Time-Dependent Plasma Protein Changes in Streptozotocin-Induced Diabetic Rats before and after Fungal Polysaccharide Treatments

Sang Woo Kim,¹ Hye Jin Hwang,¹ Eun Jae Cho,¹ Jung Young Oh,⁶ Yu-Mi Baek,⁷ Jang Won Choi,³ and Jong Won Yun*,²

Department of Biotechnology, and Department of Bioindustry, Daegu University, Kyungsan, Kyungbuk 712-714, Korea

Received May 28, 2006

Previous studies about protein modulation with chemically induced models of diabetes in animals have yielded conflicting results, in that many investigators have reported different regulation patterns for the same proteins. Therefore, it is reasonable to determine biomarkers for prognosis and diagnosis of diabetes with time profiling for the candidate proteins. In this regard, we examined the influence of hypoglycemic fungal polysaccharides (EPS) on the time-dependent plasma protein alterations in streptozotocin-induced diabetic rats. The 2-DE analysis of rat plasma demonstrated that about 50 proteins from about 900 visualized spots were found to be differentially regulated, of which 20 spots were identified as principal diabetes-associated proteins. The results of time profiling revealed that most of the identified proteins showed significant alterations in a time-dependent manner during 14 days, with notable trends. Nine out of the twenty proteins displayed very similar time profiles between normal healthy and EPS-treated diabetic rats. Interestingly, the altered profiles of several proteins by diabetes induction almost returned to control levels after EPS treatments. In particular, we found a clear distinction in differential expression of oxidative stress proteins (ceruloplasmin and transferrin) and lipid metabolism related proteins (Apo A-I, Apo A-IV, and Apo E) in the STZ-induced diabetic rats. The data presented here have identified and characterized the time-dependent changes in plasma proteins associated with EPS treatment in STZ-induced diabetic rats, thereby leading to the discovery of early-response and late-response biomarkers in diabetic and EPS-treated states.

Keywords: diabetes • plasma proteome • polysaccharide • proteomics

Introduction

The search for appropriate hypoglycemic agents has recently focused on many plants and mushrooms used in traditional medicine, due to the various risks of antidiabetic drugs currently in use.¹⁻⁴ The extract of a medicinal mushroom *Phellinus baumii* is known to have various biological activities including inhibition of pulmonary inflammation, antioxidant, and free radical scavenging activities.⁵,⁶ In the present study, we first investigated the hypoglycemic effect of the EPS produced by submerged mycelial culture of *P. baumii* in STZ-induced diabetic rats, followed by a proteome profiling of rat plasma for 2 weeks.

 Plasma is not only the primary clinical specimen but also is the largest and most varied sample available for proteome analysis in the search for protein disease markers. The proteins in plasma perform many important functions in the body, and the protein profiles of plasma vary with physiological and pathological conditions. Alteration in serum protein concentration is commonly used in clinical practice as a nonspecific indicator of underlying disease or to monitor disease activity. In this regard, the use of plasma and serum for diagnosis is thus an obvious approach, and, in fact, such attempts have been undertaken with some success for many decades.⁷⁻¹⁶ Important parallel works on the rat plasma proteome and its perturbation by experimental treatments have been carried out by many investigators.¹⁷⁻²⁴ Although proteomics has been extensively employed to investigate cancers and other diseases, there are currently few reports concerning the proteomics study of diabetes. Recently, Sanchez et al.²⁵,²⁶ studied the differential expression of diabetes-associated proteins in pancreatic islets of type 2 diabetic mice before and after treatment of an insulin sensitizer drug, Rosiglitazone. To the best of our knowledge, there is no report describing proteomic study of diabetes before and after treatment with anti-diabetic natural sources, such as mushrooms and other herbal medicines.

Studies about plasma protein modulation with chemically induced models of diabetes in animals have yielded conflicting results, in that many investigators have reported different regulation patterns for the same proteins.¹³,¹⁷,¹²,¹⁷ These results are obviously due to using various states of the blood sample taken at different experimental time periods. In this regard, it
is strictly required to determine biomarkers for prognosis and diagnosis of specific diseases based on time profiling of the candidate proteins. This could be clinically important because variations in the expected levels of circulating plasma proteins in states of diabetes might be taken to indicate underlying diseases, and, thus, knowledge of their expected levels should aid appropriate investigation, diagnosis, and treatment.

Up to date, there are no reports describing proteome time profiling for either biofluid or other biopsies of healthy and disease states of animals, except that several investigators demonstrated time-dependent changes in either specific proteins or mRNA levels.29–33

A comparison of data from time courses is best achieved when the same animals are used throughout the entire study, possibly by drawing blood on different days starting with baseline conditions. Changes as a result of experimental treatment can thus be easily distinguished from variations in the normal profiles of single proteins. Moreover, by using 2-DE, it is possible to monitor a large number of spots to search for marker proteins for the disease. The time-dependent expression of acute-phase reactants in the rat was extensively studied with 2-DE by Schreiber et al.,34 detailing both the transcription of mRNAs and the translation into serum proteins. Also, changes in the concentration of major serum proteins were monitored during 4 days in inflamed rats. To accurately search out the disease biomarkers from blood samples, it is a prerequisite to construct a time profile of candidate proteins.

