Optic Nerve Fractionation for Proteomic Analysis

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ABSTRACT

Purpose: To evaluate the efficacy of solution state isoelectricfocusing (IEF) and 2D polyacrylamide gel electrophoresis (2D PAGE) for fractionation of optic nerve for proteomic analysis.

Methods: Optic nerve was dissected from normal human eyes obtained through the Cleveland Eye Bank and the Eye Donor Program of the Foundation Fighting Blindness, Inc. Optic nerve protein extraction efficiency was evaluated in 7M urea and 2M thiourea containing either 6% C_7BzO, 3% ASB-14, 3% dodecylmaltoside (DM) or a proprietary detergent solution. Soluble protein was quantified by the Bradford and bicinchoninic assays. Following solution state IEF using the Multicompartment Electrolyzer (MCE, Proteome Systems) and 2D PAGE, gel spots were excised, digested in situ with trypsin and proteins identified by capillary LC MS/MS.

Results: Optic nerve extracts from the four detergents produced similar 1D SDS-PAGE profiles, however, ASB-14 and dodecylmaltoside yielded slightly more soluble protein. Solution state IEF in 3% DM separated optic nerve proteins into pI fractions 3-5, 5-6.5, 6.5-8, and 8-11. After IEF, 2D PAGE demonstrated distinctly different patterns for each pI fraction. A total of 153 optic nerve proteins were identified by mass spectrometric analysis of select 2D gel spots. For most of these proteins, the sequence calculated pI was in good agreement with the pH range of the trapping chamber from which it was found.

Conclusions: Optic nerve contains significant lipid-rich myelin membranes and constitutes one of the more difficult tissues from which to extract and analyze soluble protein. Solution state IEF followed by 2D gel analyses has provided a productive approach for fractionating milligram amounts of optic nerve protein. The proteins identified in this study represent the most extensive catalogue to-date of human optic nerve proteins.

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INTRODUCTION

Progressive degeneration of the optic nerve is a common feature of ocular diseases such as glaucoma, optic neuritis and ischemic optic neuropathy. Proteomic analysis of diseased and/or damaged optic nerve tissue offers a promising approach to better understanding pathogenic mechanisms in glaucoma and other optic neuropathies. However, protein fractionation of optic nerve remains challenging because it contains significant lipid-rich myelin membranes and constitutes one of the more difficult tissues from which to extract soluble protein. Here, we describe the fractionation of human optic nerve by solution state isoelectricfocusing (IEF) and two-dimensional polyacrylamide gel electrophoresis (2D PAGE) and report the identification of 149 proteins (1). The present results demonstrate the potential utility of this fractionation methodology for differential proteomic analyses of optic nerve from diseased and normal eye donors.

METHODS

Human Tissue Procurement. Thirty-five eyes from normal human donors (15 female and 20 male, ages 43-89) were used in this study. Eyes were obtained through the Cleveland Eye Bank and the Eye Donor Program of the Foundation Fighting Blindness, Inc. (Owens Mills, MD). Eyes were enucleated within 8 hours of death and either placed in Optisol GS or frozen in liquid nitrogen.

Optic Nerve Sample Preparation. The stalk region of the optic nerve was carefully dissected. Tissues were stored at –80ºC until analyzed in 20mM Tris buffer pH7.5 containing protease inhibitors (P2714, Sigma), 5 µM dithiothrietol, 10 µM butylated hydroxytoluene and 2 mM EDTA. Protein extraction efficacy was compared for four different detergent solutions containing 7M urea and 2M thiourea, namely 6% C_7BzO, 3% ASB-14, 3% dodecylmaltoside and a proprietary detergent formulation from Proteome Systems. Following extraction, protein was precipitated with acetone, resuspended in Laemmeli sample buffer for 1D SDS-PAGE.

Solution State Isoelectricfocusing. Solution State IEF was performed using the Multicompartment Electrolyzer (MCE) according to Proteome Systems (3) with 1-7.5 mg of acrylamide alkylated optic nerve protein. IEF trapping chambers of pH 3-5, 5-6.5, 6.5-8 and 8-11 were prepared with immobilized pH membranes supplied by the vendor. Protein extracts were loaded into the central pH 5-6.5 chamber and diluted with the same detergent to 3.5 ml. Detergent solution was placed in the other trapping chambers and electrode solution supplied by the vendor was placed in the terminal MCE chambers. IEF was carried out with a 100-1500V ramp over 13 hours followed by a constant 1500 V for an additional 5 hours. Maximum current was set at 0.8 mA. After IEF protein in the chamber solutions was concentrated by acetone precipitation.

