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Abstract	タンパク質は生体内において非常に重要な役割を担っている.タンパク質を網羅的に解析することは,生命現象を理解するのに重要である. 液体クロマトグラフィー質量分析計(LC-MS)を用いたショットガンプロテオミクスは,タンパク質を網羅的に解析するのに有効な手法である.ショットガンプロテオミクスでは,タンパク質を質量分析計で測定可能な断片にするために,消化酵素を用いて断片化する必要がある.しかし,タンパク質は様々な化学的性質を有しており,また,タンパク質ごとに発現量が大きく異なる.そのような性質に起因する実験的な制限により,試料中に存在しているタンパク質を短期間で網羅的に同定することは困難であった. 本論文では,ショットガンプロテオミクスにおける問題点を解決し,短期間で網羅的にタンパク質を同定できる分析手法を確立するために,大腸菌を試料として,試料の分析前処理法,および液体クロマトグラフィー分離法における新規手法の開発を行った.第1章では,プロテオーム研究の意義および本研究の目的を述べた.第2章では,前処理法に着目し,化学的消化法を開発することでタンパク質の化学的性質に偏りのない同定手法を確立し,膜タンパク質の同定効率を改善した.第3章では,液体クロマトグラフィーにおける分離法に着目し,モノリス型シリカカラムを用いたLC分析条件の検討を行うことで,1分析あたりの同定効率を大幅に改善し,大腸菌で発現している全てのタンパク質を,短期間で分析することが可能となった.
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優秀修士論文

膜

タンパク質の網羅的解析のための分析法の開発

Development of analytical methods for large-scale profiling of membrane proteins

2009年度

岩崎 未央　政策・メディア研究科 修士課程

先端生命科学プロジェクト

慶應義塾大学湘南藤沢学会

Keio SFC Academic Society

優秀修士論文推薦のことば

本論文は、タンパク質を短期間で効率的かつ網羅的に分析する手法を世界に先駆けて開発した。市販されているほとんどの薬剤がタンパク質を標的としていることから、創薬研究やその他の生物学研究にとって、細胞内でのタンパク質の変動を網羅的かつ定量的に見ることは極めて重要である。しかし、タンパク質の化学的特性や、その膨大な種類に起因する実験的な制限により、どんな生物由来のタンパク質も短期間で網羅的に分析することが、これまで極めて困難であった。本論文の技術は網羅的タンパク質解析技術の可能性を飛躍的に高め、今後は医療への応用が期待される。以上の点から、本論文を優秀修士論文として強く推薦する。

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修士論文 2009 年度（平成 21 年度）

膜タンパク質の網羅的解析のための分析法の開発

Development of analytical methods for large-scale profiling of membrane proteins

慶應義塾大学大学院 政策・メディア研究科

岩崎　未央

修士論文 2009 年度（平成 21 年度）

膜タンパク質の網羅的解析のための分析法の開発

論文要旨

タンパク質は生体内において非常に重要な役割を担っている．タンパク質を網羅的に解析することは，生命現象を理解するのに重要である．液体クロマトグラフィー-質量分析計(LC-MS)を用いたショットガンプロテオミクスは，タンパク質を網羅的に解析するのに有効な手法である．ショットガンプロテオミクスでは，タンパク質を質量分析計で測定可能な断片にするために，消化酵素を用いて断片化する必要がある．しかし，タンパク質は様々な化学的性質を有しており，また，タンパク質ごとに発現量が大きく異なる．そのような性質に起因する実験的な制限により，試料中に存在しているタンパク質を短期間で網羅的に同定することは困難であった．

本論文では，ショットガンプロテオミクスにおける問題点を解決し，短期間で網羅的にタンパク質を同定できる分析手法を確立するために，大腸菌を試料として，試料の分析前処理法，および液体クロマトグラフィー分離法における新規手法の開発を行った．第１章では，プロテオーム研究の意義および本研究の目的を述べた．第２章では，前処理法に着目し，化学的消化法を開発することでタンパク質の化学的性質に偏りのない同定手法を確立し，膜タンパク質の同定効率を改善した．第３章では，液体クロマトグラフィーにおける分離法に着目し，モノリス型シリカカラムを用いた LC 分析条件の検討を行うことで，1 分析あたりの同定効率を大幅に改善し，大腸菌で発現している全てのタンパク質を，短期間で分析することが可能となった．

キーワード

大腸菌，ショットガンプロテオミクス，化学的消化法，モノリス型シリカカラム

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Abstract of Master's Thesis Academic Year 2009

Development of analytical methods for large-scale profiling of membrane proteins

ABSTRACT

Proteins have crucial roles in all kinds of organisms. It is very important to analyze proteins comprehensively for the understanding of the life. Shotgun proteomics is a powerful method to analyze proteome using nano-liquid chromatography-mass spectrometry (nanoLC-MS/MS). In shotgun proteomics, proteins are digested to produce measurable peptides by MS. However, proteins have various chemical properties and wide differences in the concentration range. Thus, mainly because of the experimental limitations, it is difficult to analyze entire proteome in one organism in a short time LC-MS/MS analyses.

In this paper, the novel methods were developed to solve these problems and improve the identification efficiency on *E. coli* samples. In the chapter 1, the significance of proteome analysis and the purpose of this study were described. In the chapter 2, the pre-fractionation method was focused to develop the chemical digestion method. The unbiased method for the proteome analysis was achieved, and the identification efficiency of the membrane proteome was successfully improved. In the chapter 3, the LC method was focused and optimized using the long monolith silica column. The identification efficiency of a single analysis was remarkably improved, and complete identification of *E. coli* proteome was achieved.

Keywords

Escherichia coli, shotgun proteomics, chemical digestion method, monolith silica column

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Contents

CHAPTER 1

Introduction

1.1. THE ROLE OF PROTEOME IN LIVING CELLS

There are mainly three biological phases on sustaining life. These are genome, transcriptome and proteome (Figure 1). Genome stores biological information and functions as the blueprint of life, though genome alone is not enough for expressing the written information. To use the information, proper kinds of proteins and RNAs are needed. Transcriptome is the term of all RNA molecules which are the copy of protein encoded genes and is the first products of genome expression. Proteome is the term of all proteins existing in the cell derived from translated transcriptome and the second or last products of genome expression. Proteome plays main important biological functions in the cell such as working as catalysts and organizing the cell. In particular, membrane proteins are important because they first contact the surroundings of a cell and they have to transmit the information into/out of the cell. Therefore, the membrane proteins have very important functions such as cell adhesion, signal transduction, nutrient uptake, transportation, and endocytosis. For understanding the life, analyzing proteome, especially membrane proteome, is crucial because of their essential activities in the cell.

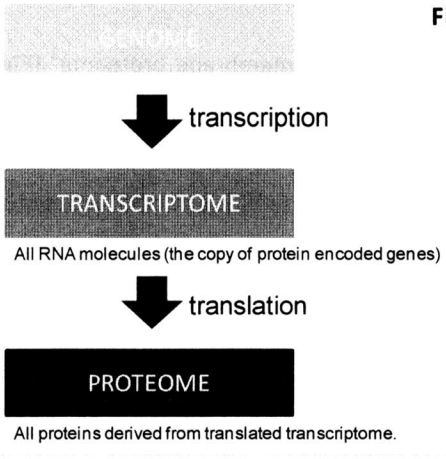

Figure 1 Genome, Transcriptome and Proteome

This is the optimized figure of Figure 1.2 in GENOMES 3rd edition. There are three main biological phases on sustaining life such as genome, transcriptome and proteome. For the understanding life, analyzing proteome is crucial because of the important biological functions.

1.2. THE IMPORTANCE OF COMPREHENSIVE ANALYSIS OF PROTEINS

To analyze the proteome comprehensively, two-dimensional (2D) gel electrophoresis is one of the conventional and powerful tools to identify and quantify proteome[1, 2]. The proteins are separated by their isoelectoric point (pI) for the 1st dimension and molecular weight (MW) for the 2nd dimension. Although this approach is good for capturing the global picture of proteome, there are some crucial limitations[2, 3]. Since the proteins are separated by their pI for the 1st dimension, the proteins which have very lower and higher pI are excluded from the gel. Thus, number of total proteins remaining the gel is reduced from the beginning condition. In addition, it is difficult to analyze membrane proteins because of their hydrophobicity and their difficulty of solubilizing in the non-detergent isoelectoric focusing buffer. Moreover, it takes a time and effort to extract proteins from gels and identify the proteins by mass spectrometry.

Shotgun proteomics is an alternative powerful tool to the gel[3, 4]. As the flowchart of shotgun proteomics shown in Figure 2, proteins are firstly extracted from the cell. The protein mixture is digested by proteases to produce measurable molecules (peptides) for MS. Then the peptides are analyzed by LC-MS/MS and proteins are immediately identified by database searching from the MS/MS results. This approach is fast and high throughput, but there are some limitations. As the gel-based method, it is difficult to identify the membrane proteins because of their hydrophobic nature. In our previous study, phase-transfer surfactants (PTS) protocol for membrane proteome analysis presented remarkable improvement in the identification of membrane proteome[5] (Figure 3). However, it still remains challenging tasks for the complete identification of proteome and membrane proteome in one organism because of the technical limitations and problems of complexity and dynamic range in the shotgun proteomics sample.

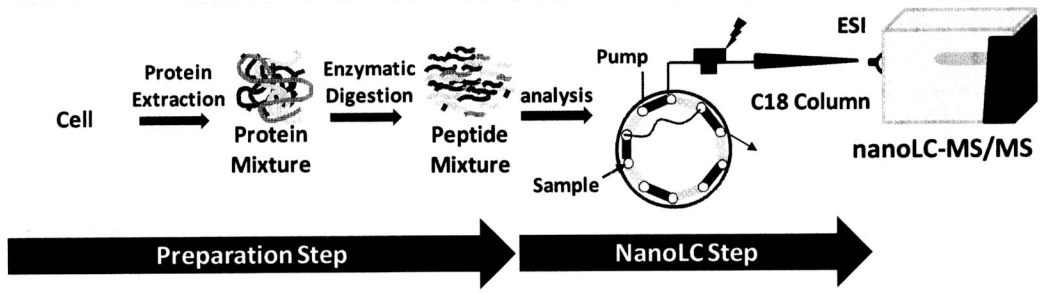

Figure 2 Flow Chart of the Shotgun Proteomics Approach

Since shotgun proteomics is very fast and high throughput approach, it is universally used for the identification of proteome. In shotgun proteomics, the cells are broken and proteins are extracted. The protein mixture is digested by enzymes to produce measurable molecules (peptides) for MS. Then the peptides are analyzed by LC-MS/MS and proteins are identified by database searching of the MS/MS results.

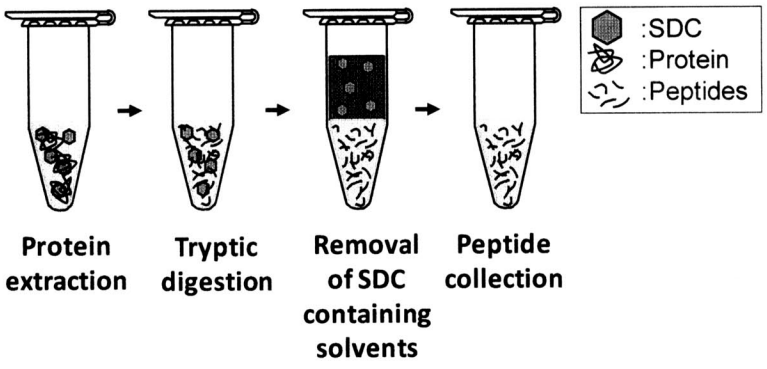

Figure 3 Phase Transfer Surfactant (PTS) Method

The PTS method greatly improved the identification efficiency of membrane proteins[5]. The PTS method uses sodium deoxycholate (SDC) to solubilize the proteins. After tryptic digestion, the removal of SDC from the solution is first performed by adding the ethyl acetate and trifluoroacetic acid (TFA) for subsequent centrifugation. Then the solution is naturally separated into 2 layers, and the upper layer has SDC. For the complete removal of the SDC from the tube, the upper layer is removed by pipette tips. The resultant solution is used as the peptides solution.

