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Liquid chromatographic separation of proteins derivatized with a fluorogenic reagent at cysteinyl residues on a non-porous column for differential proteomics analysis

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ABSTRACT

A wide-pore (30 nm) reversed-phase column (Intrada WP-RP, particle size 3 μm) was recently utilized for protein separation in differential proteomics analysis with fluorogenic derivatization-liquid chromatography–tandem mass spectrometry (FD–LC–MS/MS), and exerted a tremendous effect on finding biomarkers (e.g., for breast cancer). Further high-performance separation is required for highly complex protein mixtures. A recently prepared non-porous small-particle reversed-phase column (Presto FF-C18, particle size: 2 μm) was expected to more effectively separate derivatized protein mixtures than the wide-pore column. A preliminary experiment demonstrated that the peak capacity of the former was threefold greater than that of the latter in gradient elution of a fluorogenic derivatized model peptide, calcitonin. The FD–LC–MS/MS method with a non-porous column was then optimized and applied to separate liver mitochondrial proteins that were not efficiently separated with the wide-pore column. As a result, high-performance separation of mitochondrial proteins was accomplished, and differential proteomics analysis of liver mitochondrial proteins in a hepatitis-infected mouse model was achieved using the FD–LC–MS/MS method with the non-porous column. This result suggests the non-porous small-particle column as a replacement for the wide-pore column for differential proteomics analysis in the FD–LC–MS/MS method.

1. Introduction

High-performance liquid chromatography (HPLC) has been used for separating highly complex mixtures of compounds, such as cell and tissue extracts. However, because efficient separation of intact proteins is difficult, one-dimensional (1D) or multidimensional (mD) HPLC is usually performed with peptides generated by digesting intact proteins in proteomics analysis (reviewed in Ref. [1]). In contrast, we have developed the first reproducible quantification method using 1D HPLC for proteomics analysis, called fluorogenic derivatization-liquid chromatography-tandem mass spectrometry (FD–LC–MS/MS) with a database-searching algorithm. Intact protein mixtures were first derivatized at cysteinyl residues with a fluorogenic reagent, followed by isolation with a wide-pore reversed-phase column, Intrada WP-RP (30 nm pore size and 3 μm particle) (Imtakt, Kyoto, Japan), digestion of the derivatized proteins, and identification of the isolated proteins [2]. Application to real biological samples indicated the appearance of more than 400 or 500 proteins on a chromatogram [3–7]. Differential proteomics analysis demonstrated the existence of many proteins related to an early stage of Parkinson's disease [3], developmental stages of hepatocarcinogenesis [4], metastatic or normal breast cancer cells [5], the aging of rat brain regions [6], and the running speed of horses [7].

Differential proteomics analysis of liver proteins between hepatitis C virus (HCV) core gene transgenic (Tg) and non-transgenic (NTg) mice indicated some disease-related proteins in the developmental stages of hepatocarcinogenesis [4]. Since many of those proteins were related to the function of mitochondrial events (e.g., respiration, electron-transfer system, and β-oxidation), we further performed differential proteomics analysis of liver mitochondrial proteins between Tg and NTg mice by FD–LC–MS/MS to clarify the role of mitochondrial proteins. In a preliminary experiment, however, it was difficult to separate the mitochondrial protein mixture

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effectively using the conventional wide-pore column that was used for the FD–LC–MS/MS method. Therefore, we searched for other columns that have higher-performance separation ability than the wide-pore column.

According to recent technical developments, the use of a stationary phase of small and non-porous particles (sub-2 μm) reduces eddy diffusion and mass-transfer resistance in the mobile phase more than porous particles [8]. Chong et al. used sub-2 μm non-porous particles for separating intact proteins in biological samples [9]. However, the reproducibility of the retention time of each protein was very low, probably due to the hydrophobicity of the intact proteins and the large amount of proteins provided for ultraviolet detection, which could prevent using a non-porous column for differential proteomics analysis. In contrast, with FD–LC–MS/MS, the non-porous column seems to be useful because the proteins are derivatized into less hydrophobic ones with the hydrophilic reagent, and one or two orders of magnitude less amount of proteins is sufficient for fluorescence detection than for ultraviolet detection.

Therefore, in this study, we applied a non-porous small-particle reversed-phase column (Presto FF-C18, 2 μm particle, Imtakt) to the FD–LC–MS/MS method. Based on an investigation of column lengths and flow rates for the non-porous column, the optimized FD–LC–MS/MS method was applied to liver mitochondrial proteomics analysis, resulting in high-performance separation of the mitochondrial proteins. This result suggested the non-porous small-particle column as a replacement for the wide-pore column in differential proteomics analysis utilizing the FD–LC–MS/MS method. Also, the result of liver mitochondrial proteomics analysis indicated proteins related to hepatocarcinogenesis; thus, the roles of proteins in hepatocarcinogenesis will be investigated.

