MULTIPLEXED FRAGMENTATION AND PROTEIN INTERACTION REPORTER TECHNOLOGY
APPLICATION TO HUMAN CELLS

By
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Abstract

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Chemical cross-linking combined with mass spectrometry is a feasible approach to study the protein-protein interactions. However, system complexity and cross-linking product heterogeneity have precluded widespread chemical cross-linking use for large-scale identification of protein-protein interactions. Mass spectrometry identifiable cross-linkers protein interaction reporters (PIRs) were developed to overcome these problems. PIR couple with mass spectrometry was applied to study the protein-protein interactions of prokaryotic cells, such as Shewanella oneidensis MR-1. However, the biological features of eukaryotic cells are different with prokaryotic cells. In this report, PIR coupled with mass spectrometry was applied to human cancer cells, such as MCF7 and HeLa cells. 109 proteins in MCF7 and 127 proteins in HeLa were labeled with PIRs and identified.

The bottom-up LC/MS method involves the protein denaturation, protease digestion and LC/MS/MS to identify proteins and peptides. The most common data acquisition method for LC/MS/MS, can allow the identification of relatively abundant proteins. In biological systems, functional proteins, such as enzymes and membrane proteins are of relatively low abundance,
thus data-dependent acquisition is generally insufficient to identify many functional proteins. Multiplexed fragmentation was developed. Multiplexed fragmentation with PIR strategy is a potential approach to study protein-protein interactions. However, ambiguous identification of PIR labeled peptides is the major challenge. To overcome this problem, this report demonstrated the feasibility of LC/3-stage multiplexed fragmentation approach to study protein-protein interactions with PIRs.
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CHAPTER ONE
INTRODUCTION

Protein-Protein Interaction

Proteins can be grouped into two groups: structural and functional proteins. Structural proteins provide the structural base to cells and organisms. For instance, proteins in cytoskeletons are used to maintain cell shape. Functional proteins are related to specific cellular functions, such as enzyme catalysis, cell signaling and membrane transportation. Most of the functional proteins cannot work alone. They usually form protein complexes or interact with other proteins to conduct particular functions.

The interaction between proteins is important for many cellular functions. Protein modifications are strongly involved with protein-protein interactions. The representative examples are the interactions of protein kinases or protein phosphatases with their target proteins. When these enzymes modify their substrate proteins, interactions between enzymes and substrates are triggered [1]. Protein-protein interactions are also related with many diseases such as cancers [2, 3] and Alzheimer's disease [4, 5]. Therefore, information about protein-protein interactions better explains their biological functions and help us understand the diseases process.

Methods of Protein-Protein Interaction

There are various approaches to investigate protein-protein interactions. These techniques are divided into three categories. Each category has own strengths and weaknesses. This section discusses both aspect of each approach.

Category 1 includes the techniques requiring some manipulation at the genetic level, such
as yeast two-hybrid [6], split ubiquitin [7], in-vivo fluorescence resonance energy transfer (FRET) [8], tandem affinity purification (TAP) tag [9]. These techniques use fusion proteins, which are produced by the fusion of the foreign piece to the protein of interest. The fusion protein is used as the bait. When the protein is bound and/or interacts with the bait, the detectable signal is produced and the certain protein-protein interaction is recognized. The strengths of this category are ease of scaling up, good sensitivity, high throughput, and semi-automated experimental procedures. These methods are necessary to generate many yeast strains with genetic pieces coding for the desired attachment combined to every single open reading frame (ORF), require many pair wise screening tests to discover interactions, and to isolate and sequence DNA for identifying interacting partners. In addition, these approaches are inclined to produce the false positive and false negative results due to the fusion of foreign pieces to protein of interest. The fusion of foreign pieces to proteins of interest can change the protein tertiary structure. The structural change can make different proteins bind to the fused proteins. When the foreign protein are large enough to cover regions of the protein where a protein-protein interaction takes place, the protein-protein interaction can neither occur nor be detected. Moreover, the protein-protein interaction cannot be detected if the interaction is related to or requires the presence of post-translational modifications (PTMs) and/or enzymes, each of which does not originally exists in yeast cells.

Category 2 contains the approaches that do not require genetic manipulation. Protein-protein interactions are detected at the protein level without any changes that are introduced on the genetic level. The representative examples are protein chip [10, 11], co-immunoprecipitation (Co-IP), and Pull-down assays [12]. These approaches are based on antibody-antigen interactions. The most popular protein chip is the antibody array. Antibodies are spotted on the protein chip
and are used as bait to detect proteins from cell lysate. In CO-IP, an antibody is used to isolate the protein of interest bound with interacting partners. These techniques are high throughput and allow us to detect interactions related with PTMs. The proteins are dissolved in a certain buffer to performing the experiments. In this case, the folding of proteins can be changed. These structural changes of the proteins can produce false positive and false negative results. Also, common buffer solutions contain the detergent. The detergents in the buffer solution can affect the protein-protein interactions. Especially, the association of the detergents with hydrophobic proteins can prevent the interaction being present in vitro. The major disadvantage is the approaches require certain antibodies. If the protein of interest does not interact with any antibody, the protein-protein interaction can not be detected.

Category 3 includes chemical cross-linking coupled with mass spectrometry [13-17]. This approach is based on the close proximity of the binding partners. The proteins are labeled with the chemical cross-linker in vivo. The labeled proteins are purified, digested and identified by the mass spectrometry. The potential advantages of this method are high throughput and large-scale discovery. They can also allow discovery of protein-protein interactions without any biological hypothesis and thus, presents an unbiased approach. The major challenge is the system complexity. Various types of the cross-linked products can be produced in the cross-linking reactions, such as, dead-end, intra-, and inter-cross-linked peptides, and unlabeled peptides. The inter-cross-linked products are relatively small amount in the reaction mixture. Therefore, the detection and identification of the inter-cross-linked products are the great challenges [16-18]. The novel types of strategies [19, 20] and cross-linkers have been developed to overcome these limitations, such as isotopic labeled cross-linkers [21-23], cross-linkers contained affinity tags[17, 18, 24, 25] and mass spectrometry cleavable (MS cleavable) bonds[16-18, 26, 27].
**Protein Interaction Reporter (PIR)**

Protein Interaction Reporter (PIR) [14, 18] is a type of chemical cross-linker that contains an affinity tag and MS cleavable bonds. The low energy collisions with tandem mass spectrometry can break the MS labile bond in the cross-linkers. Then, the cross-linked products release the reporter ions and the modified peptides without disrupting peptide backbones. Figure 1-1 shows the connectional molecular design of PIRs.

Different types of the cross-linked products can generate the different fragmentation patterns. The mathematical relationships between masses of fragments are illustrated in Figure 1-2. The relationships are used to obtain the unambiguous identification of the cross-linked products [18]. Practically, the masses of the cross-linked products ($m_c$), are obtained during MS scan. On the contrary, masses of the reporter ions ($m_r$) and modified peptides ($m_{p1}$, $m_{p2}$) are measured during low energy MS/MS scan.

