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Differential expression of liver proteins in streptozotocin-induced diabetic rats in response to hypoglycemic mushroom polysaccharides

Hye Jin Hwang, Sang Woo Kim, Yu Mi Baek, Sung Hak Lee, Hee Sun Hwang, Suresh G. Kumar, Md. Atiar Rahman and Jong Won Yun†

Department of Biotechnology, Daegu University, Gyungsan, Gyungbuk 712-714, Korea
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Abstract−We investigated the influence of hypoglycemic fungal extracellular polysaccharides (EPS) on the differential expression of liver proteins in streptozotocin (STZ)-induced diabetic rats. The results of diabetic study revealed that orally administrated EPS exhibited an excellent hypoglycemic effect, lowering the average plasma glucose level in EPS-fed rats to 55.1% with enhanced glucose tolerance. In the next step, we analyzed the differential expression patterns of rat liver proteins from each group to discover potent candidates for diabetes-associated proteins. 34 proteins were upregulated and 35 proteins were downregulated upon diabetes induction. Surprisingly, the altered levels of most proteins in the diabetic group were fully or partially restored to those of the non-diabetic control group by EPS treatment. Although the molecular basis of protein modulation after EPS administration in diabetic rats was not verified, the results of the proteomic analysis provide impetus for further studies to identify reliable biomarkers for diabetic therapy.

Key words: Diabetic Rat, Liver Proteome, *Phellinus baumii*, Polysaccharides, Streptozotocin

INTRODUCTION

Recently, the search for appropriate hypoglycemic agents has focused on various traditional medicinal sources, due to the various side effects and toxicity raised by current synthetic antidiabetic drugs [1]. Mushrooms are potent exemplary sources of natural medicines with antidiabetic activity. Lately, many investigators have endeavored to study the hypoglycemic effect of either the fruiting body or the mycelia of various edible and medicinal fungi [2-5].

Phellinus baumii is a mushroom used as a folk medicine for a variety of human diseases in several Asian countries. The extract of *P. baumii* is known to have various biological activities including inhibition of pulmonary inflammation, antioxidant and free radical scavenging activities [6,7]. In our previous consecutive studies [8-10], we have found that the fungal extracellular polysaccharides (EPS) produced by mycelial culture of a medicinal mushroom *P. 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 a time-dependent manner.

To investigate differential protein expression of rat liver among the three experimental groups (normal, diabetic control, and EPS-treated diabetic groups), two-dimensional electrophoresis (2-DE) was used. Analytical technology development directed towards mapping protein expression is currently focused on biological understanding. Differential display expression analysis is directed towards events taking place in cells, tissues, and organs as well as in a number of biofluids. Therefore, proteomics technology is a suitable tool in the search for disease-associated protein/protein pathways in diabetes mellitus and other diseases with a known target organ and/or cell [11,12].

†To whom correspondence should be addressed.
E-mail: jwyun@daegu.ac.kr

Diabetes is the world's largest endocrine disease involving metabolic disorders of carbohydrates, fats, and proteins [13]. Nevertheless, there are currently only a few reports concerning the proteomics study of diabetes. Most investigators have attempted to find marker proteins associated with diabetes in tissue and biofluid. Herber et al. [14] demonstrated the differential tear protein patterns of diabetic patients by 2-DE analysis. Edvardsson et al. [15] have performed a comparative analysis of the hepatic protein expression profiles of lean and obese diabetic mice, and obese diabetic mice treated with WY14, 643 (PPARα agonist) or rosiglitazone (PPARγ agonist) using 2-DE and mass spectrometry to gain further insight into the molecular mechanisms underlying the therapeutic actions of these drugs. More recently, Sanchez et al. [16,17] investigated the differential expression of diabetes-associated proteins in pancreatic islets, liver, white adipose tissue, brown adipose tissue, and muscle of type 2 diabetic mice using an insulin sensitizer drug, rosiglitazone.

The liver is regarded as one of the central metabolic organs in the body, regulating and maintaining homeostasis. Hence, many investigators have found disease-associated target proteins in liver tissues [18-20]. Diabetes is closely linked to liver dysfunction, as well as impairment of the pancreas. It is also associated with various structural and functional liver abnormalities, including changes in glycogen [21] and lipidic metabolisms [22] as well as in the antioxidant status [23,24]. To date, to the best of our knowledge, there has been no report of expression proteomics study on diabetes before and after treatment with antidiabetic natural sources, such as mushrooms and/or other herbal medicines.

In the present study, rat liver proteome of STZ-induced diabetic rats was analyzed to investigate the differential expression patterns before and after treatment of hypoglycemic fungal polysaccharides.

MATERIALS AND METHODS

1. Preparation of the EPS

Phellinus baumii DG-07 was inoculated on potato dextrose agar (PDA) slant, incubated at 28 ℃ for 6 days, and used throughout the experiments. The stock culture was maintained by monthly subculture and the slants were stored at 4 ℃. The submerged culture of *P. baumii* for the preparation of EPS was performed in a 5-*l* stirred-tank fermenter under the following culture conditions: fructose 20 g/*l*, yeast extract 20 g/*l*, $CaCl_2$ 0.55 g/*l*; temperature, 30 ℃; aeration rate, 2 vvm; agitation speed, 150 rpm; initial pH, 5.0; working volume, 3-*l*. Culture broths were centrifuged at 10,000×*g* for 20 min, and the resulting supernatant was filtered through a Whatman filter paper No. 2 (Whatman International Ltd., Maidstone, England). The resulting culture filtrate was mixed with four volumes of absolute ethanol, stirred vigorously and left overnight at 4 ℃. The precipitated EPS was harvested by centrifugation at 10,000×*g* for 20 min, lyophilized, and used for animal experiments. 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.

2. Animal Experiments

Male Sprague-Dawley rats (Daehan Experiment 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 ℃ with 55±5% humidity) under a 12 : 12-hour light-dark cycle. A commercial pellet diet (Sam Yang Co., Seoul, Korea) and water were provided throughout the experiment. After one week of acclimatization, the rats were subjected to a 16-hour fast. Diabetes was induced by intravenous inject 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). 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 four groups with six animals in each group: normal control group (NC group), normal rats received 0.9% NaCl solution; STZ-induced diabetic control group (STZ group), diabetic rats treated with 0.9% NaCl solution; diabetic treated group (EPS group), diabetic rats treated with EPS at the level of 200 mg/kg body weight using an oral zoned daily for 14 days.

3. Oral Glucose Tolerance Test

After overnight fasting, a blood sample (0.2 ml) was taken from the rats by orbital sinus puncture. Glucose solution was administered orally (2 g/kg), 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, Milpitas, CA). Plasma insulin levels were measured with a Rat Insulin Kit (SHIBAYAGI, Gunma, Japan) with rat insulin as a standard for quantitation of insulin by sandwich technique of enzyme immunoassay.

4. Preparation of the Liver Protein Sample

Liver tissues were removed immediately after sacrifice and then excessive blood contents of liver tissues were eliminated by using a cold NaCl solution. Liver tissues were pulverized into a powder under liquid nitrogen and stored at −80 ℃ 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 methanol/chloroform method prior to electrophoretic separation. The precipitate was resuspended in rehydration buffer and then kept at −80 ℃ until use.

5. 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, USA) using the protocol suggested by the manufacturer. For a short time, 50 µg (~8 µl) of solubilized liver protein was mixed into 342 µl 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 by using silver staining.

6. 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 with a modified version of ImageMaster 2D software V4.95 (Amersham Biosciences, Little Chalfont, Buckinghamshire, England). 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.

