



iTRAQ Quantitation of Proteins in AMD Bruch's Membrane Following SCX Fractionation

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Overview

Purpose: Age-related changes in Bruch's membrane lead to thickening and loss of permeability. We seek to identify and quantify proteome changes in Bruch's membrane associated with age-related macular degeneration (AMD).
Methods: Bruch's membrane samples were isolated from human AMD and normal donor eyes. Protein was extracted in detergent, digested with trypsin, and peptides were labeled with iTRAQ tags. Labeled peptides from AMD and normal preparations were mixed, fractionated by SCX chromatography and fractions analyzed by LC MS/MS. Proteins were identified using the Mascot search engine and the Swiss-Prot protein sequence database. Relative protein quantification from iTRAQ labeling utilized a macro written in visual basic. Results: Relative quantitation of 787 unique proteins has been obtained from Bruch's membrane from 14 AMD donors. The on-line LC QTOF2 results and the off-line LC MALDI TOF/TOF analyses have been complementary and provided ~22%-38% more quantified proteins per sample compared with analyses using only one instrument. Both approaches yielded iTRAQ ratios with ~20% average relative standard deviations. As expected from biological diversity, amounts of individual proteins varied from donor to donor. Conclusions: For proteins quantified in all 14 AMD donors, the average relative amount was greater for several proteins such as vitronectin, alpha crystallin B, TIMP3, selectin complement components and clusterin. These observations are consistent with previous Western analyses (Kamei and Hollyfield, 1999) and proteomic analyses (Crabb et al., 2002) of Bruch's membrane and drusen from AMD and normal donors.

Introduction

Age-related macular degeneration (AMD) is the most common cause of legal blindness in the elderly population of developed countries. Over a third of those over 75 years currently have some form of this disease. In the USA, the prevalence of AMD in Medicare beneficiaries 65 years of age or older increased from ~5% to ~27% in the 1990s. Early stages of AMD are associated with the macular accumulation of extracellular deposits (drusen) and thickening of Bruch's membrane. Geographic atrophy develops in the later stages and is characterized by macular loss of retinal pigment epithelial and photoreceptor cells. Advanced stage dry AMD is characterized by geographic atrophy with regional loss of photoreceptor and RPE cells. Advanced stage wet AMD accounts for ~80% of AMD vision loss and is characterized by choroidal neovascularization with abnormal blood vessel growth through Bruch's membrane into the retina. Bruch's membrane is the extra cellular matrix through which nutrients and waste products exchange between the retinal pigment epithelium and the blood bearing choroid. With age and AMD, debris accumulates in this membrane, resulting in increased thickness and decreased permeability. The purpose of this study was to quantify proteome changes in Bruch's membrane associated with AMD using conventional iTRAQ technology and strong cation exchange (SCX) chromatography (Ross et al., 2004).

Methods

Isolation of Bruch's Membrane for Proteomic Analysis. Human AMD and non-AMD cadaver eyes were thawed and the anterior segment removed along with the vitreous, retina and RPE. The Bruch's membrane-choroid layer was stripped from the posterior half-globe (2002 Proc Natl Acad Sci U S A, 99:14682-7) and either 4 mm or 6 mm trephined samples were isolated from the macular and surrounding central region for subsequent analysis (Figure 1).