1.3. OBJECTIVES & SUMMARY

In this paper, I focused on the two phases in the shotgun proteomics approach to improve the identification efficiency as shown in Figure 2. In the chapter 2, I focused on the preparation phase and the novel combination digestion method of chemical and enzymatic cleavage was developed. It was computationally analyzed that it was effective for the *E. coli* membrane proteome analysis to use the digestion of cysteine and tryptic sites. Then, the *in silico* results were proved experimentally to apply the combined digestion method to *E. coli* membrane-enriched samples. In the chapter 3, I focused on the LC phase and examined the LC method with long monolith silica column to improve the separation efficiencies. Long monolith column with long gradient times exhibited great separation efficiencies, and identification efficiencies were remarkably improved.

CHAPTER 2

Chemical Cleavage-Assisted Tryptic Digestion for Membrane Proteome Analysis

2.1. INTRODUCTION

Proteins localized in the plasma membrane and other membranous organelles play critical roles in a wide range of physiological processes, including cell adhesion, signal transduction, nutrient uptake, transportation, and endocytosis. Therefore, 65% of all contemporary pharmaceuticals target membrane proteins[6]. Consequently, there is great interest in large-scale membrane proteome analysis, particularly with mass spectrometry (MS)-based approaches. For membrane proteome analysis, the sample preparation steps, including protein denaturation and digestion, are crucial. Additives such as urea as a chaotropic agent[7-11], sodium dodecyl sulfate (SDS) as a detergent[11-15], methanol as an organic solvent[8-10, 13, 14, 16-21] and other surfactants[3-5,5, 12, 22, 23] are commonly used to solubilize membrane proteins, which are then digested, usually with trypsin, to obtain peptides for MS analysis. The drawback of this approach is that the additives have to be diluted or removed to avoid deactivation of the protease and poor LC-MS performance, such as reduced chromatographic separation and impaired peptide ionization[24]. When the additives are diluted or removed, however, membrane proteins become less soluble and the structure becomes less accessible to trypsin, resulting in reduced efficiency of peptide identification. Acidlabile surfactant (ALS)[3-5,22, 23] such as RapiGest, is well-known to be effective for solubilizing membrane proteins, compatible with enzymes, and easily removed by precipitation at low pH. However, hydrophobic peptides often co-precipitate, resulting in poor recovery of membrane proteins[22]. Recently, our research group developed a trypsin digestion protocol using phase transfer

surfactants (PTS)[5], such as sodium deoxycholate (SDC). With this protocol, we could identify more membrane proteins than was possible with ALS-assisted trypsin digestion. However, it remains a challenging task to analyze membrane proteins due to the intrinsic limitations of the trypsin digestion procedure. In general, there are few basic amino acids in the transmembrane domains (TMD) of membrane proteins, so that trypsin cleavage tends to afford quite large, hydrophobic peptides. Recently, Ficher et al.[25] reported that combining different proteases with different cleavage specificities is effective to identify hydrophobic peptides. So far, cyanogens bromide (CNBr) and chymotrypsin have been used in combination with trypsin[19, 25]. These combined digestion methods provided better results than tryptic digestion alone. Chemical reagents such as CNBr offer the advantage that they can be used to solubilize hydrophobic proteins under severe conditions without the need to consider protease inactivation. Recently, Swatkoski et al.[26] applied a chemical cleavage reaction at Asp in the presence of formic acid for yeast ribosome proteome analysis.

Chemical cleavage at cyanidated cysteine has been frequently used in biochemistry and bioengineering since Catsimpoolas and Wood[27] introduced the cleavage reaction in 1966, and Jacobson et al.[28] modified it in 1973. The specific cleavage reaction of the peptide bond at the N-terminal side of cysteine residues occurs under mild alkaline conditions after cyanidation of SH groups with 2-nitro-5-thiocyanobenzoic acid (NTCB). This cleavage reaction is induced by the nucleophilic attack of hydroxyl ion on the carbonyl carbon at the N-terminal side of the cyanocysteine residue. Cyanocysteine cleavage has been widely applied in various fields, for labeling proteins[29], C-terminal amidation of synthetic peptides[30], ligation of two peptides[31], immobilization of proteins at the C-terminus[32] and backbone cyclization of proteins[33]. Because this reaction proceeds under alkaline conditions where membrane proteins can be extracted with high efficiency, it should be applicable directly to membrane proteome analysis.

In this chapter, I developed a combination approach for membrane proteome analysis, using cyanocysteine-mediated cleavage reaction followed by tryptic digestion. First, I computationally predicted the effect of combining cleavage at Cys with tryptic cleavage at Lys and Arg for *Escherichia coli* membrane proteome analysis. Then, chemical digestion based on cyanocysteine-mediated cleavage was experimentally optimized for proteome analysis. Finally, the optimized cyanocysteine digestion method was applied to *E. coli* membrane-enriched fractions in combination with subsequent tryptic digestion in the presence of SDC according to PTS protocol.

2.2. MATERIALS AND METHODS

2.2.1. Materials.

Sodium hydroxide, sodium hydrogen carbonate and 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF) were from Nakalai (Kyoto, Japan). Tris (2-carboxyethyl) phosphine hydrochloride (TCEP) was from Pierce (Rockford, IL). 2-Nitro-5-thiocyanobenzoic acid (NTCB) was obtained from Tokyo Chemical Industry (Tokyo, Japan). C18 Empore disc cartridges and membranes were from 3 M (St. Paul, MN). Water was purified by a Millipore Milli-Q system (Bedfold, MA). Sodium deoxycholate (SDC), mass spectrometry-grade lysyl endoprotease (Lys-C), ethyl acetate, acetonitrile, acetic acid, methanol, trifluoroacetic acid (TFA), urea, iodoacetamide (IAA), sodium carbonate, phenylisothiocyanate, glutathione, cyanogens bromide (CNBr) and all other chemicals were purchased from Wako (Osaka, Japan).

2.2.2. Preparation of membrane-enriched fractions of *Escherichia coli* BW25113 cells.

E. coli strain BW25113 cells grown in Luria-Bertani (LB) cultures at 37 ℃ were used in this study. The cell pellet was prepared by centrifugation at 4,500 *g* for 10 min and was resuspended in 10 mL of ice-cold 1 M KCl, 15 mM Tris (pH 7.4). A protease inhibitor AEBSF was added to the final concentration of 10 mM. The cells were lysed by ultrasonication, and unbroken cells and debris were precipitated at 2,500 *g* for 5 min. The supernatant was centrifuged at 100,000 *g* for 60 min, and the resultant pellet was resuspended in ice-cold 0.1 M Na_2CO_3 solution. After centrifugation at 100,000 *g* for 60 min, the pellet was collected as the membrane-enriched fraction.

2.2.3. Optimization of cyanocysteine cleavage reaction.

E. coli whole cell lysate was dissolved in 50 mM sodium carbonate buffer (8 M urea) to make a protein concentration of 5 μM, assuming the average molecular weight of all proteins is 35,000 Da.

Prior to the chemical modification, protein was reduced with TCEP at 37 °C for 30 min and cyanidated with NTCB. The solution was diluted 4-fold and digested overnight with trypsin at 37 °C. The solution was acidified with TFA and desalted with C18-StageTips[34] for subsequent nanoLC-MS/MS analysis. Reaction parameters, such as the cyanidation time, the digestion time, the TCEP concentration, the NTCB concentration, the buffer constituents and pH, are described in Results and Discussion.

2.2.4. In-solution Cys cleavage-trypsin digestion of membrane-enriched fraction.

The membrane-enriched fraction was dissolved in 50 mM sodium carbonate buffer at pH 11 containing 5% SDC. Proteins were reduced with a 10-fold molar excess of TCEP at 37 °C for 30 min and cyanidated with a 100-fold molar excess of NTCB at 37 °C for 30 min. The sample solution was diluted 10-fold and digested with trypsin at 37 °C for 8 h (trypsin-to-protein ratio of 1:50 (w/w)). An equal volume of ethyl acetate was added to the solution and the mixture was acidified with the final concentration of 0.5% TFA according to the PTS protocol reported previously[5]. The mixture was shaken for 1 min and centrifuged at 15,700*g* for 2 min, then the aqueous phase was collected and desalted with C18- StageTips[34]. For in-solution trypsin digestion, the same procedure was employed, except that a Cys alkylation step with IAA was performed at 37 °C for 30 min instead of the NTCB addition step.

2.2.5. Peptide fractionation of digested samples.

In accordance with the StageTip fractionation protocol[35], fractionations using StageTips with a strong cation exchange (SCX) disk and poly(styrene-divinylbenzene) copolymer (SDB) disk were performed for the digested membrane-enriched samples. For the SCX fractionation, SCX-StageTips[36] were used and 20-500 mM ammonium acetate solutions containing 15% acetonitrile were employed to elute peptides, resulting in 5 fractions. All eluted fractions, including the flow-through fraction, were desalted by means of C18-StageTips. For the SDB fractionation, 40 μL of 0.1% NH4OH, 5% ACN (fraction 1), 0.1% NH4OH, 10% ACN (fraction 2), 0.1% NH4OH, 20% ACN (fraction 3) and 0.1% NH4OH, 80% ACN (fraction 4) buffers were used as elution buffers for SDB-StageTips after the sample solution had been loaded. The resultant 5 samples, including the flow-through fraction, in addition to the unfractionated sample, were desalted with C18-StageTips.

2.2.6. NanoLC-MS system.

An LTQ-Orbitrap XL (Thermo Fisher Scientific, Bremen, Germany) or a QSTAR-XL (AB/MDS-Sciex, Toronto, Canada) with a nanoLC interface (Nikkyo Technos, Tokyo, Japan), Dionex Ultimate3000 pump with FLM-3000 flow manager (Germering, Germany), and HTC-PAL autosampler (CTC Analytics, Zwingen, Switzerland) was employed for nanoLC-MS/MS measurement. A self-pulled needle (150 mm length, 100 μm i.d., 6 μm opening) packed with ReproSil-Pur C18-AQ materials (3 μm, Dr. Maisch, Ammerbuch, Germany) was used as an analytical column with "stone-arch" frit[37]. The injection volume was 5 μL, and the flow rate was 500 nL/min. The mobile phases consisted of (A) 0.5% acetic acid and (B) 0.5% acetic acid and 80% acetonitrile. A three-step linear gradient of 5-10% B in 5 min, 10-40% B in 60 min, 40-100% B in 5 min, and 100% B for 10 min was employed throughout this study. A spray voltage of 2,400 V was applied. The MS scan range was *m/z* 300-1,500 (LTQ-Orbitrap) or 350-1,400 (QSTAR). For the LTQ-Orbitrap, the top 10 precursor ions

were selected in MS scan for subsequent MS/MS scans by ion trapping in the automated gain control (AGC) mode; AGC values of 5.00×10^5 and 1.00×10^4 were set for full MS and MS/MS, respectively. The normalized CID was set to be 35.0. A lock mass function was used to obtain stable and accurate *m/z* values within 3 ppm. For QSTAR experiments, MS scans were performed for 1 s to select three intense peaks, and subsequently three MS/MS scans were performed for 0.55 s each. An information dependent acquisition function was activated for 90 s to exclude previously scanned precursor ions. The CID energy was automatically adjusted by the rolling CID function of Analyst QS 1.1.

2.2.7. Data analysis and bioinformatics.

The raw data files were analyzed by Mass Navigator v1.2 (Mitsui Knowledge Industry, Tokyo, Japan) to create peak lists on the basis of the recorded fragmentation spectra. In order to improve the quality of MS/MS spectra, Mass Navigator discarded all peaks with an absolute intensity of less than 10, and with an intensity of less than 0.1% of the most intense peak in MS/MS spectra, and an in-house Perl script called "mgf creator" converted the *m/z* values of the isotope peaks to the corresponding monoisotopic peaks when the isotope peaks were selected as the precursor ions[38]. Peptides and proteins were identified by Mascot v2.2 (Matrix Science, London, U.K.) against the total ORF amino acids sequences of *E. coli* K-12 (BW25113) from GenoBase (http://ecoli.naist.jp/GB6/search.jsp) with a precursor mass tolerance of 3 ppm (LTQ-Orbitrap) or 0.25 Da (QSTAR), and strict specificity allowing for up to 2 missed cleavages. For trypsin digestion, carbamidomethylation of cysteine was set as a fixed modification, and methionine oxidation was allowed as a variable modification. For Cys cleavage-trypsin digestion, cyanidation and dehydroalanine conversion of cysteine, and methionine oxidation were allowed as variable modifications. Note that cysteine of Cys-cleaved C-terminal peptides (2- iminothiazoline-4-carbonyl

amino terminal peptides) has the same mass of the cyanidation modification. Peptides were rejected if the Mascot score was below the 95% confidence limit based on the "identity" score of each peptide, and a minimum of two peptides meeting the criteria was required for protein identification. False-positive rates (FPR) were estimated by searching against a randomized decoy database created by the Mascot Perl program supplied by Matrix Science. The grand average hydropathy (GRAVY) values for identified proteins and peptides were calculated according to a previous report[39]. Proteins and peptides exhibiting positive GRAVY values were recognized as hydrophobic. Mapping of transmembrane (TM) domains for the identified proteins was conducted using the TM hidden Markov model (TMHMM) algorithm available at http://www.cbs.dtu.dk/services/TMHMM-2.0/, to which FASTA files were submitted in batch mode[40]. Information on the subcellular location of identified proteins was obtained from gene ontology (GO) component terms using GOSlim (http://www.geneontology.org). *E. coli in silico* digestion was performed using an in-house Perl ver.5.6.1 script.

