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Sample preparation of human serum for the analysis of tumor markers
Comparison of different approaches for albumin and γ-globulin depletion

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Abstract

LC–MS is a powerful method for the sensitive detection of proteins and peptides in biological fluids. However, the presence of highly abundant proteins often masks those of lower abundance and thus generally prevents their detection and identification in proteomic studies. In human serum the most abundant proteins are albumin and γ-globulins. We tested several approaches to specifically reduce the level of these proteins based on either specific antibodies, dye ligands (for albumin) and protein A or G (for γ-globulins). The resulting, depleted serum was analyzed by sodium dodecylsulfate–polyacrylamide gel electrophoresis and LC–MS for the residual presence of these abundant proteins as well as for other serum proteins that should remain after depletion. To test the applicability of this method to real-life samples, depleted serum of a cervical cancer patient was analyzed for the presence of a specific tumor marker protein SCCA1 (squamous cell carcinoma antigen 1; P29508), which is present at ng/ml concentrations. The results demonstrate that SCCA1 can be detected by LC–MS in patient serum following depletion of albumin and γ-globulins thus opening the possibility of screening patient sera for other, so far unknown, tumor markers.
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1. Introduction

In recent years, various methods were successfully applied for the discovery of novel biomarkers or biomarker patterns for major human diseases, especially for various types of cancer. In the area of cervical cancer there is presently only one biomarker that is clinically used but its predictive value for early stage squamous cervical cancer is low [1–4]. Methodology used for biomarker discovery comprises two-dimensional gel electrophoresis (2D-GE) [5], surface-enhanced laser desorption time-of-flight mass spectrometry (SELDI-TOF-MS) [6,8,9] and more recently liquid chromatography–mass spectrometry (LC–MS) [7,10]. While 2D-GE is powerful in separating a wide range of proteins, it has

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shortcomings in loading capacity (difficult to detect low-abundance proteins), the separation and display of low-molecular-mass proteins and peptides and the handling of very basic or hydrophobic proteins. In addition, 2D-GE is a rather work intensive method not well suitable to analyzing large series of clinical samples. SELDI-TOF-MS is a more recent development combining sample preparation with surface ionization mass spectrometry. The SELDI technique has the advantage of being rather fast and not very labor intensive but it does not generate data that allow the identification of potential biomarkers, which makes further development of high-throughput immunological assays difficult. Finally LC–MS and LC–MS–MS have become increasingly popular because they combine protein or peptide separation with the potential to identify the separated markers and thus to allow the development of immunological screening assays. The presence of abundant proteins in most biofluids used for diagnostic purposes, however, decreases the capacity of analytical methods to detect low-abundance proteins or peptides. For instance, human serum albumin constitutes 57–71% and γ-globulins constitute 8–26% of the total of all human serum proteins [11]. Removal of these proteins alone should thus increase the loading capacity of the analytical system by a factor of 3–9 and thus improve the detection of low-abundance proteins significantly. For this purpose, various human serum albumin (HSA) and immunoglobulin G (IgG) binding columns have been developed. For HSA binding two types of stationary phases are generally used: (a) those based on dye ligands such as Cibacron-Blue and derivatives thereof [12], and (b) those based on specific antibodies against human serum albumin. Other approaches based, for example, on ultrafiltration proved to be less successful [13]. Removal of IgG is exclusively based on immobilized protein A or protein G columns due to their high affinity and selectivity [14–18]. We have tested a range of these approaches in a comparative manner to elucidate their capacity to reduce the protein load in serum in view of performing sensitive biomarker analyses by LC–MS. To test these approaches on real-life samples, we performed an analysis of serum from a cervical cancer patient for the presence of a specific tumor marker SCCA1 (squamous cell carcinoma antigen 1) [19,20], which is present at ng/ml levels. Since SCCA1 is not a reliable marker for early stage cervical SCC, it is critical to find alternative markers for early diagnosis [21]. The presented method of sample preparation is a first step in that direction and may be applicable to many other problems of biomarker analysis in human serum.