Acetone Precipitation. Four volumes of acetone were added to one volume of the detergent solubilized protein solution and incubated at room temperature for 20 min with intermittent, gentle mixing. The suspension was then centrifuged at 2500 x g for 15 min at room temperature and the clear supernatant fluid carefully removed with a pipette. The precipitate was re-centrifuged at 2500 x g for 5 min and residual supernatant removed. For 1D-PAGE, the pellet was resuspended in Laemmli sample buffer, and quantified by the bicinchoninic (BCA) protein assay (Pierce). For 2D-PAGE, the pellet was resuspended in 3% dodecylmaltoside containing 7M urea and 2M thiourea and quantified by a modified Bradford assay (2).

2D-PAGE. First dimension IEF was performed with the Pharmacia IPGphor, 11 cm precast ProteomIQ™ immobilized linear pH gradient strips (IPG, pH 3-10) from Proteome Systems and a programmed voltage gradient (4). For the second dimension, IPG strips were equilibrated in 50 mM Tris acetate pH 7.0, 3 M urea, 2% SDS, and 0.0001% bromophenol blue then embedded in 0.7% w/v agarose on the top of a 6-15% acrylamide linear gradient gel containing a 4% stacking gel (ProteomeIQ™ Gelchip, Proteome Systems). Second dimension 1D-PAGE was performed for 5h at 300 mA constant current. Gels were stained with ProteomeIQ™ Blue (Proteome Systems) and scanned with a GS-710 Imaging Densitometer (BioRad).

Protein identification. Select 2D gel spots were excised, digested *in-situ* with trypsin, and peptides were extracted and analyzed by LC MS/MS using a CapLC system and a quadrupole time-of-flight mass spectrometer (QTOF2, Waters Corp.) (1, 5, 6). Protein identifications from MS/MS data utilized ProteinLynx™ Global Server (Waters Corporation) and Mascot (Matrix Science) search engines and the Swiss-Protein and NCBI protein sequence databases.

RESULTS

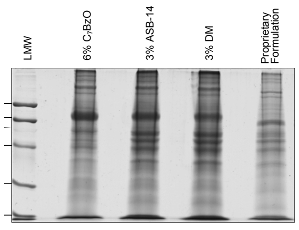

Figure 1. SDS-PAGE of Optic Nerve Proteins Extracted in Different Detergents

Protein was extracted from equal amounts of optic nerve (~20 mg wet weight) using the indicated detergents (100 µl per extraction) and 15 µg protein per lane subjected to SDS-PAGE on a 10% gel.

All four detergents produced similar 1D-PAGE profiles, however, ASB-14 and dodecylmaltoside yield greater Coomassie blue staining intensity and slightly greater recovery of soluble protein (Table 1).

Table 1. Solubilization of Optic Nerve Protein

Detergent Solution	Protein (µg) Recovered per mg Tissue	Total Protein Recovered (µg)	Relative Yield (%)
6% C_7BzO, 7M Urea, 2M thiourea	8.0 ± 0.8	160.4 ± 2.5	0.8
3% ASB-14, 7M Urea, 2M thiourea	8.7 ± 0.6	174.0 ± 1.8	0.9
3% Dodecylmaltoside, 7M Urea, 2M thiourea	8.9 ± 0.7	176.3 ± 1.5	0.9
Proprietary formulation, Proteome Systems	6.7 ± 0.4	134.4 ± 2.8	0.7

Optic nerve explants (20 ± 1.25 mg wet weight) were extracted (1x) with the indicated detergent solution (100 µl) and soluble protein quantified by a modified Bradford assay as described in Methods. Average results (± standard deviation) are shown from four independent experiments.