2.3. RESULTS AND DISCUSSION

2.3.1. *In silico* prediction of effects of digestion at Cys on membrane proteome.

Since tryptic peptides generated from membrane proteins are in general longer than those generated from soluble proteins and the scan range of MS for peptides is limited (typically *m/z* 350-1500), I first evaluated the observability of peptides from *E. coli* membrane and soluble proteins. All *E. coli* proteins were digested *in silico* at the C-terminal sides of K and R (i.e., trypsin cleavage) and at the C-terminal sides of K and R, as well as the N-terminal side of C (i.e., trypsin and cyanocysteine-mediated cleavage). Figure 4 shows the distribution of sequence coverage, assuming that MS-detectable peptides range in mass from 700 to 3,000 Da and the digestion reaction proceeds with 100% efficiency. Higher coverage (on average) and narrower distribution were obtained for soluble proteins in comparison to membrane proteins (Figure 4a). This would be one of the reasons why the membrane proteome has been less well characterized than the soluble portion of the whole proteome. By adding Cys cleavage to the tryptic cleavage sites, approximately 15-20% higher sequence coverage on average was observed for membrane proteins, whereas almost unchanged coverage was obtained for soluble proteins (Figure 4b,c). These *in silico* analyses indicate that Cys cleavage in addition to tryptic digestion would facilitate efficient membrane proteome analysis by MS, provided that other factors such as solubility and the digestion efficiency of membrane proteins are not limiting factors.

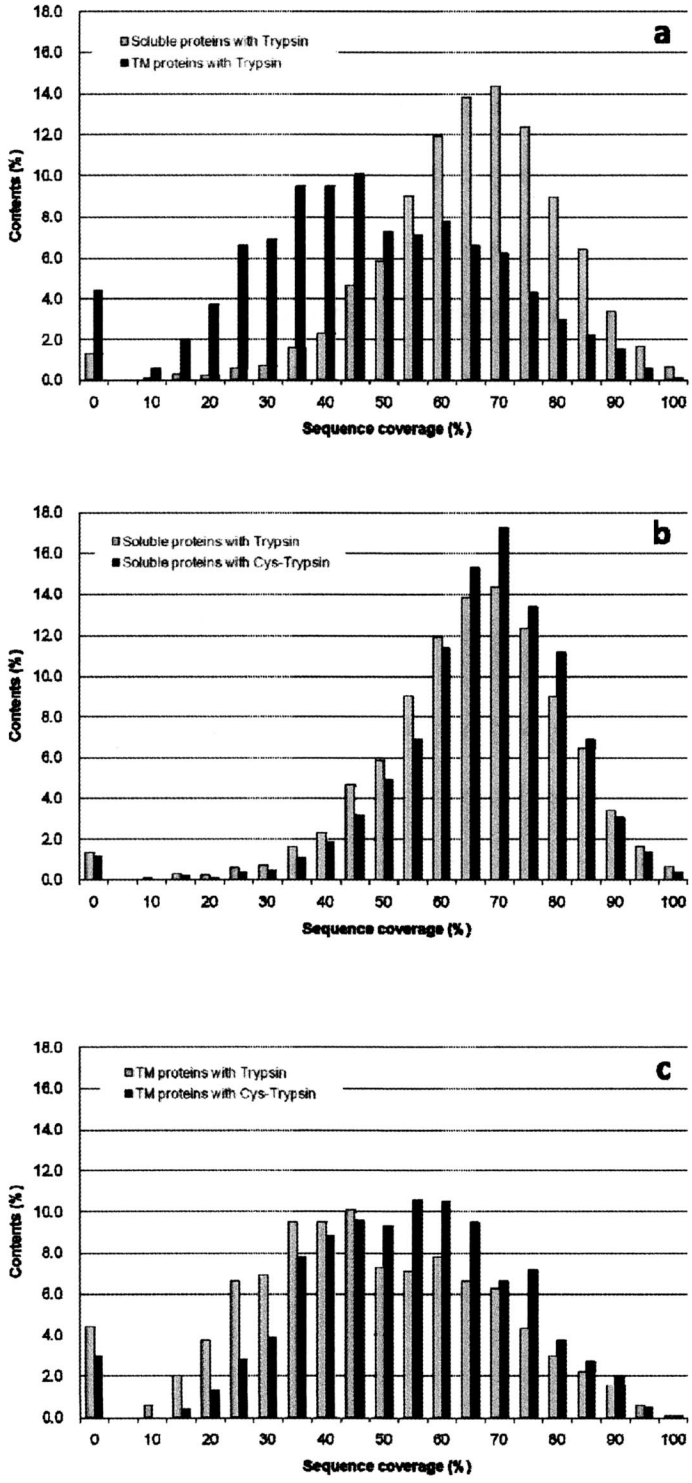

Figure 4 Distribution of sequence coverage for the *E. coli* proteome in *in silico* digestion

(a) Comparison between soluble proteins (gray bar) and TM proteins (black bar) digested with trypsin; (b) comparison between soluble proteins digested with trypsin (gray bar) and with Cys cleavage-trypsin (black bar); (c) comparison between TM proteins digested with trypsin (gray bar) and with Cys cleavage-trypsin (black bar). *E. coli* whole proteome from GenoBase was digested *in silico*, and peptides ranging from 700 to 3,000 Da were used for the analysis. In total, 3,273 soluble proteins and 1,043 TM proteins were obtained in this way from the Genobase, and these numbers were used to calculate the contents (%) of soluble and TM proteins. TM proteins were predicted using the TMHMM program.

2.3.2. Optimization of Cys cleavage protocol.

To cleave the peptide bond at Cys experimentally, I employed the cyanocysteine cleavage reaction. As shown in Figure 5, cysteine residues are cyanidated by NTCB as a first step, then the resultant cyanidated peptides are digested at the N-terminal side of cyanocysteine under alkaline conditions. Conventionally, this reaction takes 12 h, and purification of the product after the first step is required. To apply this reaction to proteome-wide analysis, I aimed to minimize the number of required steps, as well as the reaction time. In this optimization study, I used *E. coli* whole cell lysates. First, I examined whether it was possible to omit the purification step between the cyanidation and the cleavage steps. By controlling the amounts of TCEP and NTCB relative to the total protein amount (NTCB/TCEP/proteins) 100:10:1 M), I could obtain the cleaved products quantitatively without any purification step (the conventional method was used as a control). I also found that the cyanidation reaction proceeds under alkaline conditions, where the cleavage reaction occurs. In addition, higher pH resulted in a shorter reaction time, as well as a greater number of identified Cys-cleaved peptides, when I examined reaction buffers of pH 8, 9, 10, and 11. This would be because the cleavage reaction is based on the nucleophilic attack of hydroxyl ions on the carbonyl carbon at the N-terminal side of cyanocysteine residues, and a longer reaction time increases the formation of side products, such as dehydroalanine derivatives generated by β-elimination[31, 41, 42]. In the conventional protocol, proteins were cyanidated at neutral pH and digested at pH 9. After optimization, I established a single-step protocol for cyanidation and digestion in one pot at pH 11 for 30 min. NanoLC-MSMS analysis of fragments obtained with this protocol identified approximately twice as many Cys-cleaved peptides as when the conventional protocol was used (184 in this protocol, 100 in the conventional protocol).

PITC-labeled glutathione was used to estimate the recovery of this protocol. The resultant phenylthiocarbamyl glutamic acid was separated by reversed phase HPLC and was detected at 254

nm. The recovery was 73.0 ± 0.3% (n = 3). This value was supported by the results from *E. coli* samples where 68% of the total cysteine-containing peptides was Cys-cleaved peptides, while 22% and 9% were β-elimination peptides and uncleaved cyanocysteine peptides[42], respectively. Note that I did not observe potential side products as well as the residue modifications at pH 11 such as carbamylated lysine, thionitrobenzoate derivatives and β-elimination products of serine and threonine.

Since the most widely used chemical cleavage reaction is methionine cleavage by CNBr, I compared the Cys cleavage-trypsin digestion protocol with the conventional CNBr-trypsin protocol[7] for 50 μg of *E. coli* membrane fraction. As a result, CNBr-trypsin provided only 39 identified proteins (190 peptides), whereas Cys cleavage-trypsin gave 487 proteins (3,672 peptides) in total from triplicate analysis. This might be because the sample solution became turbid when pH of the sample solution changed from acidic pH to weak basic pH after the CNBr cleavage. In addition, the sample amount was relatively low compared to the conventional procedure ($\sim$ 1 mg). On the other hand, the sample solution was kept under basic conditions throughout the Cys cleavage-trypsin protocol.

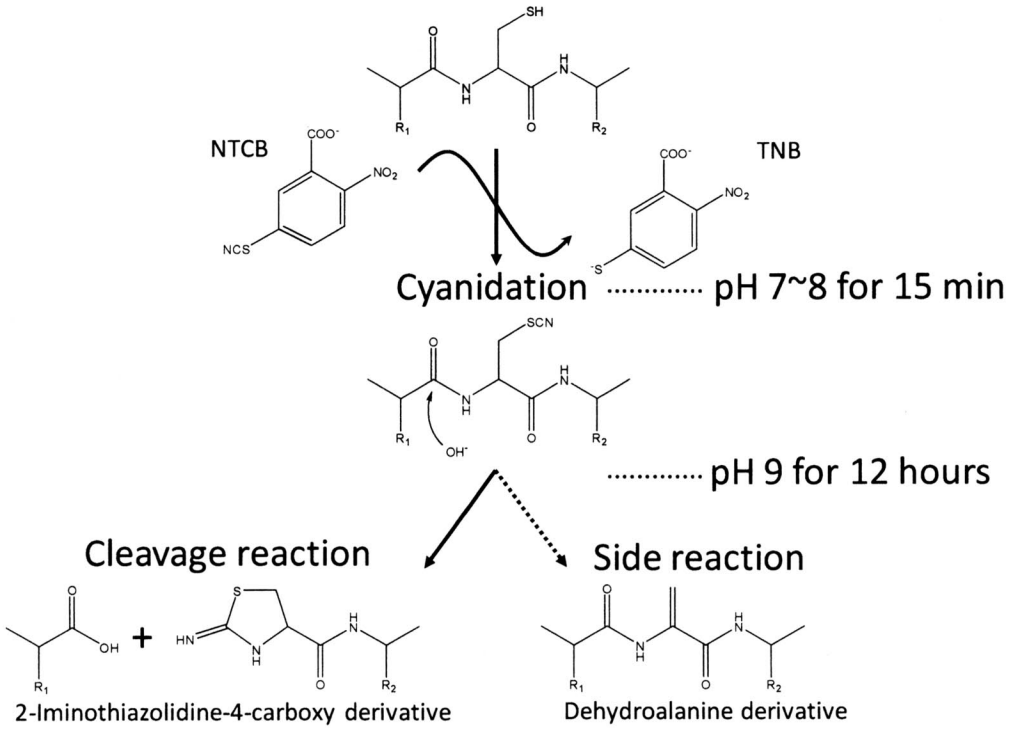

Figure 5 Reaction scheme of cysteine cleavage via cyanocysteine

Cysteine residues are cyanidated by adding NTCB as a first step, and the resultant cyanidated peptides are cleaved at the N-terminal side of cyanocysteine under alkaline conditions; conventionally this reaction is completed in approximately 12 h.

