

Sample Preparation. Samples A and B were dried *in vacuo*, resuspended in 20 μ L of LDS, reduced with DTT and heated in a 70-80°C water bath for approximately 10 min. The samples were electrophoresed approximately 2 cm into a 10% Bis Tris gel, stained with Colloidal Blue followed by destaining in water. The gel regions were excised and subjected to in-gel trypsin digestion. Briefly, the gel regions were excised and washed with 100 mM ammonium bicarbonate for 15 minutes. The liquid was discarded and replaced with fresh 100 mM ammonium bicarbonate and the proteins reduced with 5 mM DTT for 20 minutes at 55° C. After cooling to room temperature, iodoacetamide was added to 10 mM final concentration and placed in the dark for 20 minutes at room temperature. The solution was discarded and the gel pieces washed with 50% acetonitrile/50 mM ammonium bicarbonate 20 minutes, followed by dehydration with 100 % acetonitrile. The liquid was removed and the gel pieces were completely dried, re-swelled with 0.3 μ g of modified trypsin (Promega) in 100 mM NH_4HCO_3 , and digested overnight at 37°C. Peptides were extracted by three changes of 60% acetonitrile/0.1% TFA, and all extracts were combined and dried *in vacuo*. Samples were reconstituted in 30 μ L 0.1 % formic acid for LC-MS-MS analysis.

LC-MS-MS Analysis. Peptides were analyzed using a ThermoFisher Orbitrap instrument equipped with an Eksigent pump and autosampler. Peptides were separated on a packed capillary tip (Polymicro Technologies, 100 μ m X 11 cm) with Jupiter C18 resin (5 μ m, 300 Å, Phenomenex) using an in-line solid-phase extraction column (100 μ m X 6 cm) packed with the same C18 resin [using a frit generated with liquid silicate Kasil 1 (1)] similar to that previously described (2). Mobile phase A consisted of 0.1 % formic acid and Mobile phase B consisted of 0.1% formic acid in acetonitrile. A 95 min

gradient was performed with a 10 min washing period at 1.5 uL/min of 100 % A during which time the flow was diverted to waste to allow for solid-phase extraction and removal of any residual salts. After the initial ten minutes, the flow was then decreased to 700 nL/min and directed into the mass spectrometer. Following the washing period, the gradient was increased to 25% B by 50 min, followed by an increase to 90 % B by 65 min and held for 9 min before returning to the initial conditions. Tandem spectra were acquired using a data dependent scanning mode in which one full MS scan (m/z 400-2000) was acquired on the FTMS followed by 5 MS-MS scans on the LTQ. Tandem spectra were searched against the human subset of the UniREF100 database and also a combined database against the human subset of the IPI and the e coli subset of UniREF100 using the SEQUEST algorithm. The databases were concatenated with the reverse sequences of all proteins in the database to allow for the determination of false positive rates. The Sequest output was then filtered using IDPicker using a false positive ID threshold (default is 0.05 or 5% false positives) based on reverse sequence hits in the database (3). Protein reassembly from identified peptide sequences is done with the aid of a parsimony method recently described by Zhang et al. (4), which identifies indiscernable proteins (protein groups) that can account for the identified peptides.

Results. The major constituents identified in each sample were advanced glycosylation end product-specific receptor precursor and calmodulin. The advanced glycosylation end product-specific receptor precursor had 50.2% and 62.1% sequence coverage in the 5ug and 10 ug samples, respectively. The calmodulin had 33.8% and 23.6% sequence coverage in the 5 ug and 10 ug sample, respectively. Based upon spectral counts per

protein, it appears that the 5 ug sample consists primarily of the advanced glycosylation end product-specific receptor precursor protein. Although the advanced glycosylation end product-specific receptor precursor protein is also found in the 10 ug sample, there appears to be a more calmodulin as compared to the 5 ug sample.

References

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