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Chapter 16

Peptidome Analysis of Mouse Liver Tissue by Size Exclusion Chromatography Prefractionation

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Abstract

Here we report our approach to the peptidomic analysis of mouse liver. We use ultrafiltration for peptide prefractionation, which is followed by size exclusion chromatography. The low molecular weight peptides (MW below ~3 kDa) are analysed directly by nanoLC-MS/MS, and the higher molecular weight peptides (MW above ~3 kDa) are characterized with MALDI-TOF MS first and then proteolytically digested prior to nanoLC-MS/MS analyses.

Key words: Mouse liver, peptidomics, ultrafiltration, size exclusion chromatography, multidimensional separation, mass spectrometry.

1. Introduction

The unprecedented growth and development of separation and detection technologies in recent years has led to rapid progress in proteomic research (1, 2). However, only a few proteins have been validated as disease biomarkers because of the low abundance of the potential biomarkers and the complexity of biological samples (3). The low molecular weight (LMW) fraction of proteome (termed "peptidome"), considered previously as biological debris (4), has attracted increasing attention recently (5). The area of research involved in comprehensive study of peptides or LMW proteins expressed by a cell, tissue and organs of an organism has become known as Peptidomics (6). The peptidome analysis mainly focuses on the quantitative and qualitative analysis of peptides, which can be divided into two classes: (I) the

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endogenous peptides that exert vital functions in biological process such as hormones, cytokines, growth factors, MHC class I peptides and alike (6, 7); (II) the degraded fragments of proteins which reflect the proteolytic enzyme species and biological state of individual (8, 9). The endogenous peptides play crucial roles in the respiratory, cardiovascular, endocrine, inflammatory and nervous systems (7, 10). Discovering novel neuropeptides has been extensively studied and some databases have been established for the endogenous peptides (11, 12). The degraded fragments of proteins are generated by the proteolytic enzymes and can be considered as the metabolic products of proteins. Circulating protein fragments generated in the body fluid or tissues may reflect the biological events and provide a rich bank for diagnostic biomarkers (13). It is believed that peptide concentration in tissues should be higher than that in the blood and thus screening for peptide biomarkers in tissues may be another way for speeding up the biomarker discovery (14). However, unlike the abundance of publications on the peptidomic analysis of body fluids, few peptidomic analyses from other tissues were reported (15). Liver is a vital organ, which is considered as the main "chemical factory" and "energy plant" for the body (16) and can therefore provide a rich source of peptides indicative of body metabolism and hepatic function. NanoLC-MS/MS (nano-liquid chromatography tandem mass spectrometry) has high detection sensitivity and is capable of high throughput, which makes this technique suitable for the peptidomic analyses. However, few peptides over 3000 Da can be characterized directly. To fully characterize the peptidome over the whole range of molecular weights, we developed a comprehensive method which relies on a simple but highly reproducible ultrafiltration step to extract the liver peptidome, followed by prefractionation of the peptidome using size exclusion chromatography and nanoLC-MS/MS (17). The flow chart summarizing the procedure is shown in **Fig. 16.1**.

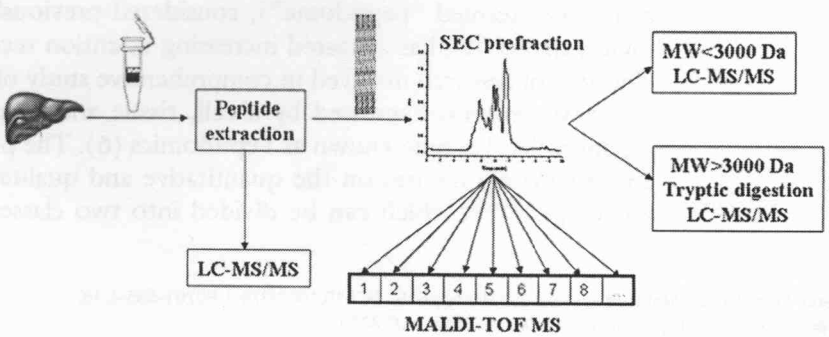

Fig. 16.1. Outline of the experimental approach to the comprehensive peptidomic analysis of mouse liver tissue.