2.3.3. Reproducibility and false positive identification rate in the Cys cleavage-trypsin protocol.

With the use of the established protocols of Cys cleavage and trypsin digestion, I evaluated the reproducibility as well as the rate of false positive identification. I carried out duplicate analyses of sample preparation including the digestion step and did duplicate nanoLC-MS/MS measurements for each sample (4 data sets in total) (Table 1). Considering the day-to-day variation in sample preparation as well as the variation in LC-MS measurement, the reproducibility in the identification number of peptides and proteins using the Cys cleavage-trypsin protocol was similar to that using the PTS-trypsin protocol in our previous study[5]. Since the Cys cleavage-trypsin protocol has to consider more variable modifications during the Mascot database searching step, it might have a higher false positive identification rate. However, a random database search identified only a few false positive peptides, and the false positive rates in peptide and protein identification were 0.24% and 0.73%, respectively. Compared with the false positive rate of the PTS-trypsin protocol in our previous study[5], introduction of variable modification of Cys did not significantly decrease the reliability of protein identification.

I also evaluated the contents of semitryptic peptides and peptides containing missed tryptic cleavage sites generated by the Cys cleavage-trypsin protocol. As a result, I did not find any difference between the conventional trypsin protocol and the Cys cleavage-trypsin protocol in terms of semitryptic cleavage (semitryptic peptides: 2.8% and 2.9% for the Cys cleavage-trypsin protocol and the trypsin protocol, respectively) and missed tryptic cleavage (missed cleavage peptides: 21.4% and 22.4% for the Cys cleavage-trypsin protocol and the trypsin protocol, respectively).

Table 1 Reproducibility and False Positive Identification Rate in the Cys Cleavage-Trypsin Protocol[a]

Cys cleavage-trypsin protocol	Batch 1		Batch 2		Average (SD)
	inj. 1	inj. 2	inj. 1	inj. 2	
no. of total peptides	2,425	2,388	2,166	1,957	2,234 (217)
no. of total proteins	364	372	347	325	352 (21)
no. of TM proteins	154	155	149	137	149 (8)
no. of other membrane proteins	67	68	61	58	64 (5)
False positive rate					
Peptide			0.24 %		
Protein			0.73 %		

[a] The *E. coli* membrane-enriched fraction (14 μg) was employed for duplicate sample preparations using the Cys cleavage-trypsin protocol and duplicate measurements were done using nanoLC-MS/MS (LTQ-Orbitrap). False positive rate was calculated for the merged results.

2.3.4. Application of the Cys cleavage-trypsin protocol to *E. coli* membrane proteome using two-dimensional LC-MS/MS.

To explore application of the new protocol to *E. coli* membrane proteome analysis, I fractionated the digested samples using both ion exchange and reversed phase modes prior to LC-MS/MS analyses. I also evaluate the effect of introducing Cys cleavage on the PTS-trypsin protocol. Duplicate analyses coupled with two different prefractionations followed by duplicate LC-MS/MS measurements for each sample were carried out, and the results were merged to provide an overall list of identified peptides and proteins. In this way, I successfully identified 667 *E. coli* membrane proteins and 5,542 hydrophobic peptides. Compared with the PTS-trypsin protocol, they represent increases of more than 10% and 18% in the identified numbers of proteins and peptides, respectively. In total, I identified 14,338 peptides and 1,530 proteins with the Cys cleavage-trypsin protocol, whereas 12,561 peptides and 1,330 proteins were found with the PTS-trypsin protocol (Table 2). Interestingly, contrary to the *in silico* prediction, more soluble proteins were observed with the Cys cleavage-trypsin protocol than with the trypsin protocol, though the identification efficiency for membrane proteins was also improved with the Cys cleavage-trypsin protocol, as predicted (Figure 6). Note that the soluble proteins identified in this study were not from the soluble fraction of *E. coli* cells, but from the membrane-enriched fraction. Therefore, I presumed that the improvement for soluble proteins arose because the releasing step of soluble proteins from the membrane-enriched pellets was limiting, unless the Cys cleavage was employed. To test this hypothesis, I reversed the order of the digestion in the Cys cleavage-trypsin protocol, that is, tryptic digestion was done before Cys cleavage reaction. As expected, this reversed protocol gave worse results than the Cys cleave-trypsin protocol and almost identical results to the trypsin protocol (Table 3). Next, I evaluated whether the Cys cleavage-trypsin protocol achieved unbiased digestion, independent of the TMD number per protein. The

experimental pattern of the distribution of TMD number for transmembrane (TM) proteins was in excellent agreement with the predicted pattern for GenoBase-registered TM proteins (Figure 7), supporting the view that our digestion protocol is unbiased, that is, that cleavage is independent of the TMD number per protein. I also evaluated the coverage of membrane proteome identified in this study by comparing our results with the GenoBase-registered membrane proteome. In addition, I examined the coverage within various subcategories of the membrane proteome, such as TM proteins predicted by TMHMM algorisms, transporters and ABC superfamily proteins categorized by 'gene product description' of GenoBase. As shown in Table 4, the coverage values were quite consistent in all subcategories at approximately 40%. Furthermore, I checked the total number of TMD of all proteins and confirmed that the coverage was also approximately 40%. These results strongly support the conclusion that unbiased identification was achieved with our new protocol based on cyanocysteine cleavage-assisted trypsin digestion.

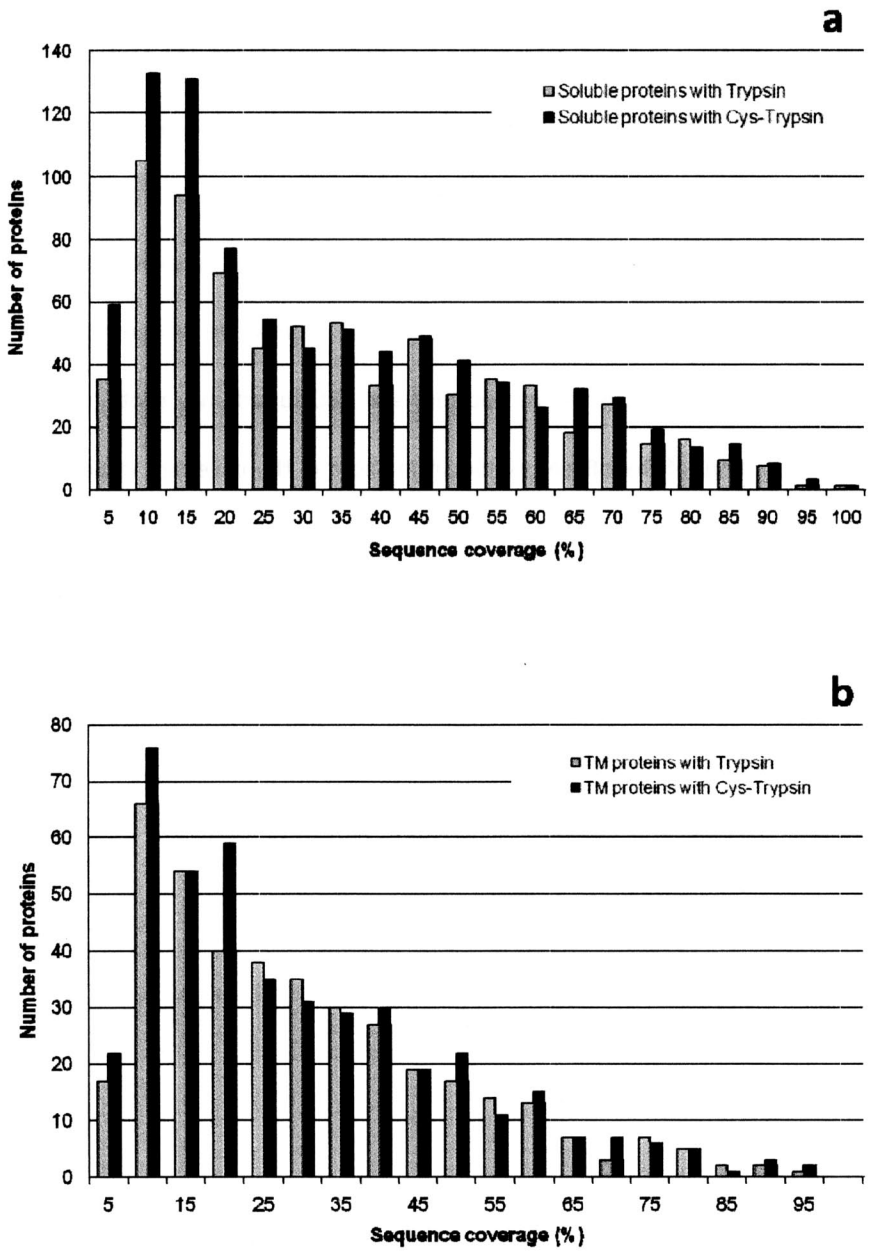

Figure 6 Distribution of sequence coverage for the identified proteins

(a) Soluble proteins identified using the Cys cleavage-trypsin protocol and the trypsin protocol are indicated by black and gray bars, respectively. (b) TM proteins identified using the Cys cleavage-trypsin protocol and the trypsin protocol are indicated by black and gray bars, respectively. The sequence coverage of each identified protein was calculated based on the experimental results shown in Table 2.

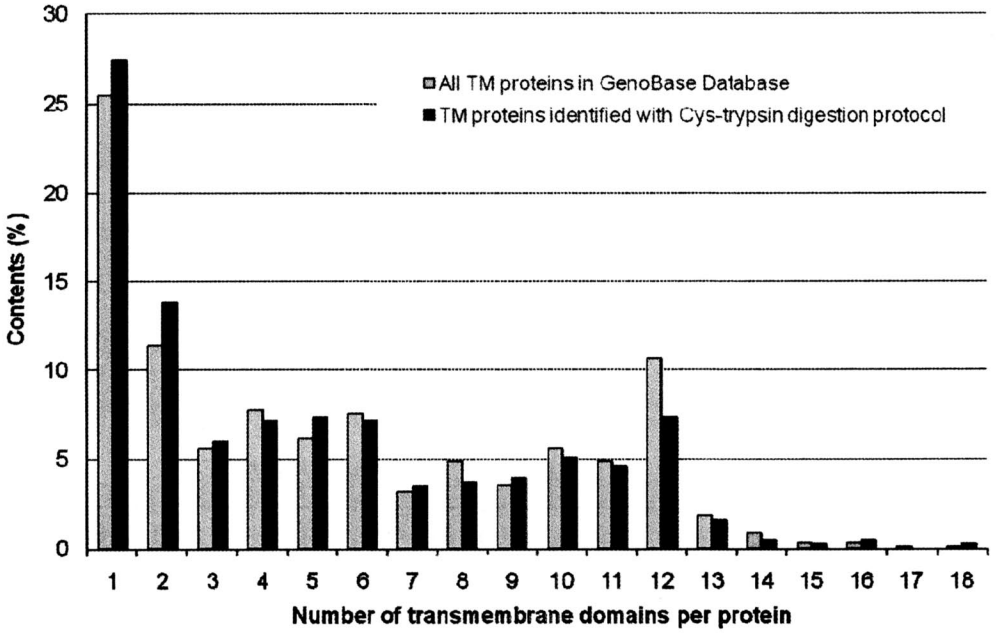

Figure 7 Comparison of the TMD numbers per protein identified according to the Cys cleavage-trypsin protocol with those from GenoBase

In total, 1,043 TM proteins were predicted from Genobase and 434 TM proteins were identified using the Cys cleavage-trypsin protocol. These total numbers and the numbers of TMD predicted by TMHMM were used to calculate the content (%) of TM proteins.

Table 2 *E. coli* Membrane Proteome Analysis Using the Cys Cleavage-Trypsin Protocol and PTS-Trypsin Protocol[a]

No. of peptides/proteins	protocol		% increase
	trypsin	Cys Cleavage-trypsin	
No. of total unique peptides	12,561	14,338	14.1
No. of hydrophobic peptides	4,689	5,542	18.2
No. of total unique proteins	1,330	1,530	15.0
No. of membrane proteins	605	667	10.2

[a] *E. coli* membrane-enriched fractions (14 μg) extracted with 5% SDC were used. Details of both protocols are described in Materials and Methods. For both SCX and SDB fractionation approaches, duplicate sample preparations coupled with duplicate nanoLC-MS measurements using LTQ-Orbitrap for each sample were carried out, and the obtained results per protocol were merged. Membrane proteins were defined by using TMHMM, GO terms and GRAVY scores. Hydrophilic and hydrophobic peptides were categorized on the basis of GRAVY scores

Table 3 Impact of the Preceding Cys Cleavage on Protein Identification

Protocol	No. of total proteins	No. of TM proteins	No. of other Membrane proteins
Trypsin	253	108	53
Cys cleavage-trypsin	270	117	53
Trypsin-Cys cleavage	247	104	51

[a] The *E. coli* membrane-enriched fraction (14 μg) was employed for each protocol. The 'Trypsin-Cys cleavage' protocol consisted of trypsin digestion followed by Cys cleavage reaction. The digested samples were analyzed by nanoLC-MS/MS (QSTAR). Triplicate analyses were performed and merged results are shown.

