Protein functional analysis is a frontier in post-genome era

Protein function has become a major research target of biological sciences because extensive DNA sequencing has been accumulating vast amounts of information on genes/proteins without any annotations in databases. Although the genomic sequencing of dozens of species have been, or are about to be, completed, the function of a fourth to half the open reading frames (ORFs) in a whole genome cannot be elucidated by primary as well as tertiary structural similarity to the known proteins. For example, forty percent of the ORFs of free living nematode Caenorhabditis elegans (C. elegans) have been functionally unknown by experiments and homology searches to other known proteins [1] although C. elegans is one of the most genetically-studied organisms. Moreover, a considerable number of essential genes for autonomous cellular lives are still unknown. The essential genes in ‘minimal genome’ were estimated from global transposon mutagenesis of the known simplest creature, Mycoplasma genitalium (M. genitalium) living with less than 500 genes [2]. The analysis revealed more than 100 genes are functionally unknown among about 300 essential genes for living in a laboratory condition. Comparative genomic analysis between M. genitalium and Haemophilis influenzae (H. influenzae) also gave a similar result [3].

To understand protein function, protein chemical analysis or its descendant ‘proteomics’, together with genomic and genetic researches, must be used because of no strict linear relationship between genome and the protein complement or ‘proteome’. Here the author describes his and other researchers’ efforts applied to three important classes of protein functional analysis from methodological viewpoint: biomolecular interaction analysis, post-translational modification analysis and proteomic profiling.

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near future) knowledge about genome.

Protein chemistry, however, has some methodological limitations such as sensitivity and throughput to gain a comprehensive understanding of complex biological processes. Hence, many efforts have been focused to improve the ability of protein chemical analysis. Here the author describes his and other researchers’ efforts applied to three important themes of protein functional analysis from methodological viewpoint: biomolecular interaction analysis, PTM analysis and proteomic profiling.

**Biomolecular interaction analysis**

Almost all protein functions are believed to be based on interaction with other biomolecule(s); e.g., antigen-antibody, extracellular ligand-cell surface receptor, transcriptional factor-DNA molecule. Detecting interactions between interested unknown protein molecule(s) and well-known protein(s) may allow us to map the unknown in the known functional network and to understand its function.

To discover interactions between unknown and known proteins and novel interactions between known proteins, genetic and biochemical methods are commonly used. The most popular method in genetics is yeast two-hybrid system (Y2H). Recently genome/proteome-wide molecular match making by Y2H has been reported for mono-cellular organisms [4, 5]. Although Y2H can essentially detect binary pair-wise interaction, genome scale screening and subsequent network construction *in silico* have identified a single genome-wide network and many clusters in which each component belongs the same or similar functional categories.

On the other hand, the most popular method in biochemistry is affinity capture methods. In the methods, targeted complexes are purified by affinity capture to a component of the complex using affinity chromatography or immuno-precipitation. Each component in the complex is then isolated using SDS polyacrylamide gel electrophoresis (SDS-PAGE) or two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) followed by in-gel proteolytic digestion for subsequent identification using Edman degradation, peptide mass fingerprinting or MS/MS [6, 7]. Because this approach can choose the experimental conditions such as pH, salt concentration and/or presence or absence of specific stimuli, the detected complex could reflect *in vivo* status.

To improve the sensitivity and the throughput of biochemical approach, we have developed a novel platform using an integration of microfluidics-based biosensor equipped with surface plasmon resonance (SPR) detector [8] and capillary high performance liquid chromatography (HPLC) - tandem mass spectrometry (MS/MS). In the system, molecules of the interest are immobilized on sensing flow cells formed on the sensor chip surface; then biological mixtures are passed over the sensor chip through the microfluidics system and specific binding activity is monitored in real time. The microfluidics as a nano-scale affinity chromatography in planer format is operated automatically by micro pumps and an autosampler; therefore, high-throughput screening can be performed, with high reproducibility. Then the bound proteins are enzymatically digested on the sensor chip by delivering proteolytic enzyme to the sensing flow cell, through the microfluidics system. After the ‘on-chip digestion’ to minimize the risk of sample loss, the resulting peptide mixture is trapped in the RP capillary precolumn by the ‘on-line recovery’ technique for subsequent HPLC-MS/MS analysis [9] (Figure 1).

