

Examination of Non-Specifically Bound Proteins and Peptides Following Depletion of Twenty High Abundance Proteins from Human Plasma

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Abstract

Depletion of high abundance proteins from human plasma and serum has become a valuable tool in the search for protein biomarkers. However, some speculate the existence of potential biomarkers associated with the depleted proteins. For example, human plasma albumin is a well-known carrier of proteins and peptides. Previous studies have identified a limited set of non-specifically bound proteins associated with proteins removed from serum and plasma, but the dynamic range of the depleted fraction may affect its accuracy and content. Therefore, an exhaustive analysis of the depleted proteins is important to address this challenge. Different elution methods and gradients will be evaluated to selectively elute non-specifically bound proteins and peptides from the depletion resin without co-eluting the twenty targeted proteins. The identifications from this analysis should produce a more complete set of non-specifically bound proteins and peptides.

Introduction

- Depletion of 20 high abundance proteins allows for observation of medium- to low- abundance proteins as a result of two effects: (1) unmasking of proteins that migrate and co-elute with the high abundance proteins, and (2) increasing the relative protein load of the depleted fraction.
- A ProteoPrep® 20 spin column was previously developed for removal of 20 high abundance proteins from 8 µl of plasma. Depletion of these proteins removes approximately 95% of total plasma protein.
- A larger format ProteoPrep 20 LC column is soon to be released for the depletion of 100 µl of human plasma.
- A major concern regarding depletion technologies is the identification of non-specifically bound proteins removed with the twenty specifically targeted high abundant proteins. It is known that several of these twenty proteins display protein-protein interactions with other plasma proteins.
- Previously, non-specifically bound proteins were identified by analyzing the entire bound fraction with 1D and 2D LC MS/MS. The highly abundant proteins make it difficult to identify non-specifically bound proteins.
- Here a different approach was taken using the same depletion technology in a 10 ml LC column. Removal of the non-specifically bound proteins was attempted with several different protein removal methods and gradients that would leave the 20 specifically bound proteins attached to the resin.
- Fifty-three bound proteins have been identified which were previously masked by the 20 high abundance proteins.

Methods

High Abundance Protein Depletion

Twenty (20) high abundance proteins were depleted from plasma (Cat. No. P9523) using a 10 ml prototype ProteoPrep 20 LC column. An AKTA FPLC (GE Healthcare) LC unit was used. Several depletions of plasma were carried out as described in the ProteoPrep 20 LC workflow. Following the wash through of the depleted plasma, several different First Elution buffers were used to remove non-specifically bound proteins from the column (table below). Five (5) ml fractions were collected. The 20 specifically bound proteins were finally eluted with ProteoPrep 20 Elution Solution (OGP).

Elution Methods	First Elution	Final Elution
1	None	ProteoPrep 20 Elution Solution (OGP)
2	1.5 M Sodium Phosphate, 5 M NaCl	ProteoPrep 20 Elution Solution (OGP)
3	2.2 M Ammonium Bicarbonate	ProteoPrep 20 Elution Solution (OGP)
4	1% OGP, PBS	ProteoPrep 20 Elution Solution (OGP)
5	pH Gradient	ProteoPrep 20 Elution Solution (OGP)

ELISA for High Abundance Plasma Proteins

The percent depletion of human plasma proteins was determined by ELISA. Whole citrated plasma and depleted plasma samples were directly coated onto 96 well ELISA plates overnight following dilution in carbonate buffer (Cat. No. C3041). The plates were washed with TBS-TWEEN® 20 and then incubated with 20 primary antibodies in TBS-BSA for 2 hr at 37 °C. The plates were washed and then incubated with HRP-conjugated secondary antibodies in TBS-BSA for 2 hr at 37 °C. The plates were washed and developed with TMB substrate (Cat. No. T0440), stopped with an equal volume of 1 M HCl and the absorption measured at 450 nm.