The present study monitored the blood plasma proteome changes of normal, STZ-induced diabetic, and EPS-treated diabetic rats. This investigation focused on the systematic analysis of time-related changes in diabetes-associated plasma proteins, particularly oxidative stress proteins (Ceruloplasmin and Transferrin) and lipid metabolism related proteins (Apo A-I, Apo A-IV, and Apo E). Next, detailed analysis of those proteins was carried out to obtain valuable information for the diagnosis and therapy of diabetes. This is the first report that addresses time-dependent expression proteomics dealing with plasma protein modulation in diabetes mellitus before and after treatments of antidiabetic natural sources.

Materials and Methods

Preparation of the EPS. The basidiomycetous fungus Phellinus baumii of our collection was isolated from a mountainous district in Korea. The stock culture of P. baumii was inoculated on a potato dextrose agar (PDA) slant, incubated at 28 °C for 6 days and maintained by monthly subculture. The slants were stored at 4 °C. The submerged culture of P. baumii for the production 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, CaCl2 0.55 g/L; temperature, 30 °C; aeration rate, 2 vvm; agitation speed, 150 rpm; initial pH, 5.0; working volume, 3 L. Culture broths were centrifuged at 10 000g for 20 min, and the resulting supernatant was mixed with four volumes of absolute ethanol, stirred vigorously, and left overnight at 4 °C. The precipitate of crude EPS was harvested by centrifugation at 10 000g and used in the 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.

Animals and Breeding Conditions. Twenty-four 5-week-old male Sprague–Dawley rats (Daehan Experiment Animals, Seoul, Korea) weighing 130–150 g were used. 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 experiment. After 1 week of acclimatization, the rats were subjected to a 16-h fast. Diabetes was induced by intravenously injecting streptozotocin (50 mg/kg body weight, dissolved in 0.01 M sodium citrate buffer, pH 4.5). Control rats were similarly injected with vehicle alone. The onset of diabetes was verified 48 h later by evaluating blood glucose levels using glucose oxidase reagent strips (Lifescan, Milpitas, CA). Rats with a blood glucose level ≥300 mg/dL (16.7 mmol/L) were considered diabetic. P. baumii EPS and buffer were administrated 48 h later. Blood glucose level was monitored using glucose oxidase reagent strips (Lifescan). 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.

Animal Experiments. The rats were randomly divided into four groups with six animals in each group: normal healthy control group (Group N), normal rats received 0.9% NaCl solution; normal EPS-treated control group (Group NP), normal rats administrated with P. baumii EPS at the level of 200 mg/kg body weight; STZ-induced diabetic group (Group S), diabetic rats treated with 0.9% NaCl solution; and EPS-treated diabetic group (Group P), diabetic rats treated with P. baumii EPS at the dose of 200 mg/kg body weight using an oral zoned daily for 14 days. Consequently, the blood glucose levels and plasma protein levels of the EPS-treated control group were the same as those of the normal control group as shown in our previous report.34 The influence of EPS treatment on the alteration of plasma proteins between those two groups was not so significant, with the results of similar blood glucose levels. Thus, we excluded the EPS-treated control group in the course of the proteomic study. On the basis of our preliminary test results (doses of 100 and 200 mg/kg) and other experimental designs by several investigators who used natural sources for diabetic therapy (normal dose ranges: 100–300 mg/kg), 200 mg/kg was chosen as a suitable dose for this experiment.

Oral Glucose Tolerance Test. The oral glucose tolerance test was performed after 14 days of EPS treatment. After overnight fasting, blood samples (0.2 mL) were taken from each rat 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 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 sandwich technique of enzyme immunoassay.

Preparation of the Blood Plasma Sample. Blood samples were obtained with anesthesia by resection of the terminal 1–2 mm of the rats’ tails at 0, 2, 4, and 14 day; a total of 0.5–0.6 mL of blood was drawn into sodium EDTA added tubes. Plasma was separated by centrifugation (3000g, 10 min) and then stored at −30 °C until analysis. The protein content of plasma samples was determined using the Bradford method with protein assay dye reagent concentrate (Bio-Rad, Hercules, CA).