![Figure 1. General scheme of the on-line BIA - MS/MS system.](image)

To study the feasibility of our system, histidine-tagged recombinant protein and 1, 4, 5-inositol trisphosphate binding protein present in the total cell lysates were isolated on the sensor chip and sequenced by HPLC-MS/MS analysis. Here the analysis of histidine-tagged xFKBP is illustrated.

We cultured *E. coli* cells transformed with pRSET-xFKBP in a small volume (1 mL), without a general induction procedure. To
detect recombinant protein produced at the basal expression level, 5 µL of *E. coli* cell lysate was injected over the Ni\(^{2+}\) chelated sensor chip NTA. The difference in response unit after the injection was 1 kRU in each flow cell, corresponding to ~160 fmol as based on the molecular weight of xFKBP (12 kDa). Additionally, the binding was not observed in blank surface and NTA surface without Ni\(^{2+}\), indicating the specific interaction between hexahistidine and Ni\(^{2+}\) chelated NTA. The bound protein was then subjected to on-chip digestion, on-line recovery and HPLC-MS/MS sequencing as described [9]. The HPLC-ESI/MS/MS profile showed three intense ions at m/z = 844.3, 918.0, and 1344.6, corresponding to peptides eluted from the RP capillary column. The peptide ion at m/z = 844.3 was fragmented in the ion trap cell and sequenced by MS/MS analysis. As shown in Figure 2, nine y and seven b series of fragment ions showed the peptide sequence G\(\text{QTVVVHYVGSL-ENGK}\) of xFKBP. Two other doubly charged ions (m/z = 918.0 and 1344.6) were also fragmented and identified as peptides of G\(\text{QQVETITTEDGRTFK}\) and D\(\text{RWIRPRDLQLVPWNSTM-DYK}\) of the same protein, respectively. Each fragmented ion spectrum of the peptide qualified solely for identification of the original protein. The sequence coverage of the observed peptides in the original protein was 33% (54/164 amino acids). In addition to these peptide ions, one relatively weak ion at molecular weight 8606, which did not match any of the completely and incompletely digested peptide of xFKBP, was observed (data not shown). This ion signal is presumably due to the digested fragment of endogenous *E. coli* protein. No other peptide was eluted from the capillary column. These results clearly demonstrate that xFKBP present in cell lysate was purified on the Ni\(^{2+}\)/NTA sensor surface via the histidine tag and that the purified protein was effectively digested on the sensor chip. The resulting on-chip digested peptides were recovered from the sensor chip, using the on-line recovery system, and unambiguous amino acid sequences were obtained.

The system buffer used in this study (running buffer, NiCl\(_2\) solution, regeneration solution, and digestion buffer) contained detergent, n-octylglucopyranoside, instead of surfactant P 20, which is a standard component of well-established conditions for BIA analysis. When the surfactant P 20 was present in the buffer system, the ions derived from digested peptides were hardly detectable because multiple intense ions derived from the surfactant were present. However, no peptide can be recovered in the absence of detergent (data not shown). Hence, a detergent that is compatible for use in HPLC-MS analysis seems necessary to inhibit peptide adsorption to microfluidics channels and to increase recovery rates. For this purpose, we found n-octylglucopyranoside although the optimal detergent is currently under investigation.