**PIR coupled with Mass Spectrometry to Study Protein-Protein Interactions**

The feasibility of PIR coupled with mass spectrometry to study protein structures and protein-protein interactions is established using the standard protein, *Shewanella oneidensis MR-1*, and Gram-negative anaerobic bacteria [14, 16-18]. But there have been few studies of the use of PIR in higher organisms like *Homo sapiens*. Human cells and bacterial cells are clearly different. The former is a large eukaryotic cell and the latter is a small prokaryotic cell shown in Table 1-1 [28]. The cell size of each cell is shown in Figure 1-3. The size of typical human cells is approximately 10µm, but the size of major bacterial cells is 1 to 5 µm. For example, *Escherichia coli* is approximately 2µm.
Figure 1-1. Conceptual modular design of Protein interaction reporter (PIR).

Figure 1-2. Specific fragmentation patterns of PIR labeled peptides helps distinguish dead-end, intra-, and inter-cross-linked peptides. The neutral mass of the precursor ion equals the sum of the neutral masses of its product ions [18].
Figure 1-3. Size range of Homo sapiens, bacteria and viruses cells. The diamond (♦) represents the size of typical Homo sapiens cell.

Table 1-1. Comparison of Prokaryotic and Eukaryotic Organisms [28].

<table>
<thead>
<tr>
<th></th>
<th>Prokaryotes</th>
<th>Eukaryotes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Organisms</td>
<td>bacteria and cyanobacteria</td>
<td>protists, fungi, plants and animal</td>
</tr>
<tr>
<td>Cell size</td>
<td>generally 1 to 10 µm in linear</td>
<td>generally 5 to 100 µm in linear dimension</td>
</tr>
<tr>
<td></td>
<td>dimension</td>
<td></td>
</tr>
<tr>
<td>Organelles</td>
<td>few or none</td>
<td>nucleus, mitochondria, chloroplasts, endoplasmic reticulum, etc.</td>
</tr>
<tr>
<td>DNA</td>
<td>circular DNA in cytoplasm</td>
<td>very long linear DNA molecules containing many noncoding regions; bounded by nuclear envelope</td>
</tr>
<tr>
<td>RNA</td>
<td>RAN and protein synthesized in</td>
<td>RAN synthesized and processed in nucleus; proteins synthesized in cytoplasm</td>
</tr>
<tr>
<td></td>
<td>same compartment</td>
<td></td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>no cytoskeleton; cytoplasmic</td>
<td>cytoskeleton composed of protein filaments; cytoplasmic streaming; endocytosis and exocytosis</td>
</tr>
<tr>
<td></td>
<td>streaming, endocytosis and exocytosis all absent</td>
<td></td>
</tr>
<tr>
<td>Cell division</td>
<td>chromosomes pulled apart by</td>
<td>chromosomes pulled apart by cytoskeletal spindle apparatus</td>
</tr>
<tr>
<td></td>
<td>attachments to plasma membrane</td>
<td></td>
</tr>
<tr>
<td>Cellular organization</td>
<td>mainly unicellular</td>
<td>mainly multicellular, with differentiation of many cell types</td>
</tr>
</tbody>
</table>
Liquid Chromatography/Tandem Mass Spectrometry (LS/MS/MS) for Proteome Research

LC/MS is a general method in the study of proteomics, such as the detection and identifications of proteins in complex mixtures like cell lysate. The bottom-up LC/MS method involves the protein denaturation, protease digestion and LC/MS/MS to identify proteins and peptides [29]. There are two necessary processes to accomplish LC/MS/MS experiments for complex protease digests. One is that mass spectra need to be analyzed in real time and the ions should be selected for collision-induced dissociation (CID). This LC/MS/MS approach is called data-dependent data acquisition [30, 31]. The advantages of this approach are high speed, high throughput and not requiring a highly accurate instrument. This method can provide information about relatively abundant proteins. In biological systems, functional proteins, such as enzymes and membrane proteins, are present at relatively low abundance. Thus, data-dependent acquisition method is generally insufficient to identify protein-protein interactions of many functional proteins. Novel MS/MS acquisition methods, such as multiplexed fragmentation method [32-34] and MS\textsuperscript{E} have been developed to overcome this problem [35, 36]. These methods are achieved by the parallel alternating scans which are acquired at either low or high collision energy.

Purposes of This Study

PIR coupled mass spectrometry is applicable to study protein-protein interactions for bacteria, such as *Shewanella oneidensis MR-1* and Gram-negative anaerobic bacteria [16-18]. In this report, PIR coupled with mass spectrometry was applied to proteome study of human cell lines to verify the feasibility of this method for studying both eukaryotic and prokaryotic cells.

As discussed in the previous section, the data-dependent LC/MS/MS technique has a
limitation. When PIR experiments are carried out, inter-cross-linked product are often of relatively low abundance. Multiplexed fragmentation approach was applied to identify low abundant species. The identification of modified peptide is performed by peptide mass fingerprinting (PMF) using mass of modified peptides obtained at high collision energy. The major challenge of this approach relates to unambiguous identification by peptide mass alone. In this report, 3-stage multiplexed fragmentation approach with PIR experiments was developed and demonstrated to help overcome this problem.
CHAPTER TWO
EXPERIMENTAL SECTION

Chemicals and Reagents

All chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA) unless otherwise noted. Fluorenylmethyloxycarbonyl-RINK (Fmoc-RINK), 4-hydroxymethyl-3-methoxyphenoxybutyric acid MBHA (HMPB-MBHA) resins, Fmoc-Arg(Pbf)-NovaSyn TGT resin, and Fmoc-protected amino acids were purchased from Novabiochem (San Diego, CA, USA). Sequencing grade modified trypsin was purchased from Promega (Madison, WI). Water used for preparing solutions and solvents was 18-MΩ deionized water produced with a Barnstead Nanopure Water System.

Synthesis of Cross-linkers

PIRs were synthesized using a 431A Peptide Synthesizer (Applied Biosystem, Foster City, CA USA). First, Fmoc-Lys(biotinyl-e-aminocaproyl)-OH was coupled to 4-hydroxymethyl-3-methoxyphenoxybutyric acid MBHA resin (HMPB-MBHA resin) using the standard symmetric anhydride method. Then, Fmoc-Lys(Fmoc)-OH was coupled to lysine and formed a branch point for PIRs. Two Fmoc-Pro-OH and Fmoc-Asp-OH were sequentially coupled to lysine to synthesize the first PIR (PIR I). Carboxyl groups were then introduced by reacting the primary amines of proline with succinyl anhydride. Subsequently, the two carboxyl groups were activated by forming the esters with N-hydroxysuccinimide (NHS). To synthesize the second PIR (PIR II), Two RINK groups were coupled to Lys by treating the Fmoc-RINK linkers. Then, succinic anhydride and NHS were coupled as described previously.
The crude product was cleaved from the superacid sensitive resin with 0.5% TFA in chloroform [37]. The chloroform and TFA pyridine salts were removed under a vacuum. The synthesized PIRs were purified using reversed-phase HPLC and the final product had a purity of approximately 90%.