Two-Dimensional Gel Electrophoresis (2-DE). 2-DE images were made in triplicate for the three rats in each experimental group (a total of 36 gel images for each group) on 0, 2, 4, and
14 days. 2-DE images were normalized prior to statistical analysis. The 12 gels of three groups were run at the same time and were performed in triplicate for run reproducibility. IPG IEF of samples was carried out on pH 4–7, 17 cm IPG DryStrips in the PROTEIN IEF cell (Bio-Rad) using the protocol recommended by the manufacturer. IPG strips were rehydrated passively overnight in strip holders in 350 µL of rehydration solution containing 3 µL of plasma sample. IEF was carried out as follows: 15 min at 250 V, 3 h at 250–10 000 V, 6 h at 10 000 V, and then held at 500 V until ready to run the second dimension. Briefly, 200 µg (<3 µL) of the plasma sample was mixed with 347 µL of rehydration solution containing 7 M urea, 2 M thiourea, 4% CHAPS, 1 mM PMSF, 20 mM DTT, and 2% IPG buffer. After focusing, the gel strips were equilibrated in a solution containing 6 M urea, 2% SDS, 1% DTT, 30% glycerol, and 50 mM Tris-HCl (pH 6.8) for 15 min, followed by further incubation in the same solution except for replacing DTT with 2.5% iodoacetamide for an additional 15 min. The equilibrated IPG strips were then gently rinsed with electrophoresis buffer. The gel strips were then placed on a 20 × 20 cm 12% polyacrylamide gel for resolution in the second dimension. The fractionation was performed with the Laemmli SDS-discontinuous system at a constant voltage of 20 mA per gel for 10 h, after which the separated gels were visualized by the silver staining.

Image Acquisition and Data 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. Intensity calibration was carried out using an intensity stepewedge before gel image capture. Comparison of the images was performed using a modified version of ImageMaster 2D software V4.95 (Amersham Biosciences, Little Chalfont, Buckinghamshire, England). A standard gel was generated out of the image with the highest spot number. Spot quantities of all gels were normalized to remove non-expression-related variations in spot intensity, so the raw quantity of each spot in a gel was divided by the total quantity of all of the spots in that gel that have been included in the standard. The results were evaluated in terms of spot OD (optical density). Statistical analysis of SPSS (ver.11.0) by t-test allowed the study of proteins that were significantly increased or decreased after the treatments of the cell line. A p < 0.05 was considered as significant. A total of 30 different spots were found to be differentially expressed. The relative optical density and relative volume were also calculated to correct for differences in gel staining. Each spot intensity volume was processed by background subtraction and total spot volume normalization; the resulting spot volume percentage was used for comparison.

In-Gel Digestion. Protein spots were enzymatically digested in-gel in a manner similar to that previously described by Shevchenko et al. using modified porcine trypsin. Gel pieces were washed with 50% acetonitrile to remove SDS, salts, and stain. The gel was then dried to remove solvent, rehydrated with trypsin (8–10 ng/µL), and incubated for 8–10 h at 37 °C. The proteolytic reaction was terminated by adding 5 µL of 0.5% trifluoroacetic acid. Tryptic peptides were recovered by combining the aqueous phase from several extractions of gel pieces with 50% acetonitrile. After concentration, the peptide mixture was redissolved in the buffer and desalted using C18 ZipTips (Millipore, Watford, Herts, UK), and the peptides were eluted with 1–5 µL of acetonitrile. An aliquot of this solution was mixed with an equal volume of a saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile, and 1 µL of the mixture was spotted onto a target plate.

Protein Identification. Protein analysis was performed using an Ettan MALDI-TOF (Amersham Biosciences). The peptides were evaporated with a N2 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. Spectra were calibrated with trypsin auto-digestion ion peak m/z (842.510, 2211.1046) as internal standards.

Western Blot Analysis. The differentially regulated levels of the five proteins of interest were further confirmed by Western blot analysis as described below. An aliquot of plasma (70 µg) was diluted in 2 × sample buffer (50 mM Tris (pH 6.8), 2% SDS, 10% glycerol, 0.1% bromophenol blue, and 5% β-mercaptoethanol) and heated for 5 min at 95 °C before SDS-PAGE gel analysis (7.5% and 12%). Subsequently they were transferred to a pure nitrocellulose membrane and incubated overnight with 5% blocking reagent (Amersham Biosciences) in Tris-buffered salt (TBS) containing 0.1% Tween-20 at 4 °C. The membrane was rinsed in four changes of TBS with Tween-20 (10 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20, pH 8.0), incubated twice for 5 min and twice for 10 min in fresh washing buffer. It was then incubated for 2 h with blocking solution containing 1:200 dilution of primary antibody (goat anti Apo A-I, A-IV, E, Cp, and rabbit anti Tf, Santa Cruz Biotechnology, Santa Cruz, CA). After four washes, the membrane was again incubated for 2 h in horseradish peroxidase-conjugated goat IgG and anti-rabbit IgG secondary antibody (1:1000, Santa Cruz Biotechnology) and developed using enhanced chemiluminescence (ECL Western blot analysis system kit, Amersham Biosciences). The Western blot was analyzed by scanning with a UMAX PowerLook 1120 (Maxium Technologies, Inc.) and digitalizing using image analysis software (KODAK 1D, Eastman Kodak Co., NY).

