Does Lipofuscin Contain Protein? Amino Acid, Protein, and Ultrastructural Analysis of Human Lipofuscin

Kutralanathan Renganathan[1, 2], Kwok-Peng Ng[1], Matthew W. Davies[3], Xiaorong Gu[1], John S Crabb[1], Malgorzata B. Rózanowska[3], Mary E. Rayborn[1], Yong Li[1], Robert G. Salomon[2], Janet R. Sparrow[4], Joe G. Hollyfield[1], Michael E. Boulton[3, 5], and John W. Crabb[1, 2]

[1]Cole Eye Institute, Cleveland Clinic, [2]Dept. Chemistry, Case Western Reserve, Cleveland, OH, [3]School of Optometry and Vision Sciences, Cardiff University, Wales, UK, [4]Dept. Ophthalmology, Columbia University, New York, NY, [5]Ophthalmology and Visual Sciences, University of Texas Medical Branch, Galveston, TX

SUPPORTED BY Research to Prevent Blindness

CASE WESTERN RESERVE UNIVERSITY

Foundation Fighting Blindness

Cleveland Clinic

ABSTRACT

Purpose: Lipofuscin (LF) accumulates in the retinal pigment epithelium (RPE) with age and may contribute to the pathogenesis of age-related macular degeneration (AMD). We are characterizing the composition of LF granules for a better understanding of the mechanism of lipofuscin formation and its potential role in AMD.

Methods: LF was isolated from human RPE by sucrose density gradient centrifugation and washed in buffer containing 0.2% SDS. Extra-granular material was removed from the LF preparations by proteinase K digestion. Undigested and proteinase K digested LF preparations were compared by microscopy, by phenylthiocarbamyl amino acid analysis (AAA) and by proteomic analyses. The dry weight of LF samples was determined prior to AAA. For proteomic analysis, the LF preparations were subjected to SDS-PAGE, gel bands excised and proteins identified by LC MS/MS.

Results: Transmission electron microscopic analysis of undigested LF revealed roughly spherical granules surrounded by substantial extra-granular proteinacous material. Digestion with proteinase K effectively removed the extra-granular material, leaving intact, debris-free LF granules. AAA of LF suggests that amino acids accounted for ~10% of the dry weight of undigested LF and ~2% of the granules following proteinase K digestion. No proteins were identified from the proteinase K digested LF granules while numerous proteins were identified from the undigested LF preparations. Many of the proteins identified from the undigested LF material exhibit mass additions and contain apparent posttranslational modifications.

Conclusions: Debris-free LF granules contain little or no protein. The protein associated with LF is essentially all extra-granular and appears to be significantly modified.

CR: N. Supported in part by NIH grants EY14239, EY14240, EY15638, GM21249, Ohio BRTT 05-29, The Foundation Fighting Blindness, Research to Prevent Blindness, The Wellcome Trust, and The Cleveland Clinic Foundation

INTRODUCTION

Lipofuscin (LF) is a heterogeneous cellular material with characteristic fluorescence emission (1). These lipophilic inclusions are located within lysosomal compartment and accumulate with age (1, 2). Formation of LF in the RPE appears to be associated with retinoids and a normally functioning visual cycle and correlates with AMD (3). We and others have proposed that oxidative modification may play a role in LF formation (1). Toward a better understanding of LF formation and its potential role in AMD, we are pursing proteomic characterization of human RPE LF. Previous proteomic analyses have not distinguished between LF granules and proteins that simply co-isolate with the fluorescent granules. Here we report proteomic, chemical, and ultrastructural analyses demonstrating that debris-free LF granules contain a minimal amount of protein/amino acids (~2% by weight). Debris-free LF granules do contain A2E and oxidative modifications and are phototoxic to ARPE19 cells.

METHODS

RPE Lipofuscin Preparation and Quantification: Human eyes were obtained from the Bristol Eye Bank, UK, and RPE cells isolated and frozen at –80° C until isolation of LF granules by high speed discontinuous sucrose density gradient centrifugations (4). Isolated granules were further purified by two approaches: (i) LF preparations were treated with proteinase K (10 µg/µl, 24h/room temperature) in 15 mM N-ethyl morpholine acetate pH 8.3, 2 mM EDTA, 100 µM BHT, 0.2% SDS; or (ii) LF preparations were washed 6x in 15 mM N-ethyl morpholine acetate pH 8.3, 2 mM EDTA, 100 µM BHT, 0.2% SDS. Aliquots of crude LF granules, proteinase K digested LF granules, and washed LF granules were quantified by dry weight prior to analysis. Granules were also quantified by counting on a hemocytometer.

Transmission Electron Microscopy: Lipofuscin preparations were fixed in a mixture of 2% paraformaldehyde, 2.5% glutaraldehyde in 0.1M cacodylate buffer. The pellet was washed in the same buffer, post-fixed in 1% OsO4, sequentially dehydrated in ethanol then embeded in Epon. Transmission electron micrographs were taken on a Tecnai 20, 200 kV digital electron microscope using a Gatan image filter and digital camera at 3600 diameters (5).

