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Ion-Exchange Sample Displacement Chromatography as a Method for Fast and Simple Isolation of Low- and High-Abundance Proteins from Complex Biological Mixtures

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Summary

Sample displacement chromatography (SDC) in reversed phase and ion-exchange modes was introduced at the end of 1980s. This chromatographic method was first used for preparative purification of synthetic peptides, and subsequently adapted for protein fractionation, mainly in anion-exchange mode. In the past few years, SDC has been successfully used for enrichment of low- and medium-abundance proteins from complex biological fluids on both monolithic and bulk chromatographic supports. If aqueous mobile phase is used with the application of mild chromatographic conditions, isolated proteins are not denatured and can also keep their biological activity. In this paper, the use of SDC in anion-exchange mode on a high-capacity chromatographic resin for separation of proteins from complex biological mixtures such as human plasma is demonstrated. By use of three and more columns coupled in series during sample application, and subsequent parallel elution of detached columns, additional separation of bound proteins was achieved. Highly enriched human serum albumin fraction and a number of physiologically active medium- and low-abundance proteins could be fractionated and detected by electrospray ionization tandem mass spectrometry (ESI-MS/MS) and matrix-assisted laser desorption/ionization time-of-flight tandem mass spectrometry (MALDI-TOF/TOF-MS). The use of the aforementioned columns that can be sanitized with 1 M sodium hydroxide for further application of SDC in biotechnology and food technology was discussed.

Key words: sample displacement chromatography, ion-exchange mode, plasma proteins

Introduction

Sample displacement chromatography (SDC) was introduced at the end of 1980s for purification of peptides in reversed-phase mode (*1*). A few years later, SDC was introduced as a method for separation of large molecules in ion-exchange mode. However, only a mixture of standard proteins was used (*2*). After further optimization, SDC was used for simple and cost-effective purification of synthetic peptides. This method was also adapted for preparative isolation of physiologically active proteins such as troponin components from the rabbit skeletal muscle in reversed-phase mode (*3*), as well as thrombin from the plasma of Atlantic salmon in affinity mode with

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Heparin Sepharose® (*4*). We have recently demonstrated the application of anion-exchange monolithic supports as well as weak hydrophobic bulk supports for separation of complex biological mixtures in SDS mode (*5,6*). When SDC was used in anion- or cation-exchange mode, as well as hydrophobic interaction chromatography (HIC) mode, several middle- and low-abundance proteins from very complex biological mixtures such as human and animal serum, plasma, cell culture supernatants and whey can be highly enriched (*4–8)*. Interestingly, mostly monolithic supports such as compact discs, cylinders and membranes were used for fractionation of complex biological mixtures in SDS mode (*5,7,8*), and the use of columns packed with bulk material is rather scarce (*4,6*). The reason is that the latter have much higher capacity, and because of the diffusion phenomena, sample displacement effects are more difficult to achieve (*6,7*). Recently, SDC in HIC mode and on the columns packed with high--capacity bulk resins was applied as a very effective method for the separation of complex biological mixtures. By use of this method, concentration of low-abundance proteins as well as the enrichment of high-abundance ones on both micropreparative and preparative scale can be achieved (*6*).

In this paper, we demonstrate that SDC in anion-exchange (AEX) mode by use of high-capacity bulk supports can also be utilized for concentration of low- and high-abundance proteins. Cryopoor human plasma that contains a high amount of different proteins in very different concentration ranges was used as a model substance for complex biological mixtures.

Materials and Methods

Starting material

The starting material for AEX chromatography was cryopoor, single-donor human plasma (Rhode Island Blood Center, Providence, RI, USA). The sample preparation was described previously (*6*). The cryopoor plasma was diluted twofold with 10 mM Tris-HCl, pH=7.4 (buffer A) and stored at 4 °C for 1 h. The pH was kept close to the physiological value (pH=7.4). After storage, the precipitated proteins were removed by centrifugation at 2500×*g* (Centrifuge 5804 R, Eppendorf, Hamburg, Germany). Protein content was determined in the supernatant, and this sample was analysed by SDS-PAGE and used for further chromatographic analysis.