Statistical Analysis. All experimental results were compared by one-way analysis of variance (ANOVA) using the Statistical Package of Social Science (SPSS, ver. 11.0) program, and the 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 ANOVA indicated an overall significant treatment effect, p < 0.05.

Results

Effect of Oral Glucose Tolerance Test. Prior to the proteomics study, the hypoglycemic effect of the fungal EPS 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 diabetic rats (Figure 1).27 Figure 1A and B shows the changes in the levels of blood glucose and insulin, respectively, during oral glucose tolerance test (2 g/kg). The STZ-treated rats showed a significant increase in the blood glucose at 60 min. In EPS-treated rats, blood glucose concentration was significantly lower 60 min after glucose administration as compared to diabetic control (p < 0.05). As shown in Figure 1B, the rise of plasma insulin observed in normal control rats was abolished in STZ-treated
A pattern similar to those of the Rat Serum Protein Study Group. Overall, the plasma maps obtained from our gels showed dimension and in a 12% SDS-PAGE gel in the second dimension and their restoration showed no clear trends (Table 2, Figures 3 and 4). The proteins significantly changed upon diabetes induction but were not restored to approximate levels of the normal healthy group after EPS treatment. (2) The proteins significantly changed upon diabetes induction but were not restored to approximate levels of the normal healthy group after EPS treatment. (3) The proteins were not significantly changed by both diabetes induction and EPS treatment. (4) The proteins significantly changed only by EPS treatment. Nine proteins were included in category (1): Ab, Apo A-I, Apo A-IV, Cp, Ft, Ig kappa chain, KBP, TTR monomer, and Vn. Seven proteins were included in category (2): α1-M, Apo A-IV, Cp, Ft, Hp, Ig kappa chain, TTR tetramer, and Tf. Two proteins were included in category (3): α1-HS and Gc. Two proteins were included in category (4): 1-AT and α1-M.

In the early stage (until day 2), the levels of the nine proteins (e.g., α1-AT, α1-I, α1-M, Apo A-I, Hp, Ig kappa chain, TTR tetramer, and Vn) were significantly decreased, whereas four proteins (e.g., Cp, Gc, Kbp, and SPI III) showed a marked rapid increase after diabetes induction. In the late stage (at day 14), five proteins (e.g., α1-AT, Apo A-I, Apo A-IV, Ig kappa chain, and Tf) sharply increased, while levels of the two proteins (e.g., Apo E and Kbp) strikingly decreased after diabetes induction. However, the effects of EPS treatment on their restoration showed no clear trends (Table 2, Figures 3 and 4).

In this study, we found a clear distinction in differential expression of many proteins, particularly oxidative stress proteins (Cp and Tf) and lipid metabolism related proteins (Apo A-I, Apo A-IV, Apo E) in the STZ-induced diabetic rats. As for