SDS-PAGE and Western Analysis: LF preparations were extracted with chloroform/methanol (2:1 v/v) and the chloroform insoluble material sonicated and boiled in SDS sample buffer for Western slot blot analyses.
Antibodies utilized in Western analyses included anti-nitrotyrosine mAb (Upstate Biotechnology, Lake Placid, NY), anti-CEP mAb (5), and anti-iso[4]LGE_2 (7).
SDS-PAGE was performed according to Laemmli on 10% gels and stained with colloidal Coomassie blue (Pierce Code Blue).

Protein Identification. LF preparations were subjected to overnight tryptic digestion and soluble components fractionated by strong cation exchange (SCX) chromatography. SCX fractions were fractionated by RPHPLC and spotted to a MALDI target using a Probot (LC Packing) and analyzed using a model 4800 MALDI TOF-TOF mass spectrometer (Applied Biosytems). Alternatively, LF SDS-PAGE gel slices were excised, digested in-situ with trypsin, and peptides extracted for LC MS/MS with a QTOF2 instrument (Waters) (5). Protein identifications from MS/MS data utilized Mascot (Matrix Science) search engine and the Swiss-Protein protein sequence databases.

Amino Acid Analysis. Lipofuscin granules from human eyes were subjected to vapor phase HCl hydrolysis and phenylthiocarbamyl (PTC) amino acid analysis (6) using Agilent 1100 HPLC system.

A2E / iso-A2E Analysis: Crude and washed LF samples were extracted with chloroform/methanol (2:1). The extracts were filtered then subjected to RP-HPLC on an Atlantis® dC18 column (3 µm, 4.6 x 150 mm, Waters) in aqueous TFA/acetonitrile solvents. A Photodiode Array Detector (Waters 2996) was used for UV monitoring at 430 and 510 nm. A2E/isoA2E were identified and quantified based on UV-visible absorbance spectra and elution times that correspond to authentic standards (8).

Phototoxicity Assay. Confluent ARPE-19 cells were fed crude and washed LF granules then maintained in basal medium. Control cells lacked granules. After 7 days basal medium was replaced with photosensitizer free medium and cells were maintained in the dark or exposed to blue light (400 - 500 nm) at 2.8 mW/cm², 35 °C for 48 hours. Cell viability was then assessed using the MTT assay (9).

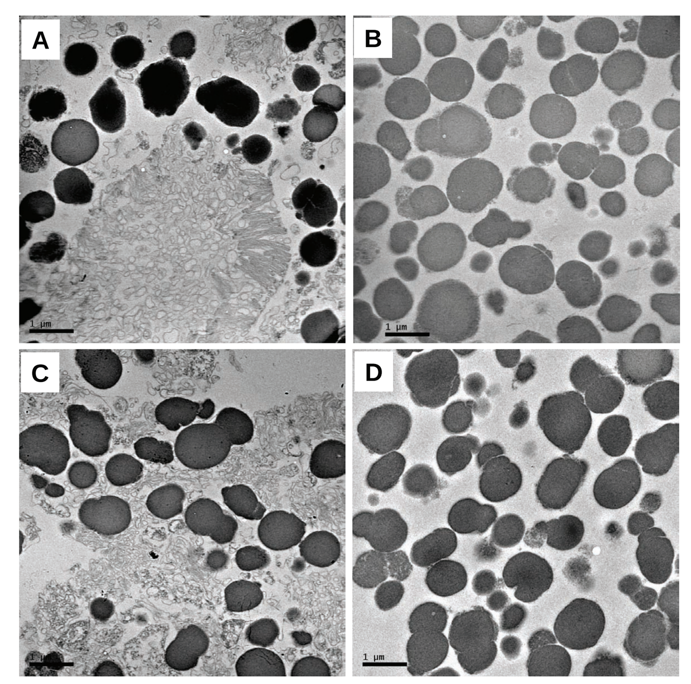


Figure 1. Transmission Electron Microscopy Lipofuscin Granules

(**A, C**) Crude LF isolated by sucrose gradient exhibit significant extra-granular debris. (**B**) Debris-free LF after proteinase K digestion. (**D**) Debris-free LF after washing with buffer containing 0.2 % SDS.

