Analysis of Protein Digests by Nano-SCX/RP/MSMS with pH/salt Gradient SCX Elution

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Introduction

As the sequenced human genome and levels of expression of mRNA do not fully describe the current state of a living cell, in order to understand complex cellular processes and pathways relevant to environmental cancer research conducted at ECD NHEERL EPA, it is important to be able to analyze the actual protein composition. Proteomics has recently emerged as a scientific discipline with the main purpose to identify, in a comprehensive manner, proteins in complex biological systems. Such comprehensive characterization of proteins is a challenging task requiring application of multiple analytical methods combining separation and detection techniques.

In this study, an automated 2D (SCX/RP) – LC/MSMS method has been developed. The technique utilizes nano-electrospray ionization to maximize sensitivity. Separation of the peptides in the first LC dimension has been the main focus of this work, and it was optimized using human serum albumin (HSA) and human lung epithelial cell lysate tryptic digests. Prior to the digestion with trypsin, the samples are reduced and alkylated with different cysteine alkylating reagents thus providing some peptides with identical amino acid sequences but differing in cysteine tags. In particular, a quaternary amine tag (APTA) has been useful for the evaluation of peptides elution order under SCX conditions. These optimized SCX peptide elution conditions combine a simultaneous pH - gradient with ammonium acetate salt – gradient elution.

Experimental

Protein Extraction

Cellular protein extract was prepared from human lung epithelial cells collected from eight 150 mm dishes. Cells were washed in PBS twice and re-suspended in 50 mM Tris-HCL, pH: 7.5. Three cycles of freezing and thawing were applied followed by 15 min centrifugation at 4ºC. Only the soluble protein fraction was used.

Human Serum Albumin (HSA) standard was purchased from Sigma-Aldrich, Inc.

Reduction, Akylation and Purification.

The test protein sample was split into 2 aliquots, each 80 ul; ca. 2 ug/ul. In the first aliquot, the cysteine residues were reduced with dithiothriethol [DTT] in 6M urea and 0.1M Tris buffer at pH 7.8, and alkylated with iodoacetic acid amide [IAM]. The sample was then purified using syringe-mounted reverse-phase-like polymeric protein cartridges (macro protein trap from Michrom Bioresources) into 2 fractions: IAM1 (eluted with 300 ul of 50% acetonitrile/49.9% water/0.1% trifluoroacid acid [TFA]) and IAM2 (eluted with 300 ul of 90% acetonitrile/9.9% water/0.1% TFA). The second 80 ul- aliquot was also similarly reduced but at Tris buffer pH 8.25, but the alkylation was conducted using (3-acrylamido propyl)trimethylammonium chloride [APTA], (*"Enrichment of Cysteine-Containing Peptides from Tryptic Digests Using a Quaternary Amine Tag"*, *Anal. Chem.* **2004**; *76*(15); 4522-4530; modified procedure). The sample was also purified on the protein macro-trap cartridge, washed thoroughly with water to remove urea and the APTA reagent, and eluted with 300 ul of 50% acetonitrile/49.9% water/0.1% TFA), generating "APTA1" sample fraction. The samples were evaporated under vacuum at 40 ºC to almost dryness (approx. 15 ul) to remove most of the volatile buffer.

Trypsin Digestion.

The three model complex protein mixture samples IAM1, IAM2 and APTA1 were each digested overnight in 100 ul trypsin solution (4 ug/100 ul trypsin in 50 mM ammonium bicarbonate buffer) at 37.4 ºC. The evaporation and volume reduction step was repeated. The sample volume was adjusted to approx. 50 ul with water and the pH was adjusted with 2M acetic acid to pH 3.7. An aliquot of 10 ul volume was removed from each of the three sample solutions and analyzed individually by 2D/LC/MSMS.

Similarly, the **HSA** sample was reduced and alkylated with the three following alkylating reagents: IAM, APTA and 4-Vinylpyridine (VP). The alkylated and digested samples (HSA - IAM, VP, APTA) were combined together and injected onto the 2D LC System.