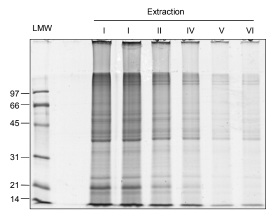

Figure 2. Multiple Protein Extractions

Protein was extracted from triplicate 200 mg (wet weight) optic nerve explants in 200 µl of 3% dodecylmaltoside, 7M urea, 2M thiourea and equal volumes (30 µl) subjected to 1D-PAGE. The average recovery (± standard deviation) for six successive extractions shown above was as follows: 793 ± 22 µg, 664 ± 12 µg, 567 ± 25 µg, 327 ± 14 µg, 101 ± 8 µg, 77 ± 11µg. The total combined recovery from the six extractions was ~2.53 mg (1.3% relative to the tissue wet weight).

For comparison, tissue samples from liver and muscle extracted under identical conditions yielded 4.9% and 2.7%, respectively, relative to tissue wet weights. Extraction of soluble protein from the membrane-protein rich optic nerve is more difficult than from many other tissues.

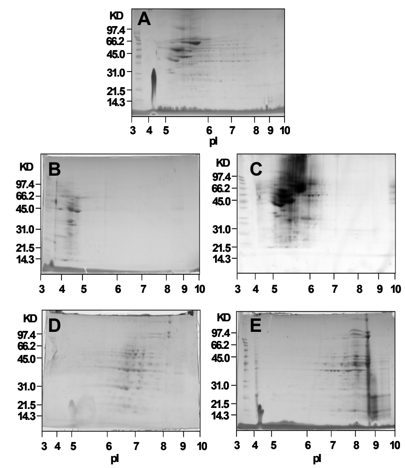

Figure 3: 2D PAGE Analyses of MCE Fractions.

Following solution state IEF of optic nerve extracts (5 mg), 2D PAGE analysis of the IEF fractions (100 mg each) was performed using immobilized linear pH gradient strips (pH 3-10, 11 cm), 6-15% gradient gels and Coomassie blue staining. **A.** Optic nerve protein before solution state IEF,100 µg; **B.** IEF fraction pH 3-5; **C.** IEF fraction pH 5-6.5; **D.** IEF fraction pH 6.5-8; **E.** IEF fraction pH 8-11.

This figure demonstrates that effective fractionation of the optic nerve extract was achieved by solution sate IEF. Recovery of protein from the IEF trapping chambers following acetone precipitation was about 31-39% (Table 2).

Table 2. Recovery of Optic Nerve Protein from Solution State IEF

IEF Chamber	3.0 mg Sample	5.0 mg Sample	7.5 mg Sample
		protein recovery (µg)	
Anode	-	-	-
pH 3-5	107	223	290
pH 5-6.5	560	1242	1100
pH 6.5-8	120	231	875
pH 8-11	105	192	590
Cathode	45	47	45
Total Protein (µg)	937	1935	2900
Percent Recovery	31	39	39

Optic nerve protein in 3% dodecylmaltoside, 7M Urea, 2M thiourea was fractionated by solution state IEF with the MCE and recovered protein quantified by a modified Bradford assay (see Methods).

Table 3. Human Optic Nerve Proteins Identified after MCE and 2D gel Fractionation[a]