2. Materials

1. HPLC pump: LC-10ADvp pump (Shimadzu, Kyoto, Japan). Large variety of instruments exist and any instrument which can operate at low flow rates (<1 mL/min) and tolerate pressure of 2000 psi can be used.
2. UV detector: SPD-M10Avp UV-vis detector (Shimadzu, Kyoto, Japan). Other suitable detectors with adjustable wavelength of 200–400 nm may be used.
3. Size exclusion column: TSK SuperSW 2000 (4 μm, 125 Å, 4.6 mm i.d. × 300 mm; TOSOH, Tokyo, Japan) (*see* **Note 1**).
4. Capillary separation column: Fused-silica AQ C18 packed capillary (5 μm, 120 Å, 75 μm i.d. × 120 mm; Michrom BioResources, CA, USA).
5. Mobile phases. Solvent 1: 45% Acetonitrile (ACN) in 0.1% trifluoroacetic acid (TFA). Solvent 2: 0.1% Formic acid in water. Solvent 3: 0.1% Formic acid in acetonitrile. All reagents should be chromatographic grade. High-purity deionized water should be used in all experiments, e.g. purified with Milli-Q system (Milford, MA, USA) (*see* **Note 2**).
6. Peptide extraction. Extraction buffer: 0.25% Acetic acid. Adult female C57 mice. High-purity deionized water should be used in all experiments, e.g. purified with Milli-Q system (Milford, MA, USA). Centrifugal filter: Amicon Ultra-15, (Millipore, Milford, MA, USA) or equivalent centrifugal filter with a nominal molecular mass limit of 10 kDa.
7. Standard proteins: 10 mg/mL cytochrome *c* in Solvent 1; 10 mg/mL insulin in Solvent 1; 10 mg/mL insulin chain B in Solvent 1.
8. Peptide digestion: 1 μg/μL TPCK-treated trypsin; 1 M dithiothreitol (DTT); 1 M iodoacetamide (IAA); 50 mM ammonium bicarbonate.
9. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS): Bruker Autoflex(TM) (Bruker, Bremen, Germany). This instrument is equipped with a nitrogen laser ($\lambda = 337$ nm) and the accelerating potential is in the range of +20/–20 kV. MALDI stainless-steel sample target (MTP 384). All mass spectra should be obtained in the positive-ion detection mode. MALDI-TOF MS is used for determining the molecular

weight of peptides in fractions obtained after size exclusion chromatography (SEC).

10. MALDI-TOF MS reagents. Matrix solvent: 33% ACN/0.1% TFA (33%/67%, v/v). Matrix: 20 mg/mL 2,5-dihydroxybenzoic acid (DHB) in matrix solvent.
11. High-performance liquid chromatography (HPLC) MS/MS: Finnigan surveyor MS pump (ThermoFinnigan, San Jose, CA) and LTQ linear ion trap mass spectrometer ThermoFinnigan (San Jose, CA, USA) with a nanospray source. HPLC MS/MS is used for separation and sequencing of peptides.
12. *SEQUEST* database search and data analysis software (Thermo Electron, San Jose, CA).

3. Methods

3.1. Sample Preparation (see Note 3)

1. Sacrifice the mouse and remove the liver, wash it in extraction buffer to remove traces of blood.
2. Mince the liver with scissors on ice and homogenize with extraction buffer at 4°C. Use 5 ml of extraction buffer per gram of liver (equivalent concentration 0.2 g of tissue per mL of buffer).
3. Sonicate the suspension for 90 s at 450 W at 4°C (*see* **Note 4**).
4. Centrifuge the suspension at 25,000×*g* at 4°C for 1 h.
5. Transfer the supernatant to Amicon Ultra-15USA and centrifuge at 5000×*g* for 30 min at 4°C (*see* **Note 5**).
6. Collect and lyophilize the filtrate.
7. Redissolve the lyophilized peptides sample in Solvent 2 (use 1 ml per 6 g of tissue) and store at –20°C until use.

3.2. SEC Prefractionation of the Peptide Sample (see Note 6)

1. Equilibrate HPLC system and the TSK SuperSW 2000 column with Solvent 1.
2. Set flow rate to 0.35 ml/min, use isocratic elution (*see* **Note 7**).
3. UV detection should be set to 214 nm.
4. Use standard proteins (cytochrome *c*, insulin and insulin chain B) for evaluating the separation efficiency. An example of the chromatogram is shown in **Fig. 16.2a**, where the standard proteins are well separated from each other.