2-1. Protein Interaction Reporter Technology Application to Human Cells

Cell Culturing, Harvesting and Labeling

HeLa and MCF7 cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Eagle’s minimum essential medium (EMEM), newborn Bovine Calf Serum (NBCS) and trypsin-EDTA were purchased from HyClone (Logan, UT, USA). Fetal bovine serum (FBS) and Penicillin-Streptomycin were purchased from GIBCO Invitrogen (Carlsbad, CA, USA).

HeLa cells were cultured in EMEM supplemented with 5% FBS, 5% NBCS and 1% Penicillin-Streptomycin. MCF7 cells were cultured in EMEM supplemented with 10% FBS, 1% Penicillin-Streptomycin and 0.1% bovine insulin. The cells were grown in a humidified chamber maintained at 37°C and 5% carbon dioxide (CO₂). After reaching 80% confluence, the culture medium was removed, and the cells were washed with phosphate buffered saline (PBS) at pH 7.4. Then, trypsin-EDTA was added for approximately 3 minutes to detach the cells. The culture medium was added to quench trypsin-EDTA digestion. Detached cells were washed 3 times with PBS. To perform the cell labeling, the cells were reacted with 1mM PIR II in PBS for 1 hour at 4°C. PIR labeled cells were washed 3 times with PBS at room temperature (RT) to remove unreacted PIRs.
Microscopy Sample Preparations

MCF7 cells were grown onto the sterilized microscopic cover glass at 37°C and 5% CO₂ until 80% confluence and washed with PBS by gently shaking. The cells were reacted with 1mM PIR II in PBS for 1 hour at 4°C. PIR labeled cells were treated with 3.7% formaldehyde in PBS for 30 minutes at RT and washed 3 times with PBS. Then, the cells were permeabilized by incubation with 1mL of 0.2% triton X-100 for 5 minutes at RT and washed 3 times with PBS. To reduce non-specific binding, the cells were blocked with 6% solution of Bovine Serum Albumin (BSA) in PBS for 30 minutes at RT and washed 3 times with PBS. The cells were incubated with 1:50 dilution of anti-biotin antibody (Sigma C7653) in 1% solution of BSA in PBS (BSA-PBS) for 30 minutes. Next, the cells were washed 3 times with BSA-PBS and incubated with 10ug/ml solution of Alexa Fluor 488 rabbit anti-mouse IgG (moluculare probes, A-11054) enough to cover all cells for 30 minutes at RT. The cells were washed 3 times with 1ml of BSA-PBS and incubated with 10ug/ml Alexa Fluor 488 goat anti-rabbit IgG enough to cover all cells for 30 minutes at RT. The cells were washed 3 times with 1mL of BSA-PBS. The cells are treated with propidium iodide to visualize the nuclei to prepare microscope slide. All images were acquired using the Zeiss LBS 510 Laser Confocal Microscope at the WSU Microscopy Center.

Sample Preparation for Identification of PIR Labeled Proteins

The unlabeled and labeled cells were lysed in Radio-Immunoprecipitation Assay (RIPA) buffer by a vortex mixer. The cell lysates were centrifuged at 1800 rpm for 45 minutes at 4°C to remove debris, which are broken cell membranes. Bradford assay was performed to determine protein concentrations.

The supernatants of cell lysates were divided into two portions. One portion was used for
SDS-PAGE and anti-biotin western blot analysis. The other was used to identify PIR labeled proteins. Monomeric avidin beads (Pierce Rockford, IL, USA) were added into the cell lysate and incubated for 12 hours 4°C to enrich the PIR labeled proteins. The beads were washed 3 times with PBS for reducing non-specific binding. The proteins bound on the beads were eluted by boiling with Laemmili sample buffer (Bio-Rad Laboratories Inc. Hercules, CA, USA) and separated by SDS-PAGE. The gels obtained from SDS-PAGE was used to identify the PIR labeled proteins.

**SDS-PAGE and Anti-Biotin Western Blot Analysis**

Cell lysate was mixed with 1:1 ratio with Laemmli sample buffer that was prepared according to manufacture’s instructions. The mixture was heated at 95°C for 10 minutes to reduce disulfide bonds. Approximately 20ug of protein was as loaded onto SDS-PAGE for separation by the gel with 5% stacking buffer and 8% resolving buffer. SDS-PAGE was performed under constant voltage condition at 180 V. The gels were washed 3 times with deionized water (DI water) for 20minutes at RT to remove detergents in the running buffer. Then, the gel was stained with Coomassie Blue R250 (Bio-Rad, Hercules, CA, USA) for 1 hour at RT. The gel was washed with DI water overnight. Finally, the gels were imaged with a Densitometer (Molecular Dynamics, Sunnyvale, CA, USA).

To acquire anti-biotin western blot images, the proteins separated by SPS-PAGE were transferred on the nitrocellulose membrane using transfer buffer by trans-blot SD semi-dry transfer cell (Bio-Rad laboratories Inc. Hercules, CA, USA). The blotting was performed for 2 hours at 15V. The membrane was air-dry overnight to fix the proteins. Then, the membrane was blocked in 15% (w/v) dry milk Tris Buffered Saline (TBS) buffer for 1 hour, incubated with
mouse anti-biotin antibody at 1:10000 dilution in 15% (w/v) dry milk TBS buffer, and washed 3 times with TBS buffer. The membrane was incubated with goat anti-mouse HRP conjugate antibody (Bio-Rad laboratory inc 170-5047) at 1:20000 dilution in 15% (w/v) dry milk TBS buffer for 1 hr, and washed 3 times with TBS buffer. The membrane was reacted with Chemoluminescent Peroxidies Substrate Kit (Sigma CPS-1-60) for approximately 1 minute to produce chemoluminescence. KODAK film for X-ray was exposed to the membrane and automatically processed. The exposed KODAK film was imaged with a Densitometer.

In-Gel Digestion

Gel bands of interest were excised and distained 50% methanol and 5% acetic acid overnight. The gel pieces were washed by acetonitrile (ACN), and completely dried in SpeedVac. The disulfide bonds of proteins in gel pieces were reduced by 10mM dithiothreitol (DTT) in 100mM ammonium bicarbonate (Ambic) for 30 minutes at RT. The reduced disulfide bonds were alkylated by 50mM iodoacetamide (IAA) in 100mM Ammonium bicarbonate for 30 minutes at RT in the dark. The gel was washed with100mM Ambic and ACN, and completely dried in SpeedVac. The gel pieces were hydrated by 20ng/µL trypsin in 50mM Ambic for 10 minutes on the ice. After the hydration of gel pieces, the excess of trypsin solution was removed. The gel was incubated at 37°C overnight. After in-gel digestion, the samples were subjected to nano LC/MS/MS.