Trypsin Digestion and LC MS/MS

The eluted bound protein plasma samples were concentrated and buffer exchanged (50 mM ammonium bicarbonate, pH 8.3) using 5,000 NMWL filters (Amicon Ultra, Millipore). The samples were reduced and alkylated (Cat. No. PROTRA) and buffer exchanged (50 mM ammonium bicarbonate, pH 8.3) again. A protein concentration was determined by the BCA Assay (Cat. No. QPBCA). The samples were digested with Trypsin (Cat. No. T6567) at a concentration of 5.0% (w/w) and allowed to incubate at 37 °C overnight. The digests were dried down in a Speed Vac and dissolved with 30 µl 5% acetonitrile, 0.1% formic acid and further diluted 5 fold. Samples of the digests (5 µl) were loaded onto a RP-HPLC column interfaced with the LTQ Mass Spectrometer (Thermo), equipped with ESI (+, 4.5Kv).

Results

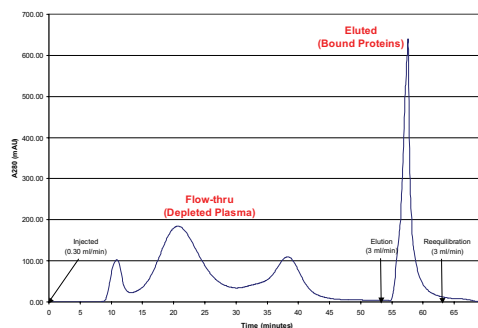


Figure 1: Representative chromatogram for 10 x 120 mm (10 ml) ProteoPrep 20 LC column.

Human citrated plasma (100 µl) was depleted of 20 high abundance proteins using the ProteoPrep 20 LC column. The absorbance was measured at A_{280} .

Depletion Efficiency

Plasma Proteins	% Depletion	Plasma Proteins	% Depletion
Albumin	99.9	Plasminogen	99.7
IgG	100.0	Haptoglobin	99.7
Transferrin	99.8	Complement C1q	99.6
IgA	99.9	Prealbumin	99.6
Ceruloplasmin	99.9	α -2-Macroglobulin	99.5
α -1-acid Glycoprotein	99.9	Apolipoprotein A2	98.9
Complement C3	99.8	Fibrinogen	98.5
Complement C4	99.8	Apolipoprotein B	98.2
α -1-Antitrypsin	99.8	IgM	97.9
IgD	99.8	Apolipoprotein A1	95.3

Figure 2: The novel antibody resin (ProteoPrep 20 technology) displays high binding capacity for 20 human plasma proteins.

- 17 high abundance proteins were removed with an average depletion of 99.6% from 100 μ l plasma.
- 3 apolipoproteins were removed with an average depletion of 97.5% from 100 μ l plasma.
- All 20 high abundance proteins were removed with an average depletion of 99.3% from 100 μ l plasma.

A depletion of 100 μ l plasma was carried out using the 10 ml PROT20 LC column as described in the Methods section. An ELISA assay was carried out on all 20 proteins as described in the Methods section.

Figure 3: Bound Proteins from prototype ProteoPrep 20 LC were evaluated using LC MS/MS.

- Of the 20 specifically bound proteins, 18 were positively identified. Complement C1q and Plasminogen were not specifically detected. (Panel A)
- Non-specifically bound proteins were eluted with various different elution solutions and gradients. (Panel B)
- Using the standard elution method with no first elution (Method 1), 9 non-targeted proteins were identified.
- First elution with high salt (Method 2) revealed 15 more protein IDs, 8 of which were Keratin. (Red)
- First elution with ammonium bicarbonate (Method 3) revealed only 3 more proteins. (Blue)
- First elution with 1% OGP (Method 4) revealed 19 unique proteins. (Green)
- A pH gradient of ProteoPrep 20 Elution Solution (OGP) revealed 12 more proteins. (Purple)

The prototype ProteoPrep 20 LC column was used for depletion of 5 samples of human plasma (100 μ l).