2. Experimental

2.1. Reagents

For this study, 7-chloro-N-[2-(dimethylamino)ethyl]-2,1,3-benzoxadiazole-4-sulfonamide (DAABD-Cl) and Buffer Solution pH 8.7 (6 M Guanidine Hydrochloride) were obtained from Tokyo Chemical Industry (Tokyo, Japan). In addition, 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate (CHAPS) and ethylenediamine-N,N,N′,N′-tetraacetic acid disodium salt (Na_2EDTA) were obtained from Dojindo Laboratories (Kumamoto, Japan). Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) and β-lactoglobulin (M.W. 18,363) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Calcitonin (M.W. 3,418) was purchased from Peptide Institute (Osaka, Japan). Trifluoroacetic acid (TFA) was obtained from Wako Pure Chemical Industries (Osaka, Japan). Acetonitrile (HPLC grade) was obtained from Kanto Chemical (Tokyo, Japan). All the other reagents were of analytical reagent grade and were used without further purification. Water was used after purification with the Milli-Q system (Nihon Millipore, Tokyo, Japan).

2.2. Columns

Non-porous spherical silica (2 μm particle and 2 m^2/g specific surface area) was utilized as packing material in the Presto FF-C18 column (Fig. 1) (Imtakt, Kyoto, Japan). Octadecylsilane (ODS) binds to functional groups on packing materials, indicating that Presto FF-C18 is useful for reversed-phase separation in HPLC. However, wide-pore spherical silica (30 nm pore size, 3 μm particle, and 100 m^2/g specific surface area) was utilized in the Intrada WP-RP column (Imtakt). Reversed-phase ligands exist on the surface of the packing materials of Intrada WP-RP, which was used as a conventional protein separation column for the FD–LC–MS/MS method. Presto FF-C18 columns were adopted with 4.6 mm i.d. and 50–250 mm length, while Intrada WP-RP with 4.6 mm i.d. and 250 mm length was usually utilized for protein separation [2–7].

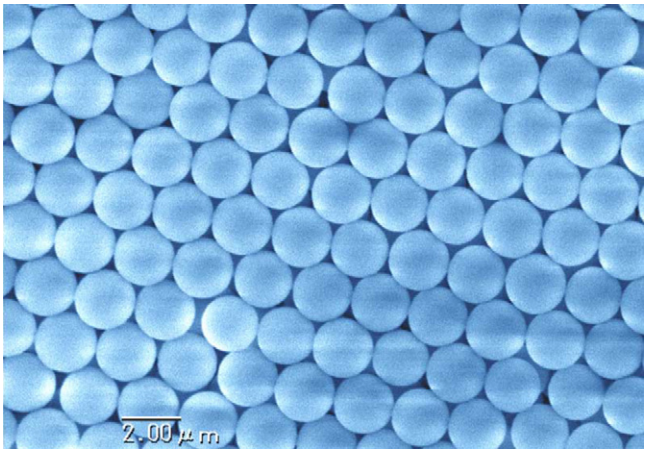

Fig. 1. Electron microscopic image of non-porous spherical silica (2 μm particle) utilized as a packing material in a Presto FF-C18 column.

2.3. FD reaction and separation of DAABD-calcitonin on the non-porous or the wide-pore column

A 10 μL aliquot of 5 μM calcitonin (Peptide Institute, Osaka, Japan) was mixed with 60 μL of 16.7 mM CHAPS/3.33 mM Na_2EDTA/0.833 mM TCEP in 6 M guanidine buffer (pH 8.7), 25 μL of 6 M guanidine buffer (pH 8.7), and 5.0 μL of 140 mM DAABD-Cl in acetonitrile. Each reaction mixture was incubated at 40 °C for 10 min, and the reaction was stopped with 3.0 μL of 20% TFAaq. The reaction mixture was then diluted threefold with the mobile phase. A 10 μL aliquot of the diluted reaction mixture was injected into an HPLC system that consisted of a pump (L-2100, Hitachi) and a fluorescence detector (L-2485, Hitachi). Fluorescence detection was carried out at 505 nm (excitation at 395 nm). Separation was performed on the non-porous column (4.6 i.d. × 50, 100, 150, or 250 mm) or the wide-pore column (4.6 i.d. × 250 mm) (Imtakt, Kyoto, Japan). The column temperature was set at 60 °C, and the flow rate was 0.2–0.5 mL/min. The gradient elution was 10–40% B over 60 min ((A) water:acetonitrile:TFA = 90:10:0.10, v/v/v; (B) water:acetonitrile:TFA = 30:70:0.20, v/v/v).