7. 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. [25] using modified porcine trypsin. 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 ℃. 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 by 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% aqueous acetonitrile and 1 µl of mixture spotted onto a target plate.

8. Protein Identification

Protein analysis was performed with an Ettan MALDI-TOF (Amersham Biosciences). The peptides were evaporated with a N_2 laser at 337 nm by 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 Rockefeller University (http://129.85.19.192/profound_bin/WebProFound.exe), 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.

9. 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±S.E. 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 polypeptide with a minimum ratio of two.

RESULTS AND DISCUSSION

1. 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 in EPS-fed rats to 55.1% of STZ-treated rats (Fig. 1). The results of diabetic study prompted us to conduct further proteomic studies.

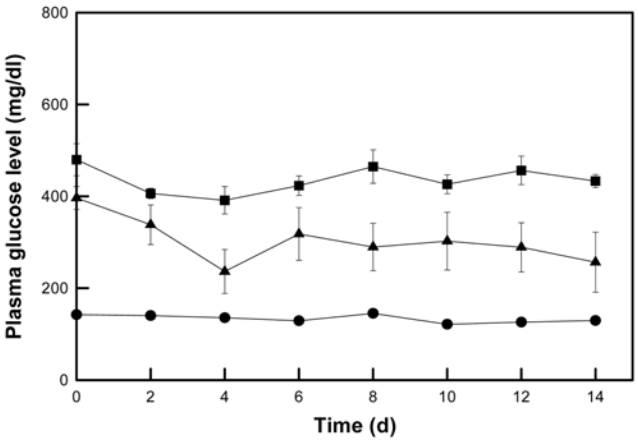

Fig. 1. Effect of fungal exo-polysaccharide (EPS) on the plasma glucose level in STZ-induced diabetic rats during two weeks on oral glucose tolerance test in STZ-induced diabetic rats. NC group (●): normal control rat group received 0.9% NaCl solution; STZ group (■): STZ-induced diabetic rat group treated with 0.9% NaCl solution; EPS group (▲): diabetic rat group treated with fungal exo-polysaccharide (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±S.E ($p<0.05$).

2. 2-DE Gel Separation and Identification of Proteins

To investigate the effect of EPS on the expression pattern of rat liver proteins, the liver proteome of three experimental groups (NC, STZ, and EPS groups) were arrayed on the 2-DE gel system, and the proteins were identified by MALDI-TOF analysis. We excluded the EPS-treated control group for proteome analysis, assuming that no significant difference would be found between the normal con-

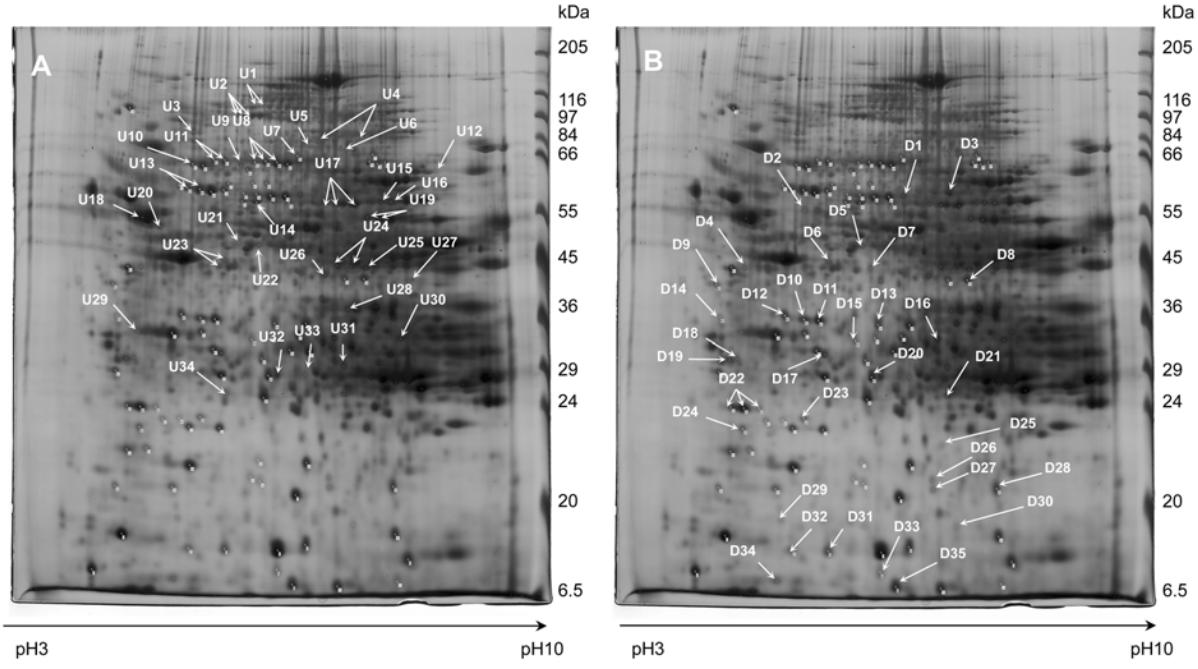

Fig. 2. A silver stained 2-DE map showing positions of putative markers identified by differential expression. Panel A: proteins upregulated in STZ group; Panel B: proteins downregulated in STZ group. All proteins are listed in Tables 2 and 3.

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

Spot ID[a]	Acc. No.[b]	Protein identity[c]	Function	Sequence coverage[d] (%)	PI[e]	Mw[f] (kDa)	Identification[g]
Carbohydrate metabolism							
879	gi|206199	L-Type pyruvate kinase	Glycolysis	20	6.2	59.02	Ms
1160	gi|119740	Fructose-1,6-bisphosphatase (FBTASE)	Gluconeogenesis	-	5.5	39.48	Gm
1179	gi|51036635	Fructose-1,6- biphosphatase 1 (FBTASE 1)	Gluconeogenesis	15	5.5	40.05	Ms
Lipid metabolism							
780	gi|1168287	Acyl-CoA dehydrogenase, very-long-chain specific, mitochondrial precursor (VLCAD)	Fatty acid (FA) oxidation	11	9.2	71.07	Ms
919	gi|38303871	Dihydrolipoamide dehydrogenase	Dihydrolipoyl dehydrogenase activity, FAD binding	17	8.4	54.59	Ms
1098	gi|1162964	Cholesterol esterase	Hydrolysis of sterol esters	11	6.0	62.47	Ms
1199	gi|1168286	Acyl-CoA dehydrogenase, short-chain specific, mitochondrial precursor (SCAD)	FA oxidation	23	8.7	45.03	Ms
1245	gi|6981184	α-Methylacyl-CoA racemase	Bile acid synthesis, lipid metabolism	20	6.2	40.04	Ms
1519	gi|113997	Apolipoprotein A-I Precursor (apo A-I)	Cholesterol transport	-	5.5	30.09	Gm
Amino acid metabolism							
162	gi|8393186	Carbamoyl phosphate synthetase I (CPS I)	Urea cycle, biosynthesis of arginine and pyrimidine	12	6.3	165.76	Ms
577, 588	gi|203576	Carbamoyl phosphate synthetase I (CPS I) precursor	Urea cycle, biosynthesis of arginine and pyrimidine	12, 9	6.3	165.76	Ms
612	gi|123760	Histidine ammonia-lyase (histidase)	Histidine degradation	32	6.1	72.95	Ms
643	gi|129684	Propionyl-CoA carboxylase α chain, mitochondrial precursor	Amino acid metabolism	22	6.3	78.32	Ms
906	gi|21431757	Formimidoyltransferase-cyclodeaminase	Histidine degradation	11	5.8	59.52	Ms
922	gi|13591997	Methylmalonate semialdehyde dehydrogenase (MMSDH)	Valine and pyrimidine metabolism	26	8.9	58.25	Ms
974, 987	gi|118543	Glutamate dehydrogenase, mitochondrial precursor (GDH)	Allosteric regulation	16	8.3	61.75	Ms
1185, 1209	gi|423740	Alloantigen F-rat	Phenylalanine and tyrosine catabolism	14, 18	6.3	43.60	Ms
1223	gi|125052	Isovaleryl-CoA dehydrogenase, mitochondrial precursor (IVD)	Leucine catabolism	19	8.5	46.87	Ms
1407	gi|9910256	3-Hydroxyanthranilate 3,4-dioxygenase	Tryptophan-nicotinic acid pathway	24	5.6	32.85	Ms
1423	gi|48428718	14-3-3 Protein γ	Activates tyrosine and tryptophan hydroxylases	-	4.8	28.17	Gm
1425	gi|52000883	14-3-3 Protein ζ/δ	Activates tyrosine and tryptophan hydroxylases	-	4.7	27.77	Gm
1598	gi|40018538	Aci-reductonedioxygenase (ARD)-like protein 1 (ALP1)	Methionine salvage pathway	42	5.3	21.36	Ms

