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Proteomics as a tool for optimization of human plasma protein separation

Spomenka Kovac[b], Xinli Yang[a], Feilei Huang[a], Douglas Hixson[a], Djuro Josic[a,*]

[a] COBRE, Center for Cancer Research Development, Rhode Island Hospital and Brown University, Providence, RI, USA
[b] J.J. Strossmayer University of Osijek, Department of Chemistry, HR-31000 Osijek, Croatia

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ABSTRACT

The application of proteomics technology in purification of proteins from human plasma and for characterization of plasma-derived therapeutics has been recently discussed. However, until now, the impact of this technology on the plasma protein fractionation and analysis of the final product has not been realized. In the present work, we demonstrate the use of proteomic techniques the monitoring of the first step of the plasma fractionation by use of anion-exchange chromatography. This chromatographic method is frequently used in the purification scheme for isolation of vitamin K dependent clotting factors II, VII, IX and X, and clotting inhibitors protein C and protein S, as well as inter-alpha inhibitor proteins (IaIp). After the removal of immunoglobulin G and non-binding proteins in the flow-through fraction, albumin and weakly bound proteins were eluted with low concentration of sodium chloride. The proteins that strongly bind to the anion-exchange column were eluted by higher salt concentrations. The fractions of interest were analyzed, and proteins were identified by LC–ESI-MS/MS. By use of this method, not only candidates for therapeutic concentrates, but also some potentially harmful components were identified. This strategy was very helpful for further process optimization, fast identification of target proteins with relatively low abundance, and for the design of subsequent steps in their removal or purification.

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1. Introduction

Since more than 50 years, human plasma has been a very important raw material for production of therapeutics, such as human serum albumin (HSA) [1], immunoglobulins (Ig) [2], von Willebrand factor (vWF) [3], clotting factors [1,3–5], prothrombin complex concentrate (PCC) [6], and other biologically active proteins such as antithrombin III [7] and inter-alpha inhibitor proteins (IaIp) [8,9]. Mostly because of virus safety, it is a serious tendency to replace some plasma proteins, especially the clotting factors VIII (F VIII) and IX (F IX), by corresponding proteins produced by recombinant technology [10]. However, it is still an enormous demand on plasma-derived therapeutics, and most products developed in late 1980s and 1990s years are still on the market. Moreover, newly developed preparations of immunoglobulins and other plasma-derived therapeutic proteins are recently introduced [2,11].

Virological safety of plasma-derived therapeutic protein preparations has been the main concern for a long time [2]. In the present time, thorough virus validation studies are necessary for the market introduction of a new therapeutic, originated from human plasma [2,11]. Other aspects of clinical safety were covered by extensive and very expensive clinical trials [12]. Although all plasma-derived therapeutic preparations that are on the market nominally belong to the group of so-called "well-characterized biologicals" [11,13], they still contain relatively high amount of foreign proteins. In contradiction to this name, most impurities are biochemically poorly characterized, or even not identified at all [5,14].

Newly, proteomics techniques are successfully used in the design and optimization of bioprocesses, also for characterization of the downstream processing, scale up and process characterization and validation [15,16]. As a part of the product development for the preparation of IaIp as potential plasma-derived therapeutics, we have already performed a thorough proteomic investigation during the isolation of this group of proteins [17].

In this paper, we demonstrate the use of proteomic techniques for design and optimization of the first step of the plasma protein separation by use of anion-exchange chromatography. Ion-exchange chromatography on weak or strong anion-exchange resins is frequently used as first step in isolation of vitamin K dependent clotting factors II, VII, IX and X, clotting inhibitors protein C and protein S (6, 18, 19). This step was also recently used for isolation of IaIP [17]. After separation, each fraction was analyzed by SDS-PAGE followed by tryptic digestion and protein identification by LC–MS/MS. In parallel experiments, no SDS-PAGE was performed, and eluted proteins were directly digested by trypsin and identified by LC–MS/MS. This strategy was very helpful for detection of pro-

* Corresponding author at: Proteomics Core, COBRE, Center for Cancer Research Development, Rhode Island Hospital, 1 Hoppin Street, Coro-West-4, Providence, RI 02904, USA. Fax: +1 401 793 8908.
E-mail address: Djuro_Josic@brown.edu (D. Josic).

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teins of interest in eluted fractions and for development of optimal salt gradient for removal of potentially harmful impurities.

2. Materials and methods

2.1. Human plasma

Human plasma (Rhode Island Blood Center, Providence, RI, USA) was used as starting material. For removal of cryoprecipitate, frozen plasma was slowly thawed (at +4 °C for 24 h) and subsequently centrifuged at 3000 rpm/90 min (Eppendorf Centrifuge 5804, Hamburg, Germany). The supernatant, cryopoor plasma, was used for further experiments.

2.2. Anion-exchange chromatography

For anion-exchange chromatography, Toyopearl DEAE 650 M gel (Tosoh Bioseparations, Stuttgart, Germany) was used. The gel was packed in a 10 mL column (10 mm I.D., BioRad, Hercules, CA, USA) or in a 32 mL glass column (XK 16, GE Healthcare Bio-Sciences AB, Piscataway, NJ, USA). After washing with the HPLC water, the column was equilibrated with a buffer with low ionic strength (10 mM Tris–HCl, pH 7.4, Buffer A). The human plasma was 5× diluted with the Buffer A and applied on the column (between 0.5 and 1.0 mL cryopoor plasma/mL gel). The non-bound proteins were collected and subsequently analyzed. The column was washed with 5 column volumes of Buffer A. The bound proteins were eluted with a step gradient of Buffer B (1 M NaCl in Buffer A). After each run, the column was sanitized with 0.5 M NaOH for at least 1 h. The flow rate for all chromatographic separation was between 2 and 10 mL/min.

