iTRAQ Quantitation of Proteins following SDS-PAGE Fractionation

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OVERVIEW

Introduction. Age-related macular degeneration (AMD) is a slow, progressive retinal disease with multiple genetic and environmental risk factors and is the leading cause of legal blindness in the elderly population of developed countries. Bruch's membrane, the extracellular matrix between the retina and blood bearing choroid, becomes thicker and less permeability with age. The purpose of this study was to quantify proteome changes in Bruch's membrane associated with AMD using iTRAQ technology and SDS-PAGE as the primary fractionation method.

Methods. Samples of Bruch's membrane (4 mm trephine discs) were isolated from the macular region of human AMD and non-AMD donor eyes. Proteins were extracted in SDS and AMD and non-AMD samples fractionated by SDS-PAGE in adjacent lanes. _Gel bands were excised, digested *in situ* with trypsin, peptides extracted, labeled with iTRAQ tags, and labeled peptides from gel bands corresponding in apparent mass mixed together and analyzed by on-line LC MS/MS with a QTOF2 mass spectrometer. LC MS/MS analyses were compared with and without SCX cartridge clean up. Proteins were identified using the Mascot search engine and the SwissProt protein sequence database. Analysis of iTRAQ labeling by QTOF2 MS utilized a script written in Perl.

Preliminary Data. Relative quantitation has been obtained for Bruch's membrane proteins from 10 AMD donors fractionated individually by SDS-PAGE adjacent to an age-matched non-AMD sample pooled from 10 donors. High abundance and low abundance proteins identified following SDS-PAGE fractionation prior to tryptic digestion were comparable to those identified in another iTRAQ study of Bruch's membrane using SCX chromatography after tryptic digestion. More proteins were quantified when iTRAQ labeled samples were injected directly to the trap column without a SCX cartridge clean step. An advantage of the gel fractionation approach to iTRAQ analyses is that samples solubilized in detergents like SDS can be applied directly to the gel, without acetone precipitation to remove the detergent prior to tryptic digestion. Another advantage is that the apparent mass of the protein can be estimated from the gel band, providing insights regarding proteolytic degradation, modification and aggregation. Disadvantages to the gel approach relative to SCX factionation appear to be higher relative standard derivations associated with the quantitation and generally fewer proteins quantified. Work continues toward obtaining additional iTRAQ analyses from other gel fractionated AMD and non-AMD Bruch's membrane preparations.

Novel Aspect. This mass spectrometric study illustrates the strengths and weaknesses of SDS-PAGE versus SCX fractionation for iTRAQ quantitative proteomic technology.

INTRODUCTION

Applications of iTRAQ technology are increasing in quantitative proteomic studies using mass spectrometry. We have applied iTRAQ technology to proteome analysis of ocular Bruch's membrane, the extracellular matrix separating the retina and blood bearing choroid (Yuan et al., 2007). Conventional iTRAQ methods utilize strong cation exchange chromatographic (SCX) separation of amine-specific iTRAQ labeled tryptic peptides followed by reverse-phase LC MS/MS (Ross et al., 2004) to generate relative peptide quantitation from the intensity of diagnostic reporter ions. A drawback to this approach for the analysis of proteins requiring detergent for solubilization is the protein losses incurred during the process used to lower the detergent concentration prior to tryptic digestion. To circumvent this potential problem, we have fractionated detergent solubilized proteins by SDS-PAGE prior to tryptic digestion and iTRAQ labeling and describe here the advantages and disadvantages of this approach to iTRAQ relative quantitation. Bruch's membrane proteins from human donor eyes with and without age-related macular degeneration (AMD) were used in this study. Quantitative analyses of other Bruch's membrane samples using conventional iTRAQ technology following SCX chromatography was performed in a separate study (Yuan et al., 2007).

METHODS

Human Tissue Procurement. Eyes from 10 normal human donors and 10 AMD donors were used in this study. Normal eyes were obtained through the Cleveland Eye Bank, (Cleveland, OH), or the National Disease Research Interchange (Philadelphia, PA). AMD donor eyes were from clinically diagnosed patients registered through the Eye Donor Program of the Foundation Fighting Blindness, Inc. (Owens Mills, MD). Eyes were enucleated between 1.5 and 7 hours after death and frozen in liquid nitrogen.