Table 1. Continued

Spot ID[a]	Acc. No.[b]	Protein identity[c]	Function	Sequence coverage[d] (%)	PI[e]	Mw[f] (kDa)	Identification[g]
Energy metabolism							
381, 386, 390	gi|1346044	10-Formyltetrahydrofolate dehydrogenase (FDH)	Energy generation	-	5.8	99.13	Gm
499	gi|1174637	Transitional endoplasmic reticulum ATPase	Catalyses hydrolysis of (γ(32)-p) ATP	-	5.1	89.35	Gm
967	gi|114562	ATP synthase βchain, mitochondrial Precursor	Energy homeostasis	-	5.2	56.35	Gm
1578	gi|38512279	Mitochondrial H^+-ATP synthase á subunit	Energy generation	29	9.3	59.85	Ms
Chaperone/oxidative stress response							
333, 337	gi|2495342	Heat shock 70-related protein APG-2	Chaperone	-	5.2	94.13	Gm
652	gi|55584140	Stress-70 protein, mitochondrial Precursor	Chaperone	-	6.0	73.84	Gm
830, 834	gi|31981679	Heat shock protein 1 (chaperonin)	Chaperone	16	5.7	61.11	Ms
867	gi|38382858	Glucose regulated protein, 58 kDa	Chaperone	19	5.9	57.06	Ms
1226	gi|115707	Catalase	Superoxide metabolism	25	7.1	60.08	Ms
1486	gi|204499	Glutathione S-transferase (GST) Y-b subunit	Metabolism of various drugs, xenobiotics, and physiological substrates	52	7.9	22.04	Ms
1587	gi|34849851	Peroxiredoxin I (Prx I)	Redox regulation	44	8.7	22.32	Ms
1588	gi|7188365	Glutathione S-transferase α(GST α)	Metabolism of various drugs, xenobiotics, and physiological substrates	17	9.0	26.03	Ms
Transport/binding proteins							
646, 669, 670	gi|19705431	Albumin	Blood osmoregulation, fatty acid transport	21	6.1	70.70	Ms
649	gi|1351943	Annexin A6	Calcium binding	-	5.4	75.62	Gm
671, 678	gi|123036	Hemopexin Precursor	Heme binding, iron transport	-	7.6	51.29	Gm
1319	gi|34234	Laminin-binding protein	Cell adhesion, morphogenesis	29	4.8	31.89	Ms
1441	gi|19743770	Mitogen-activated protein kinase activator with WD repeats binding protein (MAWDBP)	Signal transduction	28	6.5	31.98	Ms
1617	gi|2119647	α-2u-Globulin	Rodent urinary proteins, transport pheromones	27	5.4	21.00	Ms
1619	gi|127533	Major urinary protein (MUP) Precursor	Rodent urinary proteins, transport pheromones	-	5.9	20.74	Gm
1637	gi|21730472	Chain A, solution structure of cellular retinol binding protein (RBP) Type-I in complex with all-*trans*-retinol	Vitamin A transports to target cells	48	5.1	15.86	Ms
1638	gi|3212535	Chain D, rat transthyretin	Thyroid hormone-binding protein	35	6.0	13.11	Ms
Nucleotide metabolism							
1095	gi|7448824	Adenosine kinase	Phosphorylation, purine metabolism	20	5.8	40.42	Ms
1438	gi|13591947	Cyclin G-associated kinase (GAK)	Nucleotide metabolism, regulating the cell cycle	9	5.5	145.05	Ms
1606	gi|127984	Nucleoside diphosphate kinase B (NDK B)	Nucleoside synthesis	32	6.9	17.38	Ms

Table 1. Continued

Spot ID[a]	Acc. No.[b]	Protein identity[c]	Function	Sequence coverage[d] (%)	PI[e]	Mw[f] (kDa)	Identification[g]
Other functions							
1244	gi|433611	Aflatoxin B1 aldehyde reductase	Potent hepatocarcinogen	20	6.8	37.12	Ms
1299	gi|38197642	Aldo-keto reductase family 7, member A2 (aflatoxin aldehyde reductase)	Exo-, endogenous	23	6.3	37.98	Ms
1306	gi|346979	3-Methyl-2-oxobutanoate dehydrogenase (lipoamide)	Phosphorylation	20	5.2	41.43	Ms
1307	gi|13592133	Cytoplasmic β-actin	Cytoskeleton	21	5.3	42.06	Ms
1350	gi|123330	Hydroxymethylglutaryl-CoA synthase, mitochondrial precursor (HMG-CoA synthase)	Ketogenesis	15	9.2	57.35	Ms
1362	gi|17367393	Melanoma-associated antigen D1 (MAGE-D1 antigen)	Apoptosis	12	7.1	86.01	Ms
1377	gi|28948808	Chain D, structure of glycine *N*-methyltransferase complexed with *S*-adenosylmethionine and acetate	Methyltransfer reactions	30	7.3	32.80	Ms
1428	gi|6978873	guanidinoacetate methyltransferase	Creatine biosynthesis	24	5.7	26.67	Ms
1485	gi|6435547	Chain A, crystal structure of a mammalian 2-Cys peroxiredoxin, Hbp23	Heme metabolism	26	8.5	22.25	Ms
1510, 1512, 1514	gi|55890	Catechol *O*-methyltransferase (COMT)	Catecholamine metabolism	-, 29, -	5.4	29.60 29.81 29.60	Gm, Ms, Gm
1536	gi|51703318	Translationally controlled tumor protein	Histamine degranulation	-	4.8	19.46	Gm
1553	gi|2143870	Neurofibromin I	GTPase activity of Ras	3	7.0	320.66	Ms
1631	gi|46577661	Ubiquitin-conjugating enzyme E2 N	Ubiquitin conjugation	-	6.1	17.14	Gm
Unidentified							
636	gi|34856823	Similar to methylcrotonoyl-Co A carboxylase 1 (α)	Unknown	10	8.8	131.11	Ms
925, 932, 936	gi|34879551	Similar to aldehyde dehydrogenase family 7, member A1	Unknown	15, 28, 18	7.2	57.79	Ms
944	gi|34878463	Similar to toll-associated serine protease	Unknown	14	6.3	50.15	Ms
1024	gi|34854661	Similar to nebulin	Unknown	3	9.2	917.70	Ms
1183	gi|27707708	Similar to ferritin light chain	Unknown	37	5.0	20.01	Ms
1188	gi|34859421	Similar to RIKEN cDNA 2310001A20	Unknown	13	5.6	57.61	Ms
1219	gi|34854943	Similar to paraoxonase 3	Unknown	16	5.5	41.92	Ms
1249	gi|34871720	Similar to Ca^{2+}-promoted Ras inactivator	Unknown	16	8.8	89.07	Ms
1315	gi|34852506	Similar to RIKEN cDNA 2010317E03	Unknown	25	5.3	33.21	Ms
1317	gi|27660286	Similar to lactamase, β2	Unknown	30	5.9	32.75	Ms
1365	gi|34856664	Similar to actin, α, cardiac	Unknown	19	6.8	58.24	Ms
1426	gi|34869683	Similar to purine-nucleoside phosphorylase	Unknown	45	6.5	32.57	Ms