Then the column was washed with either 1.5 M Sodium Phosphate 5 M NaCl, 2.2 M Ammonium Bicarbonate, 1% OGP in PBS, or a pH gradient of ProteoPrep 20 Elution Solution (OGP) and Phosphate Buffered Saline (Panel A). Following the column wash, the remaining bound proteins were finally eluted with ProteoPrep 20 Elution Solution (OGP). Only protein IDs with a probability based MASCOT score > 45 are shown (Peptide Mass Tolerance \pm 1.5 Da, Fragment Mass Tolerance \pm 0.8 Da).

Conclusions

- A combination of selective elution methods can be used to help identify proteins associated with the 20 specifically depleted proteins.
- A unique set of proteins was revealed by washing the bound proteins with 5 M NaCl and 1% OGP.
- A unique set of proteins was also revealed utilizing a pH gradient of the elution solution.
- The newly developed 10 ml ProteoPrep 20 LC column (Cat. No. PROT20LC) displays a high binding capacity of 99.3% (average) for 20 high abundance proteins.

Figure 3, Panel A—Specifically Bound Proteins

		Elution Method																						
		Elution Method 1		Elution Method 2		Elution Method 3		Elution Method 4		Elution Method 5		Elution Method 6		Elution Method 7		Elution Method 8		Elution Method 9						
		Final Elution	Final Elution	5 M NaCl	Final Elution	2.2 M Amm.Bicarb.	Final Elution	1% OGP	Final Elution	10% Elution Solution	20% Elution Solution	30% Elution Solution	100% Elution Solution	Final Elution	Final Elution	Final Elution	Final Elution	Final Elution	Final Elution	Final Elution				
		mscrot score	# of peptides	mscrot score	# of peptides	mscrot score	# of peptides	mscrot score	# of peptides	mscrot score	# of peptides	mscrot score	# of peptides	mscrot score	# of peptides	mscrot score	# of peptides	mscrot score	# of peptides	mscrot score	# of peptides			
IP100745872	Albumin precursor	3578	31	499	15	4009	30	515	13	3860	31	472	13	3887	33	767	20	1357	22	4445	30	1919	31	
IP100022429	α -1-acid glycoprotein 1 precursor	103	4	118	4	94	4	220	5	86	3			168	6	107	3	181	5	62	2	397	7	
IP100020091	α -1-acid glycoprotein 2 precursor																	153	4			169	4	
IP100553177	α -1-antitrypsin precursor	354	6	81	2	559	12			336	6	481	8	527	13			823	13	187	5	748	14	
IP100478003	α -2-macroglobulin precursor	402	11	336	8	536	14	802	22	679	17	57	1	474	12	519	13	1369	33	189	4	1167	30	
IP100021841	Apolipoprotein A-I precursor	430	10	674	9	378	10	848	13	538	11	899	13	369	10	412	9	593	12	470	10	562	11	
IP100021854	Apolipoprotein A-II precursor	128	2	140	2	165	2	164	3	133	2	119	2	96	1			96	1	124	1	166	2	
IP100022229	Apolipoprotein B-100 precursor	78	2			64	1					747	23											