Instead of the on-line system described here, the protein elution from the sensor chip and subsequent conventional in-tube digestion was reported [10]. Using this off-line elution method, we could not recover an adequate amount of peptides for MS/MS analysis and MALDI/TOF MS from the sensor chip on which the same amount of xFKBP was captured (data not shown). These results show that unambiguous sequence information of proteins captured on the sensor chip at the mid- to low-femtomole level was obtained even with a single run of the BIA-MS/MS analysis. Since MS/MS analysis can identify proteins on the basis of single-peptide fragmentation spectra, the system should allow for analysis of not only simple one-to-one protein-protein interactions, but protein complexes and molecular components can also be identified. The system should prove to be a general and highly versatile system for discovering novel interactions or novel binding partners from a limited quantity of biological fluid.

These results should aid further reconstruction of a complex, an organelle, and eventually a cell from its components *in vitro* or *in silico* [11]. Because our SPR-based microfluidics can provide quantitative information on the interaction, such as kinetic parameters and equilibrium constant for complex formation in various conditions and MS/MS can provide information on identity of molecules as well as PTM that regulate the interaction dynamically, the system can be valuable as a platform for the reconstruction.

![Figure 2. MS/MS spectrum of on-chip digested bound protein.](image)
Post-translational modification analysis using HPLC-MS

Because of spatial and/or temporal regulation of PTM as well as splicing, proteins often have heterogeneity derived from partial modification(s). Hence, for the modification analysis, the means with high resolution and high sensitivity like (multi-dimensional) capillary HPLC-MS/MS can be valuable. In the hyphenated system, capillary HPLC can separate modified peptides from complicated proteolytic peptide mixture and MS can detect virtually all modification that results in mass change. The modification sites in a protein can also be determined using MS/MS [12]. Indeed many modifications have been detected and identified using capillary HPLC-MS/MS system.

A challenge in PTM analysis, in spite of development of capillary HPLC-MS, is proving one-to-one correspondence between the function and particular PTM(s) because biological roles of only a few PTMs are well understood among more than two hundred PTMs reported. To understand the function of these modifications there are no automated and routine methods. We have to analyze each modification in detail by various ways before large scale and highly automated analyses. Two examples of the analyses are described below.

The first is processing of a plant protease that regulates both cellular translocation and enzymatic activity. In a plant seed, some protein is accumulated in protein storage vacuoles. The storage protein is rapidly degraded to supply amino acid as nutrients for development when the seed germinates. The mechanism of the rapid protein degradation is not well understood although a number of the enzymes responsible for the degradation have been identified. One of such protease is a cysteine protease, designated SH-EP, with KDEL sequence known as endoplasmic reticulum (ER) retention signal at its C-terminal [13]. SH-EP is firstly expressed in ER in plant seed. Amino acid sequencing using Edman chemistry and HPLC-MS of the matured form together with genetic analyses revealed maturation process of the enzyme. The results show during the seed germination SH-EP is post-translationally processed at its C-terminal residues including ER retention signal (KDEL) and is transported to the protein storage vacuoles as proform. The enzyme is further processed at the N-terminal half to be resulting in the active form and degrades storage proteins [14]. This is the first example to modify genetically coded ER retention signal by processing. Recently, additional evidences revealed that some post-translational processing in plant alter predicted location and function of the proteins. Therefore, the mechanism presented here seems to be common in at least kingdom plantae.

The second example is a novel modification of a cysteine residue, cysteine sulfenic acid (Cys-SO$_2$H), which was determined to be responsible for the activity of a metalloenzyme, nitrile hydratase from Rhodococcus sp. N-771 (NHase) [15]. NHase is an αβ heterodimer enzyme with a non-heme ferric iron in the catalytic center. In the catalytic center, three cysteine sulfur atoms from αCys$_{109}$, αCys$_{112}$ and αCys$_{114}$ are coordinated to the ferric iron. αCys$_{112}$ and αCys$_{114}$ are modified to cysteine sulfenic acid (Cys-SO$_2$H) and cysteine sulfenic acid (Cys-SOH), respectively [15]. To date, these modifications have not been reported in any other metalloenzyme. How these modifications play a role in the catalytic reaction is still unclear.

