Focussing Review

Development of Highly Sensitive and Reproducible Analytical System for Biological Macromolecules with Fluorogenic Derivatization

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Abstract

Fluorogenic derivatization (FD)-HPLC is a powerful tool for analyzing biological samples due to its high sensitivity and reproducibility. In recent years, we have developed FD-LC-MS/MS, a novel proteomics method that consists of HPLC separation of fluorogenic derivatized biological macromolecules with DAABD-Cl and identification of the separated derivatives. To develop the analytical system for differential analysis, high-resolution HPLC conditions were optimized. The analytical system was then developed to clarify the flow dynamics of signaling in cells. The affinity column for extracting albumin in plasma was also evaluated. FD-LC-MS/MS method is superior to other proteomics methods in sensitivity, quantitativity and reproducibility, and enables demonstrating flow dynamics in cells.

Keywords: Fluorogenic derivatization, Proteomics, Biomolecules, Plasma

1. Introduction

Most biomolecules are few non-fluorescent. Therefore, high-performance liquid chromatography (HPLC)-fluorescence detection is a powerful tool for highly sensitive and reproducible quantification analysis. Recently, we used a water-soluble, thiol-specific fluorogenic reagent (ammonium 7-fluoro-2,1,3-benzoxadiazole-4-sulfonate (SBD-F) or 7-chloro-N-[2-(dimethylamino)ethyl]-2,1,3-benzoxadiazole-4-sulfonamide (DAABD-Cl)) for the derivatization of proteins. These reagents have no fluorescence themselves but produce fluorescent proteins when derivatized (FD), resulting in a low background noise and thus high signal-to-noise ratio in the detection of the derivatized proteins. DAABD-Cl is a rather small molecule with an ionizable group in the neutral medium at pH 7 and is highly reactive to the thiol moiety of cysteine in proteins [1]. Additionally, the detectability of DAABD derivatives is significantly enhanced, compared with SBD derivatives in MS/MS analysis, probably due to the positively charged group in the DAABD moiety.

Using DAABD-Cl, we developed FD-LC-MS/MS, a novel proteomic analysis that has better sensitivity and quantitative capability than other proteomic analyses, including two-dimensional polyacrylamide gel electrophoresis (2 D-PAGE), and evaluated its applicability to clinical proteomics.

2. Development of differential proteomic analysis [2]

It has become routine to search for and demonstrate defects in genetic disorders to provide knowledge for diagnosis, treatment, and protection against diseases. It is also important to search for and demonstrate the cause of the disease from the proteomic perspective, since the intracellular signaling system actually consists of expressed protein dynamics [3]. Demonstrating changes in protein dynamics enables us to understand abnormal phenomena, and thus leads to diagnosis, treatment, and protection against diseases.

To properly understand protein dynamics, FD-LC-MS/MS is a worthwhile method of proteomic analysis. It consists of fluorogenic derivatization (FD), LC separation and detection/quantifica-

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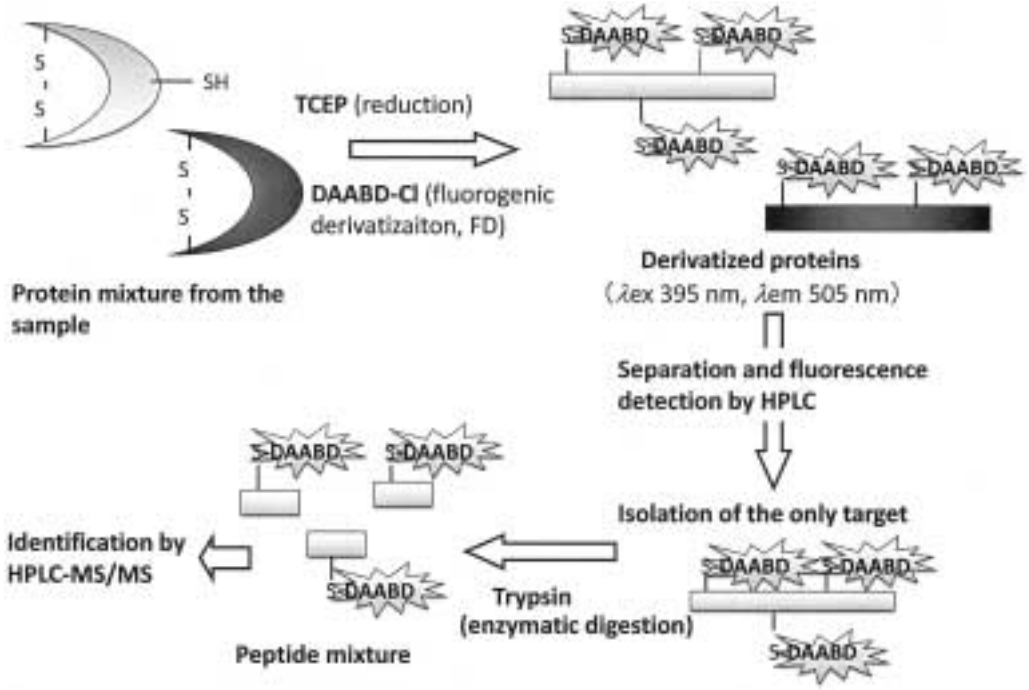

