Characterization of the low-molecular weight human plasma peptidome

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Running Title: low-molecular weight human plasma proteome

Keywords: blood; plasma; proteome; low-molecular weight; LMF; LMW; ultrafiltration; peptidome; proteomics; degradome

Abstract

The human plasma proteome represents an important secreted sub-proteome. Proteomic analysis of blood plasma with mass spectrometry is a challenging task. The high complexity and wide dynamic range of proteins as well as the presence of several proteins at very high concentrations complicate the profiling of the human plasma proteome. The peptidome (or low-molecular weight fraction, LMF) of the human plasma proteome is an invaluable source of biological information, especially in the context of identifying plasma-based markers of disease. Peptides are generated by active synthesis and proteolytic processing, often yielding proteolytic fragments that mediate a variety of physiological and pathological functions. As such, degradomic studies investigating cleavage products via peptidomics in particular, have warranted significant research interest. However, due to their molecular weight, abundance and solubility, issues with identifying specific cleavage sites and coverage of peptide fragments remain challenging. Peptidomics is currently focused towards comprehensively studying peptides cleaved from precursor proteins by endogenous proteases. This protocol outlines a standardized rapid and reproducible procedure for peptidomic profiling of human plasma using centrifugal ultrafiltration (<20,000 Da) and mass spectrometry. Ultrafiltration is a convective process that uses anisotropic semi-permeable membranes to separate macromolecular species on the basis of size. We have optimized centrifugal ultrafiltration (cellulose triacetate membrane) for plasma fractionation with respect to buffer and solvent composition, centrifugal force, duration and temperature to facilitate recovery >95% and enrichment of the human plasma peptidome. This method serves as a comprehensive and facile process to enrich and identify a key, under-represented sub-proteome of human blood plasma.

1 Introduction

Human plasma is one of the most informative and important proteomes from a clinical perspective. For example, characteristic changes in protein levels in plasma are indicative of many clinical conditions, including severe liver disease, hemolytic anemia and Down's syndrome, schizophrenia, Alzheimer's disease, amyotrophic lateral sclerosis and Creutzfeldt-Jakob disease [1]. Hence, characterization of plasma proteins (both in qualitative and quantitative terms) should provide a foundation for the discovery of candidate markers for disease diagnosis and development of new therapeutics. However, human plasma is limited by its dynamic range of protein abundances (10 orders of magnitude between the least abundant (1–5 pg/mL, e.g., interleukins, cytokines) and most-abundant (35–70 × 10^9 pg/mL, e.g., albumin, IgG [2]). For example, albumin and immunoglobulin G constitute approximately 51–71% and 8–26% of the total protein content in human plasma, respectively [3]. This complexity creates extensive difficulties in the use of many proteomic separation tools (e.g., free-flow electrophoresis, 10^5 [4]) for the identification of low-abundance species directly in plasma (overview: [2]). The strategies that have been most frequently used to overcome this issue of dynamic range are to fractionate the plasma proteome into smaller subsets (sub-proteomes), and/or to deplete one or more of the major proteins. Immunoaffinity is an established method that addresses the dynamic range of plasma by specific depletion of high-abundance proteins [5]. However, although the efficiency of immunodepletion ranges from 96-99%, the remaining concentration of albumin, for example, would still be ~50-1,000 μg/mL – a value ~10^4-fold higher than blood CEA levels (~5 ng/mL) and 5 × 10^6-fold higher than blood IL-6 levels (~10 pg/mL). Hence, mass spectrometry-based detection of most already known biomarkers in blood requires deployment of additional separation/enrichment technologies.

The peptidome (also known as degradome or low-molecular weight fraction, LMF) is defined as a subset of the proteome that consist of peptides formed through the proteolysis of proteins to release latent bioactive peptides. This targeted proteolytic digestion of proteins can release peptides that have distinct activity compared to the precursor protein. The proteolysis directed towards proteins occurs through the catalytic activity of system protease enzymes and protease inhibitors. It is well known that protease activity is extremely selective and the enzyme is directed to a site-specific sequence of amino acids to be able to cleave the protein into multiple peptides; and in addition each individual protease has optimal conditions under which it can exert its activity. Known plasma polypeptides such as the defensins, bioactive peptides like glucagon, insulin, growth hormone, and neuropeptides are involved in a variety of biological functions. The LMF also contains proteolytic peptide fragments of several abundant proteins such as albumin, transthyretin, and the apolipoproteins [6,7]. To this aim the plasma or serum proteome has been the focus of recent attempts to identify low abundance and low-molecular weight endogenous peptides which hold diagnostic and prognostic potential [8-12].