Sample Preparation and SDS-PAGE. Bruch's membrane preparations (4 mm trephined tissue buttons, 4 buttons per sample) were solubilized in 2% SDS, 62.5 mM Tris-HCl, 1 mM dithiothreitol containing protease inhibitor cocktail and phenylmethylsulfonyl fluoride. Soluble protein was quantified by the BCA assay using as a reference, amino acid analysis quantified bovine serum albumin. Approximately equal amounts of each non-AMD Bruch's membrane preparation were pooled to yield a single non-AMD donor reference sample. Each AMD preparation (~50 µg) was fractionated by 1D SDS-PAGE according to Laemmli on a 4-20% acrylamide gradient gel (8 cm x 8 cm x 1.5 mm) immediately adjacent to the pooled non-AMD reference sample (~50 µg) and stained with Coomassie blue (Code Blue, Pierce).

Tryptic Digestion and iTRAQ Labeling. Gel bands were excised from the top to the bottom of each lane, proteins were reduced, alkylated with iodoacetamide, digested *in situ* with trypsin overnight at 37oC overnight, and peptides were extracted and dried (Bhattacharya et al., 2005). Tryptic peptides were redissolved in 167 mM TEAB containing 66% ethanol and sufficient amount of one of four amine specific, isobaric iTRAQ tags according to the vendor.

Mass Analysis and Bioinformatic Quantification. The iTRAQ-labeled tryptic digests from the AMD and reference samples from gel bands of corresponding apparent mass were mixed 1:1 and analyzed by on-line reverse-phase LC MS/MS with a QTOF2 mass spectrometer. LC MS/MS analyses were compared with and without SCX cartridge clean up following iTRAQ labeling. Proteins were identified using the Mascot search engine and the SwissProt database. Quantitation of iTRAQ labeled peptides utilized a visual basic program available at http://www.lerner.ccf.org/eye/crabb/software/ (Crabb et al., 2007).

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METHODS

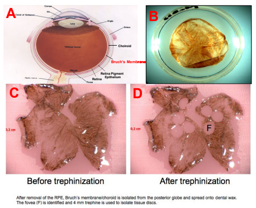

Figure 1. Bruch's Membrane Isolation

(A) Bruch's membrane is an extra cellular structure that acts like a sieve through which nutrients and waste products exchange between the retinal pigment epithelium and the blood bearing choroid. With age and age-related macular degeneration (AMD), debris accumulates in this membrane, resulting in increased thickness and decreased permeability. (B, C) Dissected Bruch's membrane/choroid before trephinization. (D) Dissected Bruch's membrane after trephinization with the fovea (F) is identified.

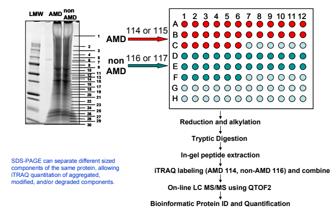

Figure 2. Methods Overview

AMD sample (50 µg) were fractionated by 1D SDS-PAGE along with the same amount of protein from a pooled non-AMD sample. Gel bands were excised, transferred to a 96 well microtiter plate, digested *in situ* with trypsin after reduction and alkylation with iodoacetamide. Tryptic peptides were extracted, dried, and labeled with iTRAQ reagent. Labeled peptides from AMD and non-AMD gel bands corresponding in the same apparent mass were combined and analyzed by on-line LC MS/MS with a QTOF2 mass spectrometer.

RESULTS

Table 1. iTRAQ Labeling Efficiency following SDS-PAGE Fractionation

Sample Name	Total # peptide ID	No tag	iTRAQ tagged	% of No tag	% of iTRAQ tagged
# 1	5988	340	5648	5.7	94.3
# 2	6217	424	5793	6.8	93.2
# 3	6029	382	5647	6.3	93.7
# 6	4268	168	4100	3.9	96.1
# 8	5963	216	5747	3.6	96.4

While conventional iTRAQ technology involves only one iTRAQ labeling reaction per sample, following SDS-PAGE fractionation, multiple iTRAQ labeling reactions are required, I.e., one for each excised and tryptic digested gel band. To evaluate iTRAQ labeling efficiency, we searched with MASCOT using iTRAQ labeled N-term and ε-lysine as variable modifications. The above table shows the number of iTRAQ tagged and untagged peptides detected in 50% of the Bruch's membrane samples analyzed. The overall yield ranged from 93% to 96%.