2.4. Preparation of liver mitochondrial sample and determination of total proteins

Sixteen-month-old Tg and NTg mice were used for analysis. Progression of disease state and morphological features were described in previous reports [4,10].

A preliminary study clearly indicated that an extraction procedure utilizing a mitochondrial isolation commercial kit was not useful, due to the low repeatability in isolation handling. Therefore, in this study, mitochondria were extracted from liver samples (100 mg) by density-gradient centrifugation using a mannitol/sucrose solution, as reported by Lopez et al. [11]. The mitochondrial pellet obtained was suspended with twice-volume of 2% CHAPS in 6 M guanidine buffer (pH 8.7). The suspension was sonicated for 15 s on ice four times at 15 s intervals. The sonicated suspension was centrifuged at 13,000 g for 2 min at 4°C. The supernatant was then collected and stored as a soluble fraction at −80°C after freezing with liquid nitrogen. The total liver mitochondrial proteins were determined with a BCA™ Protein Assay

Kit (Thermo Scientific, Rockford, IL, USA), following the written instructions. Bovine serum albumin was used as a protein standard.

2.5. FD–LC–MS/MS method for liver mitochondrial proteomics analysis

The previous method was used for the FD procedure for liver mitochondrial proteins with DAABD-Cl [4], except for the amount of total protein; in brief, 60 μg of liver mitochondrial proteins was derivatized in 100 μL reaction mixture. Twenty microliters of the reaction mixture (12 μg proteins) was subjected to HPLC. Sample proteins amount per injection was low enough as compared to the maximum (24 μg) for separation on the non-porous column. The overall system consisted of a Hitachi L-2000 series HPLC system with a non-porous column (4.6 i.d. × 250 mm) at a column temperature of 60 °C [2] and a flow rate of 0.3 mL/min. Fluorescence detection was carried out at 505 nm (excitation at 395 nm). The compositions of the mobile phases were the same as described above. The 267.5 min gradient program was used to compare the non-porous column with the wide-pore column. The gradient elution was 16% B held over 5 min, to 25% in 10 min, to 43% B in 112.5 min, to 45% B in 135 min, to 55% B in 185 min, to 65% B in 215 min, and to 100% B in 267.5 min. The 535 min gradient program was used for proteomics analysis of mitochondrial proteins in livers of the hepatitis-infected mouse model. The gradient elution was 16% B held over 10 min, to 25% in 20 min, to 43% B in 225 min, to 45% B in 270 min, to 55% B in 370 min, to 65% B in 430 min, and to 100% B in 535 min. To keep the long life-time of the non-porous column, a washing operation was performed after operation of each analysis. The gradient time program of the washing operation was 100 to 0% B in 5 min and 0 to 100% B in 10 min at 0.3 mL/min of flow rate, which was repeated four times.

The isolated derivatized proteins were identified as reported in Ref. [5] using HPLC and tandem mass spectrometry. The obtained amino acids sequence data were searched for the taxonomy *Mus musculus* against the National Center for Biotechnology Information non-redundant (NCBInr) database using MASCOT version 2.1.03 (Matrix Science, Ltd., London, UK).

3. Result

3.1. Separation of DAABD-calcitonin in gradient elution with the non-porous column

The non-porous column was applied to separate fluorogenic derivatized calcitonin, a model peptide, to investigate its separation efficiency. Calcitonin (0.5 μM, M.W. 3418) was derivatized with a fluorogenic reagent, 7.0 mM DAABD-Cl, and subjected to HPLC-fluorescence detection in gradient elution on either the non-porous or the wide-pore column. Both columns were the same size (4.6 i.d. × 250 mm). The retention times and shapes of both DAABD-calcitonin peaks suggested that the non-porous column exhibited stronger affinity for the peptide and higher resolution than the wide-pore column (Fig. 2). The retention time of the compounds less retained on the non-porous column was shorter than that on the wide-pore column. The separation efficiencies of both columns were then compared utilizing the peak capacity, since the separation efficiency of HPLC columns in gradient elution is usually evaluated with column peak capacity P, while under isocratic conditions it is evaluated with theoretical plates N. The peak capacity represents the maximum theoretical number of components that can be separated in a column within a given gradient time. Each P value was then calculated from peak width w measured at 4σ