IP100017601	Ceruloplasmin precursor	83	2			170	4			50	1			122	3			241	8			434	13	
IP100783987	Complement C3 precursor	716	18	74	2	804	16	77	2	886	19	205	6	686	16	54	1	49	1	105	3	1411	37	
IP100032258	Complement C4-A precursor	148	5			227	6			151	3			248	4					94	2			
IP100418163	Complement component 4B preproprotein																						755	22
IP100021885	Fibrinogen α chain precursor	157	3	228	3	393	5	323	2	242	4			483	5	128	1	485	4	248	3			
IP100029717	Fibrinogen α chain precursor																						282	5
IP100298497	Fibrinogen β chain precursor	267	5			199	5	107	2	225	7	61	1	240	6			342	9	209	6	352	8	
IP100021891	Fibrinogen gamma chain precursor	51	1			68	3			70	1			125	4			175	6	72	3	119	4	
IP100641737	Haptoglobin precursor	321	9	188	6	472	10	364	8	370	10			408	10	195	5	929	13	404	10	706	13	
IP100477597	Haptoglobin-related protein precursor							295	6	206	6	188	3	268	7	159	4			272	7			
IP100382455	Ig heavy chain V-I region EU					50	1																	
IP100382480	Ig heavy chain V-III region BRD	76	1			113	1			110	1			110	1							55	1	
IP100003111	Ig kappa chain V-I region AU	53	1																					
IP100387025	Ig kappa chain V-I region DEE	82	1																			46	1	
IP100385252	Ig kappa chain V-III region GDJ																						149	2
IP100385264	Ig mu heavy chain disease protein																			48	1			
IP100061977	IGHA1 protein	212	3			211	4			170	4			95	3					152	4			
IP100166866	IGHA1 protein							94	2									241	4			341	6	
IP100418422	IGHD protein																	79	1			47	1	
IP100448938	IGHG1	410	7	89	2					463	8							349	6	513	8	736	9	
IP100472345	IGHG3 protein			83	1	163	3			207	4			212	5	133	3	259	4	206	3	319	6	
IP100550640	IGHG4 protein	275	4	111	2	338	3	82	2	337	6	86	2	391	4	193	4	270	4	329	4	414	6	
IP100472610	IGHM protein					564	7	82	3			90	2	664	8	217	6							
IP100477090	IGHM protein	114	2	103	2					101	2			107	2	114	2	198	4			247	5	
IP100479708	IGHM protein					131	3	87	3															
IP100419424	IGKV1-5 protein			154	2			183	2			171	3					147	2					
IP100719373	IGLC1 protein																					202	3	
IP100719452	IGLC1 protein	99	2	81	1	145	2	56	1	100	2	72	1			64	1	125	2	108	1	283	3	
IP100178926	immunoglobulin J chain							76	1									68	1			78	1	
IP100783024	*Myosin-reactive immunoglobulin heavy chain variable region (fragment)*	77	1			111	1			112	1			110	1							54	1	
IP100022463	Serotransferrin precursor	393	8	131	3	551	11	193	6	571	11			543	11	67	1	194	5	828	14	1051	19	
IP100022432	Transferrin precursor	47	1	138	3	74	1	59	1	59	1	86	2	66	1	71	1	131	2	90	2	266	5	
Total # of the 20 Specifically Targeted Proteins		17		13		17		13		16		12		16		11		16		14		17		