Fig. 1 Schematic diagram of FD-LC-MS/MS method. After fluorogenic derivatization (FD) of proteins existing the reducing reagent, Tris (2-carboxyethyl) phosphine hydrochloride (TCEP), the protein mixtures are separated by HPLC, and proteins exhibiting significant differential expression are isolated and identified using HPLC-MS/MS and database searching.

tion of proteins in a biological sample, followed by isolation, tryptic digestion of target proteins, and then LC-MS/MS identification of the target proteins [1, 4]. A schematic diagram of differential analysis using FD-LC-MS/MS is presented in Fig. 1. The proteins in other biological samples are treated in the same way, and comparison of the LC chromatograms among two or more samples can demonstrate the different expressions of proteins among two or more biological samples (differential analysis). To evaluate this method as quantification and differential analysis, we attempted to apply it to the quantification and differential analysis of liver proteins taken from hepatitis C virus (HCV) of core gene transgenic (Tg) and non-transgenic (NTg) mice as a model. HCV is the main cause of chronic hepatitis, ultimately resulting in the progression of hepatocellular carcinoma. The proposed method demonstrated for the first time the existence of several event-marker proteins in the progression stages of hepatocarcinogenesis in transgenic mice. For validating the method in LC-chromatograms, the utilization of the soluble fraction of extracted liver proteins afforded 500 protein peaks in each analysis with of 8.0 to 40 μg per injection of total proteins without any pretreatment of the samples (e.g., precipitation). In general, other proteome analyses of biological samples require from dozens to hundreds of micrograms of protein samples [2]. The quantification of proteins by FD-LC-MS/MS has high reproducibility. For example, the respective relative standard deviation (RSD%) of each between-day peak height was less than 16 (high peak in liver tissue), 17 (medium peak), and 23% (low peak) (n = 3). An additional benefit is that the simple apparatus (consisting of a pump, a column, and a fluorescence detector) does not require a complex facility for operation. The results confirm that FD-LC-MS/MS method is worthwhile for clinical proteomic analysis.

3. Application of FD-LC-MS/MS for intracellular protein signaling analysis

We believe that the performance of intracellular protein signaling can be demonstrated with highly sensitive, reproducible, and quantitative proteomic analysis. In a study of thoroughbred horse muscle and breast cancer cell lines, the biochemical events in the faster-running horse and the presumptive mechanisms involved in tumor progression were successfully presented. Figure 2 depicts typical chromatograms obtained for thoroughbred horse muscle tissue after training and detraining. The mobile phases consisted of 0.20% trifluoroacetic acid (TFA) in acetonitrile/isopropanol/water (A) 9/1/90 and 0.15% TFA in (B) 50/20/30. The effect of isopropanol concentration in the HPLC eluent on protein separation was also investigated in the range of 0 to 20%. The low concentration (less than 10% in the eluent) suited separation at 0 to 1.0 h and 6.0 to 8.0 h, while a concentration of 10% isopropanol in the eluent was suitable for protein separations in the range of 2.5 to 6.0 h and