Nano LC/MS/MS and Data Analysis for Protein Identification

Data-dependent nano LC/MS/MS of in-gel digest was performed using ion trap mass spectrometer (Esquire HCT, Bruker Daltonics, Billerica, MA, USA) equipped with a nano ESI
source and nano HPLC systems (Ultimate, Dionex, Sunnyvale, CA, USA). The peptides were injected by autosampler and trapped into the precolumn. The peptides were eluted from precolumn by washing with solution A (0.1% formic acid, 2% ACN in water) at a flow rate of 50μL/min for 3 minutes. These peptides were trapped into nano C18 reversed phase column (C18 PepMap, 75μm X 150mm, 3μm, 100Å, Dionex, Sunnyvale, CA, USA) Then, the peptides were eluted using the following gradient: 0% Solution B (0.1% formic acid, 98% ACN in water) for 0-3 minutes, 0-15% Solution B for 3-15 minutes, 15-25% Solution B for 15-60 minutes, 25-40% Solution B for 60-90 minutes, 95% Solution B for 90-105 minutes and 0% Solution B for 135 minutes.

Data analysis was performed using Bruker Daltonics DataAnalysis software (version 3.1, Billerica, MA, USA). Proteins were identified by searching against NCBI human protein database using Mascot44 (version 2.1.0, MatrixScience Ltd., London, UK) licensed in house. Database search parameters were used as previously reported [16, 17] Database search parameters were shown in Figure 2-1. The auto hits option was selected to allow reporting of all the protein hits with the probability-based mascot scores that exceeded their thresholds (p < 0.05), indicating significance above the 95% confidence level. The subcellular locations of identified proteins were obtained from DAVID Bioinformatics Resources of National Institute of Allergy and Infectious Diseases (NIAID, http://david.abcc.ncifcrf.gov/home.jsp).
Figure 2-1. MASCOT search parameters for protein identification. Database search parameters are briefly: search database, NCBI; taxonomy, *Homo sapiens*; enzyme, trypsin; allowed missed cleavages, up to 3; fixed modifications, carbamidomethyl (C); variable modifications, oxidation (M); peptide tolerance, 1.6 Da; and MS/MS tolerance, 0.8 Da.
2-2. Multiplexed Fragmentation

Synthesis of Model Peptides

Model peptides were prepared in-house based on F-moc solid phase peptide synthesis chemistry. Fmoc amino acids were sequentially coupled to Fmoc-Arg(Pbf)-NovaSyn TGT resin (Novabiochem, San Diego, CA, USA). The first model peptide (Peptide I) was synthesized by sequential coupling with Fmoc-Ala-OH, Fmoc-Gly-OH, Fmoc-Lys(Boc)-OH, Fmoc-Leu-OH, Fmoc-Phe-OH, Fmoc-Val-OH, Fmoc-Arg(Pbf)—OH, Fmoc-Ser-OH, and Fmoc-Val-OH. The sequence of Peptide I is Val-Ser-Arg-Val-Phe-Leu-Lys-Gly-Ala-Arg (VSRVFLKGAR).

The other peptide (Peptide II) was synthesized by sequential coupling with Fmoc-Gln-OH, Fmoc-Asn-OH, Fmoc-Glu-OH, Fmoc-Phe-OH, Fmoc-Lys(Boc)-OH, Fmoc-Leu-OH, Fmoc-Gly-OH, Fmoc-Pro-OH, Fmoc-Leu-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Ser-OH, and Fmoc-Val-OH. The sequence of Peptide II is Val-Ser-Arg-Leu-Pro-Gly-Leu-Lys-Phe-Glu-Asn-Gln (VSRLPGLKFENQR).

The crude product was treated with acetic anhydride to protect N-terminus of model peptide. Acetic acid and extra acetic anhydride were removed under a vacuum. Then, peptides were treated with TFA in chloroform, and then neutralized with pyridine to cleave the resin and protecting groups on Lys and Arg. The chloroform and TFA pyridine salts were removed under a vacuum. The synthesized peptides were purified using reversed-phase HPLC and the final product had a purity of approximately 90%. The schematic mechanism of peptide synthesis is summarized in Figure 2-2. The molecular structures of Peptide I and II are shown in Figure 2-3.
1st step: activation of Fmoc amino acids

2nd step: coupling reaction between activated Fmoc amino acid and amino acid resin

3th step: deprotection of Fmoc group

4th step: acetylation of N-terminus

5th step: removal of resin

Figure 2-2. Schematic mechanisms of peptide synthesis. The solid circle (●) represents the superacid sensitive resin.
(a) Peptides I

(b) Peptides II

Figure 2-3. Molecular structures of Peptide I and Peptide II. (a) Peptide I; Ac-VSRVFLKGAR; monoisotopic mass: 1173.70 Da, (b) Peptide II; Ac-VSRLPGLKFENQR, monoisotopic mass: 1584.88 Da.
Cross-linking Reaction and In-Solution Digestion

Peptides I and II were completely dried in SpeedVac overnight. Peptides were mixed with 2:1 ratio with Disuccinimidyl suberate (DSS), or PIRs that was dissolved in dried DMSO. The reaction mixture was incubated at RT overnight. Without quenching the reaction, DMSO was removed in SpeedVac overnight. The labeled peptides were suspended in 100mM Ambic. Reduction of disulfide bonds were sequentially performed using DTT in 100mM Ambic for 30 minutes at 37°C. Then, alkylation was carried out adding IAA in 100mM Ambic for 30 minutes at 37°C in the dark. Trypsin in 50mM Ambic was added into the mixture, and incubated at 37°C overnight. The digests were subjected to the multiplexed fragmentation experiments. The efficiencies of cross-linking reaction and trypsin digestion were determined by taking Matrix-assisted laser desorption/ionization – time of flight mass spectra (MALDI-TOF MS, Omni Flex MALDI-TOF mass spectrometer, Bruker Daltonics, Billerica, MA, USA).

Instrument

Fourier Transform Ion Cyclotron Resonance Mass Spectrometer (FTICR-MS, Apex-Q FTICR MS, Bruker Daltonics, Billerica, MA, USA) with a 7.0 T superconducting magnet was used for developing multiplexed fragmentation approach with PIR labeled peptides. Schematic diagram of Apex-Q 7T FTICR MS is shown in Figure 2-4. A 2.5µL syringe pump was used to introduce sample solution to electrospray ionization (ESI) source, which was the choice of ionization source, at a flow rate of 30µL/hour. The voltage on ESI source was set at 1800V. The other voltages applied to each segment of FTICR-MS were shown in Figure 2-5. The instrument was operated using Bruker XMASS software version 7.0.6 (Bruker Daltonics, Billerica, MA, USA).
Figure 2-4. Schematic diagram of Apex-Q 7T Fourier Transform Ion Cyclotron Resonance Mass Spectrometer (Apex-Q 7T FTICR MS).

Figure 2-5. Multiplexed fragmentation method in Apex-Q 7T FTICR MS. The black line indicates the potential applied to each region of the mass spectrometer for MS scan. ISCID scan is shown by the red line and the double activation scan is shown by the red and blue line.
**In-Source Collision-Induced Dissociation (ISCID) and Double Activation Analysis**

In-Source Collision-Induced Dissociation (ISCID) was used to release report ions and modified peptides from cross-linked products. ISCID experiments were performed by changing the potential difference between Capillary Exit and Skimmer. The fragments of modified peptides are generated by the double activation. The double activation experiments were achieved by combining ISCID and collision induced dissociation (CID) without the ion isolation [38]. As explained in the instrument section, Apex-Q 7T FTICR MS was operated in positive ESI mode with an applied voltage of 1800 kV. During MS, ISCID, and double activation scanning, data were acquired for 15 scans (approximately 15 s). Figure 2-5 shows the voltages setting to perform MS, ISCID, and the double activation.

