Measurement of Protein Levels in Cerebrospinal Fluid Using Two Quantitation Strategies: ICAT-Labeling and Relative Peak Areas from Ion Chromatograms

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Overview

Purpose: The applicability of two different quantitation approaches to measure protein levels in human cerebrospinal fluid (CSF) is explored with emphasis on those proteins that are involved in migraines.

Methods: We applied the cleavable isotope-coded affinity tag (ICAT) method as well as a simple proteolyis to human CSF for protein identification via 1-D LC/MS/MS.

Results: The applicability of the ICAT method to CSF and migraine-related proteins is limited due to low reproducibility and an overall decrease in the number of proteins observed– leading to an inability to monitor the CSF proteins involved in migraine. In contrast, simple 1-D LC/MS/MS experiments of unmodified CSF digests provide data that can be used to monitor a number of migraine related proteins.

Introduction

The cerebrospinal fluid (CSF) proteome provides a readily accessible window into the health state of the central nervous system (CNS). Diseases involving the CNS markedly and characteristically alter the concentrations and isoform patterns of CSF proteins (Andreasen et al., 1999). We have recently used 2D-PAGE methods to determine that the amounts of 10 CSF proteins change significantly in the acute migraine attack (Harrington et al., 2003). The goal of this study is to determine the best LC/MS/MS methodology for monitoring changes of these and other proteins quantitatively. In this presentation we explore the use of cleavable ICAT tagging as well as a simple 1D LC/MS of unmodified CSF for reproducible detection of migraine associated proteins in CSF. Good reproducibility of the overall number of proteins observed and the ability to monitor a significant number of the CSF proteins involved in migraines are the criteria by which the techniques were evaluated.

Methods

Preparation of unmodified CSF protein digests: A sample of CSF (135 µg total protein) was dissolved in 6M urea and then reduced with dithiothreitol (DTT, 30 µg) at room temperature for 1 hour. Next, 100 µg of iodoacetamide was added and then incubated at room temperature in the dark for 1 hour. Next, excess iodoacetamide was removed by adding 80 µg of DTT followed by incubation at room temperature for 1 hour. Excess reagents were removed and the buffer exchanged (3X) to 100 mM ammonium bicarbonate (pH 8) with spin filtration (Viva Spin 500) to a final volume of 245 µL. Next, 5.4 µg of proteolytic enzyme (Princeton Separations, Inc.) was added and the solution incubated overnight at 37 °C. The reaction was quenched by adding 5 µl of formic acid the following morning.

Preparation of ICAT™ labeled peptides from CSF: Two 150 µL (135 µg) aliquots of CSF were reduced to a volume of 20 µL with Viva Spin 500 (MWCO 5,000) filters. The volume of each was then increased to 80 µL with ICAT™ (Applied Biosystems) denaturing buffer. The protocol included with the cleavable ICAT™ kit (Applied Biosystems) was followed to label one of the samples with the light (^{12}C) label and the other with the heavy label (^{13}C). The final volume of the combined, digested, ICAT-labeled CSF proteins was 500 µl.

NanoLC-ESI ion trap MS: A Finnigan Surveyor® HPLC was employed with autosampler and nanoflow solvent delivery system in front of a 75 µm ID x 10 cm Aquasil™ nanobore HPLC column with a 15 µm i.d. pulled tip (New Objective, Inc., Cambridge, MA). In a typical experiment, 5 µL of the CSF digest (1.4 µg total protein) was injected onto the column and then eluted with a linear gradient of 0 – 80% B over 180 min at a flow rate of 220 nL/min (A = 0.1% formic in water, B = 0.1% formic acid in acetonitrile). Eluting peptides were analyzed by a Finnigan LCQ™ Deca XP Plus ion trap mass spectrometer equipped with a nano-electrospray ion source (both Thermo Finnigan, San Jose, CA). The mass spectrometer was operated in a Data-Dependent MS/MS mode and Dynamic Exclusion was enabled.