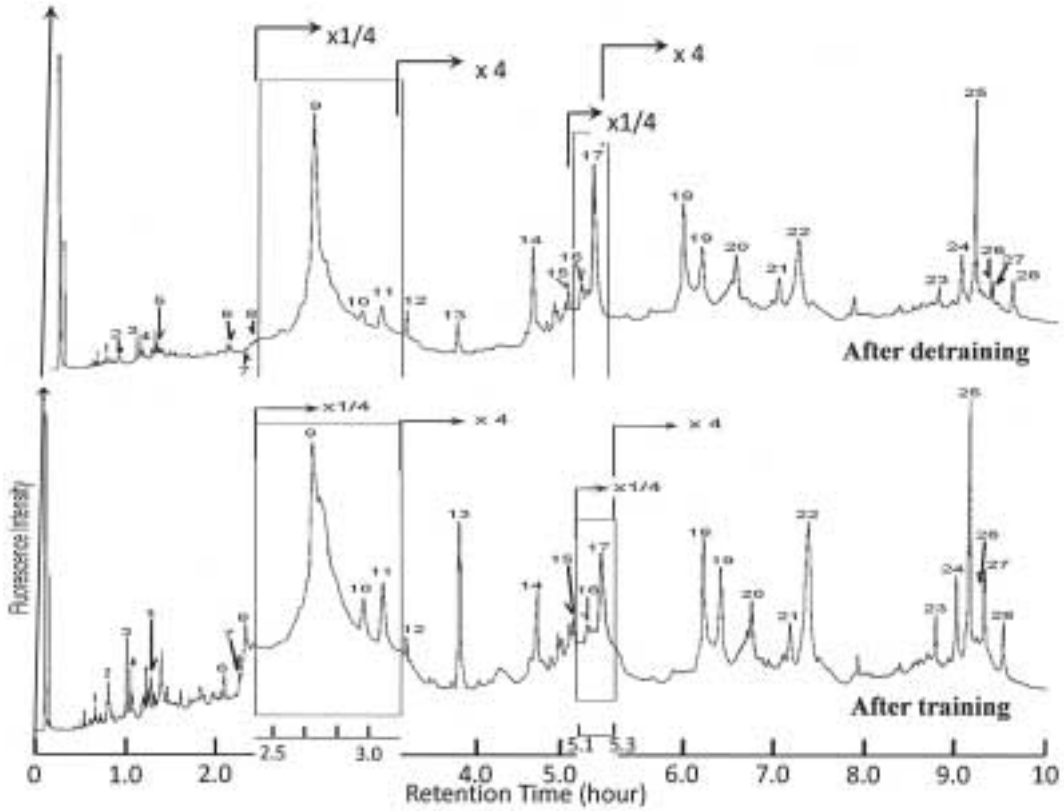

Fig. 2 Chromatograms of proteins derivatized with DAABD-Cl (8.0 μg protein per HPLC injection) in horse muscle. The chromatograms above (below) were obtained from a thoroughbred after training (detraining). The fluorescence intensity in the retention time range (from 2.5 to 3.0 h and from 5.1 to 5.3 h) is a quarter of the intensity in other ranges.

8.0 to 10 h. Therefore, the concentration in the eluent increased with increased separation time with gradient elution. Results indicated that 16 proteins involved in energy supply, especially in anaerobic energy production, increased in the faster-running horse, suggesting that the anaerobic pathways effectively activated the aerobic pathway to produce ATP efficiently. These data suggested that this method demonstrates the biochemical events in the faster-running horse and may be useful for evaluating the training effect in thoroughbred horses [5]. In the proteomic study of the breast cancer cell line [6], our comprehensive differential proteomic analysis identified 13 fluctuating proteins compared with a normal human mammary epithelial cell, and from these data we propose a model to demonstrate how the dynamic expression of these proteins affects breast cancer tumor progression (Fig. 3). Additionally, specific proteins or biomarker candidates related to breast cancer and metastatic breast cancer were successfully identified. For example, since RanGAP was solely detected in all the cancer cells, the protein might become a novel candidate for a marker. Cancer cells expressing a higher raf-1 kinase inhibitor protein (RKIP) level would be estrogen receptor-positive, while cancer cells expressing a lower level of annexin-2, galectin-1 and EF-Tu would be human epidermal growth factor receptor type 2 (Her 2)-positive. These data may be very useful for medical treatment. The presence of cooperatively expressed annexin-2 and galectin-1 without tropomyosin-1 in a breast cancer cell is suggestive of its metastatic property.