Extracellular proteolysis represents a dynamic role in cell regulation, signalling, and tissue homeostasis. In cancer, proteolytic activity is an important component regulating intercellular communication throughout the surrounding microenvironment, with altered proteolysis promoting deregulated tumour growth, tissue remodelling, inflammation, tissue invasion, and metastasis [13]. Ectodomain shedding and intramembrane proteolysis are becoming critical elements of many diverse intra- and intercellular signalling events mediated by small peptide fragments [12]. The presence of bioactive components in human plasma and serum as a consequence of cancer and

other pathological processes has been well documented [6,8-10,14-18]. Recently, efforts have been directed towards developing high throughout proteomic screens to identify protease cleavage events and protease substrates in complex biological samples. However, due to their molecular weight (<3kDa) and solubility, issues with identifying specific cleavage sites and coverage of peptide fragments remain challenging. Peptidomics is currently focused towards comprehensively studying peptides cleaved from precursor proteins by endogenous proteases [19-29].

Of the studies investigating the plasma peptidome, issues with membrane selectivity, centrifugal conditions, buffers and solvents, and filtrate heterogeneity and contamination with abundant, high-M_r plasma proteins have limited enrichment and characterization of the peptidome [11,19,30-33]. Centrifugal ultrafiltration has been the most widely-used method to extract peptides and remove proteins with higher molecular weights from plasma/serum based on a size-exclusion filtration mechanism [30,31,34-36]. Typically, membranes have a mean pore size between 10 Å and 500 Å (or 1.0 nm and 50 nm). Here, we report a strategy to selectively and rapidly isolate and identify the human plasma peptidome using centrifugal ultrafiltration and mass spectrometry (**Figure 1**).

2 Materials

Throughout the protocol, Milli-Q deionized water (HPLC grade, ≥18 MΩ) should be used for making up all aqueous solutions. All washing, lysis and HPLC buffers should be prepared using clean glassware on the day analysis is to be performed.

2.1 Blood collection, plasma preparation and storage (Notes 1-3)

1. EDTA Blood Collection Tubes (e.g., BD Vacutainer)
2. Polypropylene tubes (1.5 mL, 15 mL)
3. Freezer (-80 °C or lower)
4. Gloves, gown, eye protection
5. Pipettes
6. Disposal container for contaminated tubes
7. Centrifugation unit (either/or bench-top / swing bucket rotor – compatible with 1.5/15 mL tubes, programmable temperature setting; range 4-25 °C)
8. Labels for blood sample tubes
9. Alcohol (70% (v/v) aqueous ethanol) and swabs for cleaning venipuncture site
10. Micro BCA Protein Assay Kit, sufficient reagent to perform 480 standard tube assays or 3,200 microplate assays (Pierce)
11. Water bath, or incubator set at 37 °C

2.2 Centrifugal ultrafiltration

1. Centrifugation unit (bench top series – compatible with 1.5 mL tubes, programmable temperature setting; range 4-25 °C)

2. Centrifugation unit (swing bucket rotor – compatible with 15 mL tubes, programmable temperature setting; range 4-25 °C)
3. Centrifugal ultrafiltration membranes – Vivaspin-2® MWCO of 20,000 (Cellulose triacetate (CTA), Sartorius Stedim Biotech, Aubagne, France) (**Notes 4-5**). For selecting the correct NMWL of the filtration membrane device **Notes 6-7**
4. Acetonitrile, HPLC grade (Fisher, A998-1 or equivalent) (**Note 8**)
5. Water, HPLC grade (Fisher, W5-1 or equivalent)

2.3 SDS-PAGE

1. Laemmli non-reducing sample buffer (0.2M Tris-HCl, 40% (v/v) aqueous glycerol, 4% SDS, trace bromophenol blue)
2. Heat block – up to 95 °C (compatible with 1.5 mL centrifuge tubes)
3. NuPAGE® LDS sample buffer (Invitrogen), store at 4 °C
4. 1D-Gel apparatus (Invitrogen Novex Mini-Cell)
5. Precast SDS polyacrylamide 12-well, 1.5 mm gel (4-12 % Bis-Tris precast gel, Invitrogen)
6. 20× NuPAGE® MES SDS running buffer (Invitrogen): 50 mM MES, pH 7.2, 50 mM Tris-NaOH, 0.1% SDS, 1 mM EDTA, pH 7.3, stored at room temperature (RT). Add 25 mL 20× running buffer to 475 mL water for 1× SDS running buffer
7. Benchmark or Mark 12 protein standard mix, store at 4 °C