2. Experimental

The following columns were used in our study: Poros anti-HSA and protein G cartridges (0.2 ml, provided by Dr. Tristan Moore, Applied Biosystems, UK), Aurum Serum Protein column (Bio-Rad, USA), a novel type of Albumin Removal column (provided by Dr. Robertus Hendriks, Merck, Germany) and HiTrap Blue and HiTrap protein G columns (Amersham Bioscience, UK), 1 ml each. All columns were pre-equilibrated and used according to the manufacturer's instructions. Crude serum was filtered through a 0.2 μm filter and first applied to the albumin-binding columns as follows.

2.1. Protocol 1

Poros anti-HSA was loaded with 0.5 ml of diluted crude serum [5 μl of crude serum diluted 100 times with phosphate-buffered saline (PBS) solution]. Further, 0.2 ml PBS were applied and the eluent was collected (albumin-depleted serum) and used for further purification. The column was washed with 3 ml PBS (eluent discarded) and bound albumin was eluted with 12 m*M* HCl. Albumin-depleted serum was applied on a Poros protein G column.

2.2. Protocol 2

Amersham HiTrap Blue and HiTrap protein G columns were connected and loaded with crude serum (100 μl of crude serum diluted to 0.5 ml with 20 m*M* KH_2PO_4 buffer, pH 7.0, binding buffer). Further, binding buffer was applied and the eluent was collected (albumin- and IgG-depleted serum). For regeneration the Blue and protein G columns were disconnected. The Blue column was eluted with 1.0 ml 1.5 *M* KCl in 20 m*M* KH_2PO_4 buffer, pH 7.0 and the protein G column was eluted with 1.0 ml

0.1 *M* glycine–HCl, pH 2.7. After elution the columns were equilibrated with binding buffer.

2.3. Protocol 3

The Merck Albumin Removal column was loaded with 1 ml of diluted crude serum [100–200 μl of a crude serum diluted to 1 ml with 25 m*M* 3-(*N*-morpholino)propanesulfonic acid (MOPS)–NaOH, pH 7.5, binding buffer]. Further, 2 ml of the binding buffer were applied and the eluent was collected (albumin-depleted serum). Bound protein (albumin) was eluted with 25 m*M* Na_2HPO_4 pH 8.0 containing 2.5 *M* NaCl. Albumin-depleted serum was applied on a HiTrap protein G column (see Protocol 2).

2.4. Protocol 4

The Bio-Rad Aurum Serum Protein column was loaded with 0.24 ml of diluted crude serum (60 μl of crude serum mixed with 180 μl of 20 m*M* NaH_2PO_4 pH 7.0). The column was vortexed, incubated at room temperature for 15 min and centrifuged. Then, 200 μl of the same buffer were applied on the column and the eluate was collected by centrifugation (depleted serum).

All depleted sera (250 μl) were applied on 1-ml PD-10 columns (Amersham Biosciences, Sweden). Next, 150 μl of 25 m*M* ammonium hydrogen carbonate pH 7.8 were applied and the eluent was discarded. The final sample was eluted in 400 μl of the same buffer and collected for tryptic digestion.

Serum samples were digested with trypsin (1:100 w/w enzyme to substrate) at 37 °C overnight. Sequencing grade modified trypsin was from Promega (catalog no. V5111, USA). This digest was concentrated five times in a vacuum centrifuge and used for the LC–MS analysis.

All LC–MS analyses were performed on an Agilent 1100 capillary HPLC system coupled on-line to an SL ion trap (Agilent, Benelux) equipped with a Zorbax 300 SB-C_{18} column (150 mm×1.0 mm I.D., 3.5 μm). For each run 5 μg (~100 pmol) of total protein digest were injected. Peptides were eluted in a linear gradient from 0 to 70% acetonitrile with 0.1% aqueous formic acid at a flow-rate of 20 μl/min. Protein concentrations were determined according to Lowry [22] and calculated for an average protein molecular mass of 50 000.