4. Evaluation for applicability to plasma proteomic study [7]

Blood samples can be taken at a particular point in time with little burden on patients, and the constituents of the blood samples may reflect a developing or existing illness because tissue-specific proteins may be released into the blood stream from damaged or dead cells. Therefore, it is generally recognized in proteomics studies that blood samples represent the greatest potential source of information on the proteins related to human diseases. However, plasma proteome analysis aimed at quantitative protein profiling and biomarker discovery is not easy. Therefore, in plasma proteomics, before proteomic analysis it is essential to prepare protein samples without high-abundance proteins, including albumin, via specific preparation techniques (e.g., immunoaffinity capture) [8-11]. However, our preliminary experiments suggest that functional changes with use alter the ability of the immunoaffinity column.

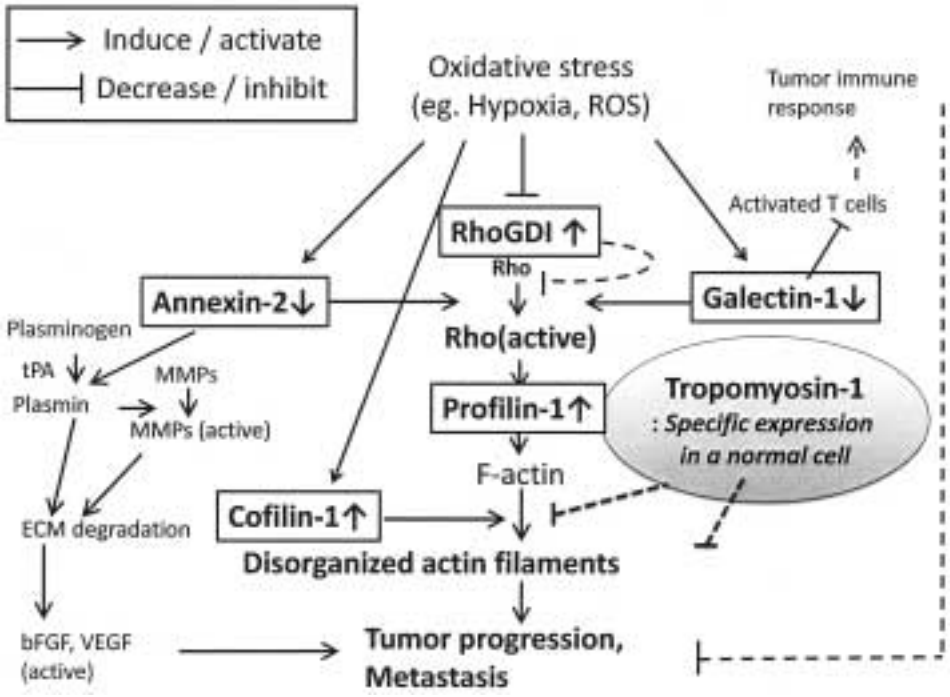

Fig. 3 Possible flow dynamics for proteins leading to invasion, metastasis, and proliferation in human breast cancer cells, especially in metastatic cells. Oxidative stress resulting from extensive activities in breast cancer cells would affect the expression of various proteins including annexin-2, galectin-1, and cofilin-1. The upper (lower) arrows indicate up- (down-) regulated proteins compared to normal cells. The full lines indicate possible signaling, and the dotted lines indicate possible nonsignaling in the cancer cells. Tropomyosin-1 did not detect in any cancer cells examined. This study demonstrates the importance of the existence of topomyosin-1 in normal cells, suggesting that a material that stimulates the expression of tropmyosin-1 could suppress metastatic breast cancer cells.

Thus, in this study, plasma proteome analysis was performed for a long-term test for reproducibility of the affinity column using FD-LC-MS/MS method combined with an IgY column to evaluate changes in the removal ability of abundant proteins from plasma by the immunoaffinity column. At first, to eliminate the non-specific adsorption to the affinity column, the protein standards mixture was divided into two parts. The one was subjected into the untreated affinity column, and the flow-though fraction was derivatized with the DAABD-Cl, and separated by the HPLC system (4.8 μg protein/HPLC injection). Another part of the standards mixture was diluted and derivatized with DAABD-Cl, and injected onto the HPLC system (4.8 μg protein/HPLC injection). The obtained chromatograms are depicted in Fig. 4. Each protein peak was collected, digested in peptide mixtures, and identified by applying the peptides to HPLC-MS/MS. The identified protein names and adsorption ratio were inserted in Fig. 4. As regard to the peak 5 and 6, the same protein name, trypsin inhibitor, was identified. Although it might be derived from the impurity of the standard solutions, this issue was not further examined because it was out of our scope.