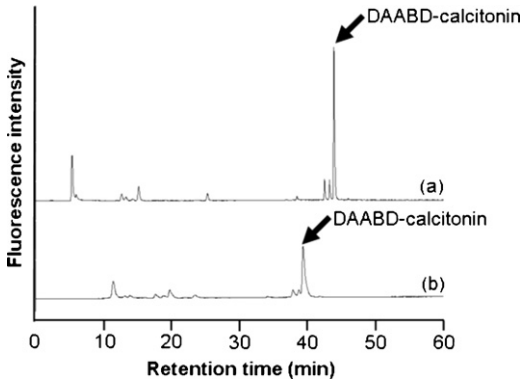

Fig. 2. Chromatograms obtained from DAABD-calcitonin separated in (a) the non-porous column or (b) the wide-pore column under gradient elution conditions. Chromatographic conditions are described in Section 2.

(13.4% of peak height) and the gradient time t_g according to Eq. (1) [12]:

$$P = 1 + \frac{t_g}{w} \quad (1)$$

The P value with the non-porous column was found to be three-fold higher than that with the wide-pore column under 60 min gradient conditions (197 with the non-porous column vs. 64 with the wide-pore column). In a similar experiment using a typical standard protein (β-lactoglobulin, M.W. 18,363), the P value exhibited the same tendency with both columns (data not shown). These results indicate that separation efficiency in the non-porous column was superior to that in the wide-pore column.

3.2. Optimized column length and flow rate with the non-porous column

In order to obtain appropriate separation efficiency, the gradient elution of DAABD-calcitonin was performed with different lengths (50, 100, 150, and 250 mm) of the non-porous column at different flow rates (0.2, 0.3, 0.4, and 0.5 mL/min). However, for the 250 mm-long column, the flow rate was limited to a maximum of 0.3 mL/min because of the durability of the HPLC flow (20 MPa) system used in the present experiment. DAABD-calcitonin was separated in the same 60 min gradient program as described in Section 3.1, and each P value was calculated according to Eq. (1). The P value increased with increasing column length and flow rate, indicating that separation efficiency was greater with a longer column and a higher flow rate (Fig. 3A). The same was true for β-lactoglobulin, a model protein (data not shown).

Moreover, mitochondrial protein extract was injected into each length of the columns at the maximum flow rate (0.3 or 0.5 mL/min) to investigate the separation of a real biological sample. The number of separated protein peaks increased with increasing column length: a 250 mm-long column at a 0.3 mL/min flow rate exhibited the highest separation efficiency for the actual protein mixture sample (Fig. 3B). Therefore, a column length of 250 mm with a flow rate of 0.3 mL/min was selected for separating the mitochondrial protein extract.

3.3. Comparison of the non-porous column with the wide-pore column for separating a mitochondrial protein extract

Based on the results above, the non-porous column (250 mm length with a flow rate of 0.3 mL/min) was applied to separate a mitochondrial protein extract. The chromatogram obtained under the appropriate conditions described in Section 2.5 was compared with that obtained from the wide-pore column (250 mm length,

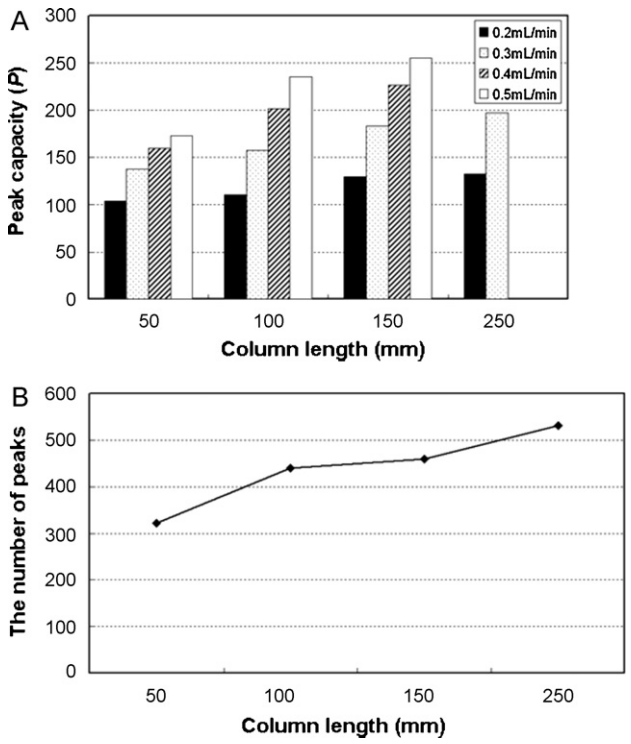

Fig. 3. (A) Peak capacities of the non-porous column with various column lengths (50, 100, 150, and 250 mm) at various flow rates (0.2, 0.3, 0.4, and 0.5 mL/min) in the gradient elution of DAABD-calcitonin. Each *P* value was calculated with Eq. (1) in Section 3.2. (B) The number of protein peaks separated in each length of the column (50, 100, 150, and 250 mm) obtained from the mitochondrial protein extract. The flow rate was 0.5 mL/min, except for the 250 mm column that had a 0.3 mL/min flow rate. The details are described in Section 2.