Recombinant SCCA1 [fusion protein with glutathione-S-transferase (GST)] was generously provided by Professor Gary Silverman (Harvard Medical School, MA, USA) and digested with trypsin as described above.

In-gel digestion was performed according to Rosenfeld et al. [23] with minor modifications. Matrix solution for matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF-MS) was prepared by dissolving 20 mg α-cyano-4-hydroxycinnamic acid (Aldrich) in 1 ml of 70% acetonitrile (gradient grade), 30% water (Milli-Q) with 0.1% trifluoroacetic acid. Two μl of the matrix were mixed with 2 μl sample. The calibration mix for MALDI mass spectra contained: bradykinin 1060.57 (monoisotopic mass); angiotensin, 1296.69 (monoisotopic mass); adrenocorticotropic hormone, 2465.2 (average mass); insulin 5734.5 (average mass). Analyses were performed on a Tofspec E time-of-flight mass spectrometer (Micromass, UK) equipped with delayed-extraction in the reflectron mode.

Sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed on a Mini-Protein III cell (Bio-Rad) using a 12% T gel with 0.1% SDS according to the instructions of the manufacturer. Prior to analysis, samples were boiled for 1 min in SDS sample buffer containing 0.1 *M* dithiothreitol (DTT), then cooled down and applied directly to the gel. Staining was performed with Coomassie Brilliant Blue R concentrate (Sigma, Zwijndrecht, The Netherlands) diluted and used as prescribed by the manufacturer.

3. Results and discussion

Three types of columns were evaluated for their capacity to remove albumin and/or IgG from human serum: (a) columns that bind human serum albumin (Poros Anti HSA, HiTrap Blue, Merck Albumin Removal column), (b) columns that bind γ-globulins (Poros protein G, HiTrap protein G), and (c) a single column that was designed to bind both albumin and γ-globulins simultaneously (Aurum Serum Protein column). The capacity of these columns to remove the target proteins was determined using crude serum