Consequently, non-specific binding to the column materials or to carrier proteins such as albumin itself was observed in the 18.3 to 45.9% range and could result in the loss of presumed biomarkers, although the affinity column removed bovine serum albumin (BSA; 83.9%) more efficiently than the other proteins in the standards. Subsequently, specific adsorption for albumin decreased with an increase in the amount of column usage before its expiration date. Moreover, hydrophobic high molecular weight compounds in plasma adsorbed onto the column materials surface contributed to functional changes from specific immunoaffinity adsorption into hydrophobic interaction. These results suggested that, in quantitative plasma proteomic studies, it is important to keep in mind the risk of not only nonselective loss but also changes in the adsorption ability of the immunoaffinity column.

5. Conclusion

We developed a novel proteomic analysis method, FD-LC-MS/MS, and applied it to several real samples in order to test its applicability to clinical proteomic analysis. In this paper, we described FD-LC-MS/MS method from the viewpoint of the development of differential proteome analysis, its application for intracellular pro-

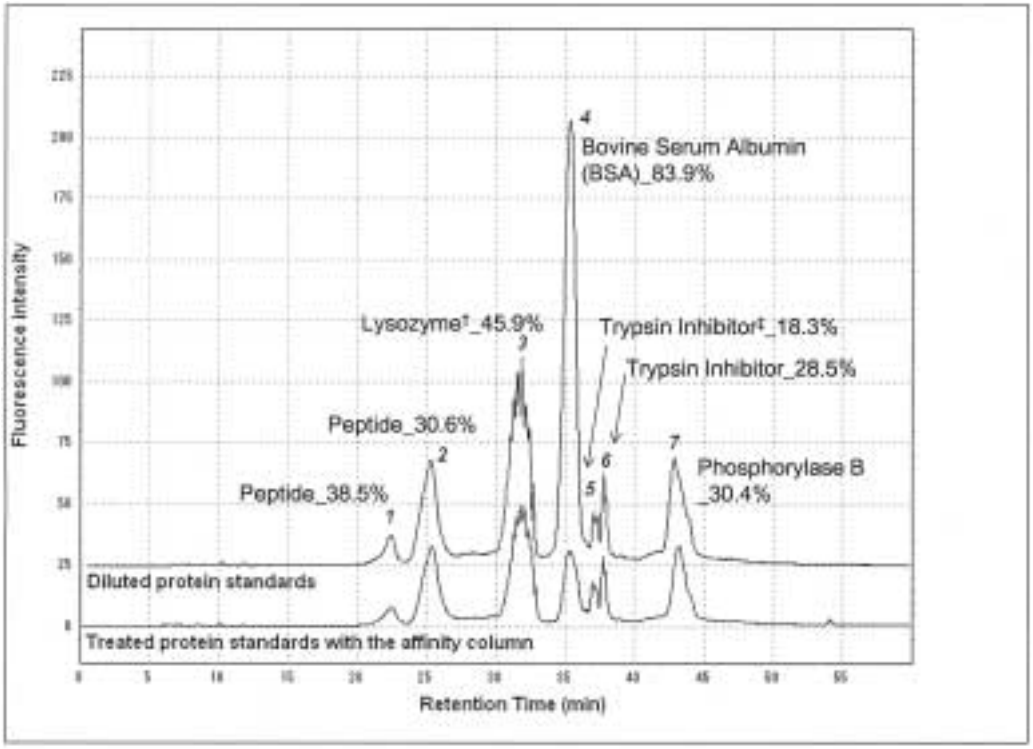

Fig. 4 Chromatograms of the protein standards which were treated with the IgY column and diluted to the same protein amount (4.8 μg per HPLC injection) as the amount for the immunoaffinity column treatment. Each identified protein name and adsorption ratio to the untreated IgY column were described in each corresponding peak. Peak 1 and 2 are peptide coexisting in the standard solutions. †, ‡: Most highest peak

tein signaling analysis, and evaluation of its applicability to plasma proteomic study. All obtained data demonstrated high sensitivity and reproducibility of the method, and no pre-treatment steps (e.g., precipitation, clean-up with the column, and enzymatic protein digestion) are required, except for plasma sample, resulting in high sensitivity and reproducibility without losing existing proteins in the sample during pre-treatment steps. We believe that proteomic studies employing FD-LC-MS/MS could help advance our understanding of intracellular protein performance.

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