Anion-exchange chromatography

For AEX chromatography, glass columns (Tosoh Bioscience, Stuttgart, Germany) with an i.d. of 6.5 mm packed with high-capacity bulk support Toyopearl GigaCap DEAE (Tosoh Bioscience) were used. The bed volume was 1 mL. After washing with HPLC water, the column was equilibrated with 10 mM Tris-HCl, pH=7.4 (buffer A), and the sample was loaded. Unbound proteins were collected and subsequently analysed. After sample application, the column was washed with at least five volumes of buffer A, and bound proteins were eluted with the high ionic-strength buffer (buffer B, 1 M NaCl in buffer A). The flow rates for all chromatographic separations were 0.5 and 1 mL/min. In some runs under overloading conditions, three identical columns coupled in series were used. After sample loading and washing with buffer A, columns were detached, and bound proteins were eluted in three parallel steps. All runs were performed at 4–8 °C, and a BioLogic DuoFlow chromatographic system (Bio-Rad, Hercules, CA, USA) was used. Proteins were detected by UV absorption at 280, 260 and 210 nm. Protein amounts in collected fractions were determined with the Bicinchoninic Acid Protein Assay kit (Pierce, Rockford, IL, USA), according to the manufacturer's procedure. Each experiment was performed at least in triplicate.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

For SDS-PAGE, about 15 μg of protein from each sample was solubilized in NuPAGE® sample buffer (Invitrogen, Carlsbad, CA, USA) and separated as described previously (*9*). The gels were stained with GelCodeBlue dye (Pierce) and visualized by a VersaDoc Imaging System (BioRad). For further analysis, the bands of interest were excised for in-gel digestion (*10*).

Sample preparation for MS analysis

For in-gel digestion, the gel bands were excised by extracting six to ten gel particles with clean glass Pasteur pipettes and digested with trypsin as described previously (*9,10*).

For in-solution digestion, 50 μg of the acetone-precipitated and denatured protein pellet were resolubilized in 100 μL of NH_4HCO_3 (pH=8.0) and 8 M urea. The resolubilized proteins were reduced with 20 mM dithiothreitol (37 °C, 45 min) and then alkylated with 50 mM iodoacetamide at room temperature for 30 min in the dark. Before tryptic digestion, 100 mM ammonium bicarbonate buffer was added to reduce the concentration of urea. Trypsin was added to the protein mixture at an enzyme to substrate ratio of 1:60 (by mass) and proteolytic digestion was performed as previously described (*7*). The resulting tryptic peptides were dried completely (Thermo Scientific Savant DNA SpeedVac®, Thermo Fischer Scientific, Inc., Waltham, MA, USA) once more and were subjected to the MALDI-TOF or liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis after being redissolved in formic acid/water/acetonitrile/trifluoroacetic acid mixture (0.1:95:5:0.01).

Protein identification by MALDI-TOF/TOF-MS

After in-gel digestion, sample preparation for protein identification using MALDI-TOF/TOF-MS also requires cleanup by the use of C_{18} Zip-Tip devices (Millipore, Billerica, MA, USA) in order to remove salts that are beyond the levels acceptable for MALDI-MS applications. The sample preparation methods were optimized for compatible and reproducible MALDI-TOF/TOF-MS/MS analyses by using the MTP Anchorchip™ 384 or Matrix HCCA (α-cyano-4-hydroxycinnamic acid) pre-spotted AnchorChip (PAC96 and PAC384) targets (Bruker Daltonics, Billerica, MA, USA).

For MALDI-TOF/TOF analyses, an Ultraflextreme mass spectrometer (Bruker Daltonics) was used. Setup of flexControl RP_PepMix.par method for successful data acquisition included optimization of specific parameters such as: initial and maximal laser power, fuzzy control,

ion source voltage, post-column ion extraction, matrix suppression, sample rate, frequency and number of shots (>200 with 50 shots) per spot, mass range (Da), peak resolution, signal intensity, and number of precursor masses. In automated runs an appropriate AutoXecute method was selected (Default Calibrate, Default Measure, Deafult AutoLIFT, PAC_Calibrate, PAC_Measure and PAC_Automsms). Further procedure for protein identification was described previously (*7*).

