Removal of microparticles and common plasma proteins

Activated platelets were removed by centrifugation at 341 x g for 15min and supernates were filtered through the Steriflip® (Millipore, Billerica, MA), sterile disposable vacuum filtration system to remove residual platelets. Microparticles were removed by centrifugation at 100,000 x g for 1 hour at 4°C. Remaining proteins were concentrated using an Amicon® Centrifugal filter device (3kDa MW cut-off; Millipore). The ProteoPrep® 20 Jumbo Plasma Immunodepletion Kit (Sigma, St Louis, MI), designed to remove 20 highly abundant proteins from biological fluids, was used to deplete highly abundant proteins from the platelet supernatants in the first proteomic approach (Strategy 1; Figure 1). In the second approach (Strategy 2; Figure 1), 12 highly abundant plasma proteins were removed using the ProteomeLab IgY Proteome Partitioning Kit (Beckman Coulter, Fullerton, CA). Depleted platelet supernatants were concentrated as described above. Protein concentration was determined using the BCA™ Protein Assay Kit (Pierce, Rockford, IL).

In-gel trypsin digestion and peptide extraction

Proteins (10µg) concentrated after immunodepletion with the ProteoPrep® 20 Immunodepletion column were separated by electrophoresis on 4-12% pre-cast BisTris gels (NuPAGE™, Invitrogen) and stained with colloidal blue (Invitrogen). Gel slices (20 per lane) were excised and the protein digested with trypsin, before being subjected to reverse-phase liquid chromatography and tandem mass spectrometry[17].

In-solution trypsin digestion of protein samples

Fractions from the flow through of the ProteomeLab IgY column were concentrated and buffer exchanged against 6M urea, 2M thiourea using 3k MWCO spin filters. Protein disulfide bonds were reduced with 5mM dithiotheitol (DTT) (Bio-Rad Life Sciences, Hercules, CA) at 56°C for 45min. The solution was then cooled to room temperature and the free cysteines alkylated with 15mM iodoacetamide (IAA, Bio-Rad Life Sciences) in the dark for 30min at 56°C. Following incubation, the solution was diluted with 50mM ammonium bicarbonate buffer until the final concentration of urea was less than 1M. In-solution digestion was performed for each of the samples using sequencing grade trypsin (Promega, Madison, WI), at an enzyme to protein ratio of 1:50 (w/w), in 50mM

ammonium bicarbonate for 18h at 37°C. Following overnight incubation the solutions were dried under reduced pressure in a Vacuum Concentrator (Jouan).

Fractionation of peptides by SCX (strong cation exchange) chromatography

Each of the dried peptide hydrosylates was resuspended in 1ml of SCX mobile phase A (0.1% formic acid in water). SCX chromatography was performed on a PolySulfethy A column (100mm × 2.3mm, 5µm 300Å, PolyLC, The Nest Group, Southborough, MA) attached to a 1100 Series HPLC (Agilent, Santa Clara, CA). Samples were loaded for 10min with mobile phase A, followed by a step/wash gradient to 100% mobile phase B (800mM ammonium formate, 25% acetonitrile, pH6.8). Ten fractions were collected for each of the mobile phase steps every 6min. The collected fractions were lyophilized and stored at −80°C until further analysis.

Reversed-phase capillary liquid chromatography tandem mass spectrometry analyses

Samples depleted using the ProteoPrep® 20 Jumbo Plasma Immunodepletion Kit and subjected to in-gel trypsin digest were subjected to reverse phase separation of the tryptic peptides and tandem MS analysis. LC-MS/MS was performed on a LTQ-Orbitrap XL™ mass spectrometer (Thermo Fisher, San Jose, CA) coupled with a nanoLC pump and autosampler (Eksigent Technologies, Livermore, CA). Tryptic peptides were separated by RP-HPLC on a nanocapillary column, 75µm id x 15cm PicoFrit (New Objective, Woburn, MA), packed with MAGIC C18 resin, 5µm particle size (Michrom BioResources, Auburn, CA). Solvent A was 0.1% formic acid in Milli-Q water, and solvent B was 0.1% formic acid in acetonitrile. Peptides were eluted at 200nl/min using an acetonitrile gradient consisting of 3-28% B over 42min, 28-50% B over 26min, 50-80% B over 5min, 80% B for 5min before returning to 3% B in 1min. To minimize sample carryover to the next LC-MS/MS run, a blank was run between each sample. The mass spectrometer was set to perform a full MS scan (*m/z* 400 – 2000) in the Orbitrap, and the six most intense ions exceeding a minimum threshold of 1,000 were selected for MS/MS in the linear trap using an isolation width of 2.5Da. The FT master scan preview and monoisotopic precursor selection were enabled, and singly charged ions were excluded from MS/MS analysis. Ions subjected to MS/MS were excluded from repeated analysis for 45sec.