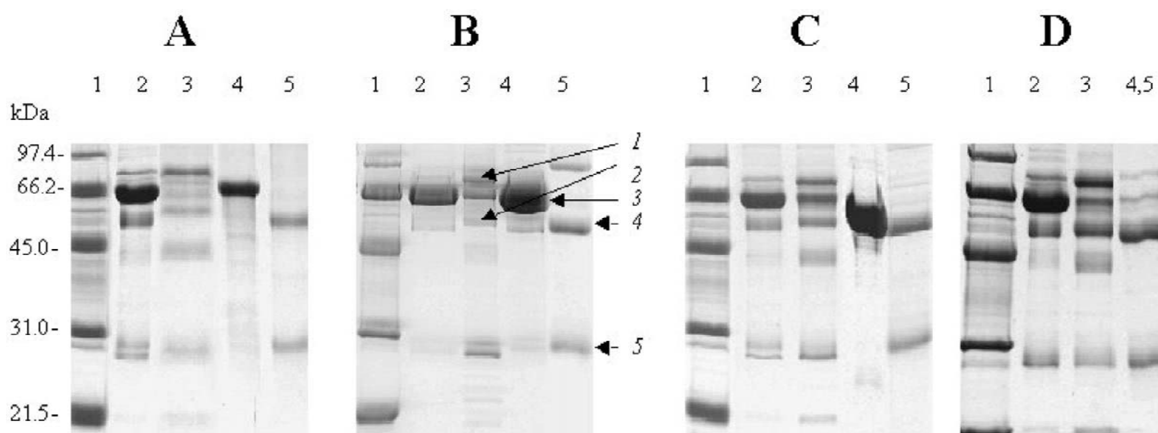

Fig. 1. Depletion of human serum of albumin and γ-globulins. In each lane 8–10 μg of protein were loaded and gels were stained with Coomassie Blue G-250. (A) Poros anti-HSA and Poros protein G columns; (B) HiTrap Blue and HiTrap protein G columns; (C) Merck Albumin Removal column and HiTrap Protein G column. (D) Aurum Serum Protein column (note, that this column is disposable, and that HSA is not quantitatively eluted). Lanes: 1, standards; 2, crude serum; 3, depleted serum; 4, bound protein eluted from albumin depleting columns with either 12 m*M* HCl (A, D), or with 1.5 *M* KCl in 20 m*M* KH_2PO_4 pH 7.0 (B), or with 25 m*M* Na_2HPO_4, 2.5 *M* NaCl, pH 8.0 (C); and lane 5, bound γ-globulins eluted from Poros protein G and Aurum columns with 12 m*M* HCl (A, D) and from HiTrap protein G with 0.1 *M* glycine–HCl pH 2.7 (B). Indicated proteins: 1, serotransferrin; 2, α_1-antitrypsin; 3, albumin; 4, 5, γ-globulins heavy and light chain, respectively.

from a healthy donor. To this end the flow-through and the adsorbed protein fractions were analyzed by SDS–PAGE (see Fig. 1). The results confirm that the larger portion of albumin and IgG is removed with generally high specificity. Although the overall patterns were similar, it appears that overall protein depletion is the highest with the Poros anti-HSA/ protein G columns (panel A, lane 3) and the combination of HiTrap Blue/HiTrap protein G columns (panel B, lane 3). From a practical point, it is also noteworthy that the dye ligand columns (panels B–D) showed a higher loading capacity for serum than the antibody column (see Table 1). Some new major bands appeared after albumin and IgG removal (see Fig. 1, panel B, lane 3) of which two were identified by in-gel tryptic digestion and MALDI-TOF analysis to be serotransferrin (band 1) and α_1-antitrypsin (band 2). To evaluate the efficiency of the depletion strategies in more detail, we performed LC–MS analyses of various albumin- and γ-globulin-depleted sera. For analysis, all sera were digested with trypsin and the resulting samples (5 μg or an estimated 100 pmol total protein digest) were subjected to LC–MS. For comparison we analyzed crude serum, which revealed 12 albumin-specific peptide fragments as the major peaks in the chromatogram (not shown). We selected five of these peptide fragments as indicators for the presence of albumin (Table 2) and found none of those fragments in the three different albumin-depleted serum samples shown in Fig. 2. A

Table 1
Major characteristics of the evaluated columns for albumin removal

Supplier	Column name	Column volume (ml)	Binding specificity		Capacity μl serum/ml column stationary phase	Serum dilution before loading to the column (fold)
			HSA	IgG		
Applied Biosystems	Poros anti-HSA	0.2	+	−	25	100
Amersham	Hi Trap Blue	1	+	−	100	5–10
Merck	Albumin Removal Column	1	+	−	100–200	5–10
Bio-Rad	Aurum Serum Protein	1	+	+	60	4

Table 2
List of selected human albumin (P02768)-specific tryptic peptide fragments used to follow albumin depletion by LC–MS

No.	$[M+H]^+$	Position	Peptide sequence
1	880.44	226–223	AEFAEVSK[a]
2	960.56	403–410	FQNALLVR
3	1017.53	65–73	SLHTLFGDK
4	1342.63	546–557	AVMDDFAAFVEK
5	1742.89	146–159	HPYFYAPELLFFAK

[a] See also Fig. 2.

small amount of this HSA-specific peptide fragment was revealed after depletion using the Aurum Serum Protein column (Fig. 2). This demonstrates that all of the employed albumin removal methods were rather efficient in light of a sensitive LC–MS analysis.