Sample Preparation and SCX Fractionation. Bruch's membrane preparations were solubilized in 100 μ l triethylammonium bicarbonate (TEAB) containing 2% SDS. Soluble protein was quantified by the BCA assay using as a reference protein, amino acid analysis quantified bovine serum albumin. Protein was precipitated with four volumes of acetone to lower the SDS concentration, redissolved in 500 μ l TEAB containing 0.1% SDS, reduced with tris-(2-carboxyethyl)phosphine (TCEP), cysteines alkylated with methyl methaniosulfonate and the alkylated protein was digested with trypsin. Two pooled non-AMD Bruch's membrane reference samples were, one each for Experiments 1 and 2 as detailed in Table 1. Typical peptides from the pooled non-AMD reference samples and the individual AMD samples were labeled with one of four amine specific, isotopic iTRAQ tags according to the vendor (Applied Biosystems). Approximately equal amounts of iTRAQ-labeled tryptic peptides from individual AMD samples and pooled non-AMD samples were mixed (see Table 1). Each combined, iTRAQ-labeled tryptic digest was subjected to SCX chromatography using a PolySulfoethyl A column (1.0 x 150 mm, 5 μ m particle size; 200 A pore size), a flow rate of 50 μ l/min and a gradient of 0-600 mM KCl in 25% acetonitrile, 10 mM KH₂PO₄, pH 3 with fractions collected at 1 min intervals. In another study, we also used SDS-PAGE as a method to fractionate Bruch's membrane proteins prior to tryptic digestion and iTRAQ labeling (Gu et al., 2007).

Protein Identification. SCX fractions were analyzed by online LC MS/MS using a QTOF2 mass spectrometer (Waters) and/or by off line LC MS/MS using a model 4800 MALDI TOF mass spectrometer (Applied Biosystems). For on line LC MS/MS, peptides from the SCX fractions were separated on a 75 μ m x 5 cm Vydac C18 column (5 μ m particle size, 300 A pore size) using a CapLC system (Waters), aqueous formic acid/acetonitrile solvents, a flow rate of ~250 μ l/min and an acetonitrile gradient and sprayed directly into the QTOF2 instrument. Protein identification from QTOF2 MS data utilized MASSLINUX 4.1 software (Waters), the Mascot (Matrix Science, version 2.1) search engine and the Swiss-Prot protein sequence database (version 51.2). For off line LC MS/MS using the MALDI TOF instrument, peptides from the SCX fractions were separated on a 75 μ m x 15 mm PeplMap100 C18 column (5 μ m particle size, 100 A pore size, Dionex) using a Ultimate 3000 LC system (LC Packings), aqueous trifluoroacetic acid/acetonitrile solvents, a flow rate of 300 μ l/min and a Probot target spotter (LC Packings). Sample spots were collected on the target at 30s intervals. Protein identification from MALDI TOF MS data utilized GPS Explorer 3.6 (Applied Biosystems) and the Mascot (Matrix Science) search engine with the Swiss-Prot protein sequence database (version 51.2). All Mascot database searches were restricted to tryptic peptides from humans with two missed tryptic cleavages allowed. N-terminal and lysine iTRAQ modifications were selected as fixed and methionine oxidation as a variable modification. In addition, database searches utilized fixed S-methyl cysteine modifications for SCX fractions.

Protein Quantification. Analysis of iTRAQ labeling by QTOF2 MS utilized a script written in Perl that incorporated as thresholds for quantitation, a minimum of two unique peptides per identified protein with iTRAQ tag ion intensities \geq 15 and peptide Mascot ion scores \geq 25. Analysis of iTRAQ labeling by MALDI TOF MS utilized GPS Explorer 3.6 (Applied Biosystems) and required a minimum of two unique peptides per identified protein, a minimum 95% confidence interval for the Mascot protein score and peptide Mascot ion scores \geq 25. For both approaches, minor correction for isotopic impurity associated with the iTRAQ reagents was performed according to the vendor (Applied Biosystems). Our script for iTRAQ quantitation is described on ASMS'07 poster MP193 (Crabb et al., 2007 and available at <http://www.clevelandclinic.org/medcenter/ophthalmology>).

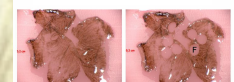
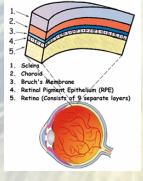


Figure 1. The Human Eye and Bruch's Membrane. Left: Schematic structures of the eye and tissues within the eye are shown. Right: Dissected Bruch's membrane/choroid before and after trephination for proteomic analysis is shown with the fovea (F) identified.