Table 1. Identification of Diabetes-Associated Proteins in Rat Plasma

<table>
<thead>
<tr>
<th>spot no.</th>
<th>protein*</th>
<th>accession no.</th>
<th>kDa</th>
<th>pl</th>
<th>coverage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>α1-AT</td>
<td>gi:112889, P17475</td>
<td>46.14</td>
<td>5.70</td>
<td>28</td>
</tr>
<tr>
<td>2</td>
<td>α1-HS</td>
<td>gi:56140, P231900</td>
<td>38.76</td>
<td>6.00</td>
<td>32</td>
</tr>
<tr>
<td>3</td>
<td>α1-I III</td>
<td>gi:128913225</td>
<td>166.68</td>
<td>5.70</td>
<td>9</td>
</tr>
<tr>
<td>4</td>
<td>α1-M</td>
<td>gi:202857, Q63332</td>
<td>167.16</td>
<td>6.46</td>
<td>10</td>
</tr>
<tr>
<td>5</td>
<td>Ab (8 spots)</td>
<td>gi:113598, P0027070</td>
<td>68.72</td>
<td>6.09</td>
<td>56</td>
</tr>
<tr>
<td>6</td>
<td>Apo A-I (3 spots)</td>
<td>gi:113997, P04639</td>
<td>30.09</td>
<td>5.52</td>
<td>48</td>
</tr>
<tr>
<td>7</td>
<td>Apo A-IV (4 spots)</td>
<td>gi:114008, P00691</td>
<td>44.46</td>
<td>5.12</td>
<td>38</td>
</tr>
<tr>
<td>8</td>
<td>Apo E</td>
<td>gi:1703318, P02650</td>
<td>35.75</td>
<td>5.23</td>
<td>46</td>
</tr>
<tr>
<td>9</td>
<td>Cp</td>
<td>gi:997109, P13635</td>
<td>120.84</td>
<td>5.34</td>
<td>13</td>
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<td>10</td>
<td>Fp β (3 spots)</td>
<td>gi:178655327</td>
<td>42.37</td>
<td>6.70</td>
<td>10</td>
</tr>
<tr>
<td>11</td>
<td>Gc (2 spots)</td>
<td>gi:139841, P04726</td>
<td>53.54</td>
<td>5.65</td>
<td>24</td>
</tr>
<tr>
<td>12</td>
<td>Hb β (3 spots)</td>
<td>gi:125313, P06866</td>
<td>38.55</td>
<td>6.10</td>
<td>26</td>
</tr>
<tr>
<td>13</td>
<td>Hpx (6 spots)</td>
<td>gi:123036, P020659</td>
<td>51.29</td>
<td>7.58</td>
<td>30</td>
</tr>
<tr>
<td>14</td>
<td>Ig kappa light chain</td>
<td>gi:18025664</td>
<td>11.76</td>
<td>8.73</td>
<td>26</td>
</tr>
<tr>
<td>15</td>
<td>Kbp (5 spots)</td>
<td>gi:266407, P05545</td>
<td>46.56</td>
<td>5.31</td>
<td>23</td>
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<tr>
<td>16</td>
<td>SPI III (5 spots)</td>
<td>gi:927388, P09690</td>
<td>46.65</td>
<td>5.32</td>
<td>25</td>
</tr>
<tr>
<td>17</td>
<td>Tf</td>
<td>gi:6715089, P12346</td>
<td>76.36</td>
<td>6.94</td>
<td>34</td>
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<tr>
<td>18</td>
<td>TTR monomer</td>
<td>gi:3212535</td>
<td>13.12</td>
<td>6.04</td>
<td>59</td>
</tr>
<tr>
<td>19</td>
<td>TTR tetramer</td>
<td>gi:3212535</td>
<td>50.28a</td>
<td>5.80a</td>
<td>48</td>
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<td>20</td>
<td>Vn (5 spots)</td>
<td>gi:9507241</td>
<td>55.46</td>
<td>5.70</td>
<td>15</td>
</tr>
</tbody>
</table>

* For protein nomenclatures, see the Abbreviations section. b Molecular weight of TTR tetramer was estimated from 2-DE gel (Figure 2). c Coverage: percent of identified sequence to the complete sequence of the known protein.

About 50 proteins from about 900 visualized spots were found to be differentially regulated, of which 20 spots were identified as principal diabetes-associated proteins by peptide mass fingerprinting (Table 1, Figure 2).

The time-dependent changes in most plasma proteins identified were observed in three groups during the entire experimental period (from day 0 to day 14). The alteration patterns of the identified 20 proteins throughout the entire periods were investigated by dividing into four main categories (Table 2, Figures 3 and 4): (1) The proteins significantly changed upon diabetes induction and were restored approximately to the levels of the normal healthy group after EPS treatment. (2) The proteins significantly changed upon diabetes induction but were not restored to approximate levels of the normal healthy group after EPS treatment. (3) The proteins were not significantly changed by both diabetes induction and EPS treatment. (4) The proteins significantly changed only by EPS treatment.

For protein nomenclatures, see the Abbreviations section.
principal lipid binding proteins (apolipoproteins), the concentration changes in Apo A-I, Apo A-IV, and Apo E were similarly regulated from 0 to 4 days in three groups; thereafter no clear trends were detected in alterations by both STZ and EPS treatments. Interestingly, the time profiles of Apo A-I and Apo E, but not of Apo A-IV, in diabetic controls were returned to those of healthy rats after EPS treatment.

Meanwhile, the plasma levels of both Cp and Tf were remarkably increased from day 2, after diabetic induction. EPS treatment led to a decrease in Cp level and constancy of Tf.