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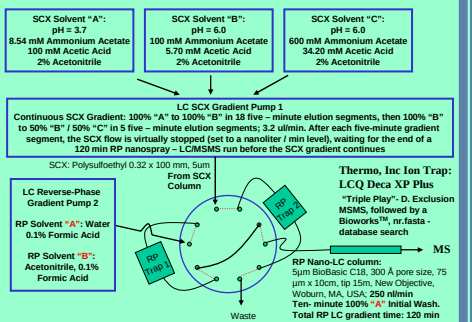
2D (SCX/RP) – LC/MSMS experimental layout: SCX – gradient pH elution followed by SCX elution at constant pH 6.0



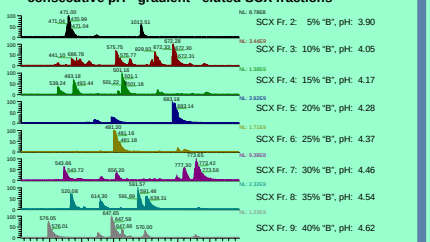
Structurally-Related peptides eluting in SCX chromatography. Retention increases with increasing number of basic residues, and decreases with increasing number of acidic residues. Adjacent residues of the same kind exert a lesser effect probably due to the same – charge repulsion.

Peptide Sequence	Elution pH	Acidic Residues # D, E	Basic Residues # H, K, R, C$^{up.}_{APTA}$
DLGEENFK	3.90	3	1
LVAASQAALGL	3.90	0	0
KLVAASQAALGL	4.46	0	1
LVRPEVDVM*CTAFHDNEETFLK	4.46	5	3
LVRPEVDVMCTAFHDNEETFLK	4.46	5	3
LVRPEVDVMCTAFHDNEETFLKK	4.86	5	4
YLYEIAR	4.28	1	1
KYLYEIAR	4.97	1	2
NEC$^{#DAM}$FLQHK	4.69	1	2
NEC$^{#DAM}$FLQHKDDNPNLPR	4.69	3	3
NEC$^{#APTA}$FLQHKDDNPNLPR	5.37	3	4
QEPERNEC$^{#DAM}$FLQHKDDNPNLPR	4.69	5	4
QEPERNEC$^{#DAM}$FLQHKDDNPNLPR	5.09	5	5
AQHVFQHAVPQEGKPITNQK	6.00	1	4

Cellular lysate protein digest: cumulative data from three 2D-LC-MSMS experiments: IAM1, IAM2, and APTA1. Most peptides elute off the SCX column before pH 6.0 - 0.1 M ammonium acetate

Acetic Acid [mM]	Ammonium Acetate [mM]	SXC Fraction pH	DTA MSMS Files
100.00	8.54	3.70	156
95.29	13.11	3.90	186
90.57	17.69	4.05	207
85.86	22.26	4.17	114
81.14	26.83	4.28	127
76.43	31.41	4.37	118
71.71	35.98	4.46	108
67.00	40.55	4.54	137
62.28	45.12	4.62	118
57.57	49.70	4.69	91
52.85	54.27	4.77	116
47.19	59.76	4.86	98
40.59	66.16	4.97	103
33.99	72.56	5.09	85
27.39	78.96	5.22	75
20.79	85.37	5.37	73
14.19	91.77	5.57	62
5.70	100.00	6.00	68
		Subtotal for pH gradient elution: 2042	
8.55	150.00	6.00	40
11.40	200.00	6.00	47
14.25	250.00	6.00	58
17.10	300.00	6.00	72
19.95	350.00	6.00	47
			Total DTAs: 2306

HSA Digest: reverse-phase "base-peak" LC/MS chromatograms for the SCX runs 5 – 40% "B", pH 3.90 – 4.62, show no carry-over between consecutive pH - gradient - eluted SCX fractions



Top 70 proteins out of the total of 900 protein hits passing Bioworks™ multiscore filter. The data set was displayed as a multi-consensus report from the IAM1, IAM2, and APTA1 cell-lysate protein 2D-LC/MSMS runs