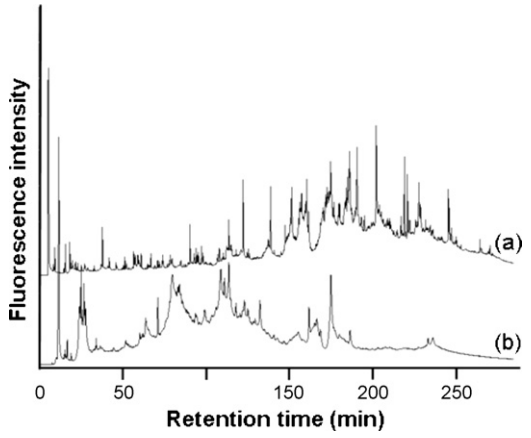

Fig. 4. Chromatograms of mouse liver mitochondrial proteins separated in (a) the non-porous column or (b) the wide-pore column. Chromatographic conditions are described in Section 2.

0.3 mL/min). Fig. 4a indicates that 420 protein peaks were obtained on the chromatogram with the non-porous column in 260 min analytical time. However, 160 protein peaks were not clearly separated in the chromatogram with the wide-pore column (Fig. 4b). This result clearly suggested that the non-porous column, rather than the wide-pore column, would be useful for proteomics analysis of mouse liver mitochondrial proteins with the FD–LC–MS/MS method.

Also, the retention times and peak shapes of the proteins injected into the non-porous column exhibited stronger adsorption and higher resolution than those injected into the wide-pore column. Furthermore, the retention time of the compounds less

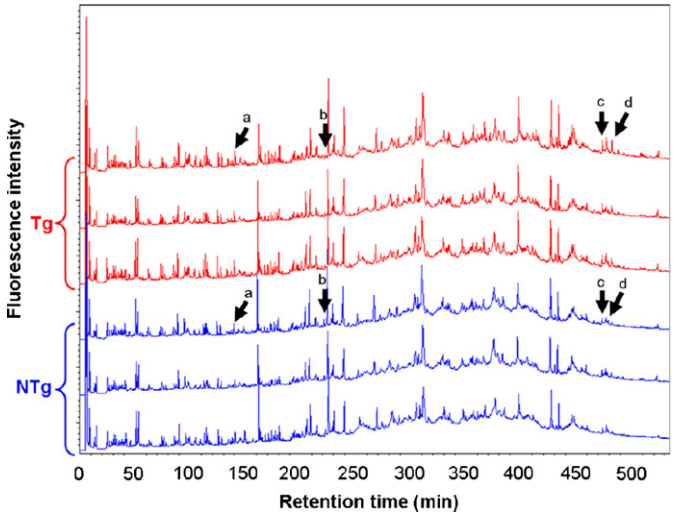

Fig. 5. Chromatograms of liver mitochondrial proteins in Tgs (red) and NTgs (blue) mice separated in the non-porous column. The peaks indicated by arrows fluctuated between Tgs and NTgs. Chromatographic conditions are described in Section 2. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

retained in the non-porous column was shorter than that on the wide-pore column.

3.4. Proteomics analysis of mitochondrial proteins in livers of hepatitis-infected mouse model

Differential proteomics analysis was performed on the non-porous column between mitochondrial protein samples extracted from livers of Tgs ($n = 3$) and NTgs ($n = 3$) mice aged 16 months. This age was selected based on the previous report that many proteins related to the function of mitochondrial events fluctuated in Tg. The appropriate separation conditions in HPLC afforded 500 protein peaks on each chromatogram for Tgs and NTgs in 9 h of analysis (Fig. 5), with each peak height representing the amount of each protein. The responsibility of this analysis was confirmed, based on the reproducibility of the retention times and the peak heights. The relative standard deviations (RSDs) of peaks a through d (Fig. 5) ranged from 0.0 to 0.5% for retention times and 0.4–18.0% for peak heights (for between-days, $n = 3$–6). The heights of peaks corresponding to specific retention times were compared between Tgs and NTgs. The expression of several peaks fluctuated, and each fluctuating peak fraction was collected, digested, and subjected to LC–MS/MS analysis to identify the protein. Table 1 summarizes the identified proteins. Three proteins were significantly up-regulated (Tg/NTg = 1.24–2.86, $0.05 \leq p < 0.10$) (Peaks a, c, and d in Fig. 5) in Tg, while one protein peak was significantly down-regulated (Tg/NTg = 0.44, $p < 0.05$) (Peak b in Fig. 5). Those four proteins were demonstrated for the first time in liver mitochondrial proteomics analysis.