[a]Spot ID 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 in NCBInr database. [d]Sequence coverage: percent of identified sequence to the complete sequence of the known protein. [e]PI: theoretical isoelectric point of the matching protein. [f]Mw: theoretical molecular weight of the matching protein in kDa. [g]Method of protein identification (Gm: gel matching with reference gel (see reference [26]); MS: mass spectrometry).

trol group and EPS-treated control group [8,9]. To illustrate the separation characteristics in this study, Fig. 2 shows the 2-DE gel images of rat liver proteome with differently expressed protein locations marked with numbers referring to spot numbers allocated in Table 1. Proteins with molecular masses of approximately 6.5-205 kDa and p*I*s of 3-10 were resolved.

A total number of 1527 well-defined protein spots were matched across all the gels by using a modified version of ImageMaster 2D software V4.95. We analyzed the differential expression patterns of rat liver proteins from each group in order to discover potent candidates for diabetes-associated proteins in rat liver. Matched 85 spots of liver tissue from each group were identified either by MALDI-TOF analysis (65 spots) or by gel matching (20 spots) with a reference gel (Table 1) [26]. These 85 spots were significantly different in their expression levels among the three experimental groups, representing 69 unique proteins (Tables 2 and 3).

Protein expression was comparatively analyzed with image analysis software. The number of protein spots of upregulated or downregulated in STZ-induced diabetic control group, as compared with non-diabetic control group, was 34 and 35, respectively. Interest-

Table 2. Rat liver proteins upregulated upon diabetes induction and normalized by EPS treatment[a]

Spot ID	No.[b]	Protein identity	Alterations (Vol%)[c]		
			NC	STZ (STZ/NC)	EPS (EPS/NC)
333 337	U1	Heat shock 70-related protein APG-2	0.0379±0.0034	0.0860±0.0060 (2.3)***	0.0642±0.0010 (1.7)**
381 386 390	U2	10-Formyltetrahydrofolate dehydrogenase (FDH)	0.2402±0.0225	0.3452±0.0115 (1.4)**	0.2417±0.0164 (1.0)**
499	U3	Transitional endoplasmic reticulum ATPase	0.0805±0.0005	0.1302±0.0085 (1.6)***	0.0771±0.0009 (1.0)***
577 588	U4	Carbamoyl phosphate synthetase I (CPS I) precursor	0.0804±0.0004	0.2532±0.0021 (3.2)***	0.0908±0.0009 (1.1)***
612	U5	Histidine ammonia-lyase (histidase)	0.0358±0.0020	0.0572±0.0029 (1.6)**	0.0324±0.0027 (0.9)**
636	U6	Similar to methylcrotonoyl-Co A carboxylase 1 (α)	0.0263±0.0029	0.0493±0.0046 (1.9)**	0.0251±0.0008 (1.0)**
643	U7	Propionyl-CoA carboxylase α chain, mitochondrial precursor	0.0612±0.0035	0.0912±0.0006 (1.5)***	0.0791±0.0005 (1.3)**
646 669 670	U8	Albumin	0.7577±0.0146	0.8801±0.0075 (1.2)***	0.8041±0.0041 (1.1)**
649	U9	Annexin A6	0.0459±0.0007	0.0572±0.0002 (1.3)***	0.0495±0.0003 (1.1)***
652	U10	Stress-70 protein, mitochondrial Precursor	0.2059±0.0060	0.2743±0.0063 (1.3)***	0.1483±0.0016 (0.7)***
671 678	U11	Hemopexin Precursor	0.0604±0.0004	0.1011±0.0006 (1.7)***	0.0796±0.0004 (1.3)***
780	U12	Acyl-CoA dehydrogenase, very-long-chain specific, mitochondrial precursor (VLCAD)	0.0658±0.0011	0.0858±0.0009 (1.3)***	0.0546±0.0008 (0.8)***
830 834	U13	Heat shock protein 1 (chaperonin)	0.6632±0.0050	0.8881±0.0563 (1.3)**	0.6649±0.0053 (1.0)**
906	U14	Formimidoyltransferase-cyclodeaminase	0.0462±0.0067	0.0793±0.0003 (1.7)**	0.0623±0.0001 (1.4)*
919	U15	Dihydrolipoamide dehydrogenase	0.0418±0.0009	0.1388±0.0008 (3.3)***	0.0925±0.0023 (2.2)***
922	U16	Methylmalonate semialdehyde dehydrogenase (MMSDH)	0.0127±0.0002	0.0707±0.0008 (5.6)***	0.0296±0.0003 (2.3)***
925 932 936	U17	Similar to aldehyde dehydrogenase family 7, member A1	0.3152±0.0086	0.5234±0.0063 (1.7)***	0.3153±0.0045 (1.0)***
967	U18	ATP synthase β chain, mitochondrial Precursor	2.2744±0.0921	3.0785±0.0625 (1.4)***	2.7092±0.0029 (1.2)**
974 987	U19	Glutamate dehydrogenase, mitochondrial precursor (GDH)	0.4136±0.0052	0.5408±0.0153 (1.3)***	0.4044±0.0033 (1.0)***
1024	U20	Similar to nebulin	0.0712±0.0075	0.1230±0.0014 (1.7)***	0.0917±0.0008 (1.3)**
1095	U21	Adenosine kinase	0.0312±0.0006	0.0761±0.0013 (2.4)***	0.0379±0.0007 (1.2)***
1098	U22	Cholesterol esterase	0.0519±0.0009	0.1111±0.0049 (2.1)***	0.0803±0.0004 (1.6)***
1160 1179	U23	Fructose-1,6-bisphosphatase (FBTASE)	0.0664±0.0006	0.0916±0.0003 (1.4)***	0.0559±0.0007 (0.8)***
1185 1209	U24	Alloantigen F-rat	0.3405±0.0415	0.7541±0.0252 (2.2)***	0.2557±0.0143 (0.8)***

