Quantification and trypsin digestion of polypeptides

Protein concentration was estimated by Bradford assay, and 100µg of total protein from each sample was subjected to in-solution trypsin digestion to generate peptides. Initially, to reduce disulfide bonds the samples were treated with 5µl of 100mM dithiothreitol in 50 mM ammonium bicarbonate for 30 min at 60ºC and alkylation with 200mM iodoacetamide in 50 mM ammonium bicarbonate at room temperature for 30 minutes. Proteins were then digested with 4µg of sequencing grade-modified trypsin (Sigma) in 50 mM ammonium bicarbonate by incubating overnight at 37ºC. The trypsin digestion reaction was stopped by 1µl of 100% formic acid. The digested peptide solutions were centrifuged at 14000 rpm for 12 minutes, and the collected supernatant was stored at -20ºC until the LC/MS/MS analysis.[14]

Liquid Chromatography

The peptide samples were analyzed by nano-LC/MS^E (MS at elevated energy) using a nano ACQUITY UPLC® System (Waters, Hertfordshire, UK) coupled to a Quadrupole-Time of Flight (Q/TOF) mass spectrometer (SYNAPT-G2, Waters). Both the systems were operated and controlled by MassLynx4.1 SCN781 software (Waters). In the nano-LC, the peptides were separated by reverse phase column chromatography. Briefly, 3 µl of each sample, equivalent to 3 µg of protein, was injected in "partial loop" mode and was loaded into the reverse phase column with 0.1% formic acid in water as mobile phase A, and 0.1% formic acid in acetonitrile as mobile phase B, using the binary solvent manager. The sample was then trapped in the trap column (Symmetry® 180 µm × 20 mm C_{18} 5 µm, Waters) to remove any salt by employing a high flow rate (15 µl/minute) with 99.9% mobile phase A and 0.1% mobile phase B for 1 min. The peptide separation was performed on a 75 µm × 100 mm BEH C_{18} column (Waters), with particle size of 1.7 µm. A gradient elution with 1–40% mobile phase B, for 55.5 min at 300 nl/minute flow rate, was employed. After separation, the column was washed with 80% mobile phase B for 7.5 min and re-equilibrated with 1% mobile phase B for 20 min. The column temperature was maintained at 40°C. Three biological replicates for each condition were performed and each sample was run in triplicates [14]

Mass Spectrometry

Peptides eluted from the nano-LC was subjected to mass spectrometric analysis on a SYNAPT® G2 High Definition MS™ System (Waters). The following parameters were used: nano-ESI capillary voltage, 3.3 KV; sample cone, 35 V; extraction cone, 4 V; transfer CE, 4 V; trap gas flow, (2 ml/minute); IMS gas (N_2) flow, (90 ml/minute). Mobility separation was performed after setting IMS T-Wave™ pulse height to 40 V during transmission and the IMS T-Wave™ velocity to 800 m/s. The traveling wave height was ramped over 100% of the IMS cycle between 8 and 20 V. Solution of 500 fmole/µl of human [Glu^1]-Fibrinopeptide B (Sigma-Aldrich) was used to calibrate time of flight analyzer (TOF), and the lock mass acquisition was performed every 30 s by the same peptide delivered through the reference sprayer of the nano-Lock Spray source at a flow rate of 500 nl/minute. This calibration set the analyzer to detect ions in the range of 50–2000 *m*/*z*. The mass spectrometer was operated in the "resolution mode" with a resolving power of 18,000 FWHM, and the data acquisition was performed in "continuum" format. The data was acquired by rapidly alternating between two functions: Function-1 (low energy) and Function-2 (high energy). In Function-1, only low energy mass spectra (MS) were acquired and in Function-2, mass spectra at elevated collision energy (MS^E) with ion mobility were acquired. In Function-1, collision energy was set to 4 V in the trap region and 2 V in the transfer region. In Function-2, collision energy was set to 4 V in the trap region and is ramped from 20 V to 45 V in the transfer region. Each spectrum was acquired for 0.9 s with an interscan delay of 0.024 s[14].

Data Analysis Tools

The LC/MS^E data was analyzed using ProteinLynx Global SERVER™ v2.5.3 (PLGS, Waters) for protein identification as well as for the relative protein quantification. Data processing included lock-mass correction post acquisition. Noise reduction thresholds for low energy scan ion, high-energy scan ion, and peptide intensity were fixed at 150, 50, and 500 counts, respectively. The database *Homo sapiens* downloaded from NCBI was used for database search. The parameters for protein identification were made in such a way that a peptide was required to have at least one fragment ion match, a protein was required to have at least three fragment ion matches, and at least two peptide matches for identification. Mass tolerance was set to 10 ppm for precursor ions and 20 ppm for fragment ions. Oxidation of methionine was selected as the variable modification and carbamidomethylation of cysteine was selected as the fixed modification. Trypsin was chosen as the enzyme with a specificity of one missed cleavage. The

false positive rate (FPR) of the algorithm for identification was set to 4% with a randomized database, appended to the original one. Only those proteins with 50% or more probability to be present in the mixture and detected with a score above 20, as calculated by the software, were selected for proteomic analysis. Data sets were normalized using the "auto-normalization" function of PLGS, and label-free quantitative analysis was performed by comparing the normalized peak area/intensity of the identified peptides. Thus, http://david.abcc.ncifcrf.gov/ parameters such as score, sequence coverage, and number of peptides were obtained for each protein. Furthermore, only those proteins with a fold change higher than 30% difference (ratio of either <0.70 or >1.3) were expressed in a significantly altered manner [14].