2.4 Protein visualization

1. SilverSNAP® Stain Kit II gel stain, sufficient reagents to stain up to 20 SDS-PAGE mini-gels (Pierce)

2. Fixing solution, 30% (v/v) aqueous ethanol containing 10% (v/v) aqueous acetic acid (> 99.7%, Sigma-Aldrich, Saint Louis, MO)
3. Personal Densitometer SI (Molecular Dynamics)
4. Coomassie R-250, 1 L sufficient reagent for up to 50 mini-gels (Imperial Protein Stain, Pierce Biotechnology) (**Note 9**)
5. ImageQuant™ software (Molecular Dynamics)

2.5 In-gel Digestion and Peptide Extraction

1. Imperial™ Protein Stain (Pierce, Thermo Fisher Scientific)
2. GridCutter (The Gel Company, San Francisco, CA)
3. Protein LoBind Tubes - 1.5 ml microcentrifuge tube (low protein binding) or Protein LoBind Plates
4. 100 mM ammonium bicarbonate: 0.4 g NH4HCO3 in 50 ml water. Prepare fresh for every digest
5. 50 mM ammonium bicarbonate/acetonitrile (1:1 v/v)
6. 50 mM ammonium bicarbonate in water
7. 10 mM DTT (dithiothreitol) in 100 mM ammonium bicarbonate (7.5 mg DTT). Pre-weighed DDT can be stored at -20 °C
8. 50 mM IAA (iodoacetamide) in 100 mM ammonium bicarbonate (10 mg IAA)
9. Trypsin solution: dissolve content of a 20 µg vial (V5111, 5 × 20 µg, Promega) in 1.5 ml of trypsin buffer (10 mM ammonium bicarbonate, 10% (v/v) acetonitrile) and keep on ice. The concentration of trypsin is 13 ng/µl. 2 ml trypsin buffer: 10 mM ammonium bicarbonate, 10% (v/v) acetonitrile

10. 5% (v/v) formic acid in water
11. Extraction buffer: 0.25 ml 5% (v/v) formic acid, 0.25 ml water, 0.5 ml acetonitrile
12. Thermomixer temperature range up to 56 °C
13. Thermostat oven at 37 °C
14. Sonicator
15. Vacuum centrifuge (lyophiliser)
16. STAGE-Tip / desalting column - remove small discs (2-3) of C18 Empore filter using a 22 G flat-tipped syringe and ejecting discs into P200 pipette tips. Ensure that the disc is securely wedged in the bottom of the tip. Condition the columns (wet membrane) for each sample by using extraction buffer.
17. MS sample vials with snap lid (#THC11141190, Snap ring vial with glass insert, Thermo Fisher Scientific)

3 Methods

3.1 Blood collection (Notes 1-3)

1. It is important to obtain the required volume of blood using each specific blood collection tube type. This is essential to ensure the blood to anticoagulant ratio is not exceeded. The blood collection should be completed within 5 ± 2 min from the starting time
2. After blood collection, gently mix the unit by inverting the tube 8-10 times
3. Label the donor collection tube(s). If storage is required, do so immediately at -20 °C

4. Thawing of the plasma sample on the day of use should be performed at 37 °C (not at room temperature or on ice) (**Notes 10-11**). This is to prevent the formation of cryoprecipitate.
5. The protein concentration of the plasma sample when thawed should be determined. For consistency the bicinchoninic (BCA) protein assay, using bovine serum albumin (BSA) as a standard should be used [37].