Table 2. Continued

Spot ID	No.[b]	Protein identity	Alterations (Vol%)[c]		
			NC	STZ (STZ/NC)	EPS (EPS/NC)
1199	U25	Acyl-CoA dehydrogenase, short-chain specific, mitochondrial precursor (SCAD)	0.1610±0.0023	0.2565±0.0034 (1.6)***	0.1409±0.0112 (0.9)***
1223	U26	Isovaleryl-CoA dehydrogenase, mitochondrial precursor (IVD)	0.1691±0.0138	0.2812±0.0482 (1.7)*	0.0854±0.0049 (0.5)**
1244	U27	Aflatoxin B1 aldehyde reductase	0.0517±0.0008	0.1305±0.0081 (2.5)***	0.0815±0.0004 (1.6)***
1299	U28	Aldo-keto reductase family 7, member A2 (aflatoxin aldehyde reductase)	0.0309±0.0002	0.1258±0.0033 (4.1)**	0.0857±0.0180 (2.8)*
1362	U29	Melanoma-associated antigen D1 (MAGE-D1 antigen)	0.0802±0.0042	0.1270±0.0208 (1.6)*	0.0815±0.0047 (1.0)*
1377	U30	Chain D, structure of glycine *N*-methyltransferase complexed with *S*-adenosylmethionine and acetate	0.0530±0.0009	0.2287±0.0191 (4.3)***	0.1041±0.0054 (2.0)***
1426	U31	Similar to purine-nucleoside phosphorylase	0.0819±0.0012	0.1170±0.0074 (1.4)**	0.0749±0.0004 (0.9)***
1438	U32	Cyclin G-associated kinase (GAK)	0.0427±0.0122	0.1421±0.0132 (3.3)**	0.0989±0.0010 (2.3)*
1441	U33	Mitogen-activated protein kinase activator with WD repeats binding protein (MAWDBP)	0.0287±0.0114	0.1292±0.0145 (4.5)**	0.0625±0.0045 (2.2)**
1485	U34	Chain A, crystal structure of a mammalian 2-Cys peroxiredoxin, Hbp23	0.0501±0.0058	0.1115±0.0068 (2.2)***	0.0868±0.0057 (1.7)**

[a]Notations for experimental group division: Group NC, normal control rat group received 0.9% NaCl solution; Group STZ, STZ-induced diabetic rat group treated with 0.9% NaCl solution; Group EPS, 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]Numbers are arbitrary assigned for depicting Figs. 2 and 3.

[c]Significant differences between the diabetic groups and the normal control group are indicated as: *p<0.05, **p<0.01,***p<0.001. For each protein, the relative intensity was averaged and expressed as a mean±SE of the three separate experiments.

ingly, the altered expression levels of most of these proteins in the diabetic control group were partially or fully restored to those of healthy rats upon EPS treatment. However, the order of magnitude of the changes differs widely. We were initially interested in proteins that showed differential expression levels at least two-fold lower or higher between normal and diabetic control groups and had an insight into these proteins for further analysis (Figs. 3 and 4).

Among the proteins that show significantly differential expression, five proteins were differently regulated in this study when compared to the results in literature (10-formyltetrahydrofolate dehydrogenase, carbamoyl-phosphate synthetase I precursor, adenosine kinase, glutathione S-transferase Y-b subunit, and peroxiredoxin 1. see Table 4). These conflicting results are probably due to use of different diabetic animal models and experimental conditions (e.g., experimental period, blood glucose level of diabetic animal, and preparation method of protein sample). However, the regulation modes of other identified proteins were in accordance with the results in literature (Tables 2, 3, 4).

3. Carbohydrate Metabolism-associated Proteins

In diabetes, elevated blood glucose is a consequence of increased hepatic glucose output in concert with reduced peripheral glucose utilization. Thus, we first focused on proteins associated with carbohydrate metabolism. A variety of enzymes involved in carbohydrate metabolism have been found to be differentially regulated in diabetic animals (e.g., glycolytic enzymes: hexokinase/glucokinase, phosphofructokinase, and pyruvate kinase; gluconeogenic enzymes: glucose-6-phosphatase, fructose-1,6-bisphosphatase, and phosphoenolpyruvate carboxykinase). However, no proteomic results are currently available showing whole carbohydrate metabolic proteins at a glance. This drove us to survey each protein one by one to study how these proteins are altered during diabetes induction. In this study, only two enzymes showing differential expression (L-type pyruvate kinase and fructose-1,6-bisphosphatase) were detected in the 2-DE map (Table 1).

Several investigators found a decrease in pyruvate kinase activity in diabetic rats [27,28]. Our result also showed that pyruvate kinase was decreased by 53% upon diabetes induction. Thereafter, the decreased levels were slightly increased after EPS administration (Table 3).

Fructose-1,6-bisphosphatase (FBTASE) is an enzyme in gluconeogenesis that catalyzes the hydrolysis of fructose-1,6-bisphosphate, producing fructose-6-phosphate and Pi. The levels of FBTASE were known to be elevated in the liver and kidney of diabetic rats [29-31]. In the present study, this enzyme was also upregulated 1.4-fold by diabetes induction and was completely restored to those of healthy rats by EPS administration (Table 2).

4. Lipid Metabolism-associated Proteins

Diabetes mellitus is frequently associated with changes in plasma lipoproteins. Alterations of lipoproteins in diabetes are related to the degree of hyperglycemia and are one of the symptoms observed in the diabetic condition [32-34]. In the present study, six proteins involved in lipid and fatty acid metabolism showed differential expression in diabetic rats. It was found that the very long-chain acyl-CoA dehydrogenases (VLCAD), short-chain acyl-CoA dehydrogenases (SCAD), dihydrolipoamide dehydrogenase, and cholesterol esterase were upregulated, while α-methylacyl-CoA racemase and

Table 3. Rat liver proteins downregulated upon diabetes induction and normalized by EPS treatment[a]