**LC/Multiplexed Fragmentation Experiment and Data Analysis**

LC/multiplexed fragmentation experiments were performed using ESI Apex-Q 7T FTICR MS with equipped with a nano HPLC systems (Ultimate, Dionex, Sunnyvale, CA, USA). HPLC was operated in the same manner as explained previously. Various cross-linked species were introduced by autosampler, and separated by nano C18 reversed phase column using the gradient elution. The eluted species from the HPLC system were directly introduced by ESI Apex-Q 7T FTICR MS system to perform multiplexed fragmentation experiment. Apex-Q 7T FTICR MS was operated using Bruker XMASS software version 7.0.6. The applied voltage on each segment of the instrument was automatically changed by Bruker XMASS software to acquire 3-stage multiplexed fragmentation data. During the data acquisition, data were acquired for 4 scans (approximately 2 seconds). LC/multiplexed fragmentation data were analyzed using ICR-2LS software [37].
False Discovery Analysis of Multiplexed Fragmentation

All simulations of false discovery rates for PIR relationship differentiation and accurate peptide mass-based protein identification were carried out using the X-links application. The term “false discovery” is used to indicate the identification of peptide masses without other information. The application X-links that was developed using Microsoft’s Visual Basic at Environmental Molecular Sciences Laboratory (EMSL), Pacific Northwest National Laboratory (PNNL) [18]. The genome of Escherichia coli K12 was downloaded from NCBI web site (http://www.ncbi.nlm.nih.gov/) and was used with the X-links application to calculate all possible tryptic peptides with variable allowed missed cleavages in the mass range from 500 to 5000 Da. Full database consists all possible tryptic peptides. Restricted database is the list of the tryptic peptides having at least one internal Lys.

The restricted database was used to verify the identification of modified peptides using 3-stage multiplexed fragmentation data. The term “candidate peptide” is used to indicate tryptic peptide having exactly same mass with Peptide I and II. The candidate peptides were selected from the restricted database. Then, the theoretical MS/MS patterns of these candidates were generated using GPMAW software version 6.00 (Lighthouse Data, Odense, Denmark).
CHAPTER THREE
RESULTS AND DISCUSSION

Protein Interaction Reporter (PIR)

PIR is a homobifunctional, MS cleavable cross-linker. It contains an amine-reactive NHS ester at each end of spacer arm. NHS esters react with primary amines at pH 7-9 to form stable amide bonds, along with release of the N-hydroxysuccinimide leaving group. Proteins generally have several primary amines in the side chain of Lys residues and the N-terminus of each polypeptide that are available as targets for NHS-ester cross-linker. The reaction mechanism between primary amines and NHS esters is shown in Figure 3-1. As explained in the introduction, PIR contains an affinity tag, and MS cleavable bonds. The structures of two PIRs (PIR I and PIR II) are shown in Figure 3-2. A tag, Biotin, is used for the affinity isolation of labeled proteins. Biotin group is specifically bound to avidin, which is a tetrameric protein produced in the oviducts of birds [39]. Two PIRs used for this research have two different MS cleavable bonds. RINK groups were utilized to make PIR I [14, 37, 40]. Asp-Pro (DP) amino acids are used to form MS cleavable bonds of PIR II [41, 42]. The fragmentation mechanisms of PIRs are shown in Figure 3-3.
Figure 3-1. Cross-linking reaction mechanism between the primary amine and NHS ester.

PIR I
Biotin-DP-NHS
Formula: C_{57}H_{77}N_{13}O_{25}S
Monoisotopic Mass: 1375.49

PIR II
Biotin-Rink-NHS
Formula: C_{74}H_{91}N_{11}O_{24}S
Monoisotopic Mass: 1548.59

Figure 3-2. Molecular structures of PIR I and II. The blue shadows represent NHS ester. The yellow shadows indicate biotin. The red dotted lines represent MS cleavable bonds.
Figure 3-3. Fragmentation mechanisms at MS cleavable bonds of (a) PIR I, and (b) PIR II.

Two labile bonds preferably cleaved by ISCID. Cleavage of both labile bonds gives rise to reporter ions at m/z (a) 752.41, and (b) 1122.48.
3-1. Protein Interaction Reporter Technology Application to Human Cells

PIR coupled with mass spectrometry was demonstrated to study protein-protein interactions of prokaryotic cells, such as bacteria. But the feasibility of this method applied to eukaryotic cells was not verified. Two human cells, MCF7 and HeLa were used to demonstrate the feasibility of PIR Strategy with eukaryotic cells. MCF7 is a breast cancer cell, and HeLa is a cervical cancer cell. These cells were cultured and labeled with PIR II to profile the proteomic data of MCF7 and HeLa cells using protein interaction reporter Strategy.

Human Cell Labeling with PIR

Confocal microscope was used to verify the feasibility of the human cell labeling with PIRs. PIR labeled cells were reacted with anti-biotin antibodies. Then, anti-biotin antibodies were sequentially interacted with Alexa Fluor 488 rabbit anti-mouse IgG and Alexa Fluor 488 goat anti-rabbit IgG. There antigen-antibody reactions produce and amplify the green fluorescence, which is biotin-specific signal. The nuclei of human cells are dyed by propidium iodide and produce the red fluorescence. The green and red fluorescence was used to visualize confocal microscopic images. Schematic mechanisms of the confocal microscopy are shown in Figure 3-4 [43].

In this report, PIR II labeled MCF7 cells were used to take microscopic images. The green and red fluorescence was separately scanned to take microscopic images. Then, two fluorescence images overlapped to obtain whole cell images. The fluorescence images of cells with and without PIR labeling are shown in Figure 3-5. Unlabeled cell produced only the red fluorescence, thus this sample does not contained biotin related species inside and outside cells. But, labeled sample produced both red and green fluorescence. The biotin-specific signal is
produced by PIR used for cell labeling. Therefore, PIRs can label human cells without lyzing cells. Also, the green fluorescence was expressed outside of cell membranes. This result can indicate that anti-biotin antibodies used to produce the fluorescence can not penetrate the cell membrane.

Figure 3-4. Schematic mechanisms of confocal microscopy [43].

Figure 3-5. Confocal fluorescence images of MCF7 cells (a) without PIR II labeling, and (b) with PIR II labeling. Both images are the superposition of fluorescent signals from Alexa Fluor 488 conjugated IgG (green; indicates biotin) and propidium iodide (red; indicates nuclei).
SDS-PAGE and Anti-Biotin Western Blot Analysis

The feasibility of human cell labeling with PIRs was verified using confocal microscopy, but these images are insufficient to determine PIR labeling efficiency. SPS-PAGE and anti-biotin western blot analysis were performed. MCF7.

