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Analysis of Peptides by Denaturing Ultrafiltration and LC-MALDI-TOF-MS

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Abstract

The dynamic range of complex biological samples represents a challenge for mass spectrometric characterization. Removal of high abundant proteins is a prerequisite for a successful mass spectrometric analysis of low abundant analytes. In particular, plasma and serum proteome span at least ten orders of magnitude and represent a major challenge for biomarker discovery. Immunoaffinity depletion is the most common methods of removal of high abundant proteins. Here we describe coupling of denaturing ultrafiltration, an alternative depletion strategy, with reverse phase fractionation and mass spectrometry for characterization of low molecular weight proteins and peptides.

Keywords

LMW proteome; peptidomics; biomarker; ultrafiltration; MALDI; mass spectrometry

1. Introduction

Mass spectrometric characterization of native peptides and low molecular weight (LMW) proteins in biological samples has evolved into a distinct field of peptidomics (1;2). Peptidomic studies are attractive because their readout represents complex pathways associated with (patho)physiology of various biological processes (3–5). The utility of peptidomics ranges from basic biology, through physiology of neuropeptides, to the biomarker discovery in serum and plasma (6;7). Even though the interpretation of the screening studies requires caution, the technologies continuously improve and there is considerable interest in the peptidomic applications (8–10). An efficient extraction of the native peptides is a prerequisite for successful mass spectrometric characterization of the biological systems. The use of denaturing ultrafiltration in peptidomic studies is the focus of this discussion.

It has been estimated that more than 10,000 proteins are present in human plasma or serum (11). Albumin, the most abundant protein, contributes around 55% of the total proteome. Twenty-two proteins constitute approximately 99% of the serum protein content and consequently limit the identification, characterization, and quantification of the lower abundant species (12). Removal of high abundant proteins narrows the dynamic range and reduces signal suppression in mass spectrometric analysis. A significant challenge for

proteomics is to enrich efficiently the lower abundant analytes (13;14). A variety of methods to deplete high abundant proteins or to capture specific subsets of peptides/proteins have been developed (15–18). We focus on the enrichment of the LMW proteins and peptides by ultrafiltration, a fast, easy to carry out and inexpensive method (19–21). By using centrifugal membranes with defined molecular weight cut-off (10–50 kDa), proteins with higher molecular weight are retained on the filter while low molecular weight species pass through the membrane. The filtrate can be desalted and further fractionated for mass spectrometric characterization. In this chapter, we describe a denaturing ultrafiltration method for enrichment of the native peptides in serum or plasma (7;22). The enriched peptides are analyzed by reverse phase liquid chromatography coupled to MALDI-MS/MS characterization of the analytes. This type of analysis results in our hands in an identification of approximately 250 native peptides from 50 different proteins.

2. Materials

2.1. Blood sample collection and serum preparation

1. Vacutainer 10 mL Red Serum tubes (BD 366430) (Becton Dickinson, Franklin Lakes, NJ) (^{Note 1})
2. Vacutainer Brand Safety-Lock Blood Collection Set (BD 367281)
3. Vacutainer Holder (BD 364815)
4. NUNC Cryotubes (094343)

2.2 Ultrafiltration

1. Amicon Ultra centrifugal filter device (15mL) with 30 kDa MWCO (Millipore, Bedford, MA) (^{Note 2})
2. Water CHROMASOLV Plus, HPLC grade (Sigma-Aldrich, Cat. 34877)
3. Guanidine hydrochloride (^{Note 3})
4. Serum samples (see blood collection)

2.3 Sep-Pak C18 desalting

1. Sep-Pak C18 cartridge (Waters)
2. Trifluoroacetic acid (TFA) (Sigma-Aldrich, Cat. T6508) (Highly corrosive)
3. Acetonitrile CHROMASOLV Plus, for HPLC 99.9% (Sigma-Aldrich, Cat. 34998) (Highly flammable and harmful)
4. Centrifuge

¹This chapter describes the preparation of peptides from serum; any biological sample can be processed by denaturing ultrafiltration with appropriate adjustments.

²The 30 kDa cutoff filters provide efficient elimination of larger proteins and good recovery of peptides. Note that the cutoff is defined by retained species and does not specify what passes the membrane. Typically, peptides with mass lower than the cutoff are eliminated. Recovery of any individual peptide needs to be verified.

³Denaturation of samples in guanidine hydrochloride gives best recovery of the peptides in our experience.

5. 15 mL centrifuge tubes
6. Tips

2.4 Reverse Phase Liquid Chromatography fractionation

1. Monolithic C18 4.6 × 100 mm (Merck)
2. Agilent 1100 HPLC (Note 4)
3. Fraction collector
4. Tubes
5. Speed Vac

2.5 MALDI Mass spectrometric peptide identification

1. a-Cyano-4-hydroxy-cinnamic acid (CHCA), 3.3 mg/ml (Bruker Daltonics, Part No. 201344) (Note 5)
2. MALDI Plate (Applied Biosystems Inc.)
3. Peptide Calibration Standard (ABI) (Store at −20°C).
4. MASCOT search engine
5. MASCOT Deamon and MASCOT Distiller

3. Method

3.1 Blood samples collection and serum preparation

1. Blood samples collected in a BD Vacutainer “red-top” tube according to standard manufacturer’s protocol are allowed to clot for 60 minutes (Note 6).
2. Serum is separated by centrifugation at 1200 g for 10 minutes and aliquoted as soon as possible in convenient volumes for freezing at −80°C in NUNC Cryotubes (Note 7).
3. Thaw a serum aliquot by submersion in room temperature H₂O; process the sample as soon as possible after thawing. All samples are processed at second thaw (Note 8).
4. Spin thawed serum using a bench top centrifuge at ~10,000 g for 10–15 seconds immediately after thawing to spin down particulates suspended in serum; process without delay on prepared ultrafiltration membranes.