All chromatographic separations were performed at 4 °C. Proteins were detected optically at 280, 260 and 210 nm, or by use of electrophoretic methods such as SDS-PAGE. For separation, a BioLogic Duo Flow chromatographic system (BioRad) was used.

2.3. SDS-PAGE

Before the analyses of by SDS-PAGE, protein content in each collected fraction was determined by use of Bicinchoninic Acid Protein Assay kit (Pierce, Rockford, IL), according to the manufacturer's procedure. Protein samples (containing between 15 and 20 μg protein/each sample) were subsequently solubilized in NuPAGE sample buffer and heated at 100 °C for 5 min. SDS-PAGE was performed with precast 4–12% Bis-Tris gels in an Xcell Sure Lock Mini-Cell (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's procedure. The gels were stained with GelCode Blue (Pierce) and visualized by a VersaDoc Imaging System (BioRad) before excising the bands of interest for in-gel digestion.

2.4. In-gel digestion

The bands of interest were excised by extracting 6–10 plugs with a micropipette and extracted and digested with trypsin as described previously [20].

2.5. Digestion of whole fractions separated by anion-exchange chromatography

In order to reduce the amount of salts, about 50 μg protein from separated fractions from anion-exchange chromatography were precipitated with the ReadyPrep 2-D Cleanup kit (BioRad), according to the manufacturer's instructions. Precipitated and denatured protein pellets were redissolved with 20 μL of 0.5 M triethylammonium bicarbonate, pH 8.5, and reduced, alkylated and tryptically digested according to the manufacturer's protocol (Applied Biosystems, Foster City, CA, USA). Subsequently, the digested proteins were dried in a vacuum centrifuge (Vacufuge, Eppendorf). The material was twice resuspended with water and dried. It was then twice redissolved in a solution of 0.5% (v/v) formic acid and 20% (v/v) acetonitrile, with vacuum drying. After resuspending in the same solvent and confirming the pH value, the peptides were isolated using a strong cation exchange TipTop™ (PolyLC, Inc., Columbia, MD, USA) according to the manufacturer's instructions. The ammonium formate eluates were dried and redissolved in formic acid:water:acetonitrile:trifluoroacetic acid mixture (0.1:95:5:0.01) in preparation for the LC–ESI-MS/MS analysis.

2.6. Identification of proteins with LC–ESI-MS/MS

Tryptic digests were fractionated with an RP column (C-18 PepMap 100, LC Packings/Dionex, Sunnyvale, CA, USA) as previously described, with the column eluate introduced directly onto a QSTAR XL mass spectrometer (Applied Biosystems, and Sciex, Concord, Ontario, Canada) via ESI [21]. Candidate ion selection, fragmentation and data collection were performed as described previously [21]. Protein identifications were performed with ProteinPilot (Applied Biosystems and Sciex), using the human and "RefSeq" databases from NCBI (http://www.ncbi.nlm.nih.gov/RefSeq/). ProteinPilot is the successor to ProID and ProGroup, and uses the same peptide/protein scoring method [22]. Briefly, given a protein score, *S*, the likelihood that the protein assignment is *incorrect* is 10^{-S}. Furthermore, scores above 2.0 require that at least two sequence-independent peptides be identified.

3. Results and discussion

Chromatography has been used for fractionation of human plasma since the seventies [1]. Since the early eighties, further development of synthetic polymer-based supports, and supports based on agarose was an important contribution for wider acceptance of chromatography in this, rather conservative branch of pharmaceutical industry [13,23]. Chromatography has been used mostly for isolation of plasma proteins with lower abundance, such as coagulation factors and inhibitors. The use of chromatography was an important contribution towards the improvement of purity and viral safety of these plasma-derived therapeutic proteins [1,24]. However, for isolation of both main proteins from human plasma HSA and IgG, chromatographic methods are rather underrepresented [1,2,13].

Toyopearl DEAE 650 M is a polymer-based chromatographic support that has been used for isolation of plasma proteins, mainly for therapeutic F VIII/vWF concentrates since more than 20 years [1,23]. This support is biocompatible, chemically resistant, and frequent sanitations and regenerations are possible without significant loss of its capacity and selectivity [13]. However, comparing to the newly developed supports, the capacity of this material is rather low [25]. Because of its frequent use in plasma fractionation, this support was chosen for the model experiments for development of a process for separation of plasma proteins. Proteomic techniques were used for the protein profiling of separated fractions.

In Fig. 1, stepwise elution of plasma proteins from a 32 mL column, packed with the Toyopearl DEAE 650M support is shown. SDS-PAGE of non-bound fraction and fractions eluted by stepwise increased NaCl concentration in elution buffer is shown in Fig. 1B. All proteins, identified by LC–ESI-MS/MS in corresponding fractions after separation by SDS-PAGE are listed in Table 1.