The human cells were cultured and divided into two portions. The one portion of cells was lysed without PIR labeling. The unlabeled sample is called “Control” sample. The other was labeled with PIR II and lysed. This sample is called “Labeled” sample. 8% SDS polyacrylamide gel was run to separate proteins in these samples. Then, the gels were visualized by Coomassie Blue stain. The gel images are shown in Figure 3-6. The cell lysates of control and labeled samples produced identical gel images, and numerous protein bands were observed on the gels. Therefore, both cell lysates contain a large variety of proteins, but SDS-PAGE is not adequate to distinguish two different samples.

To determine the labeling efficiency, anti-biotin western blot analysis was carried out using both control and labeled samples. The proteins in the cell lysates were separated by SDS-PAGE, and then transferred on the nitrocellulose membrane. This membrane was treated with anti-biotin and commercial chemoluminescent substrates. The membrane was visualized by exposing the film to chemoluminescence. Figure 3-7 shows the obtained western blot images. The images of the labeled samples contain dark spots. The dark spot is biotin-specific signal generated by chemoluminescence. The control samples did not produce biotin-specific signals. Thus, PIRs are efficiently reacted with human cells, and these results are in good agreement with the confocal microscopic images.
Figure 3-6. SDS-PAGE analysis of (a) MCF7 and (b) HeLa cell lysates.

Figure 3-7. Anti-biotin western blot analysis of (a) MCF7 and (b) HeLa cell lysates.
Identification of PIR II Labeled Proteins

PIR labeled proteins were enriched from cell lysates using monomeric avidin beads and eluted in Laemmli sample buffer by boiling to profile the proteomic data of MCF7 and HeLa. The proteins eluted from avidin beads were separated by SDS-PAGE. The gel images are shown in Figure 3-8. The control samples produce few protein bands in the gel, but labeled samples contain more than 25 protein bands. As the result of the gel images, PIR labeled proteins were efficiently captured by avidin beads. The proteins bands present in the gels were excised and destained under acidic condition. The proteins in the gel pieces were reduced and alkylated by DTT and IAA. Then, these proteins were digested by trypsin. In-gel digests were subjected to nano LC/MS/MS, and the proteins identified by Mascot search of LC/MS/MS data. In labeled MCF7 cells, 109 proteins were identified. 127 proteins are identified using labeled HeLa cells as a sample. The identified proteins were listed in Table 4-1 and 4-2 in Chapter 4.

The labeled proteins were compared with proteome data obtained from literatures [44-46]. According to the literatures, the proteins present in MCF7 and HeLa were analyzed by two-dimensional (2D) gel electrophoresis and identified by MALFI-TOF MS on the basis of PMF, following in-gel digestion. The protein profiles contain 1859 proteins for MCF7 and approximately 1200 proteins for HeLa. As a result of comparison, PIR labeled proteins identified from MCF7 and HeLa cell lines are subsets of reported proteome data. 5.86 % of the detectable proteins present in MCF7 and 10.58 % of the detectable proteins in HeLa are labeled by PIR II. Subcellular locations of PIR labeled proteins are obtained from DAVID Bioinformatics Resources and summarized in Figure 3-9. The subcellular locations of 50% of PIR labeled proteins are unknown. The other proteins are located in the variety of organelles and cytoplasm. Thus, PIR II can penetrate the cell membrane. But a small number of proteins in human cells can
be labeled with PIR II.

Figure 3-8. SDS-PAGE analysis of (a) MCF7 and (b) HeLa cell lysates after enrichment process.
Figure 3-9. Protein categorization of the identified protein by subcellular locations, (a) MCF7 and (b) HeLa cell line
3-2. Multiplexed Fragmentation

Sample Preparations using PIRs and Model Peptides

Multiplexed fragmentation method with PIR Strategy was developed to study protein-protein interaction. Cross-linked tryptic peptides is required to demonstrate PIR coupled with 3-stage multiplexed fragments experiments. Commercial standard peptides, such as angiotensin I and bradykinin, can not be used for this experiment, because the standard peptides are not tryptic peptides. Peptide I and II were synthesized to perform this experiment.

As shown in Figure 2-3, the model peptides contain one internal Arg and Lys. C-terminus of the peptides is Arg and N-terminus of the peptide is blocked by acetyl group. PIRs react with internal Lys. After cross-linking, the reaction mixture is digested by trypsin. Thus cross-linked peptides have same structure of tryptic peptides. The proposed reaction mechanisms of inter-cross-linking are shown in Figure 3-10.

Model peptide I was reacted with commercial cross-linker, disuccinimidyl suberate (DSS), to prove efficiency of sample preparation process. MALDI-TOF MS spectrum of the reaction mixture was obtained during each process. The results show in figure 3-11. The MALDI-TOF MS spectrum shows Peptide I at \( m/z \): 1174.706 before the sample preparation. The cross-linking mixture contains inter-cross-linked products at \( m/z \) 2486.501, and dead ends at \( m/z \) 1330.709. Inter-cross-lined tryptic peptides at \( m/z \) 1718.051, dead-end at \( m/z \) 945.470, and unreacted peptide at \( m/z \) 1174.710 were produced by try trypsin digestion. These MS spectra indicate that the sample preparation is efficiently carried out using model peptides.
1\textsuperscript{st} step: Cross-Linking Reaction

\[
\begin{array}{c}
\text{Ac} \\
| \\
V \\
S \\
R \\
F \\
L \\
K \\
G \\
A \\
R
\end{array}
\quad + \quad
\begin{array}{c}
\text{Ac} \\
| \\
V \\
S \\
R \\
F \\
L \\
K \\
G \\
A \\
R
\end{array}
\quad \rightarrow \quad
\begin{array}{c}
\text{Inter-cross-linked product} \\
\text{Dead-end} \\
\text{Unreacted Peptide}
\end{array}
\]

2\textsuperscript{nd} step: Trypsin Digestion

\[
\begin{array}{c}
\text{Ac} \\
| \\
V \\
S \\
R \\
V \\
R \\
V \\
G \\
A \\
R
\end{array}
\quad + \quad
\begin{array}{c}
\text{Ac} \\
| \\
V \\
S \\
R \\
F \\
L \\
K \\
G \\
A \\
R
\end{array}
\quad \rightarrow \quad
\begin{array}{c}
\text{Ac} \\
| \\
V \\
S \\
R \\
F \\
L \\
K \\
G \\
A \\
R
\end{array}
\quad + \quad
\begin{array}{c}
\text{Ac} \\
| \\
V \\
S \\
R \\
F \\
L \\
K \\
G \\
A \\
R
\end{array}
\]

Figure 3-10. Schematic mechanisms of cross-linking reaction between PIR and Peptide I and trypsin digestion.
Figure 3-11. MALDI-TOF spectra of (a) Peptide I, (b) the cross-linking mixture of Peptide I and DSS, and (c) trypsin digest of the reaction mixture.
In-Source Collision-Induced Dissociation (ISCID) and Double Activation Analysis.