Table I. Amino Acid Analysis Crude and Proteinase K treated Lipofuscin

Amino Acid	Crude Lipofuscin (n = 4) pmol Analyzed	Crude Lipofuscin (n = 4) Mole %	Proteinase K Digested Lipofuscin (n = 4) pmol Analyzed	Proteinase K Digested Lipofuscin (n = 4) Mole %
ASP (D)	166.3 ± 25.7	4.8 ± 0.5	93.7 ± 11.0	3.0 ± 0.2
GLU (E)	354.7 ± 17.5	10.3 ± 0.3	288.0 ± 36.6	9.2 ± 0.2
SER (S)	247.0 ± 21.4	7.1 ± 0.5	185.2 ± 28.9	5.9 ± 0.1
GLY (G)	332.9 ± 19.4	9.6 ± 0.4	387.3 ± 70.3	12.3 ± 0.5
HIS (H)	66.8 ± 10.4	1.9 ± 0.2	50.6 ± 4.5	1.6 ± 0.1
ARG (R)	229.2 ± 29.2	6.6 ± 0.9	227.1 ± 20.2	7.3 ± 0.5
THR (T)	363.4 ± 23.2	10.5 ± 0.5	274.5 ± 45.9	8.8 ± 0.6
ALA (A)	161.7 ± 9.6	4.7 ± 0.2	153.2 ± 11.3	4.9 ± 0.4
PRO (P)	242.2 ± 5.5	7.0 ± 0.3	156.2 ± 21.8	5.0 ± 0.2
TYR (Y)	114.8 ± 8.1	3.3 ± 0.1	104.6 ± 23.4	3.4 ± 0.9
VAL (V)	207.5 ± 10.7	6.0 ± 0.2	234.7 ± 45.5	7.5 ± 0.4
MET (M)	85.6 ± 9.7	2.5 ± 0.1	70.7 ± 21.8	2.2 ± 0.4
ILE (I)	146.4 ± 7.7	4.2 ± 0.1	179.3 ± 26.5	5.7 ± 0.2
LEU (L)	323.4 ± 22.1	9.3 ± 0.4	286.9 ± 47.5	9.2 ± 0.3
PHE (F)	175.9 ± 11.6	5.1 ± 0.2	187.1 ± 40.5	5.9 ± 0.6
LYS (K)	244.7 ± 44.9	7.1 ± 1.2	235.2 ± 57.7	7.5 ± 1.0
Total AA (pmol)	3462.3 ± 195	100	3114.3 ± 456	100
Total AA (ng)	383.1 ± 22		342.4 ± 48	
Dry Weight (µg)	4.2		18.0	
% Amino Acid (w/w)	9.2		1.9	

LF Preparations were dried and weighted, then subjected to vapour phase HCL hydrolysis and phenylthiocarbamyl amino acid analysis

Table II. Amino Acid Analysis Crude and Washed Lipofuscin

Amino Acid	Crude Lipofuscin (n = 3) nmol Analyzed	Crude Lipofuscin (n = 3) Mole %	Washed Lipofuscin (n = 3) nmol Analyzed	Washed Lipofuscin (n = 3) Mole %
ASP (D)	2.4 ± 0.1	10.6 ± 0.7	0.3 ± 0.1	7.8 ± 1.3
GLU (E)	2.5 ± 0.2	11.0 ± 0.9	0.4 ± 0.1	11.9 ± 0.3
SER (S)	1.6 ± 0.3	6.8 ± 1.0	0.2 ± 0.03	6.4 ± 0.1
GLY (G)	0.2 ± 0.2	1.3 ± 0.3	0.4 ± 0.04	10.4 ± 0.2
HIS (H)	1.5 ± 0.2	6.2 ± 0.9	0.02 ± 0.02	0.3 ± 0.5
ARG (R)	0.9 ± 0.1	4.0 ± 0.1	0.1 ± 0.01	3.1 ± 0.7
THR (T)	1.4 ± 0.04	5.9 ± 0.1	0.3 ± 0.1	8.9 ± 0.3
ALA (A)	2.0 ± 0.04	8.6 ± 0.1	0.1 ± 0.02	3.2 ± 0.7
PRO (P)	1.5 ± 0.01	6.6 ± 0.2	0.1 ± 0.02	3.3 ± 0.9
TYR (Y)	0.9 ± 0.04	4.1 ± 0.1	0.1 ± 0.01	3.9 ± 0.2
VAL (V)	1.7 ± 0.1	7.3 ± 0.03	0.2 ± 0.02	6.2 ± 0.3
MET (M)	0.7 ± 0.01	2.8 ± 0.03	0.1 ± 0.01	1.9 ± 0.1
ILE (I)	1.3 ± 0.1	5.8 ± 0.1	0.2 ± 0.02	5.1 ± 0.3
LEU (L)	2.2 ± 0.02	9.4 ± 0.3	0.3 ± 0.05	9.2 ± 0.4
PHE (F)	1.2 ± 0.3	5.0 ± 1.1	0.3 ± 0.1	7.0 ± 0.7
LYS (K)	1.2 ± 0.2	5.1 ± 0.5	0.4 ± 0.1	11.4 ± 0.6
Total AA (nmol)	23.2 ± 1.7	100	3.6 ± 0.6	100
Total AA (µg)	2.7 ± 0.1		0.5 ± 0.03	
Dry Weight (µg)	21.4		21.5	
% Amino Acids (w/w)	12.4		2.5	

LF Preparations were dried and weighted, then subjected to vapour phase HCL hydrolysis and phenylthiocarbamyl amino acid analysis

Table III. Amino Acid Analysis Different LF Preparations

	Crude (n = 16)	Proteinase K Digested (n = 7)	Washed (n = 3)
% Amino Acids (w/w)	2.0–12.3	0.6–1.9	2.5

Phenylthiocarbamyl amino acid analysis was performed on three crude LF preparations, 2 proteinase K digested LF preparations and one washed, debris-free LF preparation. These LF preparations were isolated from : (**1**) 41 donors, mean age 73; (**2**) 65 donors, mean age 70; and (**3**) 82 donors, mean age 76. The total number of amino acid analyses = n.