3.2 Centrifugal ultrafiltration

1. Prepare centrifugal filter membranes according to manufacturer's instructions by rinsing in 15 mL of HPLC grade water at 2,000 × g for 10 min (**Note 12**). Set centrifugal temperature to 20 °C. Twist off the lock cap and remove the inner tube (filtrate collector). Make sure not to touch or bend the membrane. If the device is not to be used immediately, store at 4 °C with Milli-Q water covering the membrane surface.
2. Dilute 100 μL of thawed plasma with 900 μL 10% (v/v) aqueous acetonitrile and allow to stand at RT for 2 min (**Note 8**). Centrifuge each plasma sample (with a counterbalance) at 14,000 × *g* for 2 min at RT to precipitate any insoluble material that may clog the filters.
3. Apply the supernatant to the prepared centrifugal filter(s) and samples placed in an M4 swing bucket rotor and centrifuged (with a counterbalance) at 4,000 × *g* for 35 min at 20 °C (**Note 5**). A small aliquot (50 μL) of the sample was set aside in order to assess LMF recovery. This sample is stored at -80 °C.
4. The retentate (retained fraction, ~5% initial volume) should be removed and stored separately. The filtrate (flow-through fraction, ~90-95% initial volume) volume can either be removed by pipette or the filtrate recovered by inverting the tube and centrifuging at 2,000 × *g* for 1 min.

5. The LMF recoveries of the filter membrane can be analyzed by BCA protein assay [37], comparing the initial plasma concentration to the concentration and volume of both the retained (retentate) and filtered (filtrate) samples. Typical recoveries for this experiment should be in the range 94-97 % (3 experimental replicates) (**Table 1**). Retentate samples are stored at -80 °C.
6. The plasma LMF filtrates are lyophilized to dryness by centrifugal lyophilization and re-suspended in Laemmli non-reducing sample buffer.

3.3 SDS-PAGE analyzes

1. A plasma LMF protein sample (50 μg) is mixed with pre-warmed NuPAGE® LDS sample buffer (in the ratio sample: buffer; 2:1).
2. The sample mixture is heated for 5 min on a heat block at 95 °C, and cooled (2 min) prior to sample loading.
3. Separation is performed using a precast 12-well SDS polyacrylamide gel (4-12% Bis-Tris precast gel).
4. 500 mL of 1 × MES SDS running buffer is prepared – approximately 200 mL in the upper (inner) buffer compartment and 300 mL in the lower (outer) buffer compartment.
5. Samples are loaded into defined gel lanes. Benchmark protein standards (5 μL) are used for molecular weight comparison.
6. Protein separation is performed at 150 V (constant voltage) until tracking dye reaches the bottom of the gel (approx. 75 min).

7. Immediately following electrophoresis the gel should be washed with water and stained with colloidal Coomassie R-250, as described elsewhere [38]. Destain background with water.

3.4 In-gel Digestion and Peptide Extraction

1. After staining, gel sections are excised (using either scalpel or gel-excision tool with slices ~1.0-1.5 mm) from a single lane.
2. Individual gel lanes are placed on a clean glass plate and cut into equal slices (20 × 2 mm) using a GridCutter or clean scalpel and individual gel slices subjected to in-gel reduction, alkylation and trypsinisation.
3. For sample reduction, microcentrifuge tubes are centrifuged briefly, heated using a thermomixer at 56 °C (700 rpm for 15 min), and the solution discarded. 200 µl acetonitrile is added and gel pieces should shrink and take an opaque-white colour. Remove acetonitrile and let air-dry for 5 min in thermomixer at 56 °C. Add 50 µl fresh DTT solution and incubate at 56 °C for 30 min.
4. For sample alkyation set thermomixer to 22 °C and remove DTT solution completely. Immediately add 70 µl IAA solution, incubate for 20 min in thermomixer 22 °C (700 rpm) covered by aluminium foil.
5. The IAA solution is then removed, 300 µl acetonitrile added for 2 min, then removed. Add 100 µl of 50 mM ammonium bicarbonate/acetonitrile (1/1) and incubate for 30 min in thermomixer with light mixing at RT. The sample should be lightly centrifuged and supernatant removed.
6. 300 µl acetonitrile is added to the samples, removed completely, and air dried for 5 min.

3.5 Tryptic digestion and STAGE-Tip desalting

1. For tryptic digestion, add enough trypsin to cover the dry gel pieces (typically, 50-60 µl depending on gel volume). Store all samples immediately on ice. After 30 min, check if all solution is absorbed and add more trypsin, if necessary. Gel pieces should be completely covered with trypsin.
2. Leave gel pieces for another 30 min to saturate with trypsin and add 20 µl of 50 mM ammonium bicarbonate to cover the gel pieces.
3. Place tubes with gel pieces into the thermostat oven and incubate samples overnight at 37 °C.
4. Withdraw supernatants to low protein binding tubes, use a pipette with fine gel loader tip (re-use these tips for all following peptide collection steps)
5. Add extraction buffer (100 µl) to each tube and sonicate for 15 min. Collect supernatants into the corresponding tube. Repeat
6. Dry down samples (covered with Vacufilm with 4 pin holes) in vacuum centrifuge for 15 min
7. For STAGE-Tip desalting, prepare as many desalting columns as necessary by punching out small discs (2-3) of C18 Empore filter using a 22 G flat-tipped syringe and ejecting the discs into P200 pipette tips. Ensure that the discs are securely wedged in the bottom of the tip. Careful preparation of these STAGE-Tip devices will ensure effective filtration
8. Condition columns by forcing methanol through (50 µl) and check whether the STAGE-Tips are leaky.