Identification of proteins with LC-MS/MS

Tryptic digests of whole fractions obtained by ion-exchange chromatography (in-solution digestion), or of proteins extracted from the gels after SDS-PAGE (in-gel digestion), were separated with a reversed-phase column (C-18 PepMap 100, LC Packings/Dionex, Synnyvale, CA, USA) as previously described (*10*). ProteinPilot™ Software (v. 4.0, Applied Biosystems, Inc., Foster City, CA, USA) is the successor to ProID and ProGroup, and uses the same peptide and protein scoring method. Scores above 2.0 require identification of at least two sequence-independent peptides (*10*).

In parallel experiments, an additional LC-MS/MS system was used (Agilent Technologies, Paolo Alto, CA, USA, and Thermo Electron Corporation, San Jose, CA, USA). When this system was used, tryptic peptides were separated on a 12-cm (75 μm i.d.) analytical column with 3-μm Monitor C18 resin (Orochem Technologies, Inc., Lombard, IL, USA) and containing an integrated 10-μm ESI emitter tip (PicoTip; New Objective, Woburn, MA, USA). Solvent A was 0.1 M acetic acid in water and solvent B was 0.1 M acetic acid in acetonitrile. Peptides were eluted using a linear acetonitrile gradient (0–70 % solvent B over 30 min).

Eluting peptides were introduced onto an LTQ Orbitrap Velos hybrid mass spectrometer (Thermo Scientific, San Jose, CA, USA) with a 1.8-kV electrospray voltage. Full MS scans in the *m/z* range of 300–1700 were followed by data-dependent acquisition of MS/MS spectra for the ten most abundant ions, using a 30-second dynamic exclusion time. Protein identification was performed in at least two independent experiments.

Peak list files were created from the mass spectrometer file by the program extract_msn.exe, using the following settings: the mass had to fall in the range of 600 to 4500 Da; the minimum total ion current for the scan had to be over 1000; the precursor tolerance for grouping was 0.005 Da, with no differing intermediate scans allowed and only a single scan required to create a peak file; the minimum signal-to-noise for a peak to be written to the peak file was 3, and 5 such peaks had to be found for a peak file to be created. The program determined charge states.

A program developed in-house was used to concatenate the peak files into a Mascot Generic Format (MGF; Matrix Science Inc., Boston, MA, USA) Search using a human International Protein Index (IPI) database (v. 3.79; European Bioinformatics Institute (EBI), Hinxton, UK) was performed by MASCOT. The precursor ion mass tolerance was 7 ppm and the fragment ion mass tolerance was 0.5 Da. Enzymatic digestion was specified as trypsin, with up to 2 missed cleavages allowed. The search database contained concatenated real (target) and sequence-reversed (decoy) proteins. The identifications were filtered on MOWSE score to yield a group of peptide assignments with a 1 % false discovery rate (*11*).

Results

Chromatographic separation

Different amounts of human plasma containing between 50 and 5600 mg of protein per column were applied to an AEX column. The column volume was 1 mL. After washing with five column volumes of buffer A, bound proteins were eluted with 50 % of buffer B (0.5 M NaCl in buffer A). A typical chromatogram for this kind of separation is shown in Fig. 1. The corresponding SDS-