To understand the function and the biogenic mechanism of these modified residues, we reconstituted the nitrile hydratase from recombinant unmodified subunits and analyzed by HPLC-MS/MS [16]. The αβ complex reconstituted under argon showed no activity. However, it gradually gained the enzymatic activity through aerobic incubation. HPLC-ESI/MS and MS/MS analyses confirmed that the anaerobically reconstituted αβ complex did not have the modification of αCys$_{112}$-SO$_2$H and aerobic incubation in-
duced the modification. The activity of the reconstituted αβ complex correlated with the amount of αCys112-SOH (Figure 3). Furthermore, HPLC-ESI/MS analysis of the tryptic digest of the reconstituted complex, freed of ferric iron at low pH and carboxymethylated without reduction, suggested that αCys112 is modified to Cys-SO\(_2\)H and Cys-SOH, respectively, and the modified residues are responsible for the catalytic activity solely or in combination. The importance of the modifications are also confirmed through the analysis of metal substituted NHase [17].

**Proteomic profiling**

The concept of 'profiling' is based on the fact that all genes are not expressed evenly in a prokaryotic cell at all times and the hypothesis that molecules that belong to the same machinery or functional pathway are likely to be co-expressed. The expression level of each protein is strictly regulated spatially and/or temporally at transcription, post-transcription, translation and post-translation stages. The regulation at transcription is well-studied processes. The magnitude of the regulation at post-transcription and translation is suggested by the poor correlation between mRNA and protein levels, generally lower than 0.5. The regulation mechanism at post-translation is still unclear. Therefore, to understand the biological regulation comprehensively, direct proteomic profiling is important as well as transcriptomic profiling.

To profile a proteome consisted with thousands or ten thousands of proteins (or up to one million of their proteolytic peptides), multi-dimensional separation techniques are useful. Combination of \( m \) techniques (\( m \)-dimensional separation) can separate \( N \times N \times N \times N \cdots = N^m \) species if each technique resolves \( N \), \((k = 1, 2, 3, \ldots, m)\) species at a time and if resolving parameter is independent of each other. State-of-the-art techniques in protein and/or peptide separation such as HPLC and SDS polyacrylamide gel electrophoresis can separate only several hundred species or less at a time. Whereas 2D-PAGE, the most popular technique in proteomic profiling, can separate around 10,000 species at a time with two-dimensional separation such as HPLC and SDS polyacrylamide gel electrophoresis, isoelectric point by isoelectric focusing, can separate around 10,000 species at a time with two dimensions. An alternative example is 2D-HPLC system [18, 19] that can separate thousands peptides or hundreds protein at a time.

One of the most typical examples is proteomic profiling of cerebellar development by 2D-PAGE-MS [20, 21]. Morphogenesis of the murine cerebellar cortex including the neurogenesis and synaptogenesis occurs during the early postnatal development, mostly within only three weeks after birth. To understand molecular mechanisms underlying the developmental regulation of this dynamic cellular processes, a series of proteome, expressed transiently during the morphogenesis, was analyzed using 2D-PAGE-MS system.

Crude cerebellar extracts prepared from various postnatal-day mice and rats (day 0 to 30) were separated in parallel by 2D-PAGE. By the comparison of a series of mouse cerebellar proteomes, a proteome subset (42 protein spots), expressed transiently during the early developmental stage, was detected even on CBB-stained gels. Because these results were in accord with rat cerebellar proteomic profiling, these proteins detected by the analyses seemed to be commonly important for murine cerebellar development.

The detected protein spots were then analyzed by MALDI-TOF MS and/or HPLC-MS/MS. Two interfacing techniques between PAGE and MS, i.e. liquid phase digestion of extracted protein from gel and in-gel proteolytic digestion, were used. Database searching of these spots allowed us to identify 29 proteins (out of 40 spots), which include, in addition to proteins of unknown function, many proteins known to have roles in the development of the central nervous system. These results suggest that the proteomic approach is valuable for screening of proteins involved in cerebellar morphogenesis. These results also support previous morphological observations that cerebellar morphogenesis is one of the most dynamic processes in life because in many other cases no significant changes of CBB-stained spots by various stimuli to cells and/or individuals have been detected.