Spot ID	No.[b]	Protein identity	Alterations (Vol%)[c]		
			NC	STZ (STZ/NC)	EPS (EPS/NC)
867	D1	Glucose regulated protein, 58 kDa	0.0848±0.0260	0.0280±0.0057 (0.3)*	0.0846±0.0084 (1.0)*
944	D2	Similar to toll-associated serine protease	0.1224±0.0064	0.0800±0.0093 (0.7)*	0.1290±0.0117 (1.1)*
879	D3	L-Type pyruvate kinase	0.1776±0.0067	0.0832±0.0084 (0.5)***	0.1281±0.0071 (0.7)**
1183	D4	Similar to ferritin light chain	0.0519±0.0019	0.0276±0.0075 (0.5)*	0.0577±0.0084 (1.1)*
1188	D5	Similar to RIKEN cDNA 2310001A20	0.1869±0.0117	0.0745±0.0065 (0.4)***	0.1369±0.0061 (0.7)**
1219	D6	Similar to paraoxonase 3	0.0616±0.0056	0.0280±0.0018 (0.5)**	0.0880±0.0067 (1.4)***
1226	D7	Catalase	0.1617±0.0112	0.0326±0.0084 (0.2)***	0.1370±0.0160 (0.9)**
1245	D8	α-Methylacyl-CoA racemase	0.2711±0.0416	0.1645±0.0034 (0.6)*	0.2625±0.0101 (1.0)*
1249	D9	Similar to Ca^{2+}-promoted Ras inactivator	0.1605±0.0129	0.0700±0.0095 (0.4)**	0.1257±0.0042 (0.8)**
1306	D10	3-Methyl-2-oxobutanoate dehydrogenase (lipoamide)	0.3596±0.0384	0.1680±0.0059 (0.5)**	0.3529±0.0184 (1.0)**
1307	D11	Cytoplasmic β-actin	0.4926±0.0183	0.0793±0.0042 (0.2)***	0.3861±0.0152 (0.8)***
1315	D12	Similar to RIKEN cDNA 2010317E03	0.3563±0.0187	0.2172±0.0050 (0.6)***	0.3051±0.0064 (0.9)**
1317	D13	Similar to lactamase, β2	0.1381±0.0161	0.0552±0.0094 (0.4)*	0.1154±0.0218 (0.8)*
1319	D14	Laminin-binding protein	0.1172±0.0078	0.0395±0.0050 (0.3)***	0.1094±0.0085 (0.9)***
1350	D15	Hydroxymethylglutaryl-CoA synthase, mitochondrial precursor (HMG-CoA synthase)	0.1042±0.0078	0.0680±0.0068 (0.7)*	0.0931±0.0070 (0.9)*
1365	D16	Similar to actin, α, cardiac	0.1734±0.0085	0.1142±0.0111 (0.7)*	0.2098±0.0206 (1.2)**
1407	D17	3-Hydroxyanthranilate 3,4-dioxygenase	0.2147±0.0115	0.1347±0.0135 (0.6)**	0.1804±0.0067 (0.8)*
1423	D18	14-3-3 Protein γ	0.0982±0.0148	0.0413±0.0060 (0.4)*	0.0904±0.0098 (0.9)*
1425	D19	14-3-3 Protein $\zeta\delta$	0.1371±0.0080	0.0709±0.0087 (0.5)**	0.1761±0.0117 (1.3)***
1428	D20	Guanidinoacetate methyltransferase	0.3235±0.0117	0.1918±0.0293 (0.6)**	0.3286±0.0175 (1.0)**
1486	D21	Glutathione S-transferase (GST) Y-b subunit	0.1673±0.0200	0.0565±0.0058 (0.3)**	0.1733±0.0287 (1.0)**
1510	D22	Catechol *O*-methyltransferase (COMT)	0.9550±0.1669	0.4826±0.0503 (0.5)*	0.9678±0.0401 (1.0)*
1512					
1514					
1519	D23	Apolipoprotein A-I Precursor (apo A-I)	0.1370±0.0193	0.0530±0.0068 (0.4)*	0.1625±0.0259 (1.2)**
1536	D24	Translationally controlled tumor protein	0.1216±0.0082	0.0723±0.0044 (0.6)*	0.1166±0.0136 (1.0)*
1553	D25	Neurofibromin I	0.0531±0.0119	nd[d]	0.0385±0.0058 (0.7)*
1578	D26	Mitochondrial H^+-ATP synthase α subunit	0.1239±0.0248	0.0408±0.0047 (0.3)*	0.1055±0.0173 (0.9)*
1587	D27	Peroxiredoxin I (Prx I)	0.2150±0.0360	0.0322±0.0053 (0.2)**	0.1124±0.0062 (0.5)*
1588	D28	Glutathione S-transferase α (GST α)	0.2779±0.0145	0.1812±0.0197 (0.7)*	0.3404±0.0276 (1.2)**
1598	D29	Aci-reductonedioxygenase (ARD)-like protein 1 (ALP1)	0.0640±0.0079	0.0349±0.0038 (0.6)*	0.0739±0.0077 (1.2)**
1606	D30	Nucleoside diphosphate kinase B (NDK B)	0.1142±0.0109	0.0374±0.0058 (0.3)***	0.0699±0.0059 (0.6)*
1617	D31	α-2u-Globulin	0.3514±0.0231	0.1637±0.0152 (0.5)**	0.2703±0.0233 (0.8)*
1619	D32	Major urinary protein (MUP) Precursor	0.3101±0.0209	0.1104±0.0153 (0.4)***	0.1823±0.0099 (0.6)*
1631	D33	Ubiquitin-conjugating enzyme E2 N	0.2986±0.0350	0.1017±0.0100 (0.3)**	0.1839±0.0184 (0.6)*
1637	D34	Chain A, solution structure of cellular retinol binding protein (RBP) Type-I in complex with all-*trans*-retinol	0.2497±0.0346	0.0820±0.0282 (0.3)**	0.2010±0.0282 (0.8)*
1638	D35	Chain D, rat transthyretin	0.2818±0.0150	0.2068±0.0173 (0.7)*	0.3266±0.0164 (1.2)**

[a]Notations for experimental group division: Group NC, normal control rat group received 0.9% NaCl solution; Group STZ, STZ-induced diabetic rat group treated with 0.9% NaCl solution; Group EPS, 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]Numbers are arbitrary assigned for depicting Figs. 2 and 4.

[c]Significant differences between the diabetic groups and the normal control group are indicated as: *p<0.05, **p<0.01, ***p<0.001. For each protein, the relative intensity was averaged and expressed as a mean±SE of the three separate experiments.

[d]nd means not detectable.

apolipoprotein A-I (apo A-I) were downregulated in the liver of the STZ group. The EPS was able to restore the altered expressions of the six proteins to almost control levels (Table 2).

To our knowledge, there has been no report describing the differential expression levels of dihydrolipoamide dehydrogenase related to diabetes mellitus. In the present study, an increase of over 3.3-fold was observed in the liver dihydrolipoamide dehydrogenase levels upon diabetes induction (Table 2 and Fig. 3). However, EPS treat-

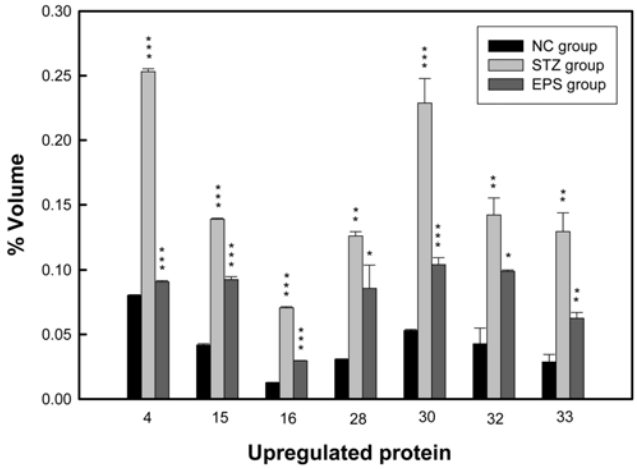

Fig. 3. Proteins upregulated upon STZ treatment and rescued by EPS administration. Animal groups are described in Materials and methods. Each spot intensity volume was processed by background subtraction and total spot volume normalization; the resulting spot volume percentage was used for comparison. All data were expressed as mean±SE (*p< 0.05, **p<0.01, ***p<0.001). Numbers for protein names were assigned in Table 2.

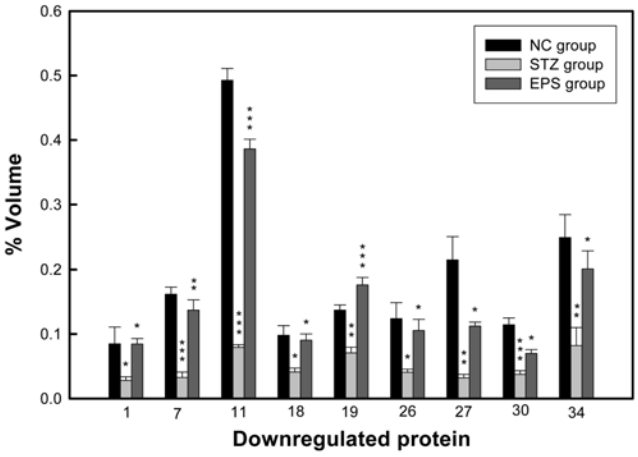

Fig. 4. Proteins downregulated upon STZ treatment and rescued by EPS administration. Animal groups are described in Materials and methods. Each spot intensity volume was processed by background subtraction and total spot volume normalization; the resulting spot volume percentage was used for comparison. All data were expressed as mean± SE (*p<0.05, **p<0.01, ***p<0.001). Numbers for protein names were assigned in Table 3.