Figure 3, Panel B—Non-specifically Bound Proteins

		Elution Method 1		Elution Method 2		Elution Method 3		Elution Method 4		Elution Method 5												
		Final Elution		5 M NaCl		Final Elution		2.2 M Amm. Bicarb.		Final Elution		10% Elution Solution		20% Elution Solution		30% Elution Solution		100% Elution Solution				
		mascot score	# of peptides	mascot score	# of peptides	mascot score	# of peptides	mascot score	# of peptides	mascot score	# of peptides	mascot score	# of peptides	mascot score	# of peptides	mascot score	# of peptides	mascot score	# of peptides			
IP100514893	Dishveeled-associated activator of morphogenesis	46	1																			
IP100478493	HP protein	273	8	168	5	374	8															
IP100430808	Hypothetical protein	179	3			217	3		2071	3		215	2	207	3		257	3	483	5		
IP100399007	Hypothetical protein DKFZp	250	4			269	3		274	5		347	4	156	4	102	3	253	4	352	7	
IP100784998	Hypothetical protein DKFZp	139	3			141	2	77	2	163	4	142	5	166	3	103	3	141	3	223	5	
IP100736860	Hypothetical protein LOC	246	4			373	4		287	4		468	4					305	4	389	4	
IP100027373	Melanoma-derived leucine zipper-containing extracellular factor	50	1			50	1		50	1		50	1					50	1	49	1	
IP100298971	Vitronectin precursor	51	1								112	2								99	2	
IP100329547	Zinc finger CCCH domain-containing protein 13	50	1			50	1		50	1		50	1					50	1	49	1	
IP100022426	AMBP protein precursor			52	1			57	1													
IP100021856	Apolipoprotein C-II precursor			55	1						60	1										
IP100021857	Apolipoprotein C-III precursor			179	2			108	2		172	2		231	2	100	2					
IP100306959	Keratin, type I cytoskeletal 7			63	1																	
IP100022488	Hemopexin precursor			97	2						109	3				70	1					
IP100022371	Histidine-rich glycoprotein precursor			129	3			350	6													
IP100103481	Keratin 72			51	1																	
IP100376379	Keratin 77			107	2																	
IP100009865	Keratin, type I cytoskeletal 10			192	5																	
IP100019359	Keratin, type I cytoskeletal 9			76	1																	
IP100220327	Keratin, type II cytoskeletal 1			424	11									99	3							
IP100021304	Keratin, type II cytoskeletal 2 epidermal			122	3																	
IP100008359	Keratin, type II cytoskeletal 2 oral			90	2																	
IP100032328	Kininogen-1 precursor			55	2			145	5													
IP100032028	PRO2831			52	1																	
IP100017696	Complement C1s subcomponent precursor							63	1													
IP100005859	Cytokeratin type II							70	1													
IP100471914	FYVE, RhogEF and PH domain-containing protein							59	1											55	1	
IP100654755	Hemoglobin subunit β			103	2	59	1	58	1					78	1					73	1	
IP100479121	153 kDa protein										48	1										
IP100032220	Angiotensinogen precursor										141	2										
IP100304273	Apolipoprotein A-IV precursor										418	8										
IP100021855	Apolipoprotein C-I precursor					46	1				69	2										
IP100021842	Apolipoprotein E precursor										509	8										
IP100299435	Apolipoprotein F precursor										124	1										
IP100030739	Apolipoprotein M										70	1										
IP100186903	Apolipoprotein L1 precursor										103	1										
IP100297181	Cadherin-7 precursor										61	1										
IP100291262	Clusterin precursor										167	5								110	3	
IP100305461	Inter-α-trypsin inhibitor heavy chain H2 precursor										62	1										
IP100007081	Interleukin-4 precursor										58	1										
IP100335089	Mps one binder kinase activator-like 2B										46	1										
IP100024900	R31180_1										45	1										
IP100646659	Serine/threonine-protein kinase 17A										50	1										
IP100019399	Serum amyloid A-4 protein precursor										92	2										
IP100218732	Serum paraoxonase/arylesterase 1										304	5								73	1	
IP100410357	Similar to Zinc finger protein 2 homolog (Zfp-2) (ZT3) isoform 3										55	1										
IP100452727	Transcription cofactor HES										53	1										
IP100796830	13 kDa protein														102	2				103	2	
IP100290562	Nuclear factor, interleukin 3 regulated														46	1						
IP100745619	Similar to 37LRPp40																			46	1	
IP100738024	26 kDa protein																				462	5
IP100402004	56 kDa protein											45	1									
IP100021258	Arlaptin-1																				46	1
IP100043270	CDNA FLJ31609 fis, clone NT2R2002852																				54	1
IP100746381	CDNA FLJ41210 fis, clone BRAL22014484																				46	1
IP100807428	Hypothetical protein											164	3									
IP100552279	N-acetyltransferase ESCO1							50	1													
IP100021812	Neuroblast differentiation-associated protein A																				50	1
IP100303770	Olfactory receptor 2G2																				46	1
IP100217512	Probable G-protein coupled receptor 115							47	1													
IP100166143	Transcription factor SOX-5																				45	1
IP100008745	Uncharacterized protein C3orf51																				45	1
Total Proteins		9		16		9		9		9		23		8		6		7		7		20