In order to check the selectivity of the removal strategies, α_1-antitrypsin (P01009) and serotransferrin (P02787) were selected as positive controls, since they should not be removed. These proteins are some of the most abundant proteins in serum after albumin and the γ-globulins [11,24]. Both proteins were identified as major bands on SDS–PAGE following depletion (see Fig. 1). While detection of peptide fragments from these proteins was difficult by LC–MS using crude serum due to the abundance of albumin-derived peptides, specific peptide fragments were clearly visible in depleted serum as illustrated for an α_1-antitrypsin-specific peptide fragment (Figs. 2 and 3). Other α_1-antitrypsin-specific peptide fragments that were found by LC–MS in depleted serum are listed in Table 3 thus confirming the beneficial effect of the depletion procedures to detect proteins that were previously masked by albumin and γ-globulins. Similar results were obtained for serotransferrin-related peptide fragments (not shown).

Since both α_1-antitrypsin and serotransferrin are still fairly abundant proteins in serum, we wanted to test our depletion procedure with a low-abundance protein that is of relevance as a cancer marker. To this end, serum from a cervical cancer patient [concentration of SCCA1 = 160.5 μg/l by enzyme-linked immunosorbent assay (ELISA)] was depleted with an Aurum column, digested with trypsin and analyzed by LC–MS for SCCA1, a typical marker of squamous cell cervical carcinoma [21]. Serum from a healthy donor was analyzed in comparison. In addition, serum (100 pmol) was spiked with a known amount (6 pmol) of recombinant SCCA1 (GST

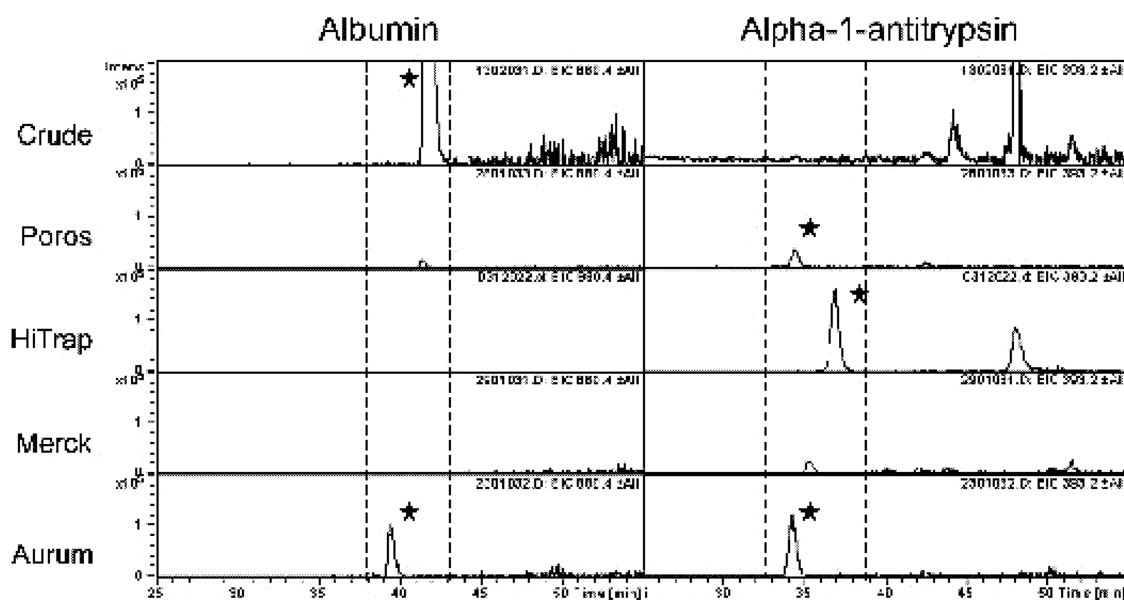

Fig. 2. Detection (selected ion monitoring; SIM) of peptides 880.4 and 393.3 for HSA (left) and α_1-antitrypsin (right), respectively, by LC–MS after tryptic digestion in crude and depleted sera. Regions of elution are boxed. *Peptide identities were confirmed by MS–MS of the respective precursor ions.