Table IV. Proteins Identified from Crude Lipofuscin after SDS-PAGE

Accession[a]	Protein[b]	Calc. Mass (kD)	Observed Mass[d] (kD) (Peptide Matches)
P68871	Hemoglobin subunit beta	16	15(3),17(4),20(3),25(6),27(8),29(7),31(2),35(4),40(2),45(5), 50(4),55(5),60(4),65(5), 75(4),87(5),125(6),150(7),250(12)
P68371	Tubulin beta	50	15(2),17(7),19(8),20(9),29(9),31(3),33(19),35(17),40(4), 45(10),50(20),55(45),60(32), 65(7),75(8),87(15),125(10),150(24),250(25)
P04406	Glyceraldehyde-3-phosphate dehydrogenase	36	27(4),29(5),31(3),33(9),35(20),45(3),50(3),55(3),60(2),65(4),75(4),87(8),125(4),150(7),250(8)
P12271	Cellular retinaldehyde-binding protein	36	27(2),29(3),31(7),33(19),35(11),50(2),65(2),75(5),87(4),150(4),250(5)
P68363	Tubulin alpha	50	17(3),20(3),23(3),27(3),29(3),33(6),35(6),45(5),55(21),60(26),65(10),75(6),87(8),125(6),150(11),50(14)
P11488	Guanine nucleotide-binding protein	40	27(2),29(3),33(4),35(10),40(14),45(8),50(2),55(3),60(3),65(4),75(6),87(8),125(4),150(8),250(12)
Q9BUF5	Tubulin beta	50	17(4),19(4),20(4),29(5),33(7),35(7),45(4),50(8),55(17),60(10),65(4),87(5),125(5),150(8),250(7)
P12277	Creatine kinase B	43	27(3),29(2),33(3),35(3),45(8),50(16),55(3),60(3),65(3),75(2),87(4),150(4),250(5)
P08100	Rhodopsin	39	33(3),35(4),40(2),45(6),50(3),55(2),60(3),65(4),75(5),87(9),125(5),150(8),250(11)
Q13509	Tubulin beta-3	50	17(6),19(5),20(7),33(11),35(11),50(15),55(29),60(22),65(6),87(10),25(9),150(17),50(19)
P04350	Tubulin beta-4 chain	50	19(5),29(8),33(14),35(13),45(8),50(13),55(36),60(23),65(6),150(16),50(18)
P07437	Tubulin beta-2 chain	50	19(5),33(13),35(13),50(20),55(43),60(28),65(7),75(6),150(18),50(21)
P14618	Pyruvate kinase isozymes M1/M2	58	45(2),50(2),60(24),65(13),75(4),87(4),150(6),250(8)
P00505	Aspartate aminotransferase	47	40(2),45(11),75(2),87(2),150(4),250(5)
P60709	beta-Actin	42	35(5),50(15),65(4),87(6),125(4),150(4),250(4)
Q12931	Heat shock protein 75 kDa	80	65(2),75(3),87(2),250(2)
P07900	Heat shock protein HSP 90-alpha	84	55(2),60(2),65(6),87(10),25(3),250(4)
P06733	Alpha-enolase	47	50(17),55(8),65(3),87(3),250(4)
P10745	Interphotoreceptor retinoid-binding protein	135	87(2),125(5),150(22),50(3)
Q16518	Retinal pigment epithelium-specific 65 kDa	61	65(14),75(2),87(5),150(3),250(6)
Q71U36	Tubulin alpha-3	50	50(16),55(22),60(26),87(8),150(11),50(14)
P62736	Actin,aortic smooth muscle	42	,45(12),55(2),60(3),
P25705	ATP synthase subunit alpha	60	55(21),60(6),65(2),150(2),250(4)
P13929	Beta-enolase	47	50(6),55(3),65(3),75(2)
Q14344	Guanine nucleotide-binding protein alpha-1	44	150(2)
P07195	L-lactate dehydrogenase B chain	36	33(4),35(2),150(2),250(2)
Q9NY65	Tubulin alpha-8 chain	50	60(13),75(3),87(3),125(3),150(6)
Q99867	Tubulin beta-4q chain	48	33(5),87(6),125(6),150(5),250(5)
P12235	ADP/ATP translocase 1	33	29(4),250(2)
P11142	Heat shock cognate 71 kDa protein	71	65(2),75(12)
P08238	Heat shock protein HSP 90-beta	83	60(2),75(5),87(9),250(4)
P00338	L-lactate dehydrogenase A chain	37	33(7),150(2),250(2)
P18669	Phosphoglycerate mutase 1	29	27(3),29(2), 250(2)
P16499	GMP-PDE alpha	99	87(7),125(2),150(2),250(2)
Q99798	Aconitate hydratase	85	75(2),87(14)
P01009	Alpha-1-antitrypsin precursor	47	55(3),60(2)
P09972	Fructose-bisphosphate aldolase C	39	45(2)
P09211	Glutathione S-transferase P	23	23(5),25(7)
P69905	Hemoglobin subunit alpha	15	65(2),150(3),250(4)
P30086	Phosphatidylethanolamine-binding protein 1	21	19(2),20(3),23(2)
P60174	Triosephosphate isomerase	27	25(7),27(11)