Protein ID	Accession No.[b]	Peptide Matches	Molecular Mass (kDa) Calculated[c]	Molecular Mass (kDa) Observed[d]	pI Calculated[c]	pI Observed[d]
MCE chamber pH 3-5						
Alpha-1-acid glycoprotein 1	P02763	6	23	25	5.0	3.5
Annexin A2	P07355	4	38	55	7.6	4.5
Apolipoprotein D	P05090	1	21	30	5.1	4.7
Cullin homolog 5	Q93034	1	91	24	8.3	3.5
Desmin	P17661	4	53	55	5.2	4.5
Desmocollin	Q02487	1	99	22	5.2	4.5
Elongation factor 1-beta	P24534	3	25	30	4.5	4.7
F-box only protein 2	Q9UK22	4	33	65	4.3	3.8
Glial fibrillary acidic protein[e]	P14136	24	50	45	5.5	4.8
Glutamine synthetase	P32288 (S)	2	42	22	6.3	4.5
Glyceraldehyde 3-phosphate dehydrogenase	P04406	8	36	55	8.8	4.5
Long-chain-fatty-acid--CoA ligase 2	P33121	1	78	45	7.2	4.8
Mimecan[e]	P20774	3	34	45	5.5	4.8
Nasopharyngeal epithelium specific protein 1	Q9UL16	1	46	30	10.3	4.7
Paired box protein Pax-3	P23760	1	53	55	9.0	4.5
PITSLRE serine-threonine kinase CDC2L1	P21127	1	93	30	5.6	4.7
Placental thrombin inhibitor	P35237	2	43	45	5.2	4.7
Plasticin	P31393 (C)	4	53	55	5.5	4.5
Proteasome subunit alpha type 5	P28066	2	26	25	4.8	4.7
Protein 14-3-3, Epsilon	P42655	26	29	30	4.7	4.7
Protein 14-3-3, eta	Q04917	8	28	25	4.8	4.7
Protein 14-3-3, tau	P27348	8	28	25	4.7	4.7
Protein 14-3-3, zeta/delta	P29312	11	28	25	4.8	4.7
Protein FAN	Q92636	1	104	30	6.1	4.7
Protein phosphatase 1 regulatory subunit 12B	O60237	1	110	45	5.6	4.8
Pyruvate kinase, M1 isozyme	P14618	3	58	20	8.2	4.5
Rho GDP-dissociation inhibitor 1	P52565	2	23	25	5.0	4.7
Vimentin	P08670	30	54	55	5.1	4.5
WD-repeat protein 9	Q9NSI6	1	257	22	8.8	3.1
MCE chamber pH 5-6.5						
Actin, Beta	P60709	22	42	40	5.3	5.5
Annexin A5	P08758	19	36	36	5.0	5.1
Apolipoprotein A-I	P02647	22	31	30	5.7	5.5
Apolipoprotein D	P05090	1	21	30	5.1	5.5
Creatine kinase	P12277	18	43	45	5.5	5.5
Cytoplasmic antiproteinase	P35237	1	43	36	5.2	5.1
Dihydropyrimidinase related protein-2[e]	Q16555	2	62	35	6.0	4.9
Enolase, Alpha	P06733	20	47	50	7.5	6.5
Extracellular superoxide dismutase	P08294	1	26	30	6.5	5.5
Ferritin heavy	P02794	2	21	30	5.5	5.5
Glial fibrillary acidic protein, astrocyte[e]	P14136	1	50	30	5.4	5.5
Glutathione S-transferase Mu 3	P21266	2	26	30	5.4	5.5
Glutathione S-transferase P	P09211	9	23	24	5.5	5.5
Heat shock 27 kDa protein	P04792	1	23	30	6.3	5.5
Histone-lysine N-methyltransferase	Q15047	1	143	36	5.9	5.5
Hypothetical protein DJ845O24.1	O60809	1	53	24	8.7	4.9
Ig gamma-1 chain C region	P01857	4	36	36	8.6	6.5
Ig kappa chain V-I region A	P01593	1	12	30	5.9	5.5
Ig kappa chain V-I region N	P01613	1	12	30	5.4	5.5
Ig lambda chain C regions	P01842	2	11	30	7.4	5.5
Junction plakoglobin	P14923	1	81	35	6.3	4.7
Mimecan[e]	P20774	4	34	36	5.5	5.1
NDRG2 protein[e]	Q9UN36	1	40	36	5.2	5.5
Neurofilament triplet M protein[e]	P07197	1	102	24	4.9	4.9
Neuron cytoplasmic protein[e]	P09936	5	25	24	5.4	5.5
Peroxiredoxin 2	P32119	1	22	30	5.9	5.5
Protein 14-3-3, Beta/alpha	P31946	7	28	24	4.8	4.9
Protein 14-3-3, Epsilon	P42655	14	29	30	4.7	4.7
MCE chamber pH 6.5-8						
Acyl-CoA dehydrogenase, very-long-chain specific	P49748	2	70	65	9.1	7.2
Aldo-keto reductase family 1 member C2	P52895	2	37	36	7.6	6.7
Aldo-keto reductase family 1 member C3	P42330	2	37	36	8.3	6.7
Annexin A2	P07355	17	38	36	8.0	6.7
Aspartyl-tRNA synthetase	Q9KDG1 (H)	2	67	65	5.1	6.7
Basic membrane protein A	O31280 (B)	2	37	36	5.2	7.0
Beta-1-syntrophin	Q13884	1	58	50	9.0	7.3
Biglycan precursor	P21810	1	41	36	7.0	6.7
cAMP-dependent chloride channele	P13569	1	168	36	9.0	6.7
Collagen alpha 1(I)	P02452	2	139	120	5.8	7.8
Collagen alpha 2(I)	P08123	2	129	116	9.2	7.8
Crystallin Alpha, B	P02511	9	20	20	7.3	6.7
Cytoskeleton-like bicaudal D protein homolog 1	Q96G01	1	110	65	5.8	7.2
G2/mitotic-specific cyclin B1	P14635	1	48	36	7.6	6.7
Glial fibrillary acidic protein, astrocytee	P14136	1	49	65	5.4	7.2
Glyceraldehyde 3-phosphate dehydrogenase	P04406	17	36	36	8.8	6.7
Hermansky-Pudlak syndrome 3 protein	Q969F9	1	113	55	6.4	7
Ig gamma-1 chain C region	P01857	1	36	50	8.6	7.3
Kinesin-like protein KIF12	Q96FN5	1	60	55	9.4	7
M-phase inducer phosphatase 2	P30305	1	65	36	6.1	6.7
Neurexin 3-alpha precursor (Neurexin III-alpha)e	Q9Y4C0	1	169	116	6.1	7.8
Nuclear pore complex protein Nup88	Q99567	1	84	36	5.6	6.7
Palmitoyl-protein thioesterase 1	P50897	1	34	36	6.7	6.7
Peroxiredoxin 1	Q06830	3	22	20	8.5	6.7
Peroxisome assembly factor-2	Q13608	1	104	50	6.3	6.8
Phosphatidylethanolamine-binding protein	P30086	10	21	20	7.6	6.7
Prolargin	P51888	2	44	50	9.6	7.3
Protein-glutamine glutamyltransferase 4	P49221	1	77	65	6.7	7.2
Pyruvate kinase, M1 isozyme	P14618	24	58	55	8.2	7.0
Restricted expression proliferation associated protein	Q9ULW0	1	86	65	9.6	7.2
Rho-related GTP-binding protein RhoG	P35238	1	21	20	8.5	6.7
Ribose 5-phosphate isomerase	P49247	1	26	116	7.6	7.8
T-complex protein 1, eta subunit	Q99832	1	59	55	7.9	7
Transcription factor BF-1e	P55315	1	65	50	9.9	7.3
Transketolase	P29401	19	68	65	7.9	7.2