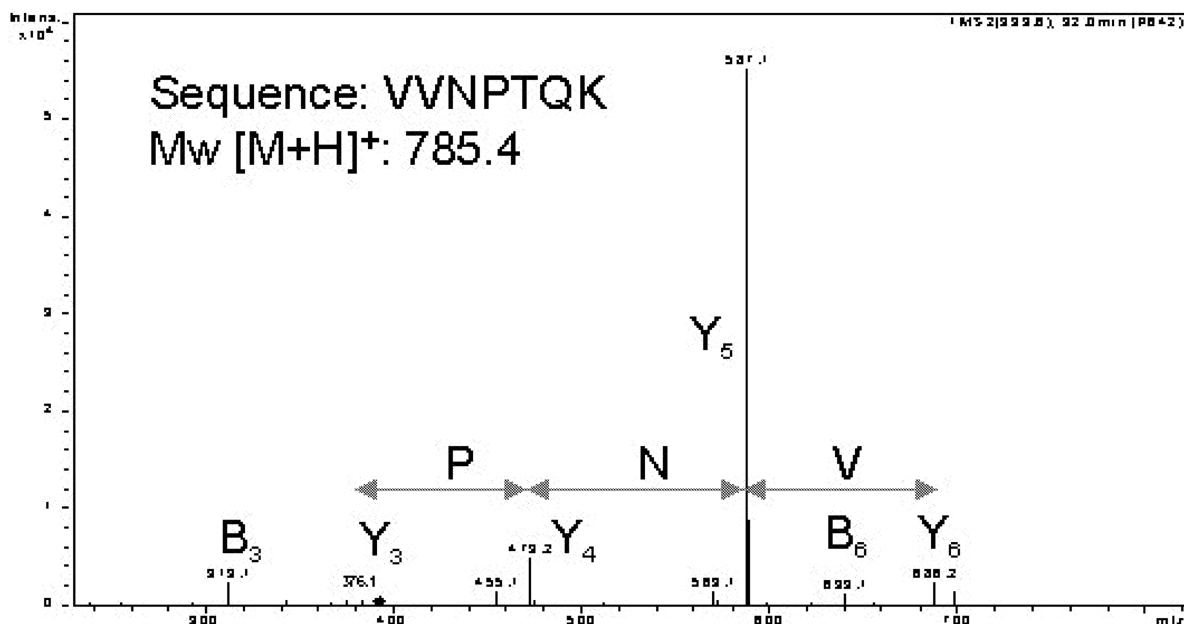

Fig. 3. Identification of an α_1-antitrypsin-specific tryptic fragment in depleted human serum (after Hi-Trap Blue and protein G columns) by MS–MS of the doubly-charged precursor ion at 393.5.

fusion protein) to check for recovery of the tumor marker after depletion and digestion.

To identify suitable tryptic peptide fragments, pure recombinant GST-SCCA1 was digested and analyzed by LC–MS, which allowed identification of seven SCCA1-specific peptides (Table 4). One of these peptide fragments ($M+H^+=861.51$) was selected to indicate the presence of SCCA1 in serum. While the presence of this peptide was clearly visible in serum of a cervical cancer patient, it was absent from the control serum of a healthy subject. This result shows that relevant tumor markers can be detected in human serum after depletion of albumin and IgG by LC–MS (selective ion monitoring) after tryptic digestion (Fig. 4) indicating that LC–MS after adequate sample preparation is a promising technique for biomarker discovery.

Table 3
List of human α_1-antitrypsin-specific tryptic peptide fragments revealed by LC–MS in albumin depleted serum (P01009)

No.	$[M+H]^+$	Position	Peptide sequence
1	686.44	193–198	IVDLVK
2	785.45	412–418	VVNPTQK
3	852.49	307–314	SASLHLPK
4	888.50	360–367	AVLTIDEK
5	1015.61	325–334	SVLGQLGITK

4. Conclusions

Effective depletion of human serum with respect to albumin and/or the γ-globulins has been achieved using dye ligand or immunoaffinity chromatography.