Table 4 Profile of Coverage in Subgroups of the *E. coli* Membrane Proteome

Category	GenoBase Database	No. of identified proteins by Cys cleavage-trypsin (coverage%)
Membrane proteins [a]	1,652	667 (40%)
TM proteins [b]	1,043	434 (42%)
Transporter, antiporter [c]	283	105 (37%)
ABC superfamily [c]	95	39 (41%)
Total no. of TMD [b]	5,740	2,181 (38%)

[a] Membrane proteins were defined using TMHMM, GO terms and GRAVY scores. [b] TM proteins were defined using TMHMM. [c] Subcategories were classified according to the 'gene product description' of GenoBase.

2.4. CONCLUSIONS

I showed that the introduction of cyanocysteine-mediated cleavage reaction in combination with trypsin cleavage increased the identification efficiency in LC-MS-based membrane proteome analysis. The optimized protocol takes no longer than the conventional trypsin protocol, and provides a higher identification efficiency than the PTS-trypsin protocol, in agreement with prediction from *in silico* analysis. The results of application of the new method to the *E. coli* membrane proteome indicated that the digestion was unbiased and independent of the TMD number per protein. Further improvement will be achieved by optimization of LC conditions for hydrophobic peptides, where approximately 13% of the MS observable peptides from TM proteins were currently out of the LC elution range according to the LC retention time prediction[43]. Since this protocol is quite simple, reproducible and unbiased, it is expected to be useful in providing wider coverage for membrane proteome analysis with various organisms.

CHAPTER 3

Toward the complete membrane proteome analysis

3.1. INTRODUCTION

Proteins have critical roles to organize the cell and process the various physiological functions. As mentioned in the chapter 1 and 2, membrane proteins play key roles in these physiological functions, and therefore the analyses of the membrane proteins are important. In the previous chapter, I focused on the sample preparation step to improve the identification efficiency. Consequently, the cyanocysteine-mediated cleavage reaction facilitated the identification efficiency with achieving unbiased identification in LC-MS based membrane proteome analysis[44]. However, I only identified about 40 % of the total *E. coli* proteins even using this new method. Compared to the other -omics method, the immaturity of the proteomics is still obvious.

There are mainly two problems which prevent complete identification of proteome by shotgun proteomics approach. Those are complexity and dynamic range problems existing in analyzing the shotgun proteomics samples. Firstly, in shotgun proteomics, proteins have to be cleaved before LC-MS analyses. The complexity of the sample is drastically increased at this process. Taking *E. coli* proteins for example, the number of tryptic peptides is about 130,000, although the number of *E. coli* proteins is about 4,000. This enormous range of peptides cannot be separated in a short time single LC, and then lots of peptide ions are co-eluted and are injected into the MS at the same time. The limited numbers of peptide ions are selected for MS/MS according to its MS signal intensity in descending order above certain criteria to identify the peptides. Large amounts of peptides get higher MS signal intensity and are identified in high efficiency. On the other hand, small amounts of

peptides get lower MS signal intensity which is hard to be selected for MS/MS, then the identification efficiency of the small amounts of peptides are very low. Secondly, there is suppression in the step of ionization and detectors in the MS. Larger amounts of peptides tend to get higher ionization rate. Moreover, because the ion capacity of the detector in the MS is limited, they occupy the detector because of their abundance. Therefore, smaller amounts of peptides become suppressed and they never seen in the results of the LC-MS/MS analyses even though they exist in the sample.

To solve such problems and improve the identification efficiency, most reports about method development on shotgun proteomics have focused on the sample preparation steps. The methods are such as using various kinds of fractionation methods before LC-MS/MS analyses to reduce the complexity and dynamic range in the sample[45-52]. In 2008, Godoy *et al.*,[51] achieved complete identification of yeast proteome through 41 day analyses of various prepared samples. However, it is impractical to spend over one month to analyze one sample. This is not the perfect solution for the problems.

In this study, I focused on the separation step in nanoLC to solve the problems and achieve the identification of complete *E. coli* proteome in short time LC-MS/MS analysis. Theoretically and experimentally, the separation efficiency is increased as the column length is increased[53-57]. The size of the beads in columns becomes smaller, the peak width becomes sharper and the peak intensity gets higher[54, 58]. However, there is drawback of this approach. The back pressure gets higher as the particle-packed columns become longer and exceeds the bearable pressure limits of HPLC machines. Monolith silica columns are different from the particle-packed columns in its 3D network structure[55-57, 59, 60] (Figure 8). The small-sized silica skeletons and the larger flow-through pores compared to the particle-packed columns allow using longer columns with lower back pressures which are bearable to conventional HPLC instruments. In 2008, Miyamoto *et al.*,[57] analyzed BSA

tryptic digested samples with 30 cm or 300 cm monolith silica column, which resulted in that the peak capacity was increased about 3.8-fold in 300 cm column. I applied monolith silica columns for *E. coli* proteome analysis, and optimized the LC parameters to increase the identification efficiency.

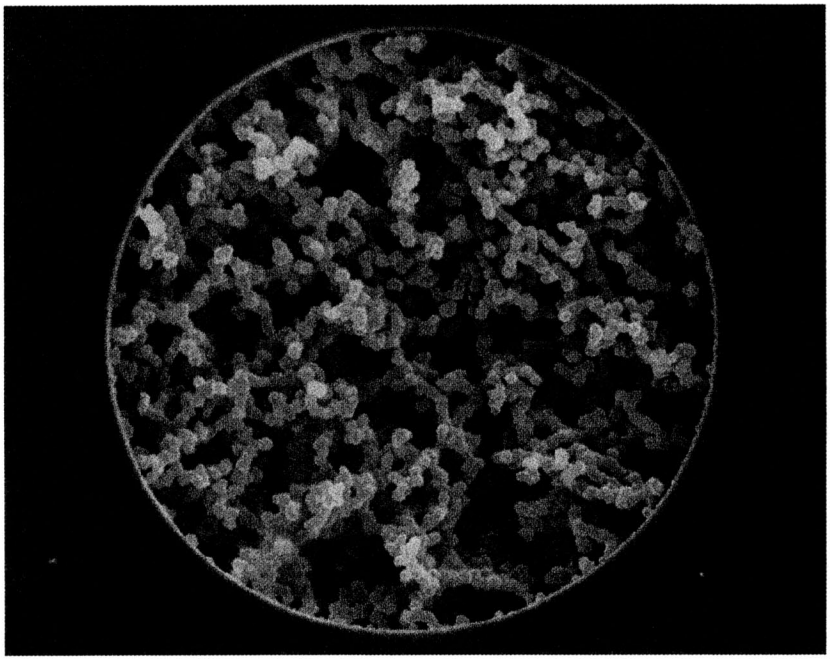

Figure 8 A SEM image of a monolithic column[60]

Monolith silica columns have small-sized silica skeletons and larger flow-through pores compared to the particle-packed columns. The monolith property allows using longer columns with lower back pressures which are bearable to HPLC instruments.

3.2. MATERIALS AND METHODS

3.2.1. Materials.

Sodium hydroxide, sodium hydrogen carbonate and 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF) were from Nacalai (Kyoto, Japan). Tris (2-carboxyethyl) phosphine hydrochloride (TCEP) was from PIERCE (Rockford, IL, USA). C18 Empore disc cartridges and membranes were from 3M (St. Paul, MN, USA). Water was purified by a Millipore Milli-Q system (Bedfold, MA, USA). Sodium deoxycholate (SDC), sodium lauroylsarcosinate (SLS), mass spectrometry-grade lysyl endprotease (Lys-C), ethyl acetate, acetonitrile, acetic acid, methanol, trifluoroacetic acid (TFA), urea, iodoacetamide (IAA), sodium carbonate and all other chemicals were purchased from Wako (Osaka, Japan). Monolith silica columns (100 μm I.D., 350 cm long) were from Dr. Tanaka and Mr. Miwa.

3.2.2. Preparation of whole cell lysate of *Escherichia coli* BW25113 cells.

Escherichia coli (*E. coli*) strain BW25113 cells grown in Luria-Bertani (LB) cultures at 37 °C were used in this study. The cell pellet was prepared by centrifugation at 4,500 g for 10 min and was resuspended in 10 mL of ice-cold 1 M KCl, 15 mM Tris (pH 7.4). A protease inhibitor AEBSF was added to the final concentration of 10 mM. The cells were lysed by ultrasonication, and the unbroken cells and debris were precipitated at 2,500 g for 5 min. The resultant pellet was used for whole cell lysate analyses.

3.2.3. In-solution trypsin digestion for the whole cell lysates.

The whole cell lysates were dissolved in 50 mM sodium carbonate buffer at pH 11 containing 12 mM SDC and 12 mM SLS. Proteins were reduced with a 10-fold molar excess of TCEP at 37 ℃ for 30 min and alkylated with a 100-fold molar excess of IAA at 37 ℃ for 30 min. The sample solution

was diluted 5-fold and digested with trypsin at 37 ℃ for O/N (trypsin-to-protein ratio of 1:50 (w/w)). An equal volume of ethyl acetate was added to the solution and the mixture was acidified with the final concentration of 0.5% TFA according to the PTS protocol reported previously[5]. The mixture was shaken for 1 min and centrifuged at 15,700*g* for 2 min, then the aqueous phase was collected and desalted with C18- StageTips[34].

3.2.4. Peptide fractionation of digested samples.

In accordance with the StageTip fractionation protocol[35], fractionations using StageTips with a strong cation exchange (SCX) disk were performed for the digested samples. SCX-StageTips[36] were used and 20-500 mM ammonium acetate solutions containing 15% acetonitrile were employed to elute peptides, resulting in 5 fractions. All eluted fractions, including the flow-through fraction, were desalted by means of C18-StageTips.

For the fractionation of isoelectric focusing, ZOOM IEF Fractionator (Invitrogen) was used and focusing buffer of pH 3.0 – pH 4.6, pH 4.6 – pH 5.4, pH 5.4 – pH 6.2, pH 6.2 – pH 7.0, pH 7.0 – pH 10.0 were employed to make 5 fractions according to the peptides pI. All eluted fractions were desalted by means of C18-StageTips.

3.2.5. NanoLC-MS system.

An LTQ-Orbitrap XL (Thermo Fisher Scientific, Bremen, Germany) or a QSTAR-XL (AB/MDS-Sciex, Toronto, Canada) with a nanoLC interface (Nikkyo Technos, Tokyo, Japan), Dionex Ultimate3000 pump with FLM-3000 flow manager (Germering, Germany), and HTC-PAL autosampler (CTC Analytics, Zwingen, Switzerland) was employed for nanoLC-MS/MS measurement. A self-pulled needle (150 mm length, 100 μm i.d., 6 μm opening) packed with ReproSil-Pur C18-AQ materials (3 μm, Dr. Maisch, Ammerbuch, Germany) was used as an analytical column of a particle-packed

column with "stone-arch" frit[37]. The injection volume was 5 μL, and the flow rate was 500 nL/min. The mobile phases consisted of (A) 0.5% acetic acid and (B) 0.5% acetic acid and 80% acetonitrile. A two-step linear gradient of 5 % to 40 % B in 70 min at a minimum, 40 % to 100 % B in 5 min, and 100 % B for 10 min was employed throughout this study. For the evaluation of gradient time, the time in the first step of the linear gradient of 5 % to 40 % B was lengthened from 70 min (Figure 9). A spray voltage of 2,400 V was applied. The MS scan range was *m/z* 300-1,500 (LTQ-Orbitrap) or 350-1,400 (QSTAR). For LTQ-Orbitrap, the top ten precursor ions were selected in MS scan by orbitrap for subsequent MS/MS scans by ion trap in the automated gain control (AGC) mode where AGC values of 5.00e+05 and 1.00e+04 were set for full MS and MS/MS, respectively. The normalized CID was set to be 35.0. A lock mass function was used to obtain stable and accurate *m/z* values within 3 ppm. For QSTAR experiments, MS scans were performed for 1 s to select three intense peaks, and subsequently three MS/MS scans were performed for 0.55 s each. An information-dependent acquisition function was activated for 90 s to exclude the previously scanned precursor ions. The CID energy was automatically adjusted by rolling CID function of Analyst QS 1.1.

