1.31)
Ubiquinol-cytochrome *c* reductase iron-sulfur subunit, Mitochondrial precursor (Rieske iron-sulfur protein) (RISP)	0.3104±0.0132	0.2038±0.0407 (0.66)	0.2779±0.0535 (0.90)

[a]Notations for experimental group division: N Group, normal healthy control rat group received 0.9% NaCl solution; STZ Group, STZ-induced diabetic rat group treated with 0.9% NaCl solution; EPS Group, EPS-treated diabetic rat group treated with *Phellinus baumii* EPS at the level of 200 mg/kg body weight using an oral zoned daily for 14 days.

[b]For each protein, the relative intensity was averaged and expressed as a mean±SE of three separate experiments.

complication. Defining the pathophysiologic mechanisms of diabetic nephropathy is necessary to identify new targets for therapeutic intervention. In this regard, the EPS developed in this study can be a potent candidate to alleviate the renal damage in diabetic nephropathy.

Many researchers compared proteins altered between normal and diabetic rat kidney to find novel biomarkers of diabetic nephropathy and other kidney diseases [64]. There are a few reports describing the comparative protein expression between the normal and diabetic or drug-treated states. However, our approach to mining potential novel biomarkers of diabetic nephropathy was to identify proteins showing altered expression in STZ-induced diabetic rat kidneys in response to therapy of fungal polysaccharides.

In the present study, 51 proteins of interest were identified in our kidney proteome map, 26 of which were

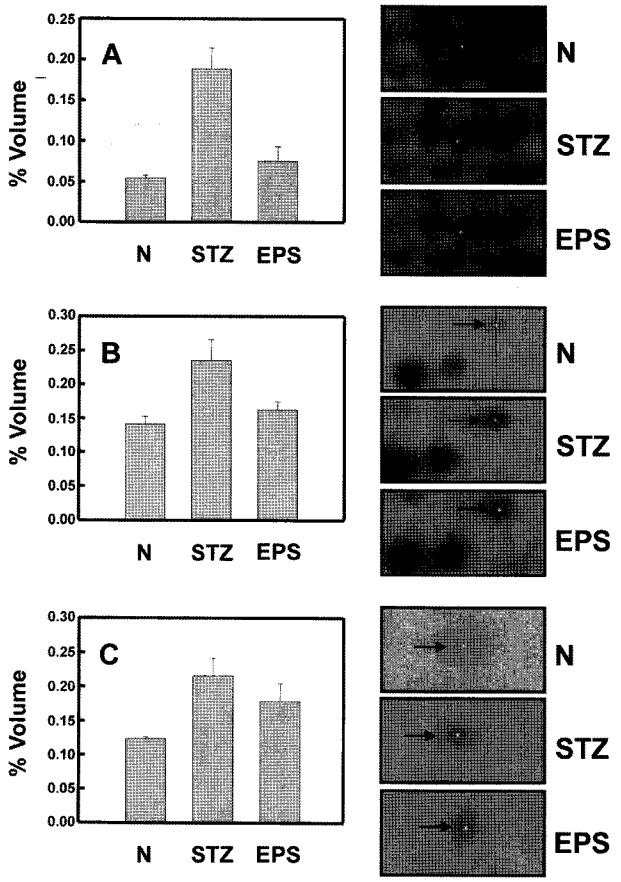

Fig. 3. Proteins showing upregulation upon diabetes induction and amelioration by EPS administration.
Notations for each group are the same as in Fig. 1. For each spot, the relative intensity was averaged and expressed as a mean±SE of three separate experiments. **A**. Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex, mitochondrial precursor (dihydrolipoamide succinyltransferase component of 2-oxoglutarate dehydrogenase complex) (E2) (E2K). **B**. Phospholipid scramblase 3. **C**. 6-Pyruvoyl-tetrahydropterin synthase/dimerization cofactor of hepatocyte nuclear factor 1 alpha.

differentially expressed during diabetes induction and EPS therapy. Six of these altered proteins were previously shown to be differentially regulated during the diabetic state by many investigators (*e.g.*, Vdac1 protein, α_{2u}-globulin, glutathione S-transferase pi 2, mitochondrial aconitase, malate dehydrogenase 1, aldo-keto reductase family 1-member A1) [53, 67, 71, 79].