Table 2 Select Proteins Increased in AMD Bruch's Membrane

Bruch's Membrane Sampl	BM-1	BM-2	BM-3	BM-4	BM-5	BM-6	BM-7	BM-8	BM-9	BM-10	Total
Total Proteins quantified	105	203	110	142	126	190	168	145	152	151	302
Normalization Factor	0.97	1.01	0.91	1.12	1.03	0.99	0.95	1.03	0.91	0.94	-
Median	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.03
Mean	1.13	1.05	1.07	1.30	1.10	1.02	1.35	1.03	1.08	1.08	1.17
StdDev	0.44	0.33	0.35	0.90	0.46	0.23	1.38	0.37	0.47	0.54	0.79
RSD	38.8%	31.3%	32.2%	69.0%	42.0%	22.4%	102.2%	36.2%	43.2%	49.6%	-

Accessior	Protein	BM-1 AMD/Non-AMD	RSD	BM-2 AMD/Non-AMD	RSD	BM-3 AMD/Non-AMD	RSD	BM-4 AMD/Non-AMD	RSD	BM-5 AMD/Non-AMD	RSD
P35625	TIMP-3	2.12	43.46%	1.71	54.61%	2.45	40.30%	1.40	53.07%	1.91	36.75%
P13645	Cytokeratin-10	1.83	62.70%	1.11	64.13%	1.35	93.21%	1.40	42.29%	1.07	29.29%
P62805	Histone H4	1.49	32.48%	1.57	80.04%	1.13	18.73%	5.82	97.72%	1.54	159.44%
P04264	Cytokeratin-1	2.15	58.18%	1.26	37.20%	1.29	62.47%	1.38	32.62%	1.36	64.52%
P04004	Vitronectin precursor	1.50	34.55%	1.34	37.15%	1.93	34.53%	1.32	45.39%	1.98	34.87%
P02748	Complement component C	1.46	35.43%	1.19	37.10%	2.15	32.85%	1.09	28.49%	1.53	31.04%
P10909	Clusterin precursor	1.84	28.35%	1.26	22.30%	2.11	33.89%	1.22	42.33%	1.44	33.88%
P01024	Complement C3 precursor	1.42	20.98%	1.73	33.14%	1.47	32.07%	0.80	25.04%	1.46	33.33%
P02511	Alpha crystallin B chain	1.70	36.88%	0.88	38.10%	1.25	22.10%	3.73	117.85%	0.67	0.22%
P08758	Annexin A5	1.00	4.94%	0.72	16.39%	0.67	10.57%	4.94	120.69%	0.52	27.05%
Q06830	Peroxiredoxin-1	1.07	37.15%	1.11	39.22%	1.01	25.90%	1.56	122.87%	1.61	59.36%
P02743	Serum amyloid P-compone	1.15	30.49%	1.29	28.79%	0.98	44.51%	2.36	104.09%	0.82	49.09%
P02545	Lamin-A/C	0.99	22.16%	0.95	50.16%	1.34	18.57%	1.04	47.85%	1.34	33.64%

Accessior	BM-6 AMD/Non-AMD	RSD	BM-7 AMD/Non-AMD	RSD	BM-8 AMD/Non-AMD	RSD	BM-9 AMD/Non-AMD	RSD	BM-10 AMD/Non-AMD	RSD	Average AMD/Non-AMD	StdDev	Frequency	AveRSD
P35625	1.42	40.43%	1.65	36.89%	1.02	65.50%	1.84	46.83%	2.67	57.53%	1.82	0.50	10	47.54%
P13645	1.10	52.48%	6.16	57.68%	1.49	131.42%	0.90	53.26%	1.28	100.86%	1.77	1.56	10	68.73%
P62805	0.99	25.72%	1.96	41.95%	0.43	30.56%	1.05	40.50%	1.38	104.02%	1.74	1.49	10	63.12%
P04264	1.30	45.54%	4.35	125.51%	1.37	70.39%	1.11	38.15%	1.12	48.62%	1.67	0.99	10	58.32%
P04004	1.23	25.40%	1.53	34.63%	1.19	35.97%	1.97	35.12%	2.37	39.24%	1.64	0.40	10	35.68%
P02748	0.79	26.11%	1.69	34.77%	1.02	34.63%	2.85	36.51%	2.46	43.50%	1.62	0.67	10	34.04%
P10909	1.12	30.01%	1.48	27.05%	0.99	36.86%	1.62	28.61%	2.04	39.83%	1.51	0.39	10	32.31%
P01024	0.97	24.76%	1.30	28.62%	1.26	29.28%	2.17	32.97%	2.44	43.28%	1.50	0.50	10	30.35%
P02511	1.34	14.46%	0.88	16.43%	1.28	62.50%	1.39	34.00%	1.21	29.30%	1.43	0.86	10	37.19%
P08758	0.96	20.31%	0.96	31.68%	1.25	152.65%	0.78	32.03%	0.62	22.16%	1.24	1.32	10	43.85%
Q06830	1.11	18.21%	0.83	25.76%	1.25	43.82%	1.24	36.51%	1.29	39.19%	1.21	0.24	10	44.80%
P02743	1.44	34.11%	1.00	23.13%	1.10	32.04%	0.80	29.74%	1.09	66.33%	1.20	0.45	10	44.23%
P02545	1.31	27.78%	1.30	33.26%	1.37	33.89%	1.25	45.04%	1.08	35.47%	1.20	0.16	10	34.78%