In the previous reports [16-18], 2-stage multiplexed fragmentation with PIR strategy was performed. This method was achieved by the parallel alternating scans which are acquired at either low or high collision energy in Hexapole #2. The information about PIR labeled peptides was obtained at low collision energy. The report ions and modified peptides were generated by high collision energy in Hexapole #2. Thus, fragmentation of modified peptides can not be performed. To perform 3-stage multiplexed fragmentation, the modified peptides and report ions need to be generated by ISCID [41]. 3-Stage multiplexed fragmentation approach consists of MS, ISCID and double activation scans. In the ISCID stage, the potential difference between capillary exit and skimmer activates MS cleavable bonds to release reporter ions and modified peptides. The double activation is achieved using ISCID and high collision energy in Hexapole #2 to fragment the modified peptides from cross-linked products.

To characterize the dissociation of MS cleavable bonds by ISCID, two PIR labeled model peptides were used. The first sample is PIR I labeled Peptide I, and the other is PIR II labeled Peptide II. Then, MS spectra of these samples were obtained under various potential differences between capillary exit and skimmer. The representative spectra are shown in Figure 3-12. The optimal conditions of MS and ISICD scans were determined using the peak intensities of inter-cross-linked products, and modified peptides. Normalized peak intensities of the species were shown in Figure 3-13. The intensities of inter-cross-linked products (charge state, 4+) present in two different samples are maximal when the potential difference between capillary exit and skimmer is 100V. The maximum intensities of modified peptides (charge state, 1+) are obtained at the potential difference, 240V. These potential differences are used to obtain the optimal spectra of MS and ISCID scans.
Figure 3-12. 3-Stage multiplexed fragmentation spectra of (a) PIR I labeled Peptide I, (b) PIR II labeled Peptide II. The bottom spectra show the fragment of modified peptides using changing m/z range.
Figure 3-13. Potential difference between capillary exit and skimmer vs. normalized peak intensities present in (a) PIR I labeled peptide I, (b) PIR II labeled peptide II. (■) inter-cross-linked product (charge state, 4+), (●) inter-cross-linked product (charge state, 3+), (▲) dead-end (charge state, 2+), (▼) modified peptide (charge state, 1+), (►) reporter ion (charge state, 1+ for PIR I, 2+ for PIR II).
The optimal condition for double activation scan mode was determined by taking MS spectra using PIR I labeled peptide I. Figure 3-14 shows the normalized intensities of modified peptide, and y ions. The intensities of these species are maximal when the applied voltage to Hexapole #2 is 30V. Thus, this voltage is used for double activation experiments.

Figure 3-14. Double activation scan experiments using PIR I labeled model peptide I. The applied voltage to the second hexapole for double activation is increasing when the potential difference between capillary exit and Skimmer is 240V. (■) modified peptide, (●) y6 ion, (▲) y5 ion, (▼) y4 ion or modified peptides.
**LC/3-Stage Multiplexed Fragmentation Experiments**

The voltages applied to each segments of instrument need to be changed automatically to perform LC/3-stage multiplexed fragmentation experiments. Alternated scan experiment was designed to demonstrate the feasibility of LC/3-stage multiplexed fragmentation experiments. The alternative scan experiments consist of direct infusion as a sample introduced method and automatic operation of instrument using Bruker XMASS software to change the applied voltages. Total number of scans for this experiment is 16. In other words, 16 spectra were obtained during this experiment using PIR I labeled Peptide I. Figure 3-15 shows the representative spectra obtained using the alternated scan experiment. The scan number of this experiment determines the scan mode using following relationships.

<table>
<thead>
<tr>
<th>Scan mode</th>
<th>Scan number</th>
<th>for alternated experiments</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS</td>
<td>3n+1,</td>
<td>1, 4, 7, 10, 13, 16</td>
</tr>
<tr>
<td>ISCID</td>
<td>3n+2</td>
<td>2, 5, 8, 11, 14</td>
</tr>
<tr>
<td>Double activation</td>
<td>3n+3 [3(n+1), or 3m]</td>
<td>3, 6, 9, 12, 15</td>
</tr>
</tbody>
</table>

Extracted ion chromatograms (EICs) of inter-cross-linked product (charge state, 4+), reporter ion (charge state, 1+), modified peptide (charge state, 1+), and y6 ion (charge state, 1+) of modified peptide are shown in Figure 3-16. EICs of these species were compared to verify the feasibility and the efficiency of alternated scan experiments. As shown in Figure 3-16, EICs of the species indicate that peak intensities are changed by scan numbers. The intensity of inter-cross-linked product is maximal at scan number, 3n+1 and sequentially decreased at the scan number 3n+2, 3n+3. When the scan number is 3n+2, the peaks of reporter ion and modified peptide appear. The intensities of these peaks are maximal at the scan number 3n+3. This EIC indicate that the mass cleavable bonds of cross-linked products are not fully fragmented. But the
intensities of reporter ion and modified peptide are sufficient to identify the species. EIC of y6 ion of modified peptide shows the opposite result to EIC of inter-cross-linked product.

As a result of this experiment, inter-cross-linked products are detected at MS scan and release reporter ions and modified peptides during ISCID scan mode. Then y ions of modified peptides are generated by double activation. Thus, 3-stage multiplexed fragmentation experiments can be carried out using instrument operation software, Bruker XMASS software.

Figure 3-15. MS, ISCID and double activation spectra of PIR I labeled Peptide I using the alternated scan experiment.
Figure 3-16. Normalized extracted ion chromatograms (EICs) of inter-cross-linked product (charge state, 4+), reporter ion (charge state, 1+), modified peptide (charge state, 1+), and y6 ion (charge state, 1+) obtained using alternative scan experiments with PIR I labeled Peptide I. The red line represents EIC of inter-cross-linked. The black lines represent EIC of (a) reporter ion, and (b) modified peptide. The blue line shows EIC of (c) y6 ion.
LC/3-stage multiplexed fragmentation experiment was carried out using PIR II labeled Peptide II. Total ion chromatogram (TIC) and EICs of inter-cross-linked product, reporter ion, modified peptide, and y ions are shown in Figure 3-17. TIC shows 3 peaks, but EICs of inter-cross-linked product have one peak at the scan number 1200~1250. Also, EICs of other species show the biggest peak at the scan number 1200~1350. To verify the relationship between inter-cross-linked product and other species, normalized EICs of these species were compared and the results are shown in Figure 3-18. The intensity of inter-cross-linked product is maximal at MS scan and sequentially decreased at ISCID and double activation scans. The peaks of reporter ion and modified peptide appear during ISCID scan. The intensities of these peaks are maximal at double activation scan. This EIC indicate that the mass cleavable bonds of cross-linked products are not fully fragmented. The intensities of y ions are increased when that of inter-cross-linked products is decreased. These results are good agreement with alternated scan experiments. Thus, LC/3-stage multiplexed fragmentation experiment with PIR strategy is feasible.
Figure 3-17. TIC and EICs obtained using PIR II labeled Peptide II from LC/3-stage multiplexed fragmentation experiment. (a) TIC, m/z range from 200 to 1800, EIC of (b) inter-cross-linked product (charge state, 4+), (c) inter-cross-linked product (charge state, 3+), (d) reporter ion (charge state, 2+), (e) reporter ion (charge state, 1+), (f) modified peptide (charge state, 1+), (g) y6 ion (charge state, 1+), (h) y8 ion (charge state, 1+) and (i) y9 ion (charge state, 1+).
Figure 3-18. Normalized extracted ion chromatograms (EICs) obtained using PIR II labeled Peptide II at scan number 1200~1250. The red line represents EIC of inter-cross-linked product (charge state, 4+), the black lines represent EIC (a) reporter ion (charge state, 1+), (b) reporter ion (charge state, 2+), (c) modified peptide (charge state, 1+). The blue line shows (d) y6 ion (charge state, 1+), (e) y8 ion (charge state, 1+), and (f) y9 ion (charge state, 1+).
False Discovery Analysis of Multiplexed Fragmentation