The increase of α_{2u}-globulin and aldo-keto reductase family 1-member A1 was directly or indirectly associated with either kidney disease or diabetic complications. α_{2u}-Globulin is filtered by the glomerulus; approximately half of the filtrate is normally reabsorbed into kidney proximal tubules, digested in cytoplasmic lysosomes, and reabsorbed back into the circulation [45]. In rats, intracytoplasmic hyaline droplet formation is predominantly associated with accumulation of the male rat-specific α_{2u}-globulin [9]. In the present study, it was found that the level of α_{2u}-globulin decreased by 0.39-fold after diabetes induction, and it was partially restored to 0.62-fold by EPS administration (Table 2).

The aldehyde reductase is linked to the polyol pathway, where it is involved in the detoxification of intermediate dicarbonyl compounds [61]. Wallner *et al.* [71] reported that aldehyde reductase and aldose reductase are relevant to the complications of diabetes mellitus, being upregulated during hyperglycemia. Our results showed an opposite result, in that the expression level of aldo-keto reductase family 1-member A1 slightly decreased (0.85-fold) after diabetes induction. Interestingly, the level of this protein in diabetic rats was partially restored by EPS administration.

Three other altered proteins, (*e.g.*, Vdac1 protein; glutathione S-transferase, pi 2; and mitochondrial aconitase), were also reported to be associated with diabetes mellitus [4, 51, 52, 70, 78, 79]. In the present study, the expression levels of glutathione S-transferase, pi 2 and mitochondrial aconitase decreased by 0.73-fold and 0.83-fold, respectively, after diabetes induction, and thereafter, the level of glutathione S-transferase, pi 2 was increased by 0.90-fold by EPS therapy. In the meantime, the decreased level of mitochondrial aconitase of the diabetic rats was not restored by EPS administration. However, the expression level of Vdac1 protein slightly increased 1.28-fold after diabetes induction and was restored to an approximately normal level by EPS treatment.

The malate dehydrogenase (MDH), which transfers cytosolic NADH into mitochondria, plays crucial roles in mitochondrial energy metabolism [39]. Lemieux *et al.* [34] demonstrated that MDH activity did not vary significantly in the renal cortex of STZ-induced diabetic rats. In this study, MDH 1 expression did not respond to either diabetes induction or EPS administration.

In the present study, the levels of most differentially expressed proteins in STZ-induced diabetic rats were returned partially or fully to those of the normal healthy rats. The levels of E2, phospholipid scramblase 3, and 6-pyruvoyl-tetrahydropterin synthase/dimerization cofactor of hepatocyte nuclear factor 1 alpha were significantly increased in the STZ-induced diabetic group (Fig. 3). The increased levels of E2 (3.45-fold) and 6-pyruvoyl-tetrahydropterin synthase/dimerization cofactor of hepatocyte nuclear factor 1 alpha (1.75-fold) were partially lowered by 1.38-fold and 1.45-fold, respectively, after EPS administration. To the best of our knowledge, there are no reports describing the differential expression of these proteins in the diabetic status.

The phospholipid scramblases (PLSCR) are a structurally and functionally unique class of proteins, which are products of a tetrad of genes conserved from *Caenorhabditis elegans* to humans [57, 72]. Of the PLSCR family, PLSCR3 is a newly recognized member responsible for phospholipid translocation between two lipid compartments in mitochondria