* Among 302 proteins quantified in Bruch's membrane following SDS-PAGE, ~4% were quantified in all 10 AMD samples with average iTRAQ ratios ≥ 1.2. Several inflammatory proteins (eg, TIMP3, vitronectin, clusterin, Complement C3 and Complement C9) appear to be elevated in AMD Bruch's membrane. These results agree with similar iTRAQ analyses using SCX fractionation (Yuan et al., 2007). This observation is consistent with genetic evidence implicating inflammatory processes in AMD pathophysiology. No proteins were clearly less abundant in AMD Bruch's membrane.

Table 3. Quantification of Molecular Species

Gel Band #	TIMP-3 AMD/Non-AMD	RSD	Vitronectin AMD/Non-AMD	RSD	α-Antitrypsin AMD/Non-AMD	RSD	C9 AMD/Non-AMD	RSD
1	1.39	18%	1.10	15%			0.66	16%
2	3.67	18%	2.70	18%			1.78	16%
3	1.80	31%	1.10	21%			0.85	18%
4	0.78	39%	1.05	13%			0.85	16%
5	1.19	41%	0.91	27%			0.89	17%
6	1.15	12%	0.95	30%			0.92	18%
7			0.99	25%			0.65	8%
8			0.60					
9			1.07	8%			0.92	
10			0.97	21%	0.86		0.98	
11			0.84	18%			0.82	
12			1.00	25%	0.80		1.19	
13			0.87	15%	0.78	14%	1.06	
14							0.95	
15			0.91	6%			0.96	24%
16			0.64	6%	0.71	20%	0.79	
17			1.17	19%	0.85	12%		
18			1.15	12%	1.12	23%	1.07	
19			0.96	12%	0.90	22%	0.93	
20			1.12	10%				
21			1.20	7%				
22			1.29	21%			0.94	
23	1.18	6%	1.09	10%				
24			1.19	11%			1.71	8%
25	1.27	20%	1.06	11%				
26	1.78	11%	1.71	13%	3.11	34%		
27	1.28	30%	1.02	17%				
28	0.80	37%	0.80					
29	1.24	19%			1.31		1.24	
30	1.11	12%	0.80				0.90	9%
Overall	1.43	51%	1.08	35%	1.16	65%	1.00	28%
Average RSD per band		23%		16%		21%		15%

RSD = Relative standard deviation = % STDEV /mean iTRAQ value.

Overall RSDs for iTRAQ values for TIMP-3, vitronectin, α-antitrypsin and complement C9 ranged from 28% to 65%, demonstrating a wide spread per protein. However, the Average RSD per band is much smaller, ie, 15% to 23%, demonstrating a much less variation in iTRAQ values per gel band.

A major factor contributing to the large overall RSD per protein following SDS-PAGE fractionation is the size variability of the components. Crosslinks, other modifications and degradation contribute to this variability.