Samples after SCX chromatography were reconstituted with 0.1% formic acid, 1% acetonitrile for reversed phase separation (Dionex Ultimate 3000 nanoflow LC system,

Sunnyvale, CA) on-line to the Thermo Finnigan LTQ-FT™ linear ion trap mass spectrometer (Thermo Fisher, San Jose, CA) using ESI. A Michrom Beta ADVANCE nanoelectrospray ionization source (Michrom Bioresources, Inc., Auburn, CA) was used to introduce HPLC eluent into the mass spectrometer. The reversed-phase separation was performed on a Halo 2.7µ 90A C18 .075 x 100mm (Michrom Bioresources, Inc., Auburn, CA) capillary column. 10µl of the reconstituted peptide solution for each fraction was loaded onto a Peptide Captrap (Michrom Bioresources) with 1% aqueous acetonitrile containing 0.1% formic acid at 10µl/ min flow rate and washed for 5min prior to elution onto the analytical column. Peptides were eluted with 95% mobile phase A for 10min at a flow rate of 1µl/min. A linear gradient in 70% mobile phase B was applied over 90min. The LTQ-FT™ mass spectrometer was operated in a data dependent scanning mode in which the seven most intense precursor ions from the FT-ICR cell full scan MS (*m*/*z* 300 − 2000) analysis were subjected to the collision-induced dissociation (CID) MS/MS analysis in the linear ion trap. Ions subjected to MS/MS were excluded from repeated analysis for 45sec.

Data analysis

Raw data from both LC-MS/MS runs was searched against a protein sequence database consisting of the Human International Protein Index (IPI) v3.51 protein sequences (74049 protein entries; 31,194,560 amino acids) and the reversed Human IPI sequences as a decoy component. Two independent algorithms, SEQUEST 3.1 (ThermoFinnigan) and MASCOT 2.1.04 (Matrix Sciences) were used. Raw mass spectra were converted to DTA peak lists using BioWorks Browser 3.2 (ThermoFinnigan) with the following parameter settings: peptide mass range 300-5000Da, threshold 10, precursor mass ±1.4Da, group scan 1, minimum group count 1, minimum ion count 15. Searches specified that peptides should have a maximum of two internal tryptic cleavage sites, with methionine oxidation and cysteine carbamidomethylation as possible modifications. SEQUEST searches specified that peptides should possess at least one tryptic terminus, and used a peptide mass tolerance of ± 0.25Da and a fragment ion tolerance of 0. MASCOT searches specified tryptic digestion, and used a peptide mass tolerance of ± 20ppm and a fragment ion tolerance of ±0.1Da. Peptide identification probabilities for both SEQUEST and MASCOT searches were calculated by executing PeptideProphet (Trans-Proteomics Pipeline) with the tags -Old -dREV for SEQUEST and -Old -dREV -l1 for MASCOT searches. Results from both searches (SEQUEST and MASCOT) and replicate analysis (1D PAGE and 2D chromatography) were combined in a single statistical analysis of protein expression per developmental stage

using the Empirical Bayes Protein (EBP) Identifier 1.0[18]. EBP is based on a statistical model that implements consensus scoring of peptide identifications from multiple search algorithms and combines information from independent replicate experiments to estimate sensitivity and false identification rate of less than 5% are reported. Spectral counts of diagnostic peptides were calculated for each identified protein in each experimental condition as a semiquantitative estimate of protein abundance.