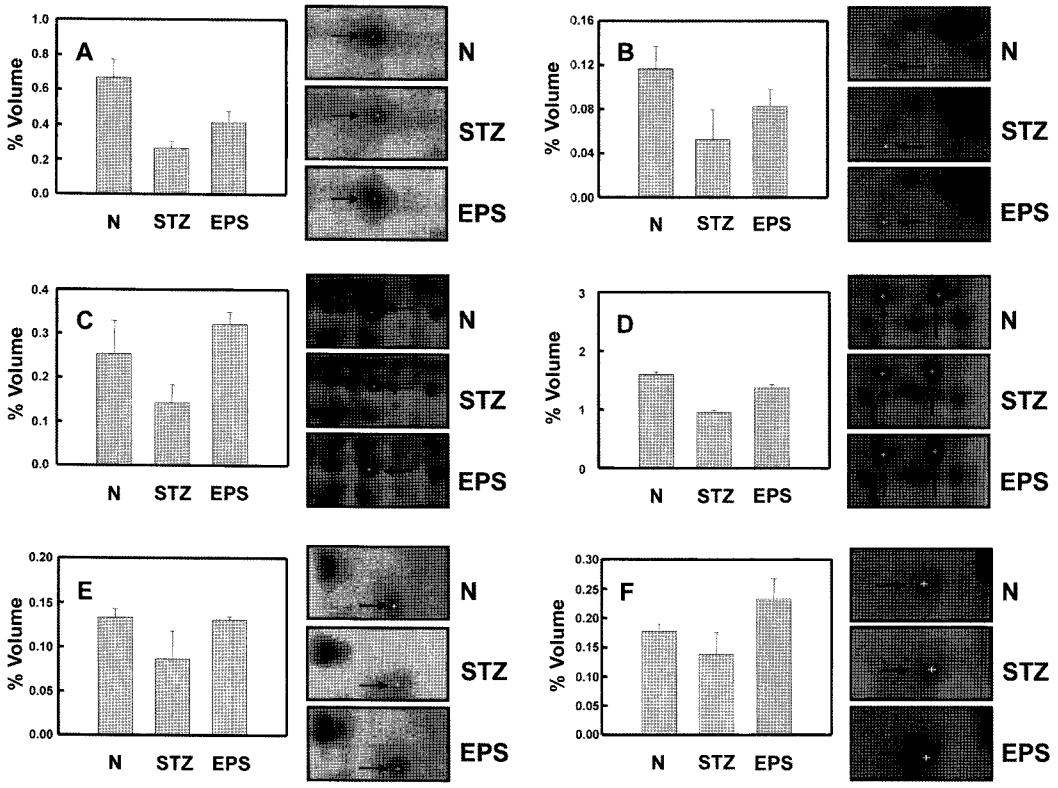

Fig. 4. Proteins showing downregulation upon diabetes induction and amelioration by EPS administration.
Notations for each group are the same as in Fig. 1. For each spot, the relative intensity was averaged and expressed as a mean±SE of three separate experiments. **A**: α_{2u}-globulin; **B**: glycoprotein 56; **C**: Hsc70-ps1; **D**: long-chain alpha-hydroxy acid oxidase=FMN-dependent alpha-hydroxy acid-oxidizing enzyme; **E**: neurogenesin 1; **F**: tropomyosin 3.

[38]. PLSCR3 also plays a critical role in mitochondrial morphology, functions, and apoptotic response. During apoptosis, activated protein kinase C-delta translocates to mitochondria and phosphorylates PLSCR3. These results suggested that PLSCR3 is a critical regulator of mitochondrial structure and respiration, and cardiolipin transport in apoptosis [15]. The present study revealed that the expression level of phospholipid scramblase 3 (PLSCR3) increased by 1.67-fold after diabetes induction, and decreased to almost control level (1.15-fold) by EPS administration.

We found that the expression levels of six proteins were decreased in diabetic rats and increased again by EPS treatment (Fig. 4). Tropomyosin is a rod-shaped protein tightly associated with fibrous actin and periodically distributed along the length of the stress fibers [59, 69]. The correlation between the level of tropomyosin in the medulla and the intensity of kidney antidiuretic reaction supposes the existence of a direct vasopressin control over tropomyosin-encoding gene expression [20]. The expression changes of the tropomyosin gene may be part of the kidney reaction to long-lasting vasopressin action, and tropomyosin seems to play one of the crucial roles in the functioning of the renal antidiuretic mechanism [20]. Dihazi *et al.* [10] reported that the tropomyosin 4 was upregulated, whereas tropomyosins 1, 2, and 3 were downregulated in the thick ascending limb of Henle's loop cells under osmotic stress. Epithelial cells of the thick ascending limb of Henle's loop cells play a major role in the urinary concentrating mechanism. However, involvement of this protein in diabetes has not been demonstrated yet.