Q92781	11-cis retinol dehydrogenase	35	29(5),31(4)
P63104	14-3-3 protein zeta/delta	28	27(2),29(3)
P09543	2',3'-cyclic-nucleotide 3'-phosphodiesterase	48	50(3)
P63261	Gamma-actin	42	45(25)
P05091	Aldehyde dehydrogenase	56	55(2),60(2)
Q00610	Clathrin heavy chain 1	191	150(4),250(7)
P06732	Creatine kinase M-type	43	50(2),250(3)
Q16555	Dihydropyrimidinase-related protein 2	62	60(5),65(6)
P01857	Ig gamma-1 chain C region	36	55(2)
P01871	Ig mu chain C region	50	75(2),87(4)
P40926	Malate dehydrogenase	36	35(4)
P23942	Peripherin	39	33(2),35(2)
P62820	Ras-related protein Rab-1A	23	23(3)
P35243	Recoverin	23	20(2),23(2)
P10523	S-arrestin	45	50(3),55(5)
P02768	Serum albumin precursor	69	65(2),75(9)
P68366	Tubulin alpha-1 chain	50	55(16),60(20)
Q9BQE3	Tubulin alpha-6 chain	50	55(20),50(10)
P22695	Complex III subunit II	48	45(5),50(2)
P52209	6-phosphogluconate dehydrogenase	53	50(2)
P12814	Alpha-actinin-1	103	87(2)
P36575	Arrestin-C	43	50(2)
P20594	Atrial natriuretic peptide receptor B	117	150(2)
P53673	Beta crystallin A4	22	23(2)
O75390	Citrate synthase	52	45(2)
P01024	Complement C3 precursor	187	75(2)
P09622	Dihydrolipoyl dehydrogenase	54	60(2)
P68104	Elongation factor 1-alpha 1	50	50(2)
P42263	Glutamate receptor 3 precursor	101	60(2)
Q03113	Guanine nucleotide-binding protein alpha-1	44	150(2)
P63096	Guanine nucleotide-binding protein G(i)	40	125(2)
P38405	Guanine nucleotide-binding protein G(olf),	44	45(2)
Q5JWF2	Adenylate cyclase-stimulating G alpha prot	101	150(2)
P19087	Guanine nucleotide-binding protein G(t)	40	45(2)
P54868	Hydroxymethylglutaryl-CoA synthase	57	50(2)
P30041	Peroxiredoxin-6	25	27(2)
Q8TC12	Retinol dehydrogenase 11	35	33(2)
Q5TZA2	Rootletin	228	250(2)
Q9BT40	Inositol phosphatase	51	50(2)
P50993	Na+/K+ ATPase 2	112	150(2)
P29401	Transketolase	68	65(2)
Q9H4B7	Tubulin beta-1 chain	50	27(2)
P21281	Vacuolar ATP synthase subunit B	56	60(2)
Q08752	40 kDa peptidyl-prolyl cis-trans isomerase	41	40(3)
P05141	ADP/ATP translocase 2	33	29(3)
P43320	Beta crystallin B2	23	25(3)
P07339	Cathepsin D precursor	45	29(3)
Q96NY7	Chloride intracellular channel 6	73	125(3)
P06396	Gelsolin precursor	86	87(3)
P08107	Heat shock 70 kDa	70	65(3)
P19367	Hexokinase-1	102	150(3)
P01834	Ig kappa chain C region	12	27(3)
Q02846	Retinal guanylyl cyclase	120	150(3)
Q01082	Spectrin beta chain	274	250(3)
P04179	Superoxide dismutase	25	23(3)
Q13748	Tubulin alpha-2 chain	50	17(3)
P38606	Vacuolar ATP synthase catalytic subunit A	68	75(3)
P46459	Vesicle-fusing ATPase	83	75(3)
P11021	78 kDa glucose-regulated protein precursor	72	75(4)
P12236	ADP/ATP translocase 3	33	29(4)
O43707	Alpha-actinin-4	105	125(4)
P09471	Guanine nucleotide-binding protein G	40	40(4)
P01876	Ig alpha-1	38	65(4)
P28331	NADH-ubiquinone oxidoreductase	79	75(4)
P49748	Very-long-chain specific acyl-CoA dehydrog	70	65(4)
P10809	60 kDa heat shock protein	61	65(5)
P24752	Acetyl-CoA acetyltransferase	45	45(6)
Q05524	Alpha-enolase	49	55(6)
P49411	Elongation factor Tu	50	50(6)
P06744	Glucose-6-phosphate isomerase	63	60(6)
P00367	Glutamate dehydrogenase 1	61	55(6)
P62873	Transducin beta chain 1	37	33(6)
P46821	Microtubule-associated protein 1B	270	65(6)
Q06830	Peroxiredoxin-1	22	23(6)
P00558	Phosphoglycerate kinase 1	44	45(6)
P05023	Na+/K+ ATPase 1	113	250(6)
P35913	GMP-PDE-beta	98	87(7)
P40939	Trifunctional enzyme subunit alpha	83	75(7)
Q8WZ42	Titin	3816	45(8)
P09104	Gamma-enolase	47	50(12)
P38646	Stress-70 protein	74	75(13)