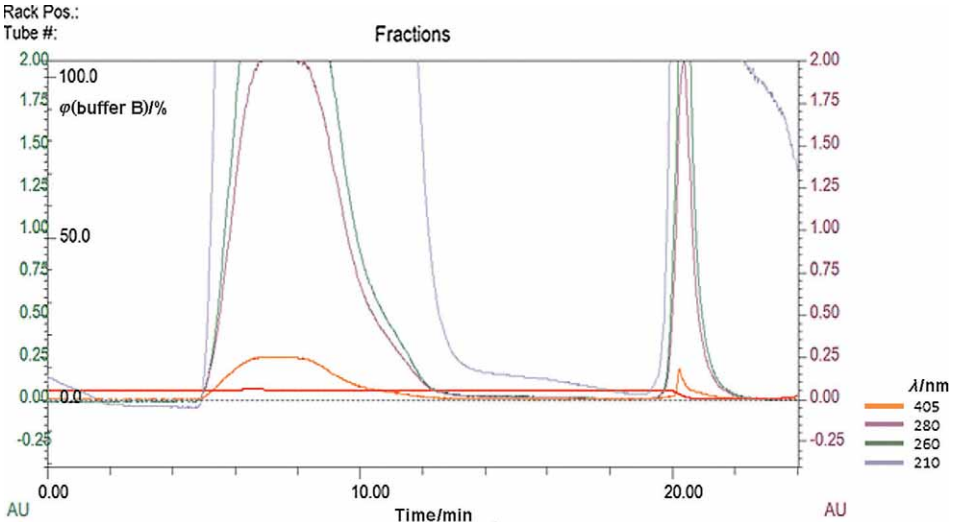

Fig. 1. Separation of proteins from human plasma. After dilution with buffer A and centrifugation, different amounts of plasma proteins were applied to a Toyopearl GigaCap DEAE column (i.d. 6.5 mm, column volume 1.0 mL). After washing with 5 mL of buffer A, bound proteins were eluted with 50 % of buffer B (0.5 M NaCl in buffer A). Absorbance was determined at 405, 280, 260 and 210 nm. Chromatographic conditions were as follows: flow rate 1 mL/min, temperature 4–8 °C and pressure $2–3 \cdot 10^5$ Pa. Collected fractions were analysed by SDS-PAGE, MALDI-TOF/TOF-MS and LC-MS/MS

-PAGE of eluted proteins after loading different amounts of sample to the column is shown in Fig. 2.

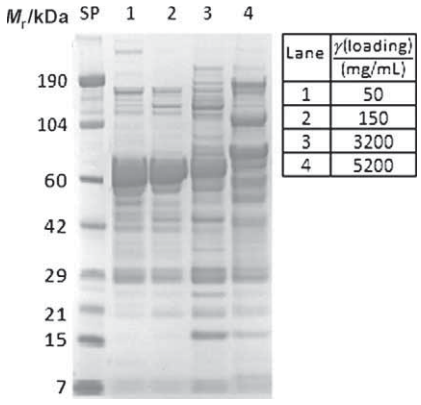

Fig. 2. SDS-PAGE of eluted proteins after loading of different amounts of plasma proteins on the AEX Toyopearl GigaCap DEAE column. For chromatographic conditions see Materials and Methods and Fig. 1 (SP=standard proteins)

Use of three-column system for SDC in AEX mode

Three columns (shown in Fig. 3a) were coupled in series during sample application. In the next step, the columns were washed with buffer A. After detachment, each column was eluted with 50 % buffer B (see previous paragraph and Fig. 3b). The eluted proteins were analysed by SDS-PAGE (Fig. 4) and identified by LC-MS/MS (Tables 1 and 2). Fast, preliminary identification was also

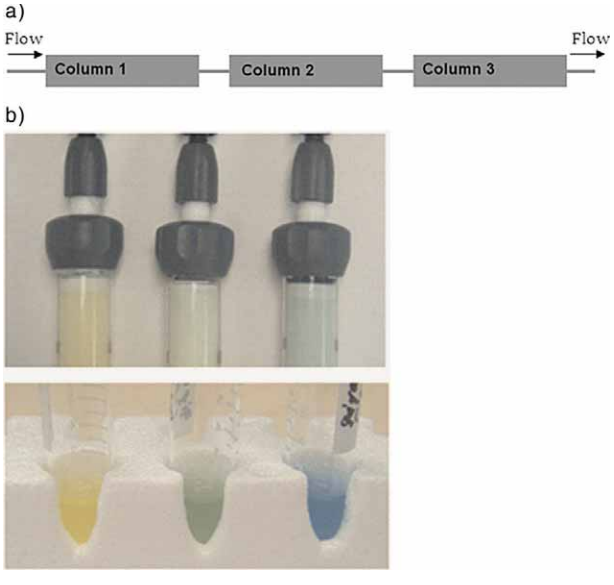

Fig. 3. SDC of plasma proteins by use of three identical columns packed with the high-capacity Toyopearl GigaCap DEAE resin (column volume: 1 mL each): a) all three columns were connected in series during sample loading and washing; b) for the elution step, the columns were detached and bound proteins were eluted from each column by a step gradient of 50 % buffer B (0.5 M NaCl in buffer A). Different proteins were eluted at very high concentrations: column 1=HSA, carrying the yellow-coloured pigment from human plasma; column 2=mixture of HSA with yellow pigment and blue coloured ceruloplasmin giving the green colour; and column 3=ceruloplasmin