4. Discussion

4.1. Comparison of the separation of DAABD-calcitonin and DAABD-β-lactoglobulin between the non-porous column and the wide-pore column

In order to determine the difference in separation between the non-porous column and the wide-pore column, a derivatized model peptide and protein (DAABD-calcitonin and DAABD-β-lactoglobulin) were separated. The retention times of the derivatized model samples were longer for the non-porous col-

Table 1
Differentially expressed liver mitochondrial proteins between Tgs and NTgs.

Peak number	Tg/NTg ratio		Protein name	Accession number	Score	Sequence coverage (%)
a	1.24	± 0.17	60S ribosomal protein L11	gi 13385408	129	12
b	0.44	± 0.33	Sterol-carrier protein 2	gi 45476581	278	10
c	1.51	± 0.35	NADH-cytochrome b5 reductase 3	gi 19745150	145	8
d	2.86	± 1.30	Hydroxysteroid (17-beta) dehydrogenase 13	gi 159573879	183	25

umn than for the wide-pore column. Since the surface of the non-porous column is covered with ODS (C18) and the surface of the wide-pore column consists of a less hydrophobic "reversed-phase" ligand than C18, stronger retention of the derivatized model samples should be caused by the hydrophobic interaction in the non-porous column. In contrast, considering the shortest retention times of the hydrophilic substances, the non-porous column should exhibit fewer void volumes than the wide-pore column. It was also shown that, considering the *P* (peak capacity) value for the non-porous column was threefold higher than that for the wide-pore column, the non-porous and small size (2 μm as compared to 3 μm of the wide-pore column) reduced eddy diffusion and mass-transfer resistance on separation and resulted in high-resolution chromatography [8]. This couldn't be caused by the narrow particle size distribution of the former column since the particle size distribution of both the columns were similar (D90/D10 < 1.4, and D90/D10 = 1.42 for the non-porous and the wide-pore, respectively, measured by laser diffraction particle size analysis and electrical sensing zone method).

4.2. Optimization of column length and flow rate for protein separation with the non-porous column

The effects of column length and flow rate on the *P* value were investigated using DAABD-calcitonin and DAABD-β-lactoglobulin to obtain optimal conditions for derivatized protein separation on the non-porous column. High *P* values were obtained using a longer column and a higher flow rate. This tendency agreed with the simulation of separation efficiency reported by Gilar et al. [12], indicating that the length of the non-porous column was proportional to separation efficiency. In this study, 0.3 mL/min was the maximum flow rate for the longest column (250 mm) because of the currently limited operating pressure (20 MPa). The LC system should be mechanically strong enough to withstand the ultrahigh pressures for further efficient separation in the non-porous Presto FF-C18 that might be 250 mm long and have a flow rate exceeding 0.3 mL/min. In this sense, in the future, further efficient separation should be examined utilizing an Ultra High Pressure Liquid Chromatography system. According to the results (Fig. 3A), the highest *P* value was obtained with a 150 mm length of column and a flow rate of 0.5 mL/min. However, more separated proteins were observed in the 250 mm-long column with a 0.3 mL/min flow rate for the mitochondrial sample (Fig. 3B). This is because the separable peaks from a large numbers of proteins in a real biological sample should be proportional to the column length. Another reason is that the dilution of the peak fraction in a 150 mm column with a high flow rate of 0.5 mL/min might be beyond the detection limit of the system. Therefore, a 250 mm column length with a flow rate of 0.3 mL/min was adopted as the optimal condition for separating liver mitochondrial proteins.

4.3. Application of the FD–LC–MS/MS method using the non-porous column for differential proteomics analysis of liver mitochondria

Nine hours of analysis indicated that the number (500) of mitochondrial protein peaks (Fig. 5) was similar to the number of extracted proteins from a whole cell separated with the wide-pore column (e.g., for mouse liver proteomics analysis) [4]. Concerning the life-time of the non-porous column, the life-time was long enough to analyze mitochondrial proteins about 40 times (400 h including the washing period) since the chromatogram obtained after 40 times analyses was the same as the initial one. That would be caused by the contribution of a washing operation after each analysis. This result suggests that the non-porous column could be substituted for the wide-pore column as a protein separation column for the FD–LC–MS/MS method.