RESULTS

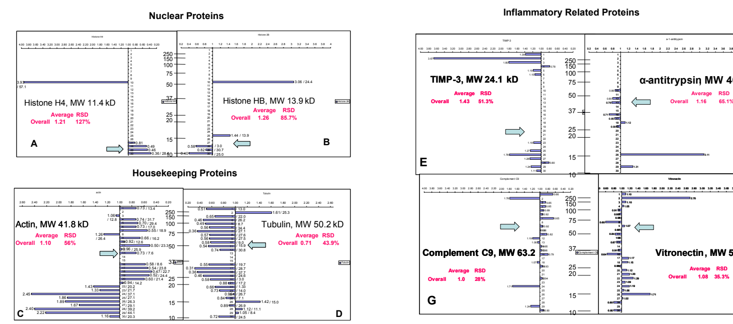

Figure 3. SDS-PAGE Distribution of iTRAQ Quantitation for Select Proteins

The X axis denotes iTRAQ value and the Y axis denotes gel band numbers. iTRAQ value > 1 indicate greater abundance in AMD than non-AMD Bruch's membrane. The position of gel molecular weight markers are shown. Blue arrows represent the calculated mass of the protein. Protein, calculated mass, and overall average iTRAQ value and RSD are also show in each panel. (A), Histone H4, (B) Histone HB, (C), actin, (D), tubulin.(E) TIMP-3, (F) vitronectin, (G) α-antitrypsin, and (H) complement C9. iTRAQ value bars in panels A-D are labeled with RSD/gel band. RSD values for gel bands in panels E-H can be found in Table 3.

These analysis suggest that the majority of Histone H4, Histone HB, TIMP-3 and vitronectin from AMD Bruch's membrane exists in an aggregated or crosslinked form and that actin, tubulin, α-antitrypsin and complement C9 are significantly degraded.

High average RSDs are apparent however the RSDs for individual bands was typically much smaller.

SDS-PAGE fractionation allows iTRAQ quantitation of the distribution of different molecular species of the same protein, providing possibly relevant insights to pathological processes.

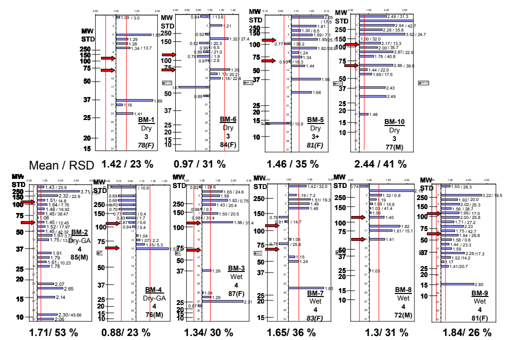

Figure 4. SDS-PAGE Distribution of iTRAQ Quantitation of Complement C3

C3 iTRAQ quantitation is shown for individual gel bands from n = 10 AMD Bruch's membrane donors. The X axis denotes iTRAQ value and the Y axis denotes gel band numbers. Vertical red lines represent iTRAQ value at 0.75 and 1.25. The age and gender of the AMD tissue donors are shown. The upper 4 panels represent intermediate stage dry AMD donors and the lower 6 panels are from late stage, advanced AMD donors. The overall average iTRAQ and RSD values are shown at the bottom of each panel. iTRAQ value bars are also labeled with RSD. Red arrows represent the calculated mass of the C3 alpha (~110 kD) and beta chains (60 kD).

While 8 of the 10 AMD samples exhibited increased complement C3 in AMD Bruch's membrane, a pattern of C3 molecular species was not apparent.

CONCLUSIONS

- Two hundred ninety-seven (297) proteins were quantified with two or more unique peptides from 10 AMD Bruch's membrane preparations following SDS-PAGE separation. The relative quantitation of these proteins was in agreement with iTRAQ analyses utilizing SCX chromatography after proteolysis (Yuan et al., 2007) and iTRAQ peptide labeling efficiency was ≥ 93%, however the number of proteins quantified was substantially lower than following SCX.

- The relative standard deviation (RSD) of iTRAQ peptide ratios averaged ~42% per protein overall for the SDS-PAGE fractionated proteins. The variability of peptides from similar sized components was much lower (eg, ~25% RSD per gel band pair). For comparison, an average RSD ~29% was obtained using SCX chromatography after proteolysis and iTRAQ labeling of other Bruch's membrane samples.

- Advantages of using SDS-PAGE separation prior to tryptic digestion and iTRAQ labeling include: (i) facilitation of sample preparation for proteins requiring high detergent for solubilization; and (ii) the capacity to quantify different sized components of individual proteins.

- Disadvanatages of using SDS-PAGE prior to tryptic digestion and iTRAQ labeling include: (i) the approach was more labor intensive; (ii) the results fewer in quantity; and (iii) the overall results more variable than the SCX based methodology. Factors contributing to these disadvantages were the required multiple peptide extractions from gel bands, the multiple iTRAQ labeling reactions and significant difference in the amounts of high mass and low mass protein components in the samples.