a. Swiss-Protein database accession number
b. Proteins were identified by Q-TOF LC MS/MS following SDS-PAGE fractionation of Crude lipofuscin.
Apparent mass additions are suggestive of posttranslational modifications, including oxidative protein modifications and cross-links
c. Based on protein sequence
d. SDS-PAGE observed mass

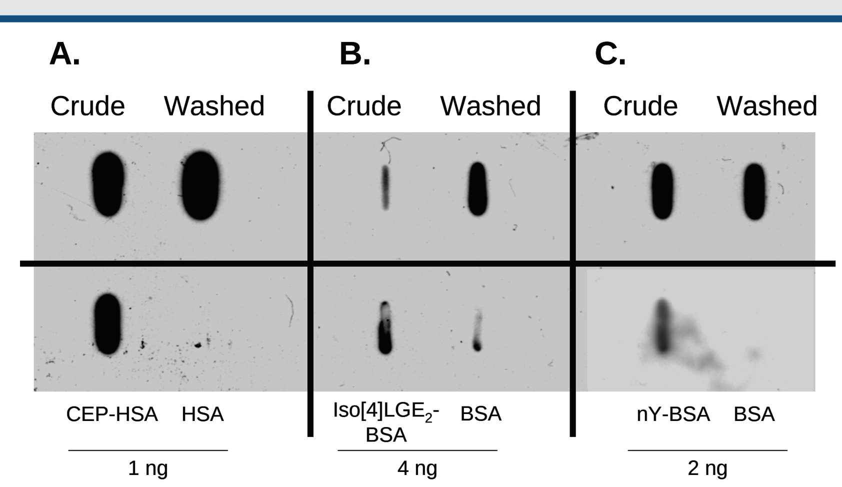


Figure 2. Oxidative Modifications in Crude and Debris-free Lipofuscin

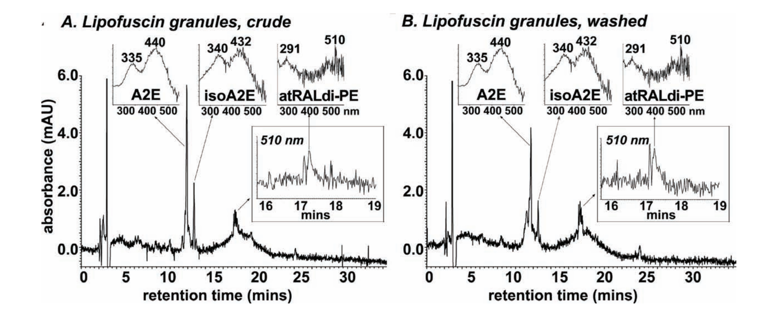
Slot blot Western analysis showed immunoreactivity for (**A**) Carboxyethylpyrrole (CEP), (**B**) iso[4]levuglandin$_2$ and (**C**) nitrotyrosine in the protein extracts from crude and debris-free LF. CEP-HSA, nitrated BSA and iso[4]LGE_2 modified BSA were used as positive controls. For both crude and washed LF granules, ~4.0E+07 granules were utilized.

CEP adducts are uniquely generated from oxidation of docosahexaenoate--containing phospholipids. The isolevuglandin family of reactive electrophiles, including iso[4]LGE_2, is generated by free radical-induced lipid oxidation and rearrangement of endoperoxide intermediates of the isoprostane pathway. Nitrotyrosine is generated from the reaction of tyrosine and free radical induced nitrogen dioxide (NO_2•) or peroxynitrite.