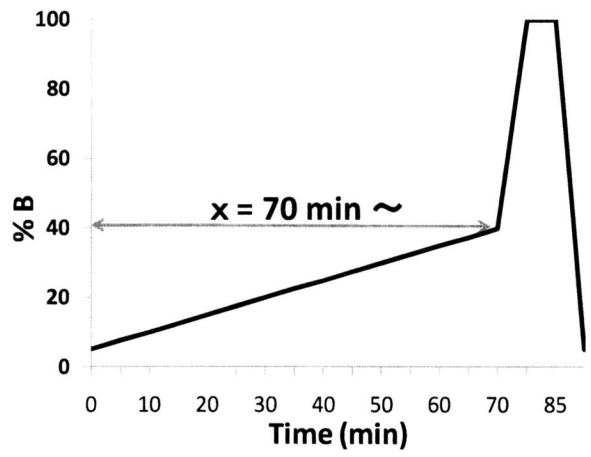

Figure 9 Optimization of Gradient Time

The length of the gradient time was evaluated from 70 minutes at a minimum to increase identification efficiencies. The mobile phases consisted of (A) 0.5% acetic acid and (B) 0.5% acetic acid and 80% acetonitrile. A two-step linear gradient of 5 % to 40 % B in 70 min which is variable, 40 % to 100 % B in 5 min, and 100 % B for 10 min was employed.

3.2.6. Data analysis and bioinformatics.

The raw data files were analyzed by Mass Navigator v1.2 (Mitsui Knowledge Industry, Tokyo, Japan) to create peak lists on the basis of the recorded fragmentation spectra. In order to improve the quality of MS/MS spectra, Mass Navigator discarded all peaks with an absolute intensity of less than 10, and with an intensity of less than 0.1% of the most intense peak in MS/MS spectra, and an in-house Perl script called "mgf creator" converted the *m/z* values of the isotope peaks to the corresponding monoisotopic peaks when the isotope peaks were selected as the precursor ions[38]. Peptides and proteins were identified by Mascot v2.2 (Matrix Science, London, U.K.) against the total ORF amino acids sequences of *E. coli* K-12 (BW25113) from GenoBase (http://ecoli.naist.jp/GB6/search.jsp) with a precursor mass tolerance of 3 ppm (LTQ-Orbitrap) or 0.25 Da (QSTAR), and strict specificity allowing for up to 2 missed cleavages. For trypsin digestion, carbamidomethylation of cysteine was set as a fixed modification, and methionine oxidation was allowed as a variable modification. Peptides were rejected if the Mascot score was below the 95% confidence limit based on the "identity" score of each peptide, and a minimum of two peptides meeting the criteria was required for protein identification.

3.3. RESULTS AND DISCUSSION

3.3.1. The effects of the gradient time on the identification efficiency.

To evaluate the effect of the gradient time on the identification efficiency, I first analyzed 1 μg of *E. coli* whole cell lysate in the lengthened gradient time from 70 min with QSTAR-XL. Figure 10a,b shows the number of identified proteins and peptides of triplicate LC-MS/MS analyses with 15 cm particle-packed column (3 μm C18 beads, 100 μm I.D.) and 350 cm monolith silica column (100 μm I.D.), respectively. As a result, both columns showed increased numbers of indentified peptides and proteins with longer gradient time. For 15 cm particle-packed column, the highest number of identified proteins was about 400 with more than 200 min gradient time analysis. On the other hand, for 350 cm monolith column, the highest number of identified proteins was about 700 with more than 400 min gradient time analysis. To explore why the identification numbers were increased with long monolith column, the peak widths at half height ($W^{1/2}$) of commonly identified 12 peptides of the analyses by the two columns were compared. Maximum 2-fold sharper peaks were obtained with long monolith column (Figure 11). Both columns showed the highest identification number at $W^{1/2}$ of about 40 sec. This is because the sensitivity was decreased with wider $W^{1/2}$ more than 40 sec with QSTAR-XL, and the numbers of peptides ions which exceed the threshold for MS/MS were decreased. Therefore, the identification efficiencies were decreased with the wider $W^{1/2}$ on QSTAR-XL.

To more improve the identification efficiency, it was considered to use increased injection amounts to get more peak intensity and more sensitive MS instrument which have higher scan speed. The injection amounts of the *E. coli* sample were increased and analyzed by LTQ-Orbitrap XL (Figure 12). For the particle-packed column, the numbers of identified proteins were increased with longer gradient time and increased injection amounts. For the monolith column, the number of identified proteins was greatly increased with 2,470 min gradient time analysis using 4 μg injection amounts.

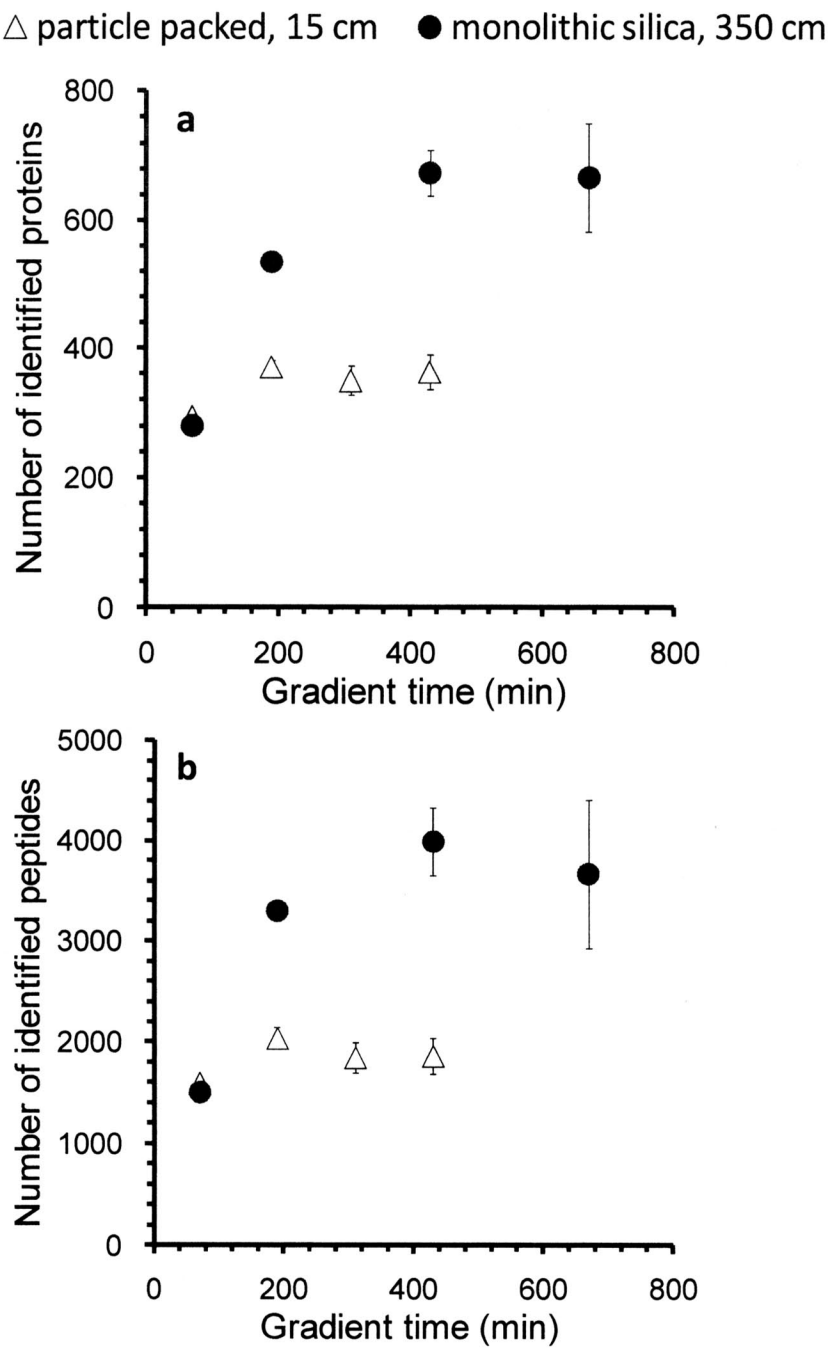

Figure 10 *E. coli* whole proteome analyses using the particle-packed column and the monolith column (QSTAR-XL)

The number of identified proteins (a) and peptides (b) of triplicate LC-MS/MS analyses with 15 cm particle-packed column (open triangle, 3 μm C18 beads, 100 μm I.D.) and 350 cm monolith silica column (closed circle, 100 μm I.D.) is shown.

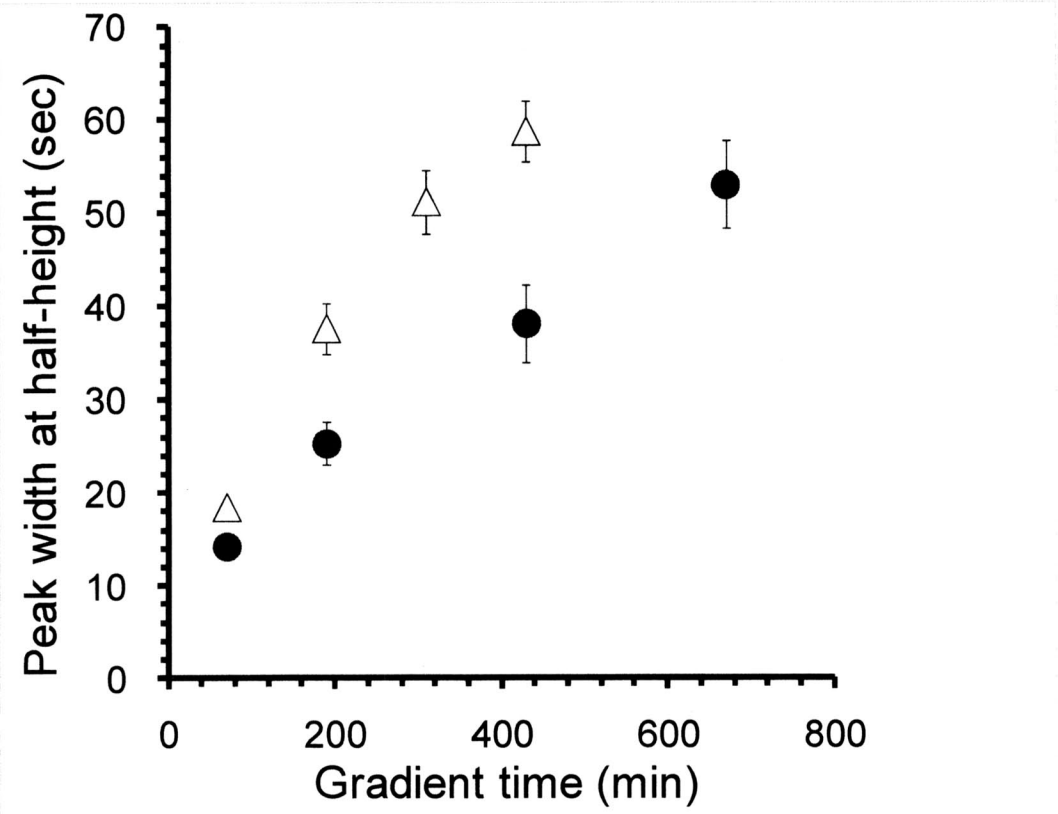

Figure 11 Relations between the peak width at half height ($W^{1/2}$) and the gradient time

Comparison of the $W^{1/2}$ of commonly identified 12 peptides of the analyses by the particle-packed column (open triangle) and monolith column (closed circle) is shown.