⁴Other HPLC systems and columns are of course appropriate for this reverse phase fractionation.

⁵Dissolve CHCA at 5 mg/ml in 50% CH₃CN, aliquot 1mg/vial (0.2 ml), vacuum dry and desiccate at −20°C. For experiments, dissolve fresh in 50% CH₃CN (0.15 ml of dH₂O and 0.15 ml of CH₃CN) at a final concentration of 3.3 mg/ml. Protect matrix from exposure to daylight at all times.

⁶Control the time of clotting as closely as possible. Differences in clotting time may affect results. Follow SOP for hazardous biological materials.

⁷Be careful not to disturb the interface between serum and red blood cell clot when aspirating serum.

⁸A: It is advisable to keep track of the time from collection to centrifugation and of any lag between centrifugation and freezing. B: avoid multiple freeze thaw cycles. Freeze-thaw cycles of serum samples can cause degradation of peptides and, consequently, changes in the mass spectra.

3.2 Ultrafiltration

1. Pipette 2 mL of dH₂O into each 30kDa filter (^{Note 9}).
2. Wash filter 5 minutes at 3,000 g and 10°C in a refrigerated centrifuge.
3. Pipette 5 mL of 8M guanidine hydrochloride solution to the washed filter.
4. Add 1 mL serum to the filter and mix by pipetting up and down.
5. Spin for 60minutes at 3,000 g and 4°C in a centrifuge. Stop when the liquid left in the filter is approximately 500 uL. (^{Note 10})
6. Collect the filtrate at the bottom of the centrifuge tube for cleanup.

3.3 Sep-Pak C18 Desalting

1. Wet the cartridge with 0.1% TFA in acetonitrile, 0.6 mL, spin 1min at 700 rpm and repeat twice.
2. Equilibrate cartridge with 0.1% TFA in water, 1 mL, spin 2 min at 700 rpm, and repeat 3 times.
3. Apply sample onto the cartridge, spin 5 min at 700 rpm, and re-apply the sample once.
4. Wash cartridge with 0.1% TFA in water, 1 mL spin 2 min at 700 rpm for 4 times.
5. Elute peptides with 0.1% TFA in acetonitrile, 0.2 mL, 1 min at 700 rpm and repeat twice (total volume 0.6 mL).
6. Dry eluent in a Speed-Vac.

3.4 Reverse phase HPLC separation and fraction collection

1. Prepare solvents.
2. Set up the Agilent 1100 HPLC system with a fraction collector. Equilibrate the column with 5% solvent B at flow rate of 1 mL/min.
3. Resuspend dried filtrate in 30 µL 0.1% TFA/water. Inject approximately 15 µL of the serum filtrate.
4. Keep the flow isocratic (5% B) for 2 min, then start a linear gradient increasing the percentage of solvent B to 40% over 23 min (^{Note 11}). Collect fractions from 2 min to 27 minutes at every 30 seconds. (^{Note 12})
5. Dry fractions for further MS analysis.

3.5 MALDI TOF/TOF mass spectrometric analysis

1. Resuspend each fraction in 1 µL matrix. (^{Note 13})

⁹Be careful not to touch the membrane with the tip!!

¹⁰Spin as slow as possible; higher speeds may compromise the separation.

¹¹Solvent A: 0.1% TFA in water; solvent B: 0.1% TFA in acetonitrile. Degas and filter solvents before use.

¹²In this experiment, we used a monolithic C18 column which provides good separation in a relatively short time. The gradient can be extended to get better separation depending on the sample.

2. Spot 1µL sample on a MALDI target, together with peptide calibration standards, and allow samples to dry (^{Note 14}).
3. Calibrate the 4800 MALDI TOF/TOF mass spectrometer (Applied Biosystems Inc.).
4. Analyze the samples by acquiring 1000 shots/spot, laser power 3000, mass range 800–4200 Da.
5. Acquire MS/MS spectra of 15 most intense ions in each sample at 1500 shots/ion, laser power 3300, 2 kV collision energy with CID gas on.
6. Perform database searches for peptide identification. Processing of the raw MS/MS spectra in Mascot Distiller followed by a MASCOT search against the NCBI human database (no enzyme was specified, MS peptide tolerance 100 ppm and MS/MS tolerance 0.3 Da) allows selection of peptides identified with greater than 99% confidence (^{Note 15}).

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¹³Pipette up and down at least 1 minute and swirl around the bottom to dissolve the sample well.

¹⁴Wear gloves to handle the plate. Wait until the spot shrinks to approximately 25% of its original size (about 10 minutes) but don't dry the spot completely.

¹⁵Mascot Distiller is an interface to convert mass spectrometry raw data files to MGF files to be used for protein database search through Mascot. Other software compatible with the instrument can be used for the same purpose.

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