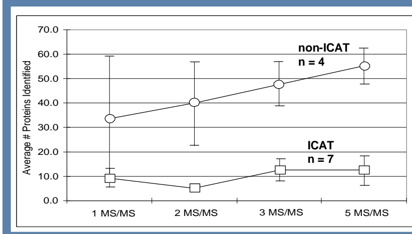
Data Analysis: MS/MS spectra obtained from the LC/MS analysis were searched against a Swiss Prot database (release 7459) using the SEQUEST® algorithm [1] implementation of BioWorks™ 3.1 (Thermo Finnigan, San Jose, CA). Search parameters or the ICAT related experiments were set as follows: static modifications for cysteine labeled peptides = 227.13 matching the ^{12}C labeled peptides and differential modifications for cysteine = +9 matching the ^{13}C labeled peptides (cleavable ICAT reagent). The list of matched peptides was then further evaluated using the following criteria: (i) the presence of a cysteine in the peptide sequence, (ii) a good Xcorr value (Eng et al., 1994), e.g. >2.2 for doubly charged ions and >3.5 for triply charged ions, (iii) a delta correlation score >0.3 and (iv) heavy and light chain ICAT pairs exhibiting closely eluting peaks (scan numbers). Search parameters for all other searches employed a Request/Unified scoring with a value of 2400 as the filter (Chelius et al., 2002).

Results

Total number of proteins identified in CSF as a function of MS/MS scans

• The number of proteins identified in the unmodified CSF digest increases linearly with increasing number of MS² scans, while variability decreases (Figure 1).
•The number and variability of proteins identified in the ICAT-modified CSF digest is essentially unaffected by the number of MS² scans and the total number of proteins identified is considerably lower than that obtained from the unmodified CSF digest.

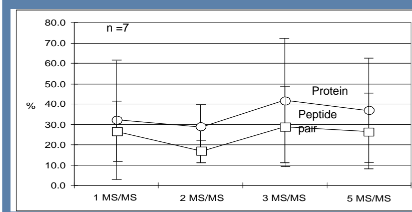
FIGURE 1. Average number of proteins identified in CSF digests as a function of the number of data-dependent MS/MS scans. Error bars represent +/- one standard deviation.



Between-run reproducibility of identifications for ICAT-labeled proteins

•ICAT Peptide Pair Reproducibility (Figure 2): Any particular ICAT peptide pair (peptides with the same amino acid sequence, but alternately tagged with ^{12}C and ^{13}C isotope-coded tags) was detected on average in only 25% of the experimental runs (n=7).

FIGURE 2. Between-run reproducibility of protein identifications as a function of the number of MS/MS scans for any particular ICAT peptide pair or protein associated with any ICAT peptide pair (e.g. 30% indicates that the protein or peptide is identified in 3 of 10 LC/MS runs). Error bars represent +/- one standard deviation.



Application of both methods to the identification of migraine specific CSF proteins.

•ICAT approach: ICAT peptide pairs were consistently found for relatively few proteins (compare Table 1 with Table 2). Only one is known to be directly involved in migraines.
•Unmodified CSF digest approach: Of the 49 unique proteins that were identified in 25% or more of the LC/MS analysis runs of the unmodified CSF digest, six are known to be directly involved in migraines (Table 2).
•Unmodified CSF digest approach: A total of 94 proteins were positively identified from peptides that were detected in 2 or more out of 16 LC/MS runs. An additional 205 proteins were identified by a single peptide identification.

TABLE 1. Proteins for which ICAT peptide pairs are observed with associated frequency of 25% or better in a total of 28 runs. Red highlighted proteins are those known to change significantly in the acute migraine attack.

%	Protein Identity
89	ALBU_HUMAN SERUM ALBUMIN
64	**TRFE_HUMAN SEROTRANSFERRIN**
36	GC1_HUMAN IG GAMMA-1 CHAIN C REGION
32	LAC_HUMAN IG LAMBDA CHAIN C REGIONS
29	HEMO_HUMAN HEMOPEXIN
25	GC4_HUMAN IG GAMMA-4 CHAIN C REGION

TABLE 2. Unique protein identifications from LC/MS analysis of unmodified CSF digests that were observed in 25% or more of the analysis within a total of 16 runs. Red highlighted proteins are those known to change significantly in an acute migraine attack.