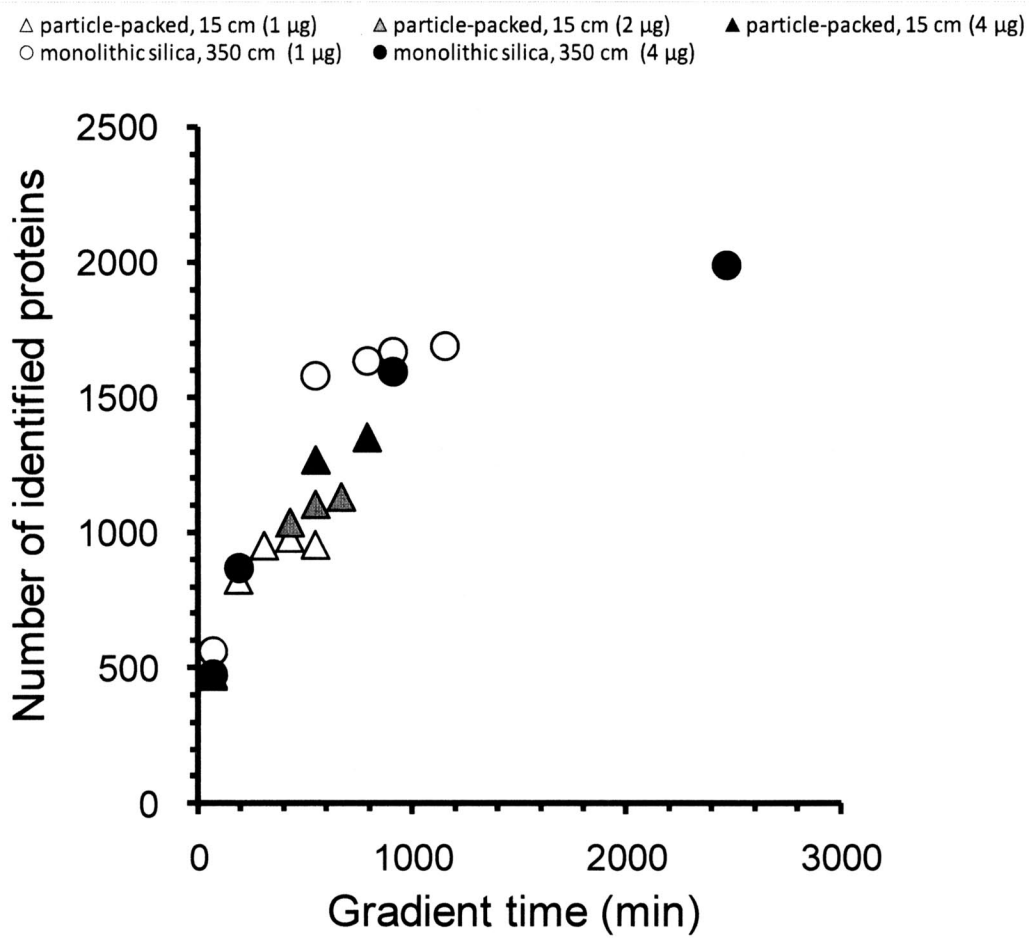

Figure 12 *E. coli* whole proteome analyses using the particle-packed column and monolith column (LTQ-Orbitrap XL)

The number of identified proteins by LC-MS/MS analyses with 15 cm particle-packed column (triangles, 3 μm C18 beads, 100 μm I.D.) and 350 cm monolith silica column (circles, 100 μm I.D.) is shown. The injection amounts to the LC-MS/MS are indicated in parentheses. For 15 cm particle-packed column, the numbers of identified proteins were increased with longer gradient time and increased injection amounts. For 350 cm monolith silica column, the number of identified proteins was greatly increased with 2,470 min gradient time analysis using 4 μg injection amounts.

3.3.2. The performance of a single analysis and analyses of pre-fractionated samples.

To evaluate the performance of a single analysis and the analyses of pre-fractionated samples, I compared the triplicate LC-MS/MS analyses of *E. coli* whole cell lysate with 15 cm particle-packed column in 70 min gradient (System A, single particle-packed), merged 10 fractionated samples of IEF and SCX with 15 cm particle-packed column (System B, MD-LC), and 350 cm monolith column in 2,470 min gradient (System C, single monolith) (Figure 13). As shown in Table 5, using the 15 cm particle-packed column, the merged number of identified proteins was about 3-fold increased from a single analysis if the pre-fractionation approaches were employed and repeated at least 3 times. However, almost the same number of proteins was identified with a single analysis by the monolith column and MD-LC analyses. The identification efficiency was extremely improved with the monolith column in terms of injection amounts (10 %) and total analytical time (11 hours less) compared to the MD-LC. The numbers of peptides were more identified with MD-LC though, that numbers were not contributed to the number of identified proteins. To investigate the differences in identified proteins of MD-LC and single monolith analyses, the proteome data were compared to the correspondence transcriptome data analyzed by microarray[61]. Figure 14 shows the proteome coverage to the correspondence expressed genes according to the mRNA expression level. There was a bias on the identification of highly expressed proteins, and lower expressed proteins were hardly identified especially by the single analysis with particle-packed column. The lower expressed proteins were more identified by the single analysis with monolith column and highly expressed proteins were more identified with MD-LC. For MD-LC approach, there were not many new peptides especially derived from the lower expressed proteins, thus the increased number of identified peptides was not contributed to the number of identified proteins. The approach using the monolith column seems to be effective to reduce the problem of dynamic range by identifying the lower expressed proteins.

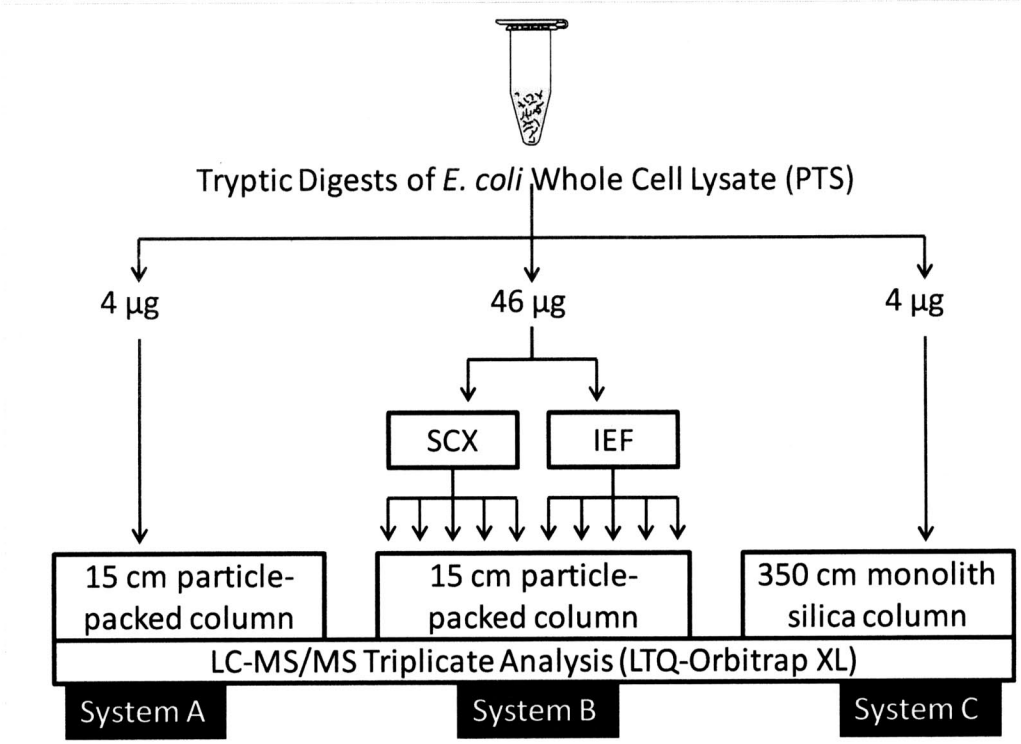

Figure 13 Flowchart of *E. coli* whole proteome analysis

Flowchart of the triplicate LC-MS/MS analyses of *E. coli* whole cell lysate is shown. System A is the LC-MS/MS analyses with 15 cm particle-packed column in 70 min gradient (single particle-packed). System B is the LC-MS/MS analyses with 15 cm particle-packed column in 70 min gradient of 10 fractionated samples of IEF and SCX (MD-LC). System C is the LC-MS/MS analyses with 350 cm monolith column in 2,470 min gradient (single monolith).

Table 5 Impact of the single analysis[a]

Approach	System A (Single)	System B (MD-LC)	System C (Single)
Column Type	15cm particle-packed	15cm particle-packed	350 cm Monolith
Single Gradient Time (min)	70	70	2,470
Total Analytical Time (min)	110	3,300	2,600
Total Injection Amounts (μg)	4	46	4
No. of identified Proteins	591 ± 24	1,930	1,925 ± 59
No. of identified Peptides	3,715 ± 77	18,989	15,993 ± 560

[a] The number of identified proteins and peptides by the triplicate LC-MS/MS analyses of system A, B, C (Figure 13) are shown. Analytical parameters such as column type, single gradient time, total analytical time and total injection amounts are also shown. Almost same number of proteins was identified with system C (single monolith) with 10% of injection amounts and 11 hours less total analytical time than that of system B (MD-LC).

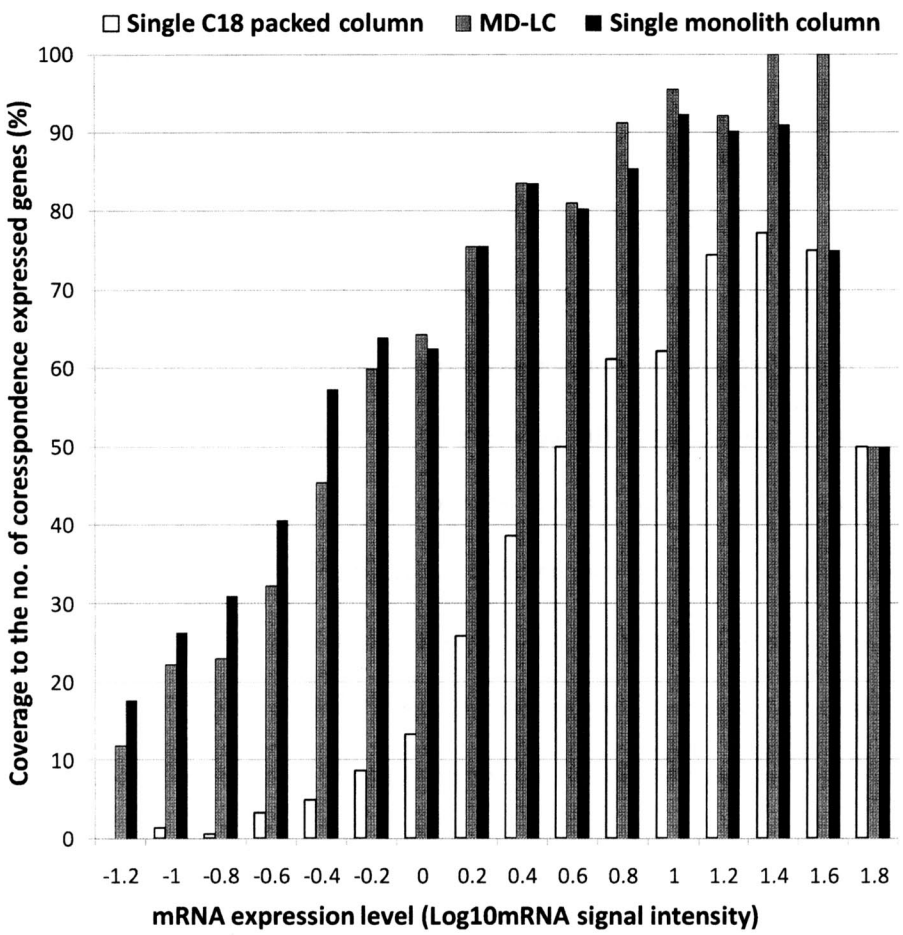

Figure 14 Comparison of the analyzed proteome to the correspondence transcriptome[61]

The proteome coverage to the correspondence expressed genes using single particle-packed column, MD-LC and single monolith column are indicated by white, gray and black bars, respectively. The lower expressed proteins were more identified with the single analysis by the monolith column than by MD-LC.