<table>
<thead>
<tr>
<th>spot no.</th>
<th>protein</th>
<th>alteration at day 2</th>
<th>alteration at day 14</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>α₁-AT (6 spots)</td>
<td>1.018 1.085 1.105</td>
<td>1.175 0.764 0.898</td>
</tr>
<tr>
<td>2</td>
<td>α₂-HS</td>
<td>0.978 1.009 0.987</td>
<td>0.999 0.976 0.975</td>
</tr>
<tr>
<td>3</td>
<td>α₁-I III</td>
<td>0.773 1.156 0.894</td>
<td>0.665 1.342 0.990</td>
</tr>
<tr>
<td>4</td>
<td>α₁-M (3 spots)</td>
<td>1.052 1.072 1.102</td>
<td>1.012 1.029 1.042</td>
</tr>
<tr>
<td>5</td>
<td>Ab (8 spots)</td>
<td>0.877 0.966 0.848</td>
<td>0.816 1.125 0.918</td>
</tr>
<tr>
<td>6</td>
<td>Apo A-I (3 spots)</td>
<td>1.274 0.887 1.131</td>
<td>1.419 0.661 0.938</td>
</tr>
<tr>
<td>7</td>
<td>Apo A-IV (4 spots)</td>
<td>1.163 0.767 0.892</td>
<td>1.374 0.849 1.167</td>
</tr>
<tr>
<td>8</td>
<td>Apo E</td>
<td>0.950 0.979 0.930</td>
<td>0.559 1.866 1.043</td>
</tr>
<tr>
<td>9</td>
<td>Cp</td>
<td>1.559 1.025 1.598</td>
<td>1.378 0.885 1.220</td>
</tr>
<tr>
<td>10</td>
<td>Ft β (3 spots)</td>
<td>0.944 1.129 1.066</td>
<td>0.944 1.054 0.995</td>
</tr>
<tr>
<td>11</td>
<td>Gc (2 spots)</td>
<td>0.815 1.046 0.852</td>
<td>0.660 1.515 1.000</td>
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<td>12</td>
<td>Hp β (3 spots)</td>
<td>1.117 1.495 1.793</td>
<td>0.979 1.023 1.001</td>
</tr>
<tr>
<td>13</td>
<td>Hpx (6 spots)</td>
<td>0.743 1.474 1.096</td>
<td>0.688 1.526 1.050</td>
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<tr>
<td>14</td>
<td>Ig kappa light chain</td>
<td>1.102 1.146 1.146</td>
<td>1.334 0.802 0.070</td>
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<tr>
<td>15</td>
<td>KBP (5 spots)</td>
<td>0.997 1.016 1.013</td>
<td>0.576 1.254 0.722</td>
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<tr>
<td>16</td>
<td>SPI III (5 spots)</td>
<td>1.383 0.610 0.843</td>
<td>1.547 0.893 1.381</td>
</tr>
<tr>
<td>17</td>
<td>Ttr</td>
<td>1.155 0.892 1.029</td>
<td>1.816 0.770 1.398</td>
</tr>
<tr>
<td>18</td>
<td>TTR monomer</td>
<td>1.178 0.949 1.118</td>
<td>0.890 1.140 1.015</td>
</tr>
<tr>
<td>19</td>
<td>TTR tetramer</td>
<td>0.586 1.110 0.650</td>
<td>0.855 1.381 1.180</td>
</tr>
<tr>
<td>20</td>
<td>Vn (5 spots)</td>
<td>0.384 2.130 1.243</td>
<td>0.787 1.604 1.263</td>
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</tbody>
</table>

* For protein nomenclatures, see the Abbreviations section.
level (Table 2, Figure 4). It is noteworthy that no significant alterations in the levels of Tf were observed in EPS-treated rats during the 2 weeks, even though their time profiles were significantly changed in normal and diabetic controls. Taken
together, except for Apo A-IV, the levels of oxidative proteins and lipid metabolism related proteins were restored to approximately those of healthy rats by EPS treatment, although the order of magnitude differed widely.

To confirm and cross-check the results obtained from the proteomics study, the time-dependent changes in the five proteins of interest (Apo A-I, Apo A-IV, Apo E, Cp, and Tf) were further examined by Western blot analysis. Interestingly, the time profiles of Apo A-IV and Apo E in Western blot data were normalized by EPS, whereas treatment of EPS did not affect Apo A-I levels. Overall, there was good correlation in the time profiles of most proteins between the results of 2-DE gel and Western blot analysis (Figure 5).

Discussion

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. Most investigators have attempted to find marker proteins associated with various diseases using 2-DE at a particular time point.\textsuperscript{18-27} Because either acute or chronic overexposure may affect the net protein balance by increasing catabolic pathways, mining disease biomarkers by proteomics should be performed carefully with additional time profiling of the candidate proteins. Nevertheless, to the best of our knowledge, there has been no report of expression proteomics study with time profiling of plasma protein modulation on diabetes with antidiabetic drugs or other natural sources.

Only a few reports are currently available, dealing with time-dependent protein changes in disease states. Previous investigations in the adult rat have demonstrated that both hyperglycemia and hypoglycemia lead to time-dependent and tissue-specific responses expressed by glucose transporter proteins (e.g., GLUT-1, GLUT-3, and GLUT-4).\textsuperscript{38-40} Das et al.\textsuperscript{30} determined the acute and chronic time-dependent and tissue-specific effects of fetal hyperglycemia and hypoglycemia on these proteins in fetal ovine insulin-insensitive (brain and liver) and insulin-sensitive (myocardium, skeletal muscle, and adipose) tissues. They derived a conclusion that fetal hyperglycemia or hypoglycemia causes time-dependent and tissue- and isoform-specific changes in fetal glucose transporter levels.\textsuperscript{30}

It has been reported that the serum levels of α\textsubscript{1}-I III reduced in diabetes, while macroglobulin did not change appreciably in diabetic rats.\textsuperscript{40,41} The serum Hp concentration in peritoneal dialysis patients with diabetes is lower than that in patients without diabetes, and it is not related to inflammation, nutrition, or adequacy of dialysis.\textsuperscript{43} Miller et al.\textsuperscript{44} tried to elucidate whether protracted diabetes in rats is associated with changes in circulating plasma Hpx levels in rats analogous to those reported in human diabetic patients with clinical evidence of complications. The Vn might be increased from activated platelets in the early stage of diabetic nephropathy.\textsuperscript{45} There were, however, conflicting results on the serum levels of α\textsubscript{1}-AT: both increased and decreased levels have been reported in patients with T1DM.\textsuperscript{27,28,46-50} These controversial results might be obtained without data of time-dependent profiling. For this reason, we must carefully choose biomarkers with time-dependent profiling of each candidate protein.