Results

Table 1 Human Age-Matched Bruch's Membrane Preparations for iTRAQ Analysis

Sample No	Donor Age	Donor Gender	Donor Stage	AMD Description	Notes	Trypsin Type	Number of Discs (mm)	Protein (ng)
Experiment 1								
137	70	F	4	Geographic Atrophy	dry	6	1	33
140	81	F	3	Moderate macular drusen	dry	6	1	369
142	77	M	4	Fibrovacular scar	wet	6	1	47
144	84	F	3	Few macular drusen	dry	6	1	389
138	73	F	non-AMD			6	1	389
141	81	F	non-AMD			6	1	389
145	74	F	non-AMD			6	1	389
143	86	M	non-AMD			6	1	389
147	85	F	non-AMD			6	1	389
Experiment 2								
148	70	F	3	Few macular drusen	dry	6	1	55
150	74	F	3	Few macular drusen	dry	6	1	55
154	87	F	3	Moderate macular drusen	dry	6	1	55
152	83	M	4	Geographic atrophy	dry	6	1	55
158	87	M	4	Fibrovacular scar	wet	6	1	55
160	76	M	4	Fibrovacular scar	wet	6	1	55
162	85	M	4	Fibrovacular scar	wet	6	1	55
164	84	F	3	Few macular drusen	dry	6	1	55
165	84	F	4	Geographic Atrophy	dry	6	1	55
166	85	M	4	Geographic Atrophy	dry	6	1	55
166	85	M	4	Geographic Atrophy	dry	6	1	55
147	85	F	non-AMD			6	2	56P
149	71	M	non-AMD			6	2	56P
151	74	M	non-AMD			6	2	56P
153	83	M	non-AMD			6	2	56P
155	87	M	non-AMD			6	2	56P
157	83	M	non-AMD			6	2	56P
159	87	M	non-AMD			6	2	56P
161	75	F	non-AMD			6	2	56P
163	86	M	non-AMD			6	2	56P

Table 2 Yield of iTRAQ Quantified Bruch's Membrane Proteins on-line QTOF LC MS/MS and off-line MALDI LC MS/MS

Sample	Total number of quantified proteins	Number of proteins quantified by QTOF	Number of proteins quantified by TOF	Increased I/Os over QTOF	Increased I/Os over TOF
BM148	427	364	233	15%	45%
BM156	447	251	389	44%	13%
BM158	443	241	405	84%	9%
BM160	257	143	226	44%	12%
BM162	453	315	369	30%	19%
BM164	345	283	222	18%	36%
BM165	415	354	285	15%	39%
BM166	400	250	489	52%	6%
Average	411	274	351	38%	22%

Table 3 Precision of iTRAQ Quantified Bruch's Membrane Proteins on-line QTOF LC MS/MS and off-line MALDI LC MS/MS

Sample	Average RSD Obtained from On-line QTOF-MS	Average RSD Obtained from Off-line MALDI-MS
BM148	21.4%	22.3%
BM156	27.1%	19.4%
BM158	21.0%	16.3%
BM160	20.6%	19.2%
BM162	23.3%	16.8%
BM164	22.6%	21.0%
BM165	20.6%	19.9%
BM166	23.5%	19.9%
Average	22.5%	19.2%