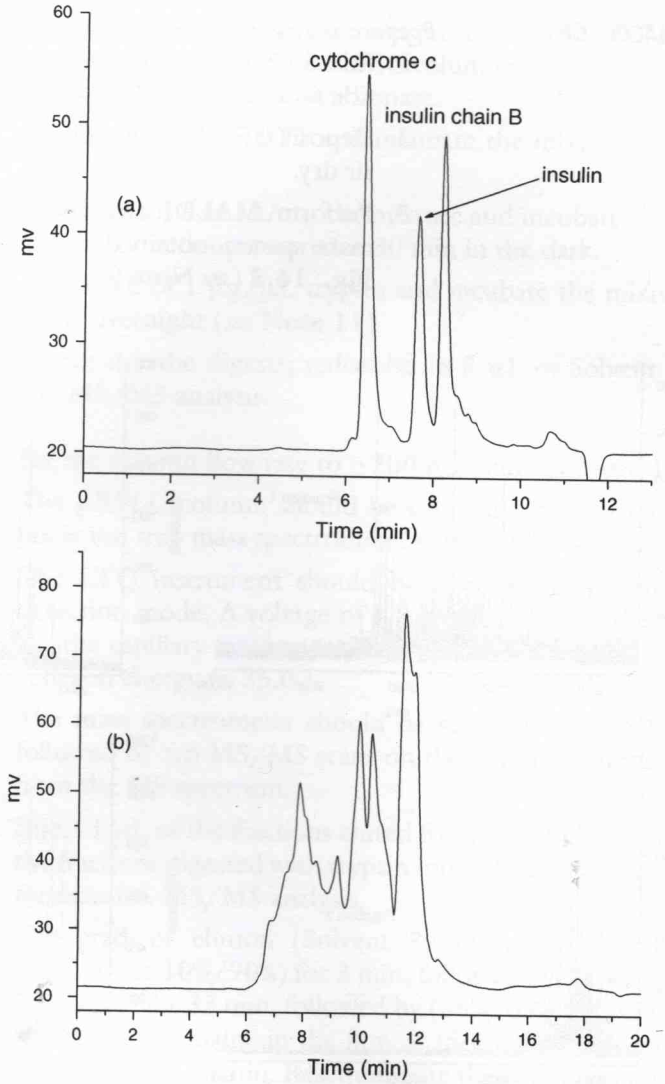

Fig. 16.2. Size exclusion chromatography separation of (**a**) standard sample containing cytochrome *c* (12327 Da), insulin (5734 Da) and insulin chain B (3496 Da) and (**b**) the extracted peptides from mouse liver tissue. Mobile phase: 45% ACN in 0.1% TFA (isocratic elution); flow rate 0.35 ml/min; UV detection at 214 nm.

5. Separate the extracted peptides (from Step 7, **Section 3.1**) on the SEC column.
6. Collect the eluted peptides (~200 µl fractions or smaller, approximately 30 s per sample). An example of the chromatogram is shown in **Fig. 16.2b**.
7. Freeze-dry the collected samples.
8. Redissolve peptides in Solvent 2 and store at –20°C until use.

3.3. MALDI-TOF MS

Prepare matrix solution:

1. Add 3 μL of Matrix (20 mg/ml DHB) to 1 μL of the peptide sample.
2. Deposit 0.5 μL of the mixture on the MALDI target plate, air dry.
3. Perform MALDI analysis (*see* **Note 8**). An example of mass spectra obtained for different fractions is shown in **Fig. 16.3** (*see* **Note 9**).