Diabetic nephropathy is also characterized by specific renal morphological and functional alterations. Features of early diabetic renal changes are glomerular hyperfiltration, glomerular and renal hypertrophy, increased urinary albumin excretion, increased basement membrane thickness, and mesangial expansion with the accumulation of extracellular matrix proteins [13, 35, 43, 46, 55]. Nonenzymatic glycosylation to glomerular proteins results in accumulation of irreversible advanced glycosylation end products in the glomerular mesangium and glomerular basement membrane [68]. This alteration leads to proteinuria and eventually causes glomerulosclerosis. Cooper [7] reported that the glomerular and vascular pathology is linked to hyperglycemia. Hyperglycemia-induced secondary mediators activation such as protein kinase C, mitogen-activated protein kinases, and cytokines production are responsible for oxidative stress-induced renal injury in the diabetic condition. By a histopathological study, Ravi *et al.* [50] confirmed

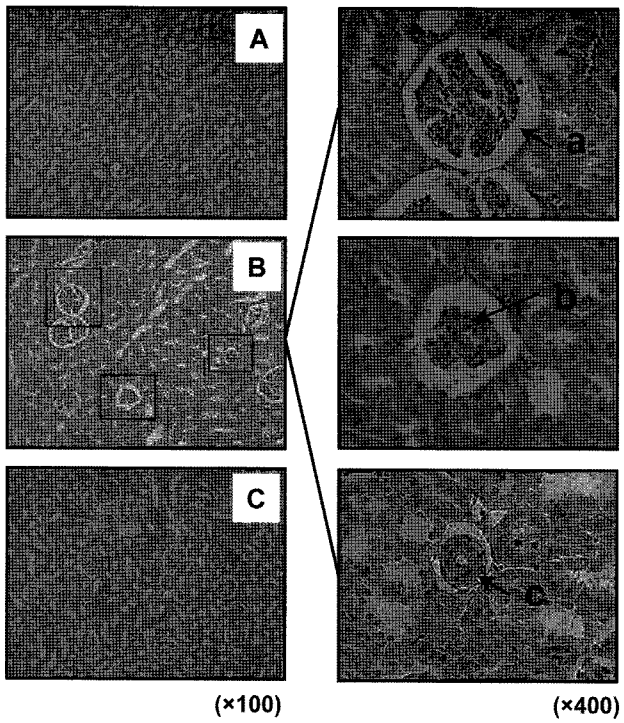

Fig. 5. Results of the histopathological studies for the kidney cortices of the three groups of rats.
(**A**) N Group, (**B**) STZ Group, and (**C**) EPS Group. Notations for each group are the same as in Fig. 1. Magnifications ×100 and ×400.

restoration of the lesioned liver and kidney of diabetic rats when treated with *Eugenia jambolana* seed kernel extract. In our experiment, we also found a normal appearance of kidney cortices in EPS-treated diabetic rats (Fig. 5). This result suggests that EPS plays a pivotal role in alleviation of the diabetic status of the rats, thereby restoring the kidney function to approximately healthy levels.

In conclusion, 2-DE polyacrylamide gel electrophoresis is obviously a powerful tool to comprehensively analyze total proteins that are differentially expressed in rat kidney. This is the first study to investigate the effect of a therapeutic natural compound on the alteration in kidney proteins before and after a diabetic state. Although only a limited number of proteins were identified according to their differentially expressed levels, the information gained from our study could provide additional data on the physiological changes that occur with diabetes. In particular, several proteins showing distinct alterations in this study should be further evaluated for the possibility of using them as nephropathy-specific biomarker proteins.

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