9. Remove any remaining organic solvent in the column by forcing buffer A through the disk (×2) (40 µl).
10. Adjust pH of peptide sample to pH <2.5 using 2% (v/v) TFA
11. Force the acidified peptide sample through the C18-StageTip column.
12. Wash the column with buffer A. Elute the peptides from the C18 material using 20-30µl buffer B. Elute directly into a microfuge tube or auto-sampler plate. Repeat elution.
13. Carefully dry samples in the speed-vac without heating, until all acetonitrile has evaporated (~2-3 µl final volume). Note to not completely overdry/dehydrate peptide sample due to issues with sample loss and resolubilisation
14. Mix the sample (1:1) with sample buffer up to 8 µl
15. Withdraw to MS specific vial for analysis (typically 3 µl loaded representing ~3 µg sample). To determine peptide concentration a spectrophotometer analysis can be obtained based on 215 nm absorbance, and comparison with known standard
16. Short-term storage at 4 °C (within 2 weeks), or long-term -80 °C (up to 18 months)

3.6 MS/MS analysis

1. RP-HPLC is performed on a nanoAcquity® (C18) 150 × 0.15-mm i.d. reversed phase UPLC column (Waters), using an Agilent 1200 HPLC, coupled online to an LTQ-Orbitrap mass spectrometer equipped with a nanoelectrospray ion source [39].
2. RP-HPLC column is developed with a linear 60 min gradient with a flow rate of 0.8 µl/min at 45 °C from 0-100% solvent B where solvent A was 0.1% (v/v) aqueous formic acid and solvent B was 0.1% (v/v) aqueous formic acid/60% (v/v) acetonitrile.

3. The mass spectrometer was operated in data-dependent mode where the top 20 most abundant precursor ions in the survey scan (300–2500 Th) were selected for MS/MS fragmentation. Survey scans were acquired at a resolution of 120,000 at m/z 400. Unassigned precursor ion charge states and singly charged species were rejected, and peptide match disabled. The isolation window was set to 3 Th and selected precursors fragmented by CID with normalized collision energies of 25. Maximum ion injection times for the survey scan and MS/MS scans were 20 ms and 60 ms, respectively, and ion target values were set to 3E6 and 1E6, respectively. Dynamic exclusion was activated for 90 s.

3.7 Data processing and analysis

1. Raw data was processed using MaxQuant [40] (v1.1.1.25) and searched with Andromeda using Human-only (UniProt) sequence database.
2. Data was searched with a parent tolerance of 10 ppm, fragment tolerance of 0.5 Da and minimum peptide length 7. Database search parameters as follows: fixed modification, carboxymethylation of cysteine (+58 Da); variable modifications, NH_2-terminal acetylation (+42 Da), methionine oxidation (+16 Da).
3. FDR was 1% at the peptide and protein levels, and data examined with label-free quantitation (LFQ) [41]. LFQ intensities for all unique and razor peptides were included, with zero intensity values replaced with a constant value of 1 to calculate fold change ratios. LFQ intensity values were normalized for protein length and fold change ratios calculated.
4. Contaminants, and reverse database identifications were excluded from further data analysis. Proteins commonly identified in both replicate experiments were used to compare against other cell samples.

5. Proteins were correlated with prediction of non-classical protein secretion (SecretomeP 2.0) (http://www.cbs.dtu.dk/services/SecretomeP/) and also the Secreted Protein Database (http://spd.cbi.pku.edu.cn/spd_search.php)
6. Other resources to classify identified proteins based on several predictive algorithms included SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/), TMHMM 2.0 (http://www.cbs.dtu.dk/services/TMHMM/), and Gene Ontology (GO) (http://www.geneontology.org/index.shtml?all/), and the UniProt database (http://www.uniprot.org/)
7. For an in-depth resource of the Human Plasma Proteome Project, including extensive collection of RAW data refer to PeptideAtlas (http://www.peptideatlas.org/hupo/hppp/)

4 Notes

1. **Sample hemolysis.** The release of cellular material due to hemolysis into serum / plasma may introduce additional confounding factors. We recommend that if hemolysis (pink to red tinge in serum / plasma sample) is observed following centrifugation, this information should be recorded. It is recommended that hemolyzed samples are not used for proteomic/peptidomic analyses.