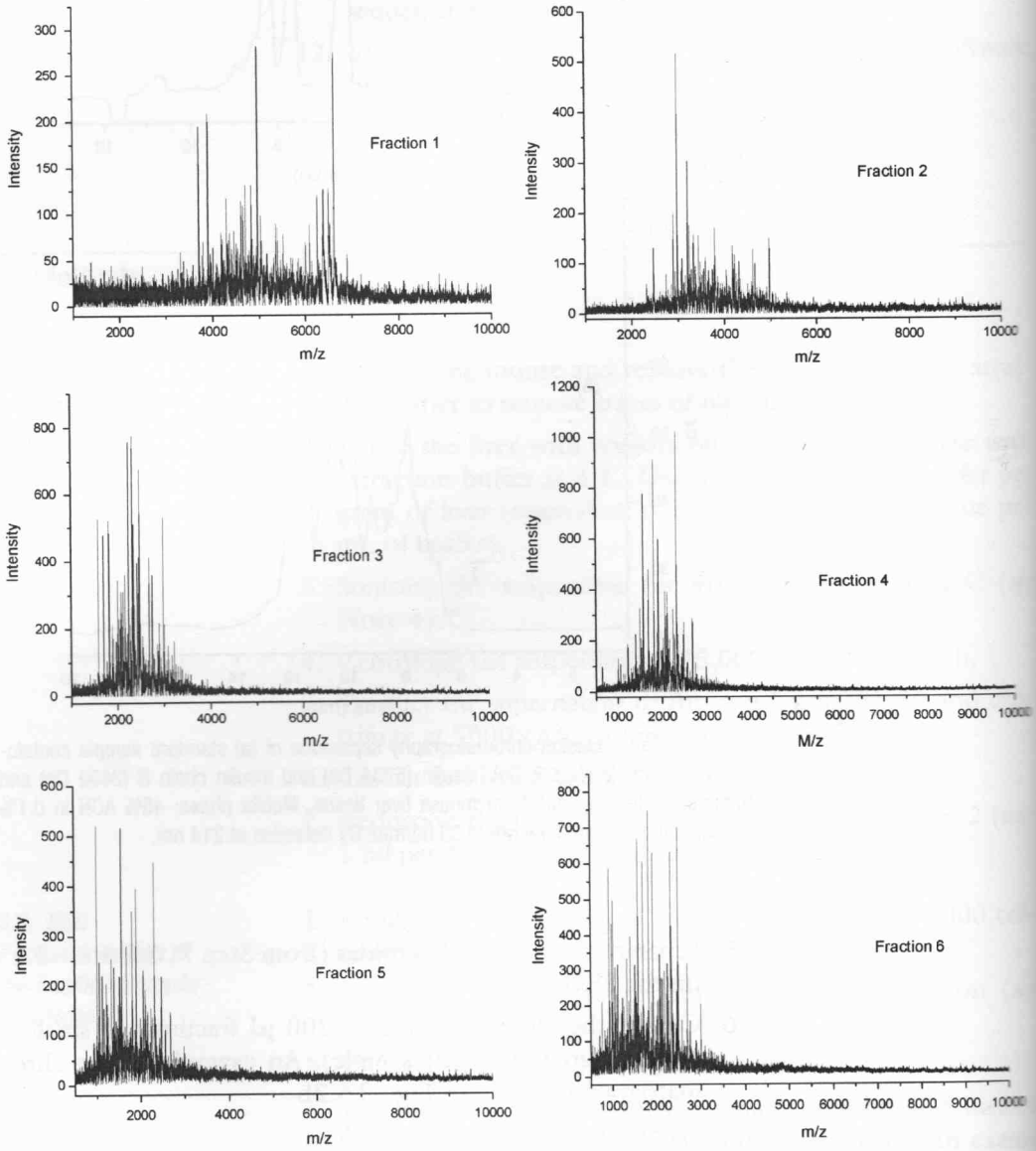

Fig. 16.3. MALDI-TOF MS analysis of the peptide fractions eluted from the size exclusion chromatography column (six consecutive fractions).

3.4. Digestion of the HMW Peptides (see Note 10)

1. Redissolve high molecular weight fractions (MW above ~ 3000 Da) collected from SEC column in 150 μL solution of 50 mM ammonium bicarbonate.
2. Add 1 μL of 1 M DTT and incubate the mixture at 37°C for at least 2 h.
3. Add 1 μL of 1 M IAA to the mixture and incubate the mixture at room temperature for 30 min in the dark.
4. Add 1 μL of 1 μg/μL trypsin and incubate the mixture at 37°C overnight (*see* **Note 11**).
5. Freeze-dry the digests; redissolve in 5 μL of Solvent 2 for LC-MS/MS analysis.