The major challenge of multiplexed fragmentation method is the unambiguous identification of modified peptides. The identification of modified peptides is accomplished using the mass of this species and its fragment. If the sample contains several peptides having a same monoisotopic mass within the certain mass measurement tolerance, the identification of a certain peptide is ambiguous. In this report, the false discovery analysis was performed using the genome data of *Escherichia coli K12* used for multiplexed fragmentation experiments. The genome data of *Escherichia coli K12* contains 4,126 proteins, obtained using ORF. These protein sequences were loaded to X-links software and theoretical tryptic peptides in the mass rage from 500 to 5000 Da were calculated and listed. All possible tryptic peptides are 285, 227 without any restriction. The tryptic peptides having at least one Lys residue in sequences are 118,340. The mass accuracy of FT-ICR MS is generally known less than 5ppm. For this reason, the mass measurement tolerances used for the false discovery analysis are ±1, ±2, ±5, ±10, ±15, and ±25 part per million (ppm). The result of the false discovery is shown in Figure 3-18. Generally, the number of candidate peptides is smaller with lower mass measurement tolerance.

LC/3-stage multiplexed fragmentation was developed to identify PIR labeled sample, thus the result of restricted database is practically important. The average of candidate peptides from restricted database is 2.744 at ± 5 ppm as a mass measurement tolerance. When FT-ICR MS is used for multiplexed fragmentation experiments, less than 3 candidate peptides can be identified using only mass of modified peptides. As shown in Figure 3-19, the unambiguous identification of modified peptides using their masses is required the high mass measurement accuracy.
Figure 3-19. Number of theoretical tryptic peptides vs. monoisotopic mass of peptides as a function of mass measurement tolerances. (a) full database resulting in 285,227 peptides, (b) restricted database with 118,340 peptides. The peptides in the restricted database contain an internal Lys.
The feasibility of identification of modified peptides using 3-stage multiplexed fragmentation data is verified by comparing the theoretical MS/MS pattern of candidate peptides with double activation spectra. First, the candidate peptides of each model peptide were search from the restricted database, and summarized in Table 3-1. Theoretical MS/MS patterns of candidate peptides were generated using GPMAW software and showed in Figure 3-20. Then the double activation spectra shown in Figure 3-12 were compared with theoretical MS/MS patterns. Asterisks (*) marked on theoretical MS/MS patterns represent the ions present in real double activation spectra. In the case of PIR I labeled Peptide I, only one theoretical pattern contains 4 fragments present in the double activation spectrum. Another pattern has 2 fragments. The other candidate peptides can not product any fragment present in the real spectrum. As a result of the comparison, the unambiguous identification of PIR labeled peptide is feasible using 3-stage multiplexed fragmentation data.

Table 3-1. Candidate peptides of Peptide I.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Candidate Peptide</th>
<th>Monoisotopic Mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypothetical Protein ECDH10B_1591</td>
<td>[349]VFKAGLR[355]</td>
<td>789.4861</td>
</tr>
<tr>
<td>Predicted Transposase</td>
<td>[349]VFKAGLR[355]</td>
<td>789.4861</td>
</tr>
<tr>
<td>Conserved Inner Membrane Protein</td>
<td>[66]FGGKLLR[72]</td>
<td>789.4861</td>
</tr>
<tr>
<td>Hypothetical Protein ECDH10B_4318</td>
<td>[40]LFNLKLR[46]</td>
<td>789.4861</td>
</tr>
<tr>
<td>Peptide I</td>
<td>VFLKGAR</td>
<td>789.4861</td>
</tr>
</tbody>
</table>
Figure 3-20. Theoretical MS/MS patterns of candidate peptides generated from (a) Hypothetical Protein ECDH10B_1591, and Predicted Transposase, (b) Conserved Inner Membrane Protein, (c) Hypothetical Protein ECDH10B_4318, and (d) Peptide I. Asterisks (*) represent fragments present in the double activation spectrum of PIR I labeled Peptide I.
Conclusion

*in vivo* identification of protein-protein interaction using PIR coupled with mass spectrometry has been reported [16, 17]. PIRs penetrate the bacteria cell membrane and react with the proteins without breaking cell membrane. In this report, PIR coupled with MS method was applied to human cell lines, such as MCF7 and HeLa. The feasibility of studying protein-protein interaction of human cell lines has been proven. The results showed that *in vivo* human cell line labeling using PIR is feasible for studying protein-protein interactions.

Chemical cross-linking combined with mass spectrometry is a common approach to study protein structure, and protein-protein interactions. This technique is primarily based on data-dependent LC/MS/MS method, however this method allows the identification of relatively abundant proteins. Consequently, this method is inadequate for identifying the protein-protein interactions of low abundant functional proteins. To overcome this problem, novel MS/MS approach, multiplexed fragmentation method has been designed. PIR coupled with multiplexed fragmentation was applied to tryptic peptides. The results established that the identification of PIR labeled peptides is feasible using the LC/3-stage multiplexed fragmentation method.
## CHAPTER FOUR

### SUPPLEMENTARY DATA

Table 4-1. Protein profile of PIR II labeled MCF7 cell line.

<table>
<thead>
<tr>
<th>Gene (gi number)</th>
<th>Annotation</th>
<th>Mascot score</th>
<th>MW (KDa)</th>
<th>Subcellular location</th>
</tr>
</thead>
<tbody>
<tr>
<td>gi</td>
<td>10803733</td>
<td>HBV pX associated protein-8; XAP-8</td>
<td>51</td>
<td>136</td>
</tr>
<tr>
<td>gi</td>
<td>1082356</td>
<td>epidermal autoantigen 450K (clone pE450-C/D)</td>
<td>82</td>
<td>73</td>
</tr>
<tr>
<td>gi</td>
<td>11024700</td>
<td>translocase of inner mitochondrial membrane 13</td>
<td>51</td>
<td>11</td>
</tr>
<tr>
<td>gi</td>
<td>113417027</td>
<td>Interleukin-7 receptor alpha chain precursor</td>
<td>49</td>
<td>-</td>
</tr>
<tr>
<td>gi</td>
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Bibliography

1. PHIZICKY, E. M.; FIELDS, S., Protein-Protein Interactions: Methods for Detection and Analysis. Microbiological Reviews 1995, 59(1) 01, 94-123.