3.3.3. The impact on the dynamic range problems.

To examine how much the dynamic range problem existing, I compared the peak areas of commonly identified 1,458 peptides between the particle-packed column and the monolith column. Figure 15a shows the peak areas of the analyses by 70 min gradient time using the particle-packed column and by 2,470 min gradient time using the monolith column with the same injection amounts. The peptide areas were 3-fold increased on average in the monolith column. A typical example is shown in Figure 15b,c. The peak area in the extracted ion current chromatogram obtained from the monolithic column was approximately 20-fold larger than that from the particle-packed column, suggesting that better chromatographic separation resulted in reduced peak suppression. Figure 16 shows the base peak chromatograms (BPC) and the number of identified proteins and peptides. Although the MS signal intensity in BPC of the analysis with monolith column (70 min gradient) was increased compared to that with particle-packed column (70 min gradient), the numbers of identified proteins and peptides were similar. And the separation patterns of BPC also look similar between the analyses of two columns. However, 17,872 peptides and 1,991 proteins were identified by 2,470 min gradient time using monolith column, and the separation pattern of BPC looks remarkably improved compared to that of the particle-packed column. This is because much space for the complex sample was needed for the comprehensive identification of proteins. Although the separation efficiencies were improved with the monolith silica column, there were still peptides eluting together because of the high complexity in shotgun proteomics samples. Then, the limited numbers of peptides were identified due to the limited numbers of MS/MS. To reduce the problem of dynamic range in the samples, it has been considered important to develop pre-fractionation approaches. However, the key to reduce the dynamic range problem is actually in the step of the LC separation. One of the effective approaches to identify the complete proteome in complex samples is to use the good column which exhibit

sharp peaks and also spare much time for the enormous number of eluting peptides.

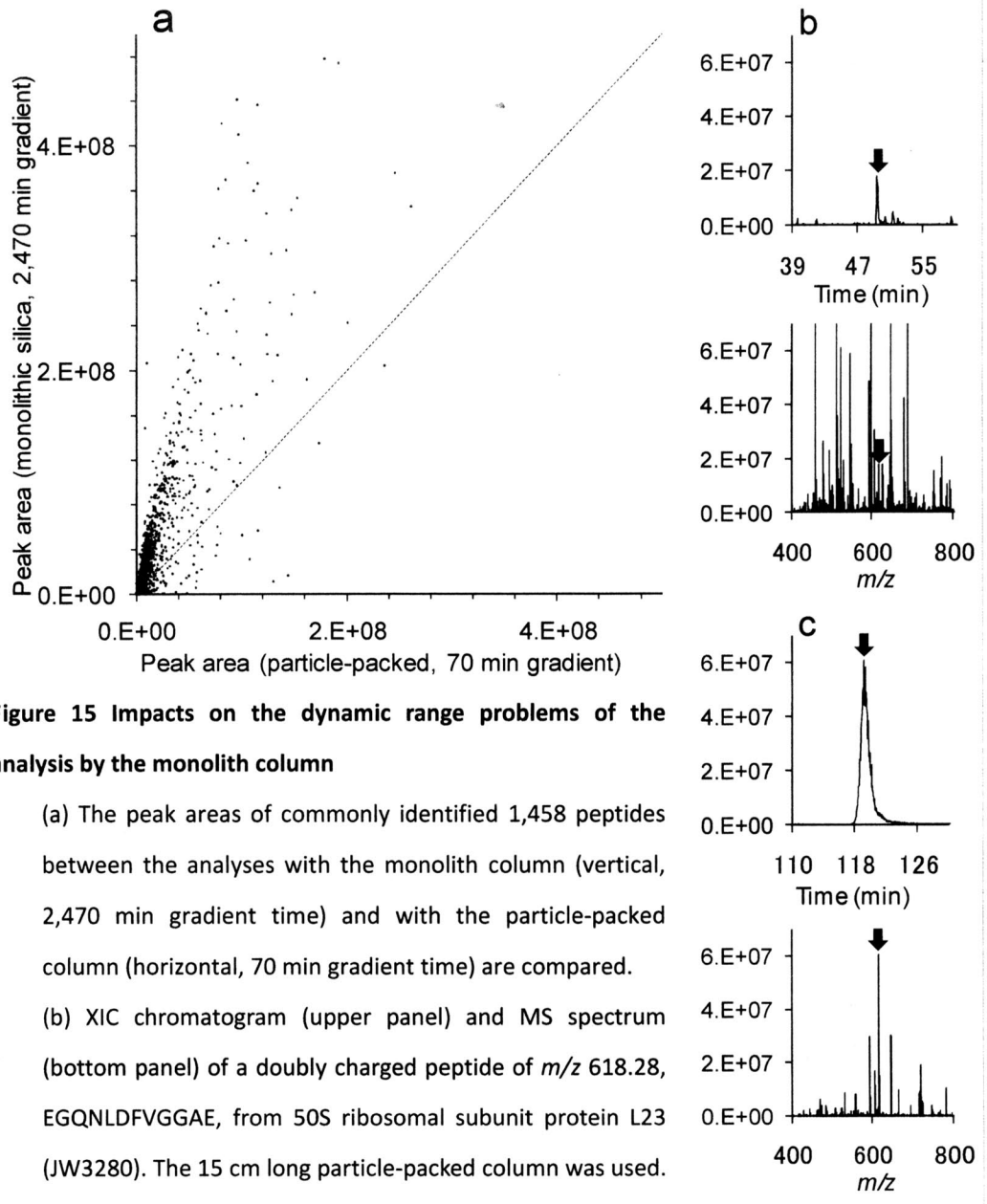

Figure 15 Impacts on the dynamic range problems of the analysis by the monolith column

(a) The peak areas of commonly identified 1,458 peptides between the analyses with the monolith column (vertical, 2,470 min gradient time) and with the particle-packed column (horizontal, 70 min gradient time) are compared.

(b) XIC chromatogram (upper panel) and MS spectrum (bottom panel) of a doubly charged peptide of *m/z* 618.28, EGQNLDFVGGAE, from 50S ribosomal subunit protein L23 (JW3280). The 15 cm long particle-packed column was used. The MS spectrum was collected at the peak top in the XIC chromatogram. Other conditions are described in Figure 2.

(c) XIC chromatogram (upper panel) and MS spectrum (bottom panel) of the same peptide as in (b). The 350 cm long monolithic silica column was used.

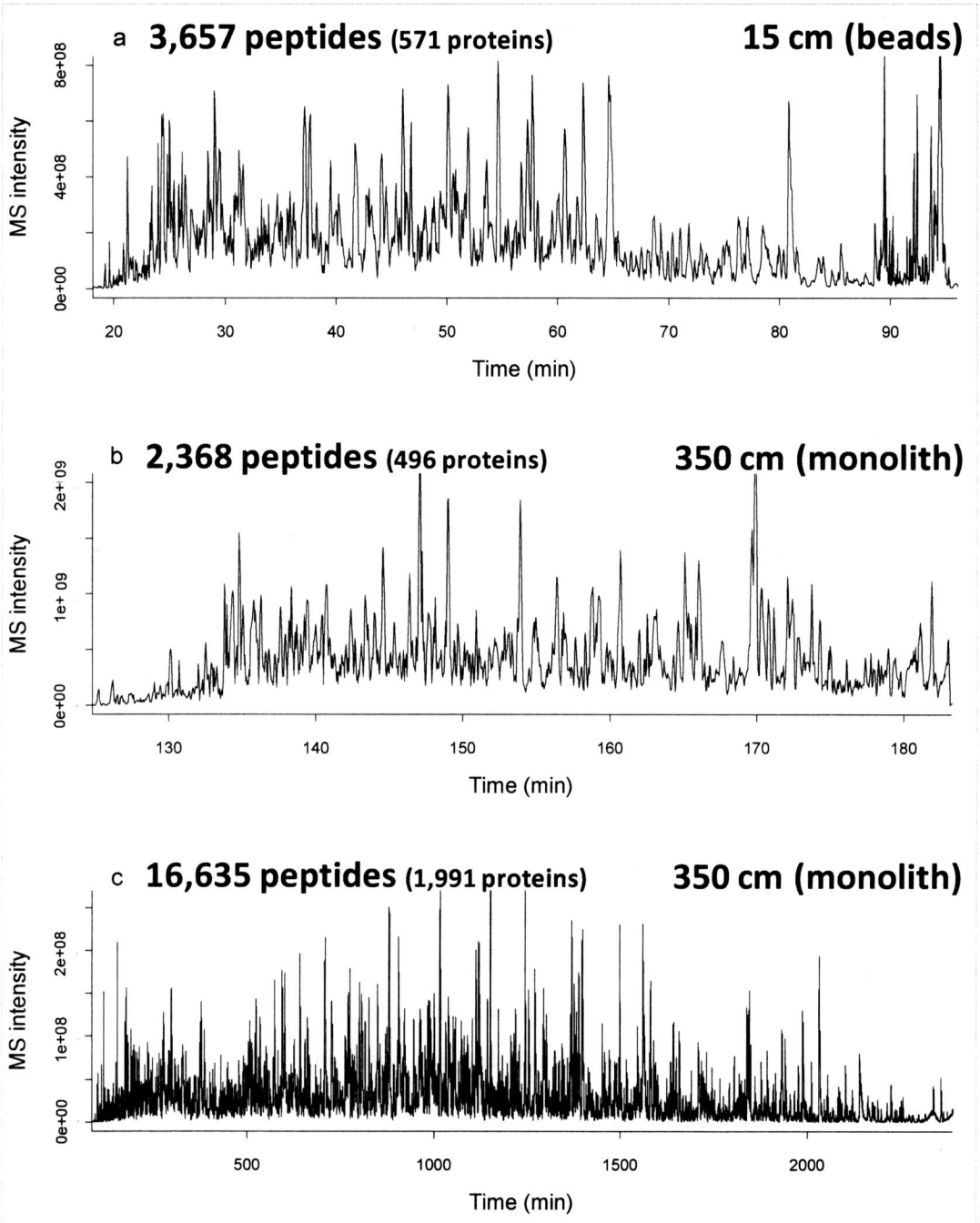

Figure 16 Total ion current of analyses of particle-packed column and monolith column.

The total ion currents of the analyses with particle-packed column (a: 70 min gradient) and monolith column (b: 70 min gradient, c: 2,470 min gradient) using 4 μg injection amounts are shown. The number of identified proteins and peptides are also shown.

3.3.4. *E. coli* membrane proteome analysis with monolith silica column.

To explore *E. coli* membrane proteome analysis, the results of 11 LC-MS/MS analyses of *E. coli* whole cell lysate using the monolith column were merged. Table 6 shows the number of identified proteins, membrane proteins and correspondence number of expressed genes. As a result, I successfully identified 2,880 proteins and 931 membrane proteins. The numbers exceeded the number of correspondence expressed genes. This is because transcriptome and proteome are considered not to be perfect matched because of the difference in the biological and technical manner (i.e., variation in the translation speed from mRNAs to proteins, and the difference in the detector used in the transcriptome studies and proteomics studies). These numbers of identified proteins are the largest among published studies on the *E. coli* membrane proteome[13, 62]. Moreover, in our approach, the complete *E. coli* membrane proteome was achieved even not with the membrane-enriched fractions, although previous *E. coli* membrane proteome reports used membrane-enriched fractions toward the identification of membrane proteins. The complete expressed proteome was achieved by this system using monolith column.

Table 6 Profile of *E. coli* transcriptome and proteome[a]

Approach	Transcriptome	This System (11 times)
Total No. of Identified Proteins	2,543	2,880
No. of Membrane Proteins	919	931

[a] The *E. coli* whole cell lysates were analyzed 11 times by nanoLC-MS/MS using monolith column (LTQ-Orbitrap). The transcriptome data was from the reference 61.

3.4. CONCLUSIONS

I showed that the new approach by the analyses with the monolith column facilitated the identification efficiency and achieved complete identification of *E. coli* proteome and membrane proteome. The monolith column showed improved separation efficiency in comparison to the particle-packed column. This approach was more efficient to the pre-fractionation approaches in terms of the total injection amounts and total analytical time. Moreover, complete identification of membrane proteome was achieved even not with the *E. coli* membrane-enriched fractions. It is novel to use this approach for the comprehensive analysis of *E. coli* proteome, and achieve the complete identification of *E. coli* expressed proteome. Further application will be considered for the more complex organisms such as human and plants. It would be very promising to identify complete proteome in more complicated samples with remarkably short time LC –MS/MS analyses using this approach.

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膜タンパク質の網羅的解析のための分析法の開発
Development of analytical methods for large-scale profiling of membrane proteins

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