2. **Monitoring pre/post-analytical variation**. In 2005, the HUPO PPP report detailed an extensive analysis of the variables affect the stability of plasma [42]. These included - (i) the anticoagulant used in collection tube types (e.g., EDTA and ascorbate), - (ii) sample processing times and - (iii) temperatures at which blood specimens were processed and stored, - (iv) sample storage parameters, and - (v) thaw/refreeze cycles, associated with obtaining human plasma and serum samples for proteomic analyses directed towards clinical research. It is of upmost importance that for diagnostic use, these variables are controlled and monitored at all times, from blood collection as an anticoagulated or coagulated source, processing, handling and storage [43-45]. Recently, it has been shown that biomarker validation studies should use standardized collection conditions, and use multiple control groups to detect and correct for potential biases associated with sample collection [46].

3. **Data points.** For blood handling it is important to note also (a) the date and time of blood collection, (b) the number and volume of samples/aliquots prepared, (c) the date and time

placed at -80 °C, (d) the date and time of shipping, (e) any freeze-thaw cycles that occur, and (f) variations or deviations from the standard-operating protocol, problems, or issues which arise.

4. **Centrifugal ultrafiltration membrane devices.** A wide range of centrifugal filters are commercially available for concentrating and filtrating protein solutions, removing small solutes, and/or buffer exchanging. These devices consist (mostly) of two chambers separated by a semi-permeable membrane. These membranes can be composed of different chemistries and different orientations depending on their application (**Note 7**). Under centrifugal force, solvent and solute molecules smaller than the NMWL readily pass through the membrane (filtrate) (**Note 6**). Vertical or angular membrane configuration reduces concentration polarization (membrane fouling) and allows high flow rates for optimal solvent passage even with high proteinaceous solutions. The direction of the centrifugal force and flow-rate of solute differs between membrane devices used. We evaluated plasma peptidome fractionation efficiency using various centrifugal ultrafiltration devices, noting significant differences in the protein recoveries of different devices and membrane types (**Table 1**). The separation efficiency of these four filtration devices were assessed by 1D-SDS-PAGE using a starting volume of 100 μL of human plasma. As noted in **Figure 2** that significant amounts of high-molecular and abundant plasma proteins are retained by the optimized ultrafiltration membrane device (device D), enabling selective enrichment and recovery of the low-molecular weight plasma proteome. Additional information regarding centrifugal ultrafiltration membrane devices and their membrane chemistry can be obtained from www.millipore.com/ and www.sartorius.com/

5. **Optimized centrifugal ultrafiltration.** Conditions for each plasma sample should be optimized. Conditions provided in this protocol are the combined effect of analyzing multiple filter membrane units, with conditions optimized with respect to plasma buffer and solvent compositions, centrifugal force, duration and temperature. Typically, plasma LMF should represent 95% of the initial supernatant applied to the filtration devices. The amount of protein recovered in the filtrate and retentate can be calculated as a percentage of the initial plasma protein concentration loaded (**Table 1**).

6. **Appropriate membranes – Selecting the NMWL.** Ultrafiltration membranes are not absolute in their pore size (NMWL) ratings. Separation occurs as a result of differences in the filtration rate of different components across the membrane in response to a given pressure. Unlike UF membranes, microporous membranes have a precisely controlled pore size that ensures quantitative retention of particles, and biomolecules greater than the pore size of the membrane. In selecting the most effective membrane for filtration applications, a rule has been developed to rapidly calculate the appropriate membrane pore size (NMWL). It is a simple calculation based on the molecular weight of the desired protein to be concentrated or removed in the retentate unit (upper level of the membrane apparatus). The "rule of 1.5-2" requires a membrane cut-off approximately two times smaller than the desired proteins molecular weight. For example, to remove proteins of ~65,000 MW and greater use a 30,000 NMWL regenerated cellulose membrane. Typically, this results in >90-95% recovery of the filtrate, containing proteins/peptides

<65,000 MW. Other factors to consider when determining an optimal membrane include flow rate, also known as flux, solute concentration, solute composition, and temperature.