3.5. NanoLC-MS/MS

1. Set the column flow rate to ~200 nL/min (*see* **Note 12**).
2. The μRPLC column should be coupled directly to a LTQ linear ion trap mass spectrometer with a nanospray source.
3. The LTQ instrument should be operated at positive-ion detection mode. A voltage of 1.8 kV is applied to the cross. Set the capillary temperature to 170°C. Set the normalized collision energy to 35.0.
4. The mass spectrometer should be set at one full MS scan followed by ten MS/MS scans on the ten most intense ions from the MS spectrum.
5. Inject 1 μL of the fractions eluted from the SEC columns or the fractions digested with trypsin into C18 nanoLC column for nanoLC-MS/MS analysis.
6. Use gradient elution (Solvent 3/Solvent 2) as follows: (2%/98% to 10%/90%) for 3 min, followed by (10%/90% to 35%/65%) for 33 min, followed by (35%/65% to 80%/20%) for 2 min and maintain the flow at (Solvent 3/Solvent 2 = 80%/20%) for 10 min. Re-equilibrate the column as follows: use a fast gradient (80%/20% to 2%/98%) for 3 min, then maintain flow at (Solvent 3/Solvent 2 = 2%/98%) for 9 min.

3.6. Data Processing and Analysis

1. Peptides can be identified by searching against sequence databases. We use *SEQUEST* database search and data analysis software and download sequence data from ftp://ftp.ebi.ac.uk/pub/databases/IPI/old/MOUSE/ipi.MOUSE.v3.08.fasta.gz.
2. Search parameters should be set as follows: no enzyme, set variable modification to oxidation of Met. If the samples were digested with trypsin, set fixed modification to carbamidomethylation of Cys and specify the enzyme as partially tryptic (*see* **Note 13**).
3. Specify the mass as monoisotopic.

4. Combine output data and remove keratins and any redundant data.
5. Filter the search results by setting lowest X_{corr} as 1.9, 2.2 and 3.75 (corresponding to 1+, 2+ and 3+ charge states, respectively).
6. A minimum delta correlation (ΔC_n) of 0.2 is required for the identification to be considered positive. Determine the false discovery rate (FDR) of the peptide identification (*see* **Note 14**).

4. Notes

1. There are different SEC columns for different samples with different ranges of molecular weight of proteins. For example, in the TSK series there are TSK 2000 (MW range 500–100,000 Da), TSK 3000 (2,000–500,000 Da), TSK 4000 (20,000–7,000,000 Da), etc.
2. All the mobile phases for chromatography should be filtered through a 0.22-μm or 0.45-μm membrane to avoid clogging the columns.
3. Extraction is the first step in the peptidomic analysis, and, therefore, the reproducibility of extraction is crucial in determining the outcome of the whole process. All procedures should be carried at temperatures below 4°C to reduce protein degradation. Delays and unnecessary sample storage steps should be avoided.
4. The tube should be immersed in ice water and the liquid level of the suspension should be lower than the ice water to keep the suspension at 4°C (7, 10, 18, 19).
5. The speed limit is different for different type of filters. The speed limit should be observed to avoid damaging the filter membrane.
6. LMW peptides (MW below ~3 kDa) should be separated from HMW components (MW above ~3 kDa) prior to the proteolysis or direct MS analysis.
7. If the SEC column has been used with other solvents such as the phosphate buffer, the column should be first equilibrated with water at a flow rate of 0.2 mL/min and then with the mobile phase (45% ACN in 0.1% TFA). For storage (over a week), the solvent should be replaced with 0.05% NaN_3 (use the same flow rate of 0.2 mL/min) to prevent bacterial growth.
8. Procedures outlined in the instrument manual must be followed.

9. At this stage one should expect to see different masses in different SEC samples. Larger molecular weight peptides should be eluted faster, whilst later collections should contain lower molecular weight peptides, as shown in **Fig. 16.3**.
10. In order to gain sequence information and identify the progenitor proteins, HMW polypeptides should be proteolytically digested prior to the nanoLC-MS/MS analysis.
11. The amount of trypsin depends on the size of sample. Typical weight ratio for trypsin:peptide is 1:50.
12. The pump flow may be split using a micro-splitter valve to achieve the required flow rate of ~200 nL/min.
13. For searching and identification of the modified peptides, search parameters (i.e. modifications) should be set accordingly.
14. FDR can be determined by performing *SEQUEST* search against a composite database that includes regular as well as reversed protein sequences. FDR = $2 \times n(\text{rev})/(n(\text{rev})+n(\text{forw}))$, where n(forw) and n(rev) are the number of peptides identified in proteins with forward (normal) and reversed sequence, respectively. (20, 21) FDR is an important parameter for the evaluation of mass matching results. Settings should be chosen such that FDR is kept below 5%.

Acknowledgments

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