Protein ID	Accession No.[b]	Peptide Matches	Molecular Mass (kDa) Calculated[c]	Molecular Mass (kDa) Observed[d]	pI Calculated[c]	pI Observed[d]
MCE chamber pH 8-11						
2',3'-cyclic nucleotide 3'-phosphodiesterase[e]	P09543	18	48	50	9.4	8.6
Acylphosphatase, muscle type isozyme	P14621	1	11	14	9.8	9.5
ATP synthase, Alpha	P25705	5	60	60	9.4	8.6
Caspase recruitment domain protein 4	Q9Y239	1	107	65	7.1	7.9
Collagen, Alpha 1(I)	P02452	3	139	150	5.8	9.0
Collagen, Alpha 1(II)	P02458	2	134	250	8.7	9.0
Collagen, Alpha 2(I)	P08123	4	129	116	9.2	9.1
Disco-interacting protein 2 homolog[e]	Q14689	1	165	250	8	9
DNA polymerase gamma subunit 1	P54098	1	139	60	6.8	8.6
DNA-directed RNA polymerase	O00411	2	139	50	9.2	8.6
Dystrobrevin alpha (Dystrobrevin-alpha)[e]	Q9Y4J8	1	84	30	6.8	9.5
Elongation factor 1-alpha	P18624 (D)	3	50	250	9.1	9.0
Fibrinogen alpha/alpha-E	P02671	2	95	20	5.9	9.5
FK506-binding protein 1A	P20071	1	12	14	8.5	8.6
Fructose-bisphosphate aldolase A	P04075	6	39	50	8.5	8.6
Glyceraldehyde 3-phosphate dehydrogenase	P80534	11	39	40	9.0	7.1
Guanine nucleotide-binding protein[e]	Q9UBI6	1	8	14	9.4	9.5
Hemoglobin, Alpha	P01922	7	15	15	9.0	8.6
Hemoglobin, Delta	P02042	3	16	15	8.4	8.6
Heterogeneous nuclear ribonucleoprotein C	P07910	2	34	15	5.0	9.5
Histone deacetylase 5	Q9UQL6	1	122	250	6.2	9
Histone H2A2	P28001	2	14	14	11.2	9.5
Histone H2B.d (H2B/d)	Q99877	4	14	14	10.6	9.5
Histone H3	P06351	3	15	14	11.3	9.5
Histone H4	P02304	3	11	14	11.4	9.5
Hypothetical protein JHP1324	Q9ZJI5 (P)	2	36	116	8.8	9.1
Ig gamma-1 chain C region	P01857	3	36	60	8.6	8.6
Mast/stem cell growth factor receptor	P10721	1	110	60	6.8	8.6
Myelin basic protein[e]	P02686	6	33	30	10.1	9.5
Na(+)-translocating NADH-quinone reductase	P00387	2	34	15	7.3	8.6
NADH-ubiquinone oxidoreductase, 15 kDa	O43920	1	12	11	9.5	9.5
NADH-ubiquinone oxidoreductase, B8	O43678	1	10	14	9.9	9.5
Nuclear pore complex protein Nup88	Q99567	1	83	65	5.6	7.9
Phosphoglycerate kinase	P07205	2	45	50	9.0	8.6
Phosphoglycerate kinase 1	P00558	7	45	50	8.5	8.6
Potential phospholipid-transporting ATPase	P98198	1	137	60	7	8.6
Prolargin	P51888	4	44	116	9.6	9.1
Protein rdxB	P54932 (R)	2	54	45	8.5	7.1
Putative HTH-type transcriptional regulator HI0570	P44757 (E)	2	23	250	9.6	9.0
Putative transmembrane protein NMB	Q14956	1	63	11	6.6	9.5
Pyruvate kinase, M1 isozyme	P14618	3	58	60, 36	8.2	9.5
Ribosomal protein L12, 60S	P30050	2	18	20	9.8	9.5
S-arrestin	P10523	1	45	14	6.5	9.5
Septin 7 (CDC10 protein homolog)	Q16181	1	49	60	9.1	8.9
Serum albumin	P02768	2	69	15	6.2	9.5
Shwachman-Bodian-Diamond syndrome protein	Q9Y3A5	1	28	14	9.1	9.5
Signal recognition particle 14 kDa protein	P37108	1	14	14	10.4	9.5
Trifunctional enzyme, Alpha	P40939	2	83	150	9.4	9.0
Ubiquinol-cytochrome C reductase complex	O14949	1	9	11	10.2	9.5