Reference File_Scan(s)	Score Delta Cn	Accession	Peptides (Hits)
Human Serum Albumin in A Complex With Myristic Acid And Tri_Iodobenzoic Acid	530.2911	4389275	53 (53 0 0 0 0)
PRO1708 [Homo sapiens]	392.2923	7992791	36 (31 5 0 0 0)
alpha enolase [Homo sapiens]	302.3379	2863209	32 (24 7 1 0 0)
heat shock 70kDa protein 5 (glucose_regulated protein_78kDa); BiP; he	298.291	16507237	30 (29 1 0 0 0)
EnoA' cytokeratin (5' end put.); putative	266.2389	309215	27 (25 2 0 0 0)
pyruvate kinase_muscle [synthetic construct]	258.332	33303751	26 (25 1 0 0 0)
Heat_Shock Cognate 70kd Protein (44kd Atpase N_Terminal Fragment) (E.C.3.6.1.3	246.2923	6402929	26 (19 7 0 0 0)
unnamed protein product [Homo sapiens]	240.3144	32486	24 (24 0 0 0 0)
Filamin B (FLN_B) (Beta_filamin) (Actin_binding like protein) (ABP	238.2561	38257404	24 (23 1 0 0 0)
transketolase [Homo sapiens]	220.3823	4507521	22 (22 0 0 0 0)
Aldolase A (E.C.4.1.2.13)	218.2972	229674	22 (21 1 0 0 0)
ACTIN 1_MUSCLE_SPECIFIC	200.2809	1168323	24 (14 5 2 1 2)
Endoplasmin precursor (Heat shock 108 kDa protein) (HSP108) (HSP 108	192.4557	119359	20 (18 0 2 0 0)
unnamed protein product [Homo sapiens]	176.2368	28193204	18 (16 2 0 0 0)
90_KDa heat shock protein alpha	170.279	34392343	17 (17 0 0 0 0)
beta tubulin	164.3458	32816015	18 (16 0 0 0 2)
tropomyosin 5; TM_5	160.6249	3653293	16 (16 0 0 0 0)
G01880 fatty_acid synthase (EC 2.3.1.85) (version 2) _human	160.3701	7433799	16 (16 0 0 0 0)
heat shock protein 90_beta [validated] _human	160.2816	72222	16 (16 0 0 0 0)
filamin 1 (actin_binding protein 280); filamin 1; filamin A_alpha (act	150.2224	4503745	15 (15 0 0 0 0)
KPYK_XENLA Pyruvate kinase_muscle isozyme (Cytosolic thyroid hormone binding	146.4116	2497536	15 (13 2 0 0 0)
cytokeratin [Homo sapiens]	146.2401	1419564	15 (13 2 0 0 0)
keratin 18 [Homo sapiens]	140.3312	4557888	14 (14 0 0 0 0)
clathrin heavy chain; clathrin heavy polypeptide_like 2 [Homo sapiens]	140.2535	4758012	14 (14 0 0 0 0)
A Chain A, Crystal Structure Of A Mammalian 2_Cys Peroxiredoxin _Hbp23.	128.309	6435547	13 (13 1 0 0 0)
vimentin [Homo sapiens]	120.3481	5030431	12 (12 0 0 0 0)
gamma filamin; filamin 2; filamin C_gamma (actin_binding protein_280);	120.3269	4557507	12 (12 0 0 0 0)
phosphoglycerate mutase 1	116.215	37362264	12 (10 2 0 0 0)
similar to glyceraldehyde_3_phosphate dehydrogenase (phosphorylating)	112.377	34809870	26 (0 6 5 20 1)
JQ1260 nucleoprotein _simian paramyxovirus SV5	110.2988	77143	11 (11 0 0 0 0)
similar to interferon_inducible protein 10 (IP_10) receptor	110.2034	20984919	11 (11 0 0 0 0)
ubiquitin carboxyl_terminal esterase L1 (ubiquitin thiolesterase); Ubi	108.2461	21361091	11 (10 1 0 0 0)
nonmuscle myosin heavy chain_A	108.2132	189030	11 (10 1 0 0 0)
similar to glyceraldehyde_3_phosphate dehydrogenase (phosphorylating)	106.2722	38090339	12 (7 3 2 0 0)
prolyl 4_hydroxylase_beta polypeptide; Protein disulfide isomerase (P	100.3598	6981324	10 (10 0 0 0 0)
actinin_alpha 1; non_muscle alpha_actinin 1	100.2948	13591902	10 (10 0 0 0 0)
Stress_induced phosphoprotein 1	100.2059	13277819	10 (10 0 0 0 0)
thioredoxin reductase GRIM_12 [Homo sapiens]	90.26575	3820535	9 (9 0 0 0 0)