Table 4. Experiment 1 : Protein Abundance in AMD vs Non-AMD Bruch's Membrane

Accession	Protein	BM-137		BM-140		BM-142		BM-144		Total			
		AMD	Non-AMD	AMD	Non-AMD	AMD	Non-AMD	AMD	Non-AMD				
P04004	Vitronectin	1.01	17.60%	2.10	30.73%	2.11	28.03%	1.15	18.28%	1.57	0.57	4	23.84%
P35625	TIMP-3 (Tissue inhibitor of metalloproteinases 3)	0.97	17.92%	2.29	20.17%	1.33	11.61%	1.45	13.47%	1.48	0.51	4	15.84%
P19099	Clusterin	1.12	22.24%	1.49	22.47%	1.83	23.14%	1.02	13.31%	1.37	0.37	4	20.29%
Q95526	Synaptic vesicle membrane protein VAMP-1 homolog	1.54	37.00%	1.29	20.10%	1.17	5.89%	1.17	5.89%	1.33	0.18	3	15.02%
P02748	Relaxin domain component C9	0.74	19.21%	1.20	22.63%	2.25	19.57%	1.10	8.89%	1.32	0.65	4	15.32%
Q14958	Heterogeneous nuclear ribonucleoprotein A2/B1	1.41	15.57%	1.76	14.67%	1.03	13.88%	1.08	9.15%	1.32	0.34	4	13.32%
P0D352	Retinal dehydrogenase-1	1.54	37.00%	1.00	16.50%	1.00	16.50%	1.29	13.21%	1.28	0.27	3	22.13%
P62805	Histone H4	1.17	13.44%	1.63	20.26%	1.31	31.84%	0.83	13.47%	1.26	0.29	4	27.26%
Q14958	Transmembrane glycoprotein NMB	1.15	23.55%	1.87	9.73%	1.00	16.82%	0.95	17.54%	1.24	0.42	4	16.94%
P04792	Heat shock 70 kDa protein (HSP 70)	1.17	14.99%	1.70	16.41%	1.01	13.99%	1.07	13.39%	1.23	0.32	4	16.54%
P81224	Ras-related protein Rap-1b	1.11	8.26%	1.25	17.11%	1.27	16.16%	1.21	10.09	3	19.52%		