Table 4. List of proteins whose expression has previously been associated with diabetes

Function/Protein identity	Regulation mode	Diabetic subjects	References
Carbohydrate metabolism			
Fructose-1,6-bisphosphatase (FBTASE)	Up	Rat	[29-31,60]
Glucose-6-phosphatase	Up	Rat	[29-31,60,61]
Phophoenolpyruvate carboxykinase (PEPCK)	Up	Rat	[61,62]
Pyruvate carboxylase	Up	Rat	[63]
Glucokinase	Up	Rat	[64]
	Down		[27,62,65]
Hexokinase	Down	Rat	[29-31,60,64,65]
Hexokinase isozyme (Type II, III)	Down	Rat	[61]
Lactate dehydragenase	Down	Rat	[60]
Phosphofructokinase	Down	Rat	[60,63,64]
Pyruvate kinase	Down	Rat	[27,28,60,62]
Glycogen synthase	Up	Rat	[66,67]
	Down		[68]
Glycogen phosphorylase	Down	Rat	[67]
Aconitate hydratase	Up	Rat	[69]
Citrate synthase	Up	Rat	[69]
Isocitrate lyase	Up	Rat	[69]
Malate dehydrogenase	Up	Rat	[69]
	Down		[68]
Malate synthase	Up	Rat	[69]
Lipid metabolism			
Glucose 6-phosphate-dehydrogenase	Down	Rat	[60,61,64,68]
Malic enzyme	Down	Rat	[60,61]
Acetoacetyl-CoA synthetase (acetoacetate-CoA ligase)	Down	Rat	[70]
GM1 sialyltransferase (Sialt-4)	Down	Rat	[71]
GM2 galactosyltransferase (GalT-2)	Down	Rat	[71]
GM3 N-Acetylgalactosaminyltransferase (GalNAcT)	Up	Rat	[71]
Angiopoietin-like protein 3 (ANGPTL3)	Up	Mouse	[72]
Fatty acid binding protein (FABP)	Down	Rat	[73]

Table 4. Continued

Function/Protein identity	Regulation mode	Diabetic subjects	References
GPI (glycosylphosphatidylinositol)-specific phospholipase D	Up	Mouse	[74]
Cytosolic Acyl-Coenzyme A hydrolase/thioesterase (CTE-I)	Up	Rat	[75]
Mitochondrial Acyl-Coenzyme A Hydrolase/thioesterase (MTE-I)	Up	Rat	[75]
Amino acid metabolism			
Carbamoyl phosphate synthetase I (CPS I)	Up	Rabbit	[76]
Phenylalanine hydroxylase	Up	Rat	[77]
Aminocarboxymuconate-semialdehyde decarboxylase	Up	Rabbit	[78]
3-Hydroxyanthranilate 3,4-dioxygenase	Down	Rabbit	[78]
Kynureninase	Down	Rabbit	[78]
Kynurenine 3-monooxygenase	Down	Rabbit	[78]
Kynurenine-oxoglutarate transaminase	Down	Rabbit	[78]
Energy metabolism			
Succinate dehydrogenase	Down	Rat	[68]
Chaperone/oxidative stress response			
Alinine hydroxylase	Up	Rat	[79]
Aminopyrene *N*-demethylase	Down	Rat	[79]
Microsomal Cytochrome P450	Down	Rat	[80]
Mitochondrial Cytochrome P450 2E1	Up	Rat	[81]
Ethoxycoumarin-*O*-deethylase	Down	Rat	[79]
Ethoxyresorufin-*O*-deethylase	Up	Rat	[79]
Mitochondrial Glutathione S-transferase A4-4	Up	Rat	[81]
Glutathione S -transfearse (GST)	Up	Rat	[79]
		Mouse	[82]
	Down	Rat	[83]
Catalase	Up	Rat	[54]
	Down		[55,56]
Glutathione peroxidase	Up	Rat	[54]
	Down		[55,56,84]
Glutathione reductase	Down	Rat	[84]
Selenium glutathione peroxidase	Down	Rat	[85]
Superoxide dismutase	Up	Rat	[54]
	Down		[55,56]
Cu/Zn Superoxide dismutase	Up	Rat	[54]
Mn Superoxide dismutase	No alteration	Rat	[54]
Xanthine oxidase	Up	Rat	[84]
Protein disulfide isomerase A3 precursor (Disulfide isomerase ER-60) (ERp60)	Up	Rat	[86]
Heat shock protein 70	Down	Rat	[51]
Transport/binding proteins			
α-2u-Globulin	Down	Rat	[87]
Stearoyl Coenzyme A desaturase	Down	Rat	[88]
Retinol-binding protein (RBP)	Down	Rat	[89]
Transthyretin (TTR)	Down	Rat	[89]
Nucleotide metabolism			
Aden osine kinase	Down	Rat	[90,91]
Other functions			
Catechol-*O*-methyltransferase (COMT)	Down	Rat	[92]
δ Aminolevulinate dehydratase (ALA-D)	Down	Rat, Human	[93]
Farnesoid X receptor (FXR)	Down	Rat	[94]
3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase	Down	Rat	[95]
Hepatocyte nuclear factor (HNF)-4a	Up	Rat	[96]
α-Ketoisocaproate dioxygenase (KICD)	Up	Rat	[97]
Mitochondrial Coenzyme Q	Up	Rat	[98]
Phosphotyrosine phosphatase	Up	Rat	[99]

ment did not fully restore the expression levels to control values.

In general, the total cholesterol and triglyceride concentrations in the serum and liver were significantly higher in the STZ-induced diabetic rats than in the nondiabetic control animals [35]. In our investigation, the expression level of cholesterol esterase was also found to be increased by 2.1-fold after induction of diabetes. Treatment with EPS returned the expression levels to those seen in the control rats (Table 2).

In the present study, expression levels of apo A-I, which participates in the reverse transport of cholesterol from tissues to the liver, were decreased by 61% in diabetic rats and, surpassed those of control rats by 19% after treatment with EPS (Table 3). Our results, therefore, evidenced different levels of apo A-I between liver and blood samples in diabetic animals and support a role for EPS in the regulation of lipid metabolism. The plasma apo A-I levels of diabetic rats were significantly higher than those of the control group, while the apolipoprotein E levels of the diabetic rats were significantly lower [36]. Haas et al. [37] reported that hepatic apo A-I mRNA levels were reduced by 42% in diabetic rats relative to nondiabetic controls.

5. Amino Acid Metabolism-associated Proteins

Since gluconeogenesis in liver is closely related to amino acid metabolism, our attention has also been given to this category of proteins. In this study, several enzymes involved in amino acid metabolism were found to be differently expressed in diabetic rats when compared to levels of normal rats (Tables 2 and 3).

Ryall et al. [38] reported that the levels of carbamoyl phosphate synthetase I (CPS I) and ornithine carbamoyltransferase (OCT) mRNA in livers of adults responded to glucagon in normal animals (1.5-fold and 2.2-fold increases, respectively) and to dexamethasone in experimentally induced diabetic animals (3-fold increase in CPS1 mRNA with no change in OCT mRNA). Our results revealed that the expression levels of CPS I precursor increased by 3.2-fold after induction of diabetes (Table 2 and Fig. 3). In turn, EPS treatment significantly lowered the expression level of this protein. Our results are in contradiction to the results of the study by Sanchez et al. [17] who reported a 4-fold decrease in expression of this protein in diabetic mice. These different results are presumably due to the discrepancy in the diabetic animal models used (obese C57BI/6J *lep/lep* mice).

The present study revealed that the expression level of histidase (histidine ammonia-lyase) increased 1.6-fold after diabetes induction (Table 2). Histidase gene expression is known to be regulated in the liver by a dietary protein at the pretranslational level in rat liver [39]. The increased histidase expression in STZ group might be due to polyphagia, which is one of the symptoms to appear in STZ-induced diabetes condition. In this study, the increases of both histidase level and food intake in diabetic rats were partially restored by EPS administration.

Methylmalonate semialdehyde dehydrogenase (MMSDH; EC 1.2.1.27), involved in valine catabolism, was found to be one of the most significantly altered proteins in this investigation (5.6-fold increase) during diabetic induction (Table 2 and Fig. 3). To our knowledge, this is the first report describing the relation between MMSDH and diabetes mellitus.