Compared to the low reproducibility for the retention times of peaks in the previous report [9], the present differential proteomics analysis of liver mitochondria obtained reproducible retention times and peak heights (RSD less than 0.5% for retention times and less than 18.0% for peak heights). The reason for this superior reproducibility may be that in the FD–LC–MS/MS method, proteins were derivatized with the hydrophilic reagent and a low amount of proteins. These results indicate that the FD–LC–MS/MS method using the non-porous column can be used for differential proteomics analysis of liver mitochondria and results in identification of four fluctuating proteins between Tgs and NTgs.

4.4. Functions of the fluctuating liver mitochondrial proteins related to hepatocarcinogenesis

All the identified mitochondrial proteins in this study were demonstrated for the first time in liver proteomics analysis. Since mitochondria were extracted from mouse liver and analyzed for expression of proteins, it is assumed that the proteins inside the mitochondria were concentrated to a detectable level of each expression fluctuation.

Only one down-regulated peak in Tg was identified as sterol carrier protein 2 (SCP2), considering differences in localization between the deduced different types (SCP2 and SCPx) (reviewed in ref. [13]). SCP2 is related to intracellular lipid transport (e.g., cholesterol) from other intracellular membranes to mitochondria [14], as well as from the outer to the inner mitochondrial membrane for oxidation. Since lipids have accumulated in the hepatocytes of 16-month-old Tg mice, causing steatosis as previously reported [10], lipid transport to mitochondria might no longer be required. Thus, SCP2 was decreased in Tg through a negative feedback pathway. Also, SCP2 is reportedly involved in regulation of the signal pathway for lipids (reviewed in ref. [13]). These findings suggest that the decrease of SCP2 in Tg may suppress lipid transport to mitochondria, leading to inhibition of lipid signaling. In contrast, three proteins were demonstrated to be up-regulated in Tg. Hydroxysteroid (17-beta) dehydrogenase 13 (17βHSD13) is specifically expressed in liver [15]. It has been reported that the intracellular localization of 17βHSD13 is similar to that of HCV core protein in endoplasmic reticulum (ER), lipid droplets (LDs), and mitochondria [16–18], while it is unknown whether 17βHSD13 localizes in mitochondria or not. In general, 17βHSD family proteins catalyze the dehydrogenation reactions of the steroid skeleton with an excess of NADH or electrons. Although 17βHSD13 may play a key role in the next step of detoxification and/or utilization of lipid metabolites through the reaction, its specific substrate is not identified. At least, the increase of 17βHSD13 in Tgs would acti-

vate lipid metabolism. NADH-cytochrome b5 reductase 3 (CYB5R3) was observed in the plasma membrane, mitochondrial outer membrane, and ER. CYB5R3 in the mitochondrial electron-transfer system catalyzes the oxidation of NADH to NAD^+ (reviewed in Ref. [19]). For mitochondrial dysfunction, CYB5R3 is up-regulated due to an increase of the NADH/NAD^+ ratio, resulting in enhanced oxidation of NADH to NAD^+. Thus, an increase of CYB5R3 in Tg would accelerate aerobic respiration in mitochondria. In addition, 60S ribosomal protein L11 (RPL11) is associated with Mdm2, which is an E3 ligase for promoting p53 ubiquitination, resulting in prevention of the degradation of p53 [20,21]. The undegraded p53 in mitochondria reportedly causes apoptosis of cancer cells [22,23]. This finding suggests that up-regulated RPL11 would suppress the growth of hepatocarcinoma in transition out of hepatitis C. If hepatocarcinogenesis activates metabolism in Tg mice at the age of 16 months, 17βHSD13 and CYB5R3 might increase and accelerate lipid metabolism and aerobic respiration. Furthermore, a decrease of SCP2 might control lipid transport to mitochondria and thus maintain equilibrium through a negative feedback pathway. Considering these results, fluctuation of these proteins suggests that activation and suppression of hepatocarcinogenesis occur simultaneously in Tg mice at 16 months of age.

In conclusion, a novel non-porous column (Presto FF-C18) achieved good separation of liver mitochondrial proteins, which was hardly achieved on a wide-pore column such as Intrada WP-RP. Moreover, the FD–LC–MS/MS method with Presto FF-C18 demonstrated for the first time several fluctuating proteins performing differential proteomics analysis of liver mitochondrial proteins in a hepatitis-infected mouse model.