Table 4
List of SCCA1-specific tryptic peptide fragments revealed by LC–MS

No.	$[M+H]^+$	Position	Peptide sequence
1	765.37	209–214	SIQMMR
2	849.49	280–286	VDLHLPR
3	861.50	231–237	VLEIPYK[a]
4	864.48	88–94	LLTEFNK
5	926.44	95–102	STDAYELK
6	932.45	70–77	AATYHVDR
7	1022.63	322–331	GLVLSGVLHK

[a] See also Fig. 4.

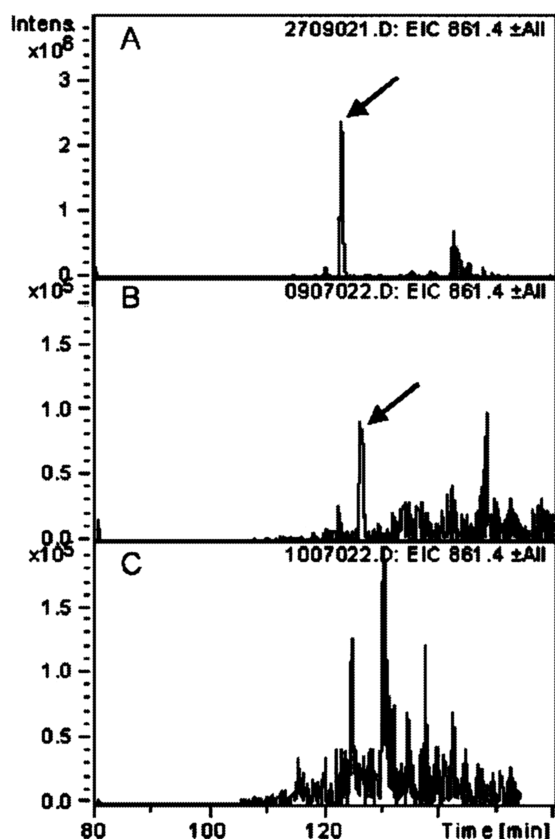

Fig. 4. Analysis of depleted human serum after tryptic digestion by LC–MS (SIM of 861.4) for the tumor marker SCCA1. (A) Purified GST-SCCA1 digest; (B) patient 5, depleted serum digest; (C) healthy control, depleted serum digest (arrow: SCCA1-related peptide). Background peaks in panel C were shown not to be related to SCCA1.

A prototype column (Merck) showed the highest capacity, while the hybrid column (Aurum, Bio-Rad) removing both serum albumin and γ-globulins in one step proved to be the most convenient approach, since it is adapted to low-speed centrifugation. Depletion on the Aurum Serum Protein column yields a relatively high protein concentration in the depleted serum. However it does not remove albumin completely as shown by albumin-specific peptides in a tryptic digest after depletion. While an immunoaffinity column (Poros anti-HSA) was found to be highly specific for human albumin, the column capacity was significantly lower than for the other approaches (0.025 ml compared to 0.1–0.2 ml).

HiTrap Blue and protein G columns in combination were quite effective in depleting crude serum. The fact that only 5% of total protein remained in the depleted serum as compared to 15% for the Aurum column indicates that other proteins may have been removed as well. It is well known that Cibacron F3GA-derived columns (including HiTrap Blue) bind a wide variety of proteins including not only albumins, but also ATP and other nucleotide-binding proteins. Therefore it is likely that its specificity is somewhat lower than for the other depletion columns, a fact that is not necessarily a disadvantage, especially when looking for specific proteins that are not adsorbed to this kind of stationary phase.

Using depleted serum, detection of other serum proteins (α_1-antitrypsin and serotransferrin) was clearly improved and a relevant, low-abundant tumor marker (SCCA1) was detectable in depleted patient serum, while it was undetectable in crude serum. In summary, our investigations show that dye ligand affinity chromatography is a viable and robust alternative to immunoaffinity approaches for the depletion of human serum from albumin and thus an important step in sample preparation for biomarker studies. Since albumin is also by far the most abundant protein in other biofluids such as cerebrospinal fluid or urine, we expect that the described methodology will improve detection limits for low-abundance markers also in these applications.

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