glucosidase II alpha subunit [Homo sapiens]	90.3554	7672977	9 (9 0 0 0 0)
myosin_heavy polypeptide 9; Myosin_heavy polypeptide 9 _non_muscle [R	88.3343	6981236	9 (8 1 0 0 0)
alpha_tubulin [Homo sapiens]	88.31571	32015	9 (8 1 0 0 0)
UV excision repair protein RAD23 homolog B; XP_C repair complementing p	88.2713	4506387	9 (8 1 0 0 0)
ACT_COSCS ACTIN	88.26859	399003	9 (8 1 0 0 0)
14_3_3protein	86.3618	2896518	9 (8 0 1 0 0)
EDARADD protein [Homo sapiens]	84.229	28422655	9 (8 0 1 0)
chaperonin subunit 2 (beta)	80.32716	6671700	8 (8 0 0 0 0)
ACT2_NAEFO Actin II	80.30848	113239	8 (8 0 0 0 0)
Endoplasmin (94 kDa glucose_regulated protein)	80.2654	6015101	8 (8 0 0 0 0)
filamin B_beta (actin binding protein 278); beta filamin; filamin 1 (a	80.24666	10079205	8 (8 0 0 0 0)
Homo sapiens profilin 1 [synthetic construct]	80.24197	30584265	8 (8 0 0 0 0)
enolase 2 (gamma, neuronal) [synthetic construct]	80.23016	32680095	8 (8 0 0 0 0)
filamin B_beta (actin binding protein 278); beta filamin; filamin 1 (a	80.21455	4503747	8 (8 0 0 0 0)
Human Thioredoxin Mutant With Cys 73 Replaced By Ser (Reduced Form)	80.19379	1827675	8 (8 0 0 0 0)
glucose regulated protein; endoplasmic reticulum protein; glucose regul	80.17952	6679687	8 (8 0 0 0 0)
albumin	78.27688	30794280	8 (7 1 0 0 0)
uracil DNA glycosylase [Homo sapiens]	78.26559	89053	10 (5 2 1 1 1)
vinculin	78.23221	17979613	8 (7 1 0 0 0)
unnamed protein product	78.22465	26327721	8 (7 1 0 0 0)
A Chain A, Human Heart L_Lactate Dehydrogenase H Chain_Ternary Complex With N	78.21955	13768647	8 (7 1 0 0 0)
actin [Allogromia sp.]	74.27876	4837604	10 (8 1 1 0)
glyceraldehyde_3_phosphate dehydrogenase	70.3253	27356762	7 (7 0 0 0 0)
Homo sapiens lactate dehydrogenase A [synthetic construct]	70.31244	30584487	7 (7 0 0 0 0)
NPM1 protein [Homo sapiens]	70.29186	16307090	7 (7 0 0 0 0)
acetyl CoA transferase_like protein [Homo sapiens]	70.28783	18880019	7 (7 0 0 0 0)

Conclusions:

This new automated 2D-LC/MSMS method, designed for protein digests analyses, is based on the principle of simultaneous pH - gradient and salt – gradient – induced SCX elution, followed by peptide trapping and RP nanospray-LC/MSMS. It is characterized by low carry-over between neighboring SCX fractions and predictable SCX elution pattern, mainly dependent on the number of acidic and basic amino acid residues present in a peptide. Most tryptic peptides elute at relatively low ammonium acetate content (below 100 mM), which makes it particularly appealing for MUDPIT – type applications. In this solvent system, ammonium acetate serves both as the salt used for displacement of peptidic cations from the SCX column and as the pH – controlling buffer. Continuous increase in the pH of this buffer system during the SCX elution apparently gradually induces negative charges within the peptides possessing acidic residues. These negative charges offset positive charges present on basic residues of a given peptide and weaken its affinity toward the SCX column.

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***** The material described in this poster is a part of a bigger study which will soon be submitted for publication as a research article in *Analytical Chemistry*.