References

[1] H.J. Issaq, J. Blonder (Eds.), J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. Netherlands (2009) 1222.
[2] M. Masuda, H. Saimaru, N. Takamura, K. Imai, Biomed. Chromatogr. 19 (2005) 556.
[3] T. Ichibangase, H. Saimaru, N. Takamura, T. Kuwahara, A. Koyama, T. Iwatsubo, K. Imai, Biomed. Chromatogr. 22 (2008) 232.
[4] T. Ichibangase, K. Moriya, K. Koike, K. Imai, J. Proteome Res. 6 (2007) 2841.
[5] K. Imai, T. Ichibangase, R. Saitoh, Y. Hoshikawa, Biomed. Chromatogr. 22 (2008) 1304.
[6] H. Asamoto, T. Ichibangase, K. Uchikura, K. Imai, J. Chromatogr. A 1208 (2008) 147.
[7] T. Ichibangase, K. Imai, J. Proteome Res. 8 (2009) 2129.
[8] N. Wu, Y. Liu, M.L. Lee, J Chromatogr A, Netherlands (2006) 142.
[9] B.E. Chong, D.M. Lubman, F.R. Miller, A.J. Rosenspire, Rapid Commun. Mass Spectrom. 13 (1999) 1808.
[10] K. Moriya, H. Yotsuyanagi, Y. Shintani, H. Fujie, K. Ishibashi, Y. Matsuura, T. Miyamura, K. Koike, J. Gen. Virol. 78 (Pt 7) (1997) 1527.
[11] M.F. Lopez, B.S. Kristal, E. Chernokalskaya, A. Lazarev, A.I. Shestopalov, A. Bogdanova, M. Robinson, Electrophoresis 21 (2000) 3427.
[12] M. Gilar, A.E. Daly, M. Kele, U.D. Neue, J.C. Gebler, J. Chromatogr. A 1061 (2004) 183.
[13] F. Schroeder, B.P. Atshaves, A.L. McIntosh, A.M. Gallegos, S.M. Storey, R.D. Parr, J.R. Jefferson, J.M. Ball, A.B. Kier, Biochimica Et Biophysica Acta-Mol. Cell Biol. Lipids 1771 (2007) 700.
[14] G.G. Martin, H.A. Hostetler, A.L. McIntosh, S.E. Tichy, B.J. Williams, D.H. Russell, J.M. Berg, T.A. Spencer, J. Ball, A.B. Kier, F. Schroeder, Biochemistry 47 (2008) 5915.
[15] Y. Horiguchi, M. Araki, K. Motojima, Biochem. Biophys. Res. Commun. 370 (2008) 235.
[16] K. Moriya, H. Fujie, Y. Shintani, H. Yotsuyanagi, T. Tsutsumi, K. Ishibashi, Y. Matsuura, S. Kimura, T. Miyamura, K. Koike, Nat. Med. 4 (1998) 1065.
[17] R. Suzuki, S. Sakamoto, T. Tsutsumi, A. Rikimaru, K. Tanaka, T. Shimoike, K. Moriishi, T. Iwasaki, K. Mizumoto, Y. Matsuura, T. Miyamura, T. Suzuki, J. Virol. 79 (2005) 1271.
[18] B. Schwer, S.T. Ren, T. Pietschmann, J. Kartenbeck, K. Kaehlcke, R. Bartenschlager, T.S.B. Yen, M. Ott, J. Virol. 78 (2004) 7958.
[19] R. de Cabo, E. Siendones, R. Minor, P. Navas, Aging (Albany NY) 2 (2010) 63.
[20] K. Itahana, H. Mao, A.W. Jin, Y. Itahana, H.V. Clegg, M.S. Lindstrom, K.P. Bhat, V.L. Godfrey, G.I. Evan, Y.P. Zhang, Cancer Cell 12 (2007) 355.
[21] S. Fumagalli, A. Di Cara, A. Neb-Gulati, F. Natt, S. Schwemberger, J. Hall, G.F. Babcock, R. Bernardi, P.P. Pandolfi, G. Thomas, Nat. Cell Biol. 11 (2009) 501.
[22] N.D. Marchenko, A. Zaika, U.M. Moll, J. Biol. Chem. 275 (2000) 16202.
[23] M. Mihara, S. Erster, A. Zaika, O. Petrenko, T. Chittenden, P. Pancoska, U.M. Moll, Mol. Cell 11 (2003) 577.