Glutamate dehydrogenase is ubiquitously expressed and it is involved in the regulation of insulin secretion, especially in amino acid-stimulated insulin secretion [40]. Tanizawa et al. [41] also suggested that constitutively activated glutamate dehydrogenase enhances oxidation of glutamate, which is intracellularly converted from glutamine to α-ketoglutarate, a tricarboxylic acid cycle substrate, thereby stimulating insulin secretion. Opposite to these results, the altered expression level of this enzyme in the present study was not so significantly altered by diabetes induction (1.3-fold increase) and was restored to the levels close to those of healthy rats when treated with EPS (Table 2). No clear association between reduced expression of glutamate dehydrogenase and diabetes mellitus could be proposed from only this observation.

Here, it is interesting to mention about the expression alterations of 14-3-3 proteins. The 14-3-3 family of proteins are ubiquitously expressed and involved in the regulation of diverse intracellular processes of several signaling proteins in all eukaryotic organisms [42]. The 14-3-3 proteins can regulate their binding partners by altering their intracellular localization catalytic activity or complex formation with other proteins [43,44]. The interaction of 14-3-3 with target proteins has been shown to encode a variety of functions including subcellular redistribution, altered protein conformation, protection from proteolysis, impaired interaction with other proteins, and scaffolding, regulation of apoptosis [45,46]. The roles of protein 14-3-3 on diabetes have been documented by several investigators. For example, Ogihara et al. [47] suggested that 14-3-3 proteins might play a role in the regulation of insulin sensitivity by interrupting the association between the insulin receptor and insulin receptor substrate 1 (IRS-1). Ramm et al. [46] reported that the insulin-dependent association of 14-3-3 with AS160 (Akt substrate of 160 kDa) plays an important role in GLUT4 trafficking in adipocytes. In the present study, 14-3-3 protein γ was 2.4-fold decreased by diabetes induction and was increased again by EPS administration (Table 3). As for 14-3-3 protein ζ/δ, the expression levels became half after diabetes induction and were fully restored to control levels upon EPS treatment. Therefore, we suggest that EPS administration might play a role in increasing of insulin efficiency by regulation of 14-3-3 proteins expression in rat liver, thereby regulating glucose metabolism.

6. Energy Metabolism-associated Proteins

The physiological role of the 10-formyltetrahydrofolate dehydrogenase (FDH) is still not clear, but it probably serves to recycle 10-formyltetrahydrofolate not required for purine synthesis back to tetrahydrofolate, where it would be available for other single-carbon reactions [48]. Recently, Sanchez et al. [17] reported that FDH was reduced by 3-fold in the liver of *lep/lep* mice (obese mice). However, in this study, the expression level of FDH was increased only by 1.4-fold in diabetic rats and was completely normalized by EPS treatment (Table 2).

Sakurai et al. [49] reported that addition of alloxan on insulin-secreting cells resulted in a decrease of intracellular ATP in a dose-dependent manner. In this study, ATP synthase β chain, mitochondrial precursor was slightly overexpressed (35%) upon diabetes induction, whereas the expression of mitochondrial H^+-ATP synthase α subunit was significantly suppressed (5.7-fold) in diabetic rats. The levels of these proteins were fully restored to those of healthy rats by EPS treatment (Tables 2 and 3, Fig. 4).

7. Oxidative Stress Response Proteins

It was reported that the expression and induction of HSC70/HSP70

may be altered in the course of diabetic disease and may result in impairment of the cytoprotective ability of diabetic rats [50]. Atalay et al. [51] reported that diabetes may increase susceptibility to oxidative damage and impair heat shock protein (HSP) protection, but endurance training may offset some of the adverse effects of diabetes by upregulating tissue HSP expression. Their results suggested that diabetes impairs HSP protection, possibly via transcriptionally mediated mechanisms. In our investigations, all three stress proteins detected (e.g. heat shock 70-related protein APG-2, stress-70 protein, and heat shock protein 1) were overexpressed in diabetic rats and EPS modulated the expression of these proteins in different magnitudes (Table 2). The elevated level of heat shock 70-related protein APG-2 was slightly alleviated by EPS treatment, whereas stress-70 protein and heat shock protein 1 were fully restored to those of normal control rats.

In STZ-induced diabetes, hyperglycemia and oxidative stress have been implicated in the etiology and pathology of disease complications [52-57]. Ravi et al. [56] reported that diabetic rats showed low activity of superoxide dismutase, catalase, and glutathione peroxidase, as well as a reduced glutathione content in the liver and kidneys. All were restored to almost normal levels by treatment with seed kernel extract. Similarly, Ramachandran et al. [55] observed a significant decrease in both glutathione content and activities of antioxidant enzymes, such as superoxide dismutase, catalase, and glutathione peroxidase in diabetic rats, as well as a concomitant increase in the level of lipid peroxides. The observed alterations in the antioxidant levels in the tissues reverted back to near normal levels after the oral administration of macrocyclic vanadium complex. In the present study, catalase was also decreased by 5.0-fold upon diabetes induction and surpassed the levels of control rats when treated with EPS (Table 3 and Fig. 4).

In addition, STZ has also been shown to cause a reduction in the glutathione-dependent antioxidant potentials of tissues, thereby making them more susceptible to increased oxidative damage [58]. A reduced glutathione S-transferase (GST)-dependent removal of toxic metabolites accompanied by an increased production of reactive oxygen species and increased membrane lipid peroxidation would have serious consequences for cellular functions of tissues in diabetes. Our results showed a 3.0-fold decrease in the expression levels of GST Y-b subunit for the STZ group when compared to the normal group. The levels were restored to those of healthy rats after EPS treatment. Moreover, the levels of GST α were also decreased by 1.5-fold in the diabetic group and surpassed those of control animals after EPS treatment. It is conceivable that EPS may play an important role in glutathione metabolism and GST distribution in the tissues of diabetic rats.

Peroxiredoxin (Prx) I and II are differentially regulated at the transcriptional and translational level under various stress conditions [59]. In this study, Prx I was strikingly downregulated (6.8-fold) in diabetic rats and restored by EPS treatment (Table 3 and Fig. 4). In contrast, the levels of chain A, a crystal structure of a mammalian 2-Cys Prx, Hbp23 were increased (2.2-fold) in diabetic rats and their levels were slightly decreased by EPS treatment (Table 2). Taken together, these results suggest that EPS obviously contributed to the detoxification activities of various toxic metabolites and xenobiotics in diabetic rats by suppressing the expression levels of the stress-related proteins.

CONCLUSIONS

In the present proteomic study of diabetic rat liver, we identified 69 significantly changed proteins (34 upregulated and 35 downregulated after diabetes induction). 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 the levels of those for non-diabetic control rats upon EPS treatment. Many of the alterations of protein levels in this study are in accordance with observations noted previously by other investigators, and 16 proteins are proved to be associated with diabetes mellitus for the first time in this study. Nevertheless, the possible relationship between the antidiabetic effect of EPS and the changes in liver protein profiles is still not clear. Therefore, the molecular basis of protein modulation by EPS in diabetic rats should be explored further, building on the present results and aiming for future clinical purpose.

In conclusion, this is the first proteomic study comparing hepatic protein expressions between the diabetic rats induced by STZ and those restored by hypoglycemic fungal polysaccharides. Furthermore, several new hypotheses have been generated through this work that may be addressed in future studies using other technologies. We believe our work on rat liver proteome has established a useful database for further mining of biomarkers for diabetes mellitus.

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