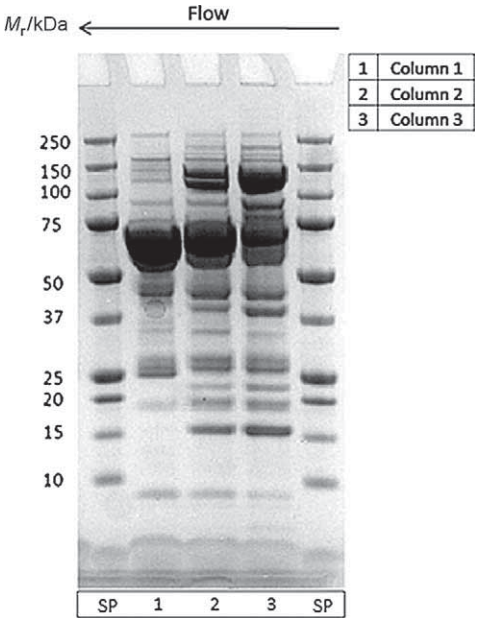

Fig. 4. SDS-PAGE of the eluates of three columns after SDC of plasma proteins (see also Fig. 3)

performed by MALDI-TOF/TOF-MS. From the first column, highly enriched human serum albumin was eluted. This fraction contained some impurities such as complement proteins (complements C3 and 4B), kininogen 1 and α-1-antitrypsin. Proteins that were eluted from the second and third columns are listed in Tables 1 and 2. The eluate from the first column contains pigment from human plasma bound to human serum albumin (HSA) and is yellow, the eluate from the third column is blue and contains highly enriched copper-binding protein ceruloplasmin (Fig. 3b).

Discussion

Already in the early 1990s, Veeraragavan *et al.* (*2*) demonstrated the use of SDC for separation of proteins by AEX chromatography. However, only standard proteins were used, and this chromatographic mode was not applied long for separation of complex biological mixtures. The real potential of SDC for separation of proteins was recognized more than 20 year later, when Mant and Hodges (*3*) and Manseth *et al.* (*4*) used this chromatographic mode for preparative separation of rabbit troponin and thrombin. In these experiments, large amounts of highly pure proteins were fractionated by use of this simple method and application of step gradient. Optimization of SDC separation can be achieved by use of a multicolumn system in a series followed by parallel elution from individual columns, as suggested by Agner (*12*). Manseth *et al.* (*4*) significantly improved the process for isolation of thrombin from the plasma of Atlantic salmon by use of SDC on Heparin Sepharose® instead of the conventional gradient elution chromatography (*13*). A multicolumn system was successfully used for the separation of this protease that is used as fish meat-binding agent (*4,13*).

Table 1. Major plasma proteins eluted from the second column after detachment from the three-column system (see also Figs. 3 and 4) identified by MALDI-TOF/TOF-MS and LC-MS/MS

Acc. No.	Protein name	Score	Uniq. pept.	Apparent M_r/kDa
IPI00783987.2	complement C3	7477.67	132	120
IPI00783987.2	complement component 4B	4221.51	79	96
IPI00745872.2	**serum albumin**	4076.72	76	68
IPI00017601.1	**ceruloplasmin**	3077.24	55	125
62739186	complement factor H	2399.35	46	155
IPI00553177.1	α-1-antitrypsin	2003.51	35	44
IPI00032291.2	complement C5	1890.93	40	115
IPI00215894.1	LMM kininogen-1	1722.17	31	60
IPI00847635.1	α-1-antichymotrypsin	1479.81	25	68
IPI00641737.1	haptoglobin	1467.45	29	38

Other highly enriched, potentially interesting, physiologically active proteins: prothrombin, plasma protease C1 inhibitor, IaIp, IaI heavy chain H4, antithrombin III. These proteins were identified by LC-ESI-MS/MS after in-solution digestion of the column eluate