Of the identified proteins in this study, TTR and Ig kappa light chain are proteins that are rarely discussed in connection with diabetes biomarkers. TTR is a transport protein for thyroxine, which is synthesized in liver and pancreas.\textsuperscript{51} In the present study, the time profiles of TTR monomer (inactive form) and TTR tetramer (active form) displayed opposite modulation of plasma protein modulation on diabetes with antidiabetic drugs or other natural sources.

![Figure 4](image-url) Time profiles of five plasma proteins of interest (Apo A-I, Apo A-IV, Apo E, Ceruloplasmin, and Transferrin) in normal and diabetic rat groups during experiment periods. *p < 0.05 Group N versus Group N, **p < 0.001 Group N versus Group N; †p < 0.05 Group S versus Group N, ††p < 0.001 Group S versus Group N; ‡p < 0.05 Group P versus Group N, ‡‡p < 0.001 Group P versus Group N. Group N, in top panels; Group S, in middle panels; Group P, in bottom panels. Time 0 means the time 48 h after intravenous injection of streptozotocin or NaCl solution. Notations for each group are the same as in Figure 1.
T1DM, whereas that of the monomeric form was increased. They also demonstrated that conversion of the tetrameric form of TTR to the monomeric form might serve as an early step associated with the development of \( \beta \)-cell failure/destruction in T1DM. There are several reports mentioning a significant decrease in the serum TTR concentration in T1DM patients compared to that in normal subjects.\(^52\)\textsuperscript{55} In patients with diabetes mellitus, increased urinary excretion of kappa light chain has been reported by Groop et al.\(^56\) However, the accurate mechanism responsible for the increased Ig kappa light chain level in diabetes remains uncertain at present, even though many investigators have studied its roles in cellular events.\(^57\)\textsuperscript{61}

Figure 5. Detection of five proteins of interest (Apo A-I, Apo A-IV, Apo E, Ceruloplasmin, and Transferrin) by Western blotting. Band density was digitized with software, and mean \( \pm \) SE of three independent experiments are shown. *\( p < 0.05 \) Group N versus Group N, **\( p < 0.01 \) Group N versus Group N; \( \dagger \dagger \ p < 0.001 \) Group S versus Group N, \( \dagger \dagger \dagger \ p < 0.001 \) Group P versus Group N. Time 0 means the time 48 h after intravenous injection of streptozotocin or NaCl solution. Notations for each group are the same as in Figure 1.
In this study, we found a clear distinction in differential expression of oxidative stress proteins (Cp, Tf) and lipid metabolism related proteins (Apo A-I, Apo A-IV, Apo E) (Figures 4 and 5). Cp and Tf are well-known antioxidative molecules in mammals. It has been suggested that Cp inhibits the autoxidation of lipids of ox-brain homogenate. However, the mechanism of the antioxidative effect of Cp has not been clarified in complex systems such as in vivo because the antioxidative effect of Cp may depend on the activities of ferrooxidase, ascorbate oxidase, O₂- scavenging, and GSH-dependent peroxidase. Increased oxidative stress has been implicated in the pathogenesis of diabetes. Free iron is capable of stimulating the production of free radicals, which causes oxidative damage such as lipid peroxidation. One of the most important mechanisms of antioxidant defense is thus the sequestration of iron in a redox-inactive form by Tf. Cp permits the incorporation of iron into Tf without the formation of toxic iron products. Of the extracellular antioxidants, Cp oxidizes Fe²⁺ to ferric iron (Fe³⁺) and facilitates the binding of Fe³⁺ to Tf and also has a marked accelerating effect on the rate of Tf–Fe³⁺ interactions.

TF inhibits iron ion-dependent hydroxyl radical formation from H₂O₂. Tf is also synthesized in the liver and circulates in the blood as a carrier protein of iron, two iron atoms per molecule. The positive correlation between Cp and Tf in healthy control subjects may suggest collaboration between these two extracellular antioxidants. However, unexpectedly, any possible roles of EPS in the modulation of plasma levels of Cp and Tf were observed in our investigation.