7. **Centrifugal ultrafiltration membrane chemistries.** For a detailed overview please refer [47].

Polyethersulfone **-** General purpose membrane, providing excellent performance with most solutions when retentate recovery is of primary importance. Polyethersulfone membranes exhibit no hydrophobic or hydrophilic interactions and are usually preferred for their low fouling characteristics, exceptional flux and broad pH range.

Cellulose triacetate **-** High hydrophilicity and very low non-specific binding characterize this membrane. These membranes are preferred for sample cleaning and protein removal and when high recovery of the filtrate solution is of primary importance.

Regenerated cellulose/Hydrosart **–** These membranes demonstrates the same properties as regenerated cellulose, but with the added benefit of enhanced performance characteristics and extremely low protein binding, making it the membrane of choice for applications such as concentration and desalting of immunoglobulin fractions.

8. **Disrupting protein–protein interactions.** A low concentration of organic solvent (typically, 5-10 % acetonitrile) is added to buffers to disrupt high-M_r protein–protein interactions. For chemical compatibility of membranes be careful to read each companies manual prior to operation (based on 2 h membrane contact time). Normally, small uncomplexed proteins and peptides (i.e., less than 30 K) are rapidly cleared from the circulation through enzymatic degradation, uptake by the reticuloendothelial system or by

glomerular filtration, which discriminates on the basis of molecular size and charge [48]. It is believed that the circulation half-life of the LMF fraction is directly related to its binding affinity to large high-abundance carrier proteins [7,10].

9. **Coomassie dye staining.** The Coomassie dyes (R-250 and G-250) bind to proteins through ionic interactions between dye sulfonic acid groups and positive protein amine groups. Coomassie R-250, the more commonly used on the two dyes, can detect protein levels down to 0.1 µg. Additionally, Coomassie R-250 does not require methanol/acetic acid fixation and destaining.

10. **Cryoprecipitate formation.** A cryoprecipitate is often formed if the fresh-frozen plasma unit is slowly thawed at temperatures just above freezing (1-6 °C), typically in a water bath or a refrigerator. The product is then centrifuged at low-speed (typically 5,000 × *g*) to remove the majority of the precipitate. The formation of the cryoprecipitate can be avoided by thawing at 37 °C.

11. **Plasma thaw process.** Thawing of plasma can be achieved various ways, the most common of which uses a re-circulating water bath. This carries a risk of bacterial contamination and must be maintained according to a controlled sterile environment. Denaturation of plasma proteins can be avoided by using a dry-heating apparatus.

12. **Pre-rinsing membranes.** Most ultrafiltration membrane devices contain trace amounts of glycerine/sodium azide. If this interferes with subsequent sample analysis, pre-rinse the

device extensively with buffer or Milli-Q water through the concentrator. If interference still persists, rinse the membrane with 0.1 M NaOH followed by repeated centrifugation with buffer or Milli-Q water.

Figure Legends

Figure 1 - Isolation and characterization strategy for the comprehensive analysis of the low-molecular weight human plasma peptidome. The efficiency of the optimized method is demonstrated by the identification of the low-abundance classically-secreted proteins cystatin-C (plasma concentration 0.62–1.02 μg/mL), CXCL7, serine protease inhibitor A3, and cystatin-M. In all, 48% of the proteins identified in the rapid processing and analysis of the plasma peptidome are known secreted proteins.

Figure 2 - Low molecular weight / peptidome analysis of human plasma. One hundred microliters (100 μL) of platelet-poor plasma was diluted 1:9 with 900 μL of 10% ACN, pH 8.5 v/v. This sample was fractionated using a prepared low-protein binding Vivaspin-2 20K MWCO membrane filter at 4,000 × *g* until 95% of the input plasma had passed through the 20K (cellulose triacetate) filter. Aliquots of whole (Lane P) or ultrafiltered plasma (filtrate, Lane F and retentate, Lane R) were subjected to 1-DE, and stained using silver staining. Lane M, Benchmark molecular weight marker.

Acknowledgements

This work was supported, in part, by the National Health and Medical Research Council (NHMRC) of Australia project grant #1057741 (RJS), La Trobe University Leadership RFA Grant (DWG), and La Trobe Institute for Molecular Science Biomedical Fellowship (DWG). We acknowledge the La Trobe University-Comprehensive Proteomics Platform for providing infrastructure and expertise.

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