Table 5. Experiment 2: Protein Abundance in AMD vs Non-AMD Bruch's Membrane

Accession	Protein	BM-137		BM-140		BM-142		BM-144		Total			
		AMD	Non-AMD	AMD	Non-AMD	AMD	Non-AMD	AMD	Non-AMD				
P02748	Relaxin domain component C9	1.22	21.62%	0.94	23.95%	1.39	18.48%	1.00	16.50%	1.14	0.51	4	21.62%
P04792	Heat shock 70 kDa protein (HSP 70)	2.37	30.71%	1.82	20.26%	1.33	10.70%	0.83	10.49%	1.21	0.27	4	27.26%
P01011	Alpha-2-macroglobulin	1.24	29.50%	0.81	20.4%	1.83	18.2%	1.84	24.1%	2.27	27.0%	1.84	24.1%
P01033	Complement C3	1.26	27.6%	1.25	30.9%	1.91	27.5%	1.99	43.5%	1.62	1.62	4	24.8%
P01030	Beta-2-microglobulin	1.21	26.5%	0.81	24.4%	0.81	18.2%	0.81	18.2%	1.09	24.6%	2.06	29.2%
P01857	Myoglobin	1.21	26.5%	1.29	29.2%	1.29	29.2%	1.29	29.2%	1.29	29.2%	1.29	29.2%
P01858	Myoglobin	1.21	26.5%	1.29	29.2%	1.29	29.2%	1.29	29.2%	1.29	29.2%	1.29	29.2%
P01859	Myoglobin	1.21	26.5%	1.29	29.2%	1.29	29.2%	1.29	29.2%	1.29	29.2%	1.29	29.2%
P01860	Myoglobin	1.21	26.5%	1.29	29.2%	1.29	29.2%	1.29	29.2%	1.29	29.2%	1.29	29.2%
P01861	Myoglobin	1.21	26.5%	1.29	29.2%	1.29	29.2%	1.29	29.2%	1.29	29.2%	1.29	29.2%
P01862	Myoglobin	1.21	26.5%	1.29	29.2%	1.29	29.2%	1.29	29.2%	1.29	29.2%	1.29	29.2%
P01863	Myoglobin	1.21	26.5%	1.29	29.2%	1.29	29.2%	1.29	29.2%	1.29	29.2%	1.29	29.2%
P01864	Myoglobin	1.21	26.5%	1.29	29.2%	1.29	29.2%	1.29	29.2%	1.29	29.2%	1.29	29.2%
P01865	Myoglobin	1.21	26.5%	1.29	29.2%	1.29	29.2%	1.29	29.2%	1.29	29.2%	1.29	29.2%
P01866	Myoglobin	1.21	26.5%	1.29	29.2%	1.29	29.2%	1.29	29.2%	1.29	29.2%	1.29	29.2%
P01867	Myoglobin	1.21	26.5%	1.29	29.2%	1.29	29.2%	1.29	29.2%	1.29	29.2%	1.29	29.2%
P01868	Myoglobin	1.21	26.5%	1.29	29.2%	1.29	29.2%	1.29	29.2%	1.29	29.2%	1.29	29.2%
P01869	Myoglobin	1.21	26.5%	1.29	29.2%	1.29	29.2%	1.29	29.2%	1.29	29.2%	1.29	29.2%
P01870	Myoglobin	1.21	26.5%	1.29	29.2%	1.29	29.2%	1.29	29.2%	1.29	29.2%	1.29	29.2%
P01871	Myoglobin	1.21	26.5%	1.29	29.2%	1.29	29.2%	1.29	29.2%	1.29	29.2%	1.29	29.2%
P01872	Myoglobin	1.21	26.5%	1.29	29.2%	1.29	29.2%	1.29	29.2%	1.29	29.2%	1.29	29.2%
P01873	Myoglobin	1.21	26.5%	1.29	29.2%	1.29	29.2%	1.29	29.2%	1.29	29.2%	1.29	29.2%
P01874	Myoglobin	1.21	26.5%	1.29	29.2%	1.29	29.2%	1.29	29.2%	1.29	29.2%	1.29	29.2%
P01875	Myoglobin	1.21	26.5%	1.29	29.2%	1.29	29.2%	1.29	29.2%	1.29	29.2%	1.29	29.2%
P01876	Myoglobin	1.21	26.5%	1.29	29.2%	1.29	29.2%	1.29	29.2%	1.29	29.2%	1.29	29.2%
P01877	Myoglobin	1.21	26.5%	1.29	29.2%	1.29	29.2%	1.29	29.2%	1.29	29.2%	1.29	29.2%
P01878	Myoglobin	1.21	26.5%	1.29	29.2%	1.29	29.2%	1.29	29.2%	1.29	29.2%	1.29	29.2%
P01879	Myoglobin	1.21	26.5%	1.29	29.2%	1.29	29.2%	1.29	29.2%	1.29	29.2%	1.29	29.2%
P01880	Myoglobin	1.21	26.5%	1.29	29.2%	1.29	29.2%	1.29	29.2%	1.29	29.2%	1.29	29.2%
P01881	Myoglobin	1.21	26.5%	1.29	29.2%	1.29	29.2%	1.29	29.2%	1.29	29.2%	1.29	29.2%
P01882	Myoglobin	1.21	26.5%	1.29	29.2%	1.29	29.2%	1.29	29.2%	1.29	29.2%	1.29	29.2%
P01883	Myoglobin	1.21	26.5%	1.29	29.2%	1.29	29.2%	1.29	29.2%	1.29	29.2%	1.29	29.2%
P01884	Myoglobin	1.21	26.5%	1.29	29.2%	1.29	29.2%	1.29	29.2%	1.29	29.2%	1.29	29.2%
P01885	Myoglobin	1.21	26.5%	1.29	29.2%	1.29	29.2%	1.29	29.2%	1.29	29.2%	1.29	29.2%
P01886	Myoglobin	1.21	26.5%	1.29	29.2%	1.29	29.2%	1.29	29.2%	1.29	29.2%	1.29	29.2%
P01887	Myoglobin	1.21	26.5%	1.29	29.2%	1.29	29.2%	1.					