Profiling using 2D-PAGE-MS has some shortcomings: (1) generality; hydrophobic and high molecular weight proteins are hardly analyzed, (2) narrow dynamic range; low abundant proteins expected to be functionally unknown cannot be analyzed even from silver stained gels of total cell lysate, (3) labor intensive and low throughput. These shortcomings remained in spite of tireless efforts of many researchers over tens of years. Thus, many researchers came to recognize two-dimensional electrophoresis was inappropriate for proteomics in the future [22].

They are searching and developing novel strategy for proteomic profiling. A candidate is direct analysis of proteolytic peptide mixture generated from biomolecular complex, organelle or whole cell using multi-dimensional HPLC-MS/MS and database searching. In the case of yeast, 1,484 proteins out of about 6,000 genes have been identified by a series of such analysis [23], while the largest 2 D-PAGE-based proteomic study to date identified 502
unique proteins of *H. influenzae* [24]. Moreover, the direct analysis based on HPLC-MS/MS and database searching can detect probable minor proteins (CAI value below 0.2) that were not detected on 2D gel. The dynamic range of the direct analysis is at least over $10^8$ because we have detected proteins in the range between 700 and 100,000 copies per *E. coli* cell using simple HPLC-MS/MS analysis (Nakayama, H. et al. unpublished data).

### Perspective

For years to come, mass spectrometer will remain the most sensitive and informative detector for protein analysis. At present most advanced mass spectrometers have potential to detect and analyze low zeptomole-quantity pure proteins [25]. On the other hand, the detection limit of ‘real’ biological samples is still mid femtomole to picomole level. This huge difference between ideal and real sample (1,000,000-100,000,000 folds) is mainly due to sample preparation and ionization process before MS. Because the sensitivity of MS depends on sample concentration, how to concentrate samples to small volume [26] and to introduce concentrated pure samples to MS is essential for the ultra-sensitive analysis.

Thus, miniaturization and integration of the analytical system before MS is one of the essential issues in proteomics. The simplest and most successful system of this concept is (multi-dimensional) capillary HPLC-MS/MS system. The more miniaturized and integrated system must be used for the analysis of less abundant biological samples. In this context, microfluidics will play an important role. Using microfluidics formed on a glass, silicon or polymer chip, small volume of samples can deliver, mix, react, separate and detect like usual ‘large-scale’ experiments in a laboratory. Hence, microfluidics is often called ‘lab-on-a-chip’ [27]. As described above, we have developed a prototypes of the integration system between lab-on-a-chip and MS.

Another important issue is the prevention of non-specific adsorption to surfaces used such as vials, test tubes, beads and flow-lines because miniaturizing devices increases the relative surface area to contact. To prevent non-specific adsorption, three classes of additives are commonly used: (1) hydrophilic or basic oligomers, (2) hydrophilic polymers, (3) ionic or nonionic detergents [28]. Most of these additives have insufficient ability for the prevention of non-specific adsorption, without affecting specific interaction or protein function, or inhibiting MS analysis, although numerous studies have been done. Hence, additives for proteomics have to be established. Recently we have found a series of HPLC-MS compatible detergents that prevent hydrophobic adsorption to microfluidic line (elastic silicone) and polypropylene tube (H. Nakayama et al., unpublished result).

A possible alternative to control adsorption of proteins to surfaces is using self-assembled monolayers (SAM). SAM is known in interfacial sciences as relatively easy way to form well-controlled surfaces. Many model experiments revealed the SAM having hydrophilic surface effectively prevented non-specific adsorption to the surfaces [29, 30]. In such model experiments, SAM was formed on the planer glass surfaces with gold thin layer. To form SAM on any shapes and materials may expand the usefulness in proteomics.

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### References


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