Furthermore, it is clear that diabetes is associated with marked alterations in the levels and distributions of the major apolipoproteins associated with circulating lipoproteins. These lipoproteins in diabetic patients exhibit alterations in composition of apolipoproteins of both intestinal and hepatic origin. In this connection, increased production of intestinal lipoproteins during experimental diabetes has been reported in rats. This might account, in part, for increased levels of plasma apo A-I and Apo A-IV in diabetes. Moreover, Apo E has been implicated in the activity of lipoprotein lipase, and its diminished level in VLDL-like particles in diabetic plasma could be a factor. Several studies have shown that the level of apolipoprotein changes in T1DM and other diseases.

Consequently, insulin treatment of STZ-induced diabetic rats significantly decreased plasma Apo A-I levels and normalized plasma apo E levels, but did not cause any changes in both plasma Apo A-I and Apo E levels in normal rats. O’Looney et al. demonstrated that diabetes was associated with significant increases in Apo A-I, Apo A-IV, and Apo B, but with a great decrease in Apo E in the whole plasma. The changes of these three apolipoproteins in this work are in accordance with those of earlier results. The elevated level of Apo E after EPS treatment (Group P) in this study implies that the increase in Apo E is probably attributable to the enhanced lipid metabolism. Apo A-I participates in the reverse transport of cholesterol from tissues to the liver for excretion by promoting cholesterol efflux from tissues and by acting as a cofactor for lecithin cholesterol acyltransferase. It is noteworthy that the altered levels of Apo A-I and Apo E according to proteomic data, but not of Apo A-IV, after diabetes induction were returned to approximately the normal plasma profiles by EPS treatment (Figures 4 and 5). In the Western blot data, the time profiles of Apo A-IV and Apo E in Western blot were normalized by EPS, whereas treatment of EPS did not affect Apo A-I levels. Therefore, the most convincing data are around Apo E in this study (Figures 4 and 5).

We believe that our experimental animal model may be a useful tool for the primary screening of molecules with potential antidiabetic activity. Although technically demanding, the detailed protocol appears cost-effective for the large-scale screening of new drugs, because information is provided at once on a large number of proteins, and unusual/unexpected modifications will not escape detection.

From the proteomic analysis of rat plasma, we first came to the important conclusion that the fungal polysaccharides contribute to normalization of the levels of several oxidative stress proteins and lipid metabolism related proteins even though the molecular mechanisms for such changes are not clear yet. However, these results suggest that the EPS of mushroom origin can decrease to the increased oxidative stress and lipid peroxidation observed in diabetes.

In this study, monitoring the effect of hypoglycemic fungal polysaccharides on the plasma proteins in diabetic rats was successfully established by global protein profiling on 2-DE. As expected, significant time-dependent changes were observed in most identified plasma proteins during experimental periods (14 days) in three experimental groups.

Of the 20 proteins identified, the plasma time profiles of nine proteins (e.g., Ab, Hp β, Ig kappa chain, KBP, TTR monomer, TTR tetramer, Vn, Apo A-I, and Apo E) in EPS-treated rats were very similar to those of the normal healthy rats. This result provided evidence of the improvement in glucose metabolism in STZ-induced diabetic rats upon EPS consumption. Furthermore, these plasma proteins probably mediated the effects of EPS on hyperglycemia in the diabetic state.

The concentrations of many plasma proteins were dramatically changed from the early stage and were maintained or markedly changed in the late stage in diabetic rats, while the EPS treatment reverted their levels to around normal protein profiles.

These results encouraged us to believe that oral administration of mushroom polysaccharide may have potential benefit in the early stage of diabetes, because pancreatic damage induced by environmental chemicals and factors is a cause of diabetes. However, it should be mentioned here that plasma protein concentrations are obviously influenced by the metabolic control of diabetes, but may not directly reflect the concentration of blood glucose.

In conclusion, the data presented here have identified and characterized the time-dependent changes in plasma proteins associated with EPS treatment in STZ-induced diabetic rats. This time-dependent study led to the discovery of early-response and late-response biomarkers in diabetic and EPS-treated states.

**Abbreviations:** α₁-I III, α₁-inhibitor III; α₁-AT, α₁-antitrypsin; α₁-M, α₁-macroglobulin; α₂-HS, α₂–HS-glycoprotein; Ab, albumin; Apo, apolipoprotein; Cp, ceruloplasmin; EPS, exopolysaccharide; Ft β, fetuin beta; Gc, Gc-globulin; Hp β, haptoglobin beta; Hpx, hemopexin; Ig, immunoglobulin; KBP, kallikrein-binding protein; SPI, serine protease inhibitor; STZ, streptozotocin; Tf, transferrin; TTR, transthyretin; T1DM, type 1 diabetes mellitus; T2DM, type 2 diabetes mellitus; Vn, vitronectin; 2-DE, two-dimensional gel electrophoresis.

**Acknowledgment.** This work was supported by grant no. R01-2005-000-10326-0 from the Basic Research Program of
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PR0602601