Table 2. Major plasma proteins eluted from the third column after detachment from the three-column system (see also Figs. 3 and 4) identified by MALDI-TOF/TOF-MS and LC-MS/MS. The eluate contains tightly bound, medium and low-abundance proteins

Acc. No.	Protein name	Score	Uniq. pept.	Apparent M_r/kDa
IPI00783987.2	complement C3	5469.48	104	120
IPI00017601.1	**ceruloplasmin**	2744.40	54	125
IPI00783987.2	complement component 4B	2335.48	46	96
IPI00745872.2	**serum albumin**	2048.21	37	68
IPI00798430.1	serotransferrin	1467.66	28	75
IPI00553177.1	α-1-antitrypsin	1369.55	28	44
IPI00032291.2	complement C5	1301.49	28	115
IPI00641737.1	haptoglobin	1240.79	24	38
IPI00021841.1	kininogen-1	1119.02	21	60
IPI00022395.1	complement component C9	1116.62	19	60
IPI00478003.3	α-2-macroglobulin	1113.93	23	180

Other highly enriched, potentially interesting, physiologically active proteins: α-1-antichymotrypsin, fibronectin, prothrombin, plasminogen, IaIp, IaI heavy chain 4, coagulation factor V. These proteins were identified by LC-ESI-MS/MS after in-solution digestion of the column eluate

We have recently used monolithic AEX and CEX supports for separation of proteins from human plasma in SDC mode (*5*). If the anion-exchange monolithic support was applied, the highly abundant protein HSA and other weakly binding proteins were displaced by stronger binding lower-abundance proteins. Consequently, a concentration of proteins with lower-abundance was achieved (*5*). However, we did not use high-capacity bulk AEX supports for such experiments. In following investigations, similar experiments were performed in hydrophobic interaction mode, and columns packed with bulk support were used. It was demonstrated that sample displacement effects were achieved by much higher loadings – up to one order of magnitude (over 4500 mg/mL compared to about 180 mg/mL (*5,6*)). However, direct comparison of AEX monoliths with AEX bulk supports was not performed. As shown here, more than 2000 mg of protein from human plasma per mL of high capacity bulk support is necessary to achieve the sample displacement (Fig. 2). This loading is more than one order of magnitude higher than in the experiments with monolithic supports (*5*).

Anion-exchange resins are used as the first step in the isolation of vitamin K-dependent clotting factors from cryopoor human plasma (so-called solid-phase extraction) (*14,15*). The resin was directly suspended in human plasma, recovered by filtration, washed and finally eluted with 2 M NaCl in order to recover the fraction containing tightly bound physiologically active vitamin K--dependent plasma proteins. Human serum albumin is barely present in the eluate, if the solid-phase extraction is performed under such heavily overloading conditions (up to 500 mL of cryopoor plasma per mL of support) (*14,15*). This process can be considered as an early example of SDC. Although relatively simple conditions were applied with minimal instrumental effort, the process is very reproducible (*15*). The plasma of Atlantic salmon is a relatively complex protein mixture which was used as a starting material for isolation of highly pure and enzymatically active thrombin, commonly used as fish meat--binding agent in food industry (*13*). The SDC was demonstrated as a rapid, simple and cost-effective method in this production process (*4*).

Conclusions

As presented here, if high-capacity AEX supports are used in SDC mode, displacement of weakly binding proteins by stronger binding ones can be achieved. If human plasma was applied, the weakly binding protein HSA was displaced by other stronger binding lower-abundance proteins (Fig. 3). Similar effect was observed when monolithic AEX supports were applied. However, the displacement effect was achieved at much higher (over one order of magnitude) loading, when high-capacity bulk supports are applied (Fig. 3). SDC offers an additional, very effective alternative for separation of complex biological mixtures and both isolation and detection of low-abundance proteins, as well as the removal of trace impurities from enriched solutions of high-abundance proteins, *e.g.* HSA and immunoglobulins, mostly monoclonal antibodies (*5*,*8*). In food processing and chemistry, SDC can be applied for the production of enzymes (*4*), and for fractionation of whey proteins and enrichment of low-abundance component from this nutritionally important raw material (*16*).

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