Table V. Proteins Identified from Crude Lipofuscin after SCX Chromatography

Accession[a]	Protein[b]	Calc Mass[c] (kD)	Peptide Matches
P07437	Tubulin beta	49.6	60
P05023	Na/K - transporting ATPase	112.8	43
P08100	Rhodopsin	38.9	41
P04406	Glyceraldehyde-3-phosphate dehydrogenase	36	35
P11488	Guanine nucleotide-binding protein	40	35
Q9BQE3	Tubulin alpha	49.9	29
P60709	Actin	41.7	27
P68871	Hemoglobin subunit beta	16	27
P14618	Pyruvate kinase isozymes M1/M2	57.9	23
P07339	Cathepsin D precursor	44.5	21
P60201	Myelin proteolipid protein	30.1	19
P02768	Serum albumin precursor	69.3	15
P12271	Cellular retinaldehyde-binding protein	36.5	14
P09543	2',3'-cyclic-nucleotide 3'-phosphodiesterase	47.5	13
P12277	Creatine kinase B-type	42.6	13
P62805	Histone H4	11.4	13
P78363	Retinal-specific ATP-binding cassette transporter	255.8	11
P06733	Alpha-enolase	47.1	10
P25705	ATP synthase subunit alpha	59.7	10
P13591	Neural cell adhesion molecule	93.3	10
Q16518	Retinal pigment epithelium-specific 65 kDa	60.9	10
P21796	Voltage-dependent anion-selective channel protein 1	30.8	10
Q05639	Elongation factor 1-alpha 2	50.4	9
Q5QNW6	Histone H2B	13.9	9
P02686	Myelin basic protein	33.1	9
P80723	Brain acid soluble protein 1	22.7	8
Q00610	Clathrin heavy chain 1	191.5	8
Q03395	Rod outer segment membrane protein 1	37.2	8
P10523	S-arrestin	45.1	8
P23634	Plasma membrane calcium-transporting ATPase 4	137.8	7
Q92781	11-cis retinol dehydrogenase	35	6
Q16555	Dihydropyrimidinase-related protein	62.3	6
P07900	Heat shock protein HSP	84.6	6
P35913	Rod cGMP-specific 3',5'-cyclic phosphodiesterase	98.3	6
P11166	Solute carrier family 2	54	6
P07195	L-lactate dehydrogenase	36.6	5
P00558	Phosphoglycerate kinase 1	44.6	5
P35243	Recoverin	23.1	5
Q02846	Retinal guanylyl cyclase 1 precursor	120	5
P47804	RPE-retinal G protein-coupled receptor	31.9	5
P08133	Annexin	75.8	4
P62988	Ubiquitin	8.6	4
Q15836	Vesicle-associated membrane protein 3	11.3	4
P08237	6-phosphofructokinase	85.1	3
P02511	Alpha crystallin	20.1	3
P00505	Aspartate aminotransferase	47.4	3
P43320	Beta crystallin	23.4	3
P43003	Excitatory amino acid transporter 1	59.5	3
P09972	Fructose-bisphosphate aldolase C	39.4	3
P06737	Glycogen phosphorylase	97.1	3
P11142	Heat shock cognate 71 kDa protein	70.9	3
Q14108	Lysosome membrane protein 2	54.3	3
P35625	Metalloproteinase inhibitor 3 precursor	24.1	3
P30041	Peroxiredoxin-6	25	3
Q9H0U4	Ras-related protein Rab-1B	22.2	3
P60174	Triosephosphate isomerase	26.7	3
Q08752	40 kDa peptidyl-prolyl cis-trans isomerase	40.7	2
P08195	4F2 cell-surface antigen heavy chain	57.9	2
Q13510	Acid ceramidase precursor	44.6	2
P01009	Alpha-1-antitrypsin precursor	46.7	2
P29973	cGMP-gated cation channel alpha 1	79.1	2
P00403	Cytochrome c oxidase subunit 2	25.5	2
P09104	Gamma-enolase	47.2	2
P06744	Glucose-6-phosphate isomerase	63.1	2
P17066	Heat shock 70 kDa protein 6	71	2
Q12931	Heat shock protein 75 kDa	80.1	2
Q6P9F7	Leucine-rich repeat-containing protein 8B	92.3	2
Q14894	Mu-crystallin homolog	33.8	2
Q9P2S2	Neurexin-2-alpha precursor	184.9	2
Q00325	Phosphate carrier protein	40.1	2
P07602	Proactivator polypeptide precursor	58.1	2
Q8N0Y7	Probable phosphoglycerate mutase 4	28.8	2
O43490	Prominin-1 precursor	97.1	2
Q8TC12	Retinol dehydrogenase 11	35.4	2
Q13813	Spectrin alpha chain, brain	284.4	2
P29401	Transketolase	67.8	2
P21281	Vacuolar ATP synthase subunit B	56.5	2

a. Swiss-Protein database accession numbers
b. Proteins were identified by MALDI TOF-TOF MS/MS following off-line SCX and RP-HPLC fractionation of a tryptic digest of crude lipofuscin
c. Based on protein sequence



	Isolated RPE Lipofuscin Granules (15% of Fraction; Equivalent to ~ 5 Human Donor Eyes) Crude Sample (*pmoles* / Total Extract Volume)	Isolated RPE Lipofuscin Granules Washed Sample (*pmoles* / Total Extract Volume)	Extract of Isolated RPE/Choroid from Single Donor Eye (64 Year Old) *pmoles* / eye
A2E + isoA2E	379.6	328.0	3500

Figure 3. Quantification of A2E Crude and Washed Lipofuscin

RP-HPLC profiles of LF extracts are shown from (**A**) crude LF and (**B**) debris-free LF that was washed with 0.2% SDS. A2E and isoA2E are indicated based upon spectra and retention times of authentic synthetic standards. Top insert, UV-visible spectra of A2E, isoA2E, and atRALdi-PE in acetonitrile/water. Lower right insert, a magnified HPLC profile for atRALdi-PE. The amount of A2E + isoA2E in these LF preparations is summarized in the Table. The results show that debris-free and crude LF contain essentially the same amount of A2E.