%	Protein Identity
100	ALBU_HUMAN SERUM ALBUMIN
94	**ANGT_HUMAN ANGIOTENSINOGEN**
94	APE_HUMAN APOLIPOPROTEIN E
94	CLUS_HUMAN Clusterin
94	**CYTC_HUMAN CYSTATIN C**
94	HEMO_HUMAN HEMOPEXIN (BETA-1B-GLYCOPROTEIN)
94	**PGHD_HUMAN PROSTAGLANDIN-H2 D-ISOMERASE**
94	**TRFE_HUMAN SEROTRANSFERRIN**
88	APA1_HUMAN APOLIPOPROTEIN A-I
88	PEDF_HUMAN PIGMENT EPITHELIUM-DERIVED FACTOR
81	CO4_HUMAN COMPLEMENT C4
75	A2HS_HUMAN ALPHA-2-HS-GLYCOPROTEIN
75	DKK3_HUMAN DICKKOPF RELATED PROTEIN-3
75	GC4_HUMAN IG GAMMA-4 CHAIN C REGION
75	GELS_HUMAN GELSOLIN
75	VTDB_HUMAN VITAMIN D-BINDING
69	OSTP_HUMAN OSTEOPONTIN
69	TTHY_HUMAN Transthyretin
63	A1AT_HUMAN Alpha-1-antitrypsin
63	**B2MG_HUMAN BETA-2-MICROGLOBULIN**
63	CO3_HUMAN COMPLEMENT C3
50	ALC1_HUMAN IG ALPHA-1 CHAIN C REGION
50	CMGA_HUMAN CHROMOGRANIN A
44	APA4_HUMAN APOLIPOPROTEIN A-IV
44	CMGA_HUMAN CHROMOGRANIN A
44	GC1_HUMAN IG GAMMA-1 CHAIN C REGION
44	M3K1_HUMAN MITOGEN-ACTIVATED PROTEIN KINASE KINASE KI
44	ZYX_HUMAN ZYXIN (ZYXIN 2)
38	APA4_HUMAN APOLIPOPROTEIN A-IV
38	FBL3_HUMAN EGF
38	PTPZ_HUMAN PROTEIN-TYROSINE PHOSPHATASE ZETA
38	SG1_HUMAN SECRETOGRANIN I PRECURSOR
31	A2MG_HUMAN ALPHA-2-MACROGLOBULIN
31	CD20_HUMAN B-LYMPHOCYTE ANTIGEN CD20
31	CERU_HUMAN CERULOPLASMIN (FERROXIDASE)
31	CFAB_HUMAN COMPLEMENT FACTOR B
31	VTNC_HUMAN Vitronectin
25	**AACT_HUMAN ALPHA-1-ANTICHYMOTRYPSIN**
25	APA2_HUMAN APOLIPOPROTEIN A-II
25	ATRX_HUMAN Transcriptional regulator
25	BRS3_HUMAN BOMBESIN RECEPTOR SUBTYPE-3 (BRS-3)
25	KAC_HUMAN IG KAPPA CHAIN C REGION
25	LAC_HUMAN IG LAMBDA CHAIN C REGIONS
25	LDHC_HUMAN L-lactate dehydrogenase C chain
25	NCR2_HUMAN Nuclear receptor co-repressor 2 (N-CoR2)
25	OA48_HUMAN OKADAIC ACID-INDUCIBLE PHOSPHOPROTEIN
25	SODC_HUMAN SUPEROXIDE DISMUTASE [CU-ZN]
25	TISD_HUMAN Butyrate response factor 2
25	Y296_HUMAN HYPOTHETICAL

Conclusions

- Reduction in sample complexity and accurate quantitation of peptides with concurrent sequence identification are stated advantages of the ICAT strategy for quantitative proteomic analysis (Han et al., 2001; Gygi, et al., 2001). In contrast with previous results on eukaryotic cells and cellular subfractions, the application of the ICAT strategy to human CSF appears to be impractical for accurate protein quantitation.
- Our results strongly suggest that the kinetics of the ICAT reaction limits protein coverage, producing peptides for only the most abundant proteins in the mixture. Removal of these proteins (i.e. albumin, transferrin and IgGs) prior to ICAT labeling might improve the results.
- An additional drawback of this method in the application to CSF is the significantly higher noise found in many of the MS/MS spectra of ICAT peptides (not shown). It strongly suggests that reaction byproducts are present in the sample that significantly contribute to an increase in sample complexity of an already complex mixture.
- The number of peptides identified is lower in ICAT experiments when compared to the non-ICAT sample, thereby reducing the confidence in correct protein identification.
- At present, our data indicate that the utility of the ICAT approach to monitor quantitative changes in CSF proteins during migraine is limited. Only one of the ten proteins known to change in concentration in the migraine state produces an observable ICAT peptide pair with a low and fixed reproducibility of 30%. On the other hand, the simple 1-D LC/MS experiments show peptides for six of the ten proteins with reproducibility between 25% and 94%. In future experiments, quantitation of these migraine relevant proteins should be possible by evaluation of peak areas from reconstructed ion chromatograms (Bondarenko et al.; 2002).
- The dependence of the number of proteins identified on the number of MS/MS scans strongly suggests that a decreased ion scan time in combination with an increase in column efficiency or chromatographic dimensionality should enhance protein coverage with concurrent accurate quantitation, without the use of ICAT labeling techniques.

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