a. Spots were excised from the 2D gel shown in Figure 3 and proteins identified by LC MS/MS as described in Methods.
b. Swiss-Protein database accession numbers are shown. Links to Swiss-Protein accession numbers use the EXPASY server at http://us.expasy.org/sprot/. Parenthesis following accession numbers designate identifications based on homology with other species: B=Borrelia afzelii, C=Carassius auratus, D=Dictyostelium discoideum, E=Haemophilus influenzae, H=Bacillus halodurans, P=Helicobacter pylori J99, R=Rhodobacter sphaeroides, S=Saccharomyces cerevisiae.
c. Based on protein sequence.
d. Approximate mass and pI observed on 2D gels.
e. Also found in brain tissue (19).

CONCLUSIONS

- ASB-14 and dodecylmaltoside were relatively effective in extracting optic nerve proteins in the presence of 7M urea and 2M thiourea, and multiple extractions improved the overall protein recovery from the tissue.
- In this study, larger sample amounts (5 - 7.5 mg) yielded better protein recovery from IEF. Optimization of the MCE instrument for smaller sample amounts and a more efficient protein concentration method than acetone precipitation could improve recovery.
- 149 proteins were identified from 2D gel spots including many neuronal proteins such as myelin basic protein, mimecan and neuron cytoplasmic protein. For most of the identified proteins, the sequence calculated pI was in good agreement with the pH range of the trapping chamber from which it was found.
- Solution state IEF has provided a productive approach to the fractionation of milligram amounts of optic nerve proteins for proteomic analyses.
- Differential proteomic analyses of diseased and normal optic nerve offers a rationale approach to deciphering pathogenic mechanisms in glaucoma and other optic neuropathies.

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