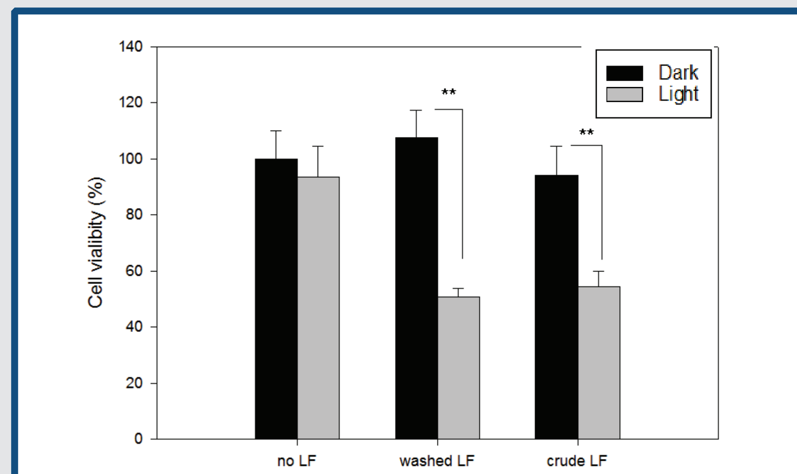


Figure 4. Phototoxicity Washed versus Crude Lipofuscin Granules

Cultured ARPE-19 cells were treated with washed or crude LF granules, exposed to intense blue light for 48 hrs or maintained in the dark then cell viability assayed. Treatment with debris-free LF reduced ARPE-19 viability by about the same amount as treatment with crude LF, namely ~50%. **p<0.0001, one-way ANOVA. n = 6 except for dark treated washed LF where n = 3.

CONCLUSIONS

- ◊ Human RPE LF isolated by conventional sucrose density gradient procedures contains substantial extra-granular material.
- ◊ Amino acid analysis demonstrated that debris-free LF granules contain ~2 % amino acids by weight compared with ~12 % by weight for crude LF.
- ◊ A significant number of proteins were identified by mass spectrometric analysis of crude LF while essentially none were identified from debris-free LF granules. LC MS/MS analysis of crude LF following SDS-PAGE support substantial posttranslational modification of extra-granular proteins (Table IV).
- ◊ Western slot blot analysis demonstrated the presence of CEP, iso[4]LGE_2 and nitrotyrosine in both crude and debris-free LF granules. These and other oxidative modification may be sufficiently extensive within granules to prevent tryptic digestion and MS/MS identification of proteins from debris-free LF.
- ◊ Debris-free LF granules and crude LF contain about the same amount of A2E.
- ◊ Debris-free LF granules are phototoxic to ARPE 19 cells

REFERENCES

1. Boulton, M. 1991. Ageing of the retinal pigment epithelium. In Progress in Retinal Research. N. Osborne, and G. Chader, editors. Oxford, New York: Pergamon Press. 125-151
2. Eldred, G.E. 1998. Lipofuscin and Other Lysosomal Storage Deposits in the Retinal Pigment Epithelium. In The retinal pigment epithelium. M.F. Marmor, and T.J. Wolfensberger, editors. New York: Oxford University Press. 651-668
3. Sparrow, J.R. and Boulton, M. (2005) Exp Eye Res 80:595-606.
4. Wassell, J., Davies, S., Bardsley, W., and Boulton, M. 1999. J Biol Chem 274:23828-23832.
5. Crabb, J.W., Miyagi, M., Gu, X., Shadrach, K., West, K.A., Sakaguchi, H., Kamei, M., Hasan, A., Yan, L., Rayborn, M.E., Salomon, R. G., and Hollyfield, J. G. (2002) Proc Natl Acad Sci U S A 99:14682-14687.
6. Crabb, J.W., West, K.A., Dodson, W.S., and Hulmes, J.D. (1997). Amino Acid Analysis. In Current protocols in protein science, Unit 11.9, Supplement 7. J.E. Coligan, H.L. Ploegh, J.A. Smith, and D.W. Speicher, editors: John Wiley & Sons, Inc. 11.09.01-11.09.42
7. Salomon, R. G., Sha, W., Brame, C., Kaur, K., Subbanagounder, G., O'Neil, J., Hoff, H. F., and Roberts, L. J., 2nd. (1999) J Biol Chem 274: 20271-20280
8. Parish, C. A., Hashimoto, M., Nakanishi, K., Dillon, J., and Sparrow, J. (1998) Proc Natl Acad Sci U S A 95: 14609-14613
9. Schutt, F., Davies, S., Kopitz, J., Holz, F. G., and Boulton, M. E. (2000) Invest Ophthalmol Vis Sci 41: 2303-2308.