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High-abundant protein depletion strategies applied on dog cerebrospinal fluid and evaluated by high-resolution mass spectrometry

Mårten Sundberg, Jonas Bergquist, Margareta Ramström *

Department of Chemistry – BMC, Analytical Chemistry and Science for Life Laboratory, Uppsala University, Box 599, 751 24 Uppsala, Sweden

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ABSTRACT

As the number of fully sequenced animal genomes and the performance of advanced mass spectrometry-based proteomics techniques are continuously improving, there is now a great opportunity to increase the knowledge of various animal proteomes. This research area is further stimulated by a growing interest from veterinary medicine and the pharmaceutical industry. Cerebrospinal fluid (CSF) is a good source for better understanding of diseases related to the central nervous system, both in humans and other animals.

In this study, four high-abundant protein depletion columns, developed for human or rat serum, were evaluated for dog CSF. For the analysis, a shotgun proteomics approach, based on nanoLC-LTQ Orbitrap MS/MS, was applied. All the selected approaches were shown to deplete dog CSF with different success. It was demonstrated that the columns significantly improved the coverage of the detected dog CSF proteome. An antibody-based column showed the best performance, in terms of efficiency, repeatability and the number of proteins detected in the sample. In total 983 proteins were detected. Of those, 801 proteins were stated as uncharacterized in the UniProt database. To the best of our knowledge, this is the so far largest number of proteins reported for dog CSF in one single study.

1. Introduction

Animal proteomics is a field of growing interest both from a veterinary perspective [1–7] and in the field of animal models used to investigate human diseases [8–15]. In human medical research there is an ever increasing amount of publications on CSF analysis. Some articles focus on a specific disease but there are also reports on large scale mapping of the human CSF proteome which have resulted in up to 3081 identified proteins [16–20]. Animal CSF studies are still relatively uncommon and involve mostly model animals like rat and mouse [10–14,21–23]. Dog CSF is of high clinical interest due to the fact that dogs, like humans, are affected by e.g. epilepsy, brain tumors, inflammation in the brain and other brain and neurological related diseases [24–26]. Dogs have proven to be very good model animals for human Alzheimer's disease and aging [27–29]. There are also dog models for more rare diseases such as Hurler's syndrome, Sanfilippo syndromes and Duchenne muscular dystrophy [30–32].

There are several challenges associated with CSF analysis. First of all, the protein concentration in CSF is relatively low (in humans 0.2–0.8 mg/mL) [33,34]. Secondly, the dynamic range of proteins has been reported to be up to twelve orders of magnitude [16,35]. Another issue is the high concentration of abundant proteins like albumin and immunoglobulins which constitute 50% and 15% of the total human CSF protein content, respectively [36]. If transferrin is added to the list, more than 70% of the total protein amount is already covered [33]. No established analytical method can today fully cover the whole dynamic range of proteins that is present in CSF or plasma/serum. Instead, there are several methods available to fractionate or remove proteins in the sample to decrease the dynamic range [37–39]. So-called depletion columns are constructed to remove the most abundant proteins from body fluids, in general from human plasma [33]. Existing columns are based on antibodies, recombinant modified variants of antibodies or other kinds of affinity matrices, removing up to 20 proteins [40]. Even though the total protein concentration in human plasma is 100–200 times higher than in CSF [33], several depletion columns have also successfully been applied on human CSF since many of the high-abundant components are the same in both body fluids [34,41–43]. Another strategy to reduce the dynamic range is to use enrichment approaches. A limiting factor, at least for CSF samples, is that rather high protein concentrations are needed. Even if the

* Corresponding author. Fax: +46 18 471 3692.
E-mail address: margareta.ramstrom@kemi.uu.se (M. Ramström).

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protocols are miniaturized, these methods require around 2 mL of CSF [44,45].

Today, only 811 out of the 25,485 sequences in the UniProt dog reference proteome (taxonomy Canis lupus familiaris (Taxon identifier: 9615)), are reviewed. This implies that most of the dog proteome is based on homology studies to proteomes of other species. Most of the proteomics research performed on samples from dog is based on plasma or serum. There are, however, some reports on dog urine, bronchoalveolar fluid and follicular fluid, but studies on CSF are still very rare [46]. Mass spectrometry is the overall mostly applied method in human CSF proteomics due to good sensitivity and the large amount of data that can be extracted from each sample. However, so far, the published mass spectrometry-based dog CSF studies are small in size. In one study, CSF from healthy dogs were compared to CSF from dogs with meningoencephalitis using 2D gel electrophoresis followed by analysis of interesting spots with MALDI-TOF MS. In total, 134 protein spots were detected on the gels and from those gel spots, 36 proteins were identified with MALDI-TOF MS [47]. In another MALDI MS-based proteomic study, CSF samples from dogs with degenerative myelopathy were compared with a control group in a search for potential biomarkers for the disease. In that study, the authors only mention transthyretin as an interesting protein [48]. Besides mass spectrometry, there are some reports on dog CSF samples performed with antibody-based technologies such as Luminex technology, Western Blot or ELISA with a small number of proteins studied [49–51].

Mass spectrometry-based methods optimized for protein analysis are often applicable in proteomics studies of samples from all species. However, affinity-based sample preparation methods should be more species-dependent. To the best of the authors' knowledge, no depletion columns have so far been developed to process body fluids from dog. Therefore, one important objective of this study was to verify if some of the methods available for other species could be used on dog CSF. Four different high-abundant protein depletion columns developed for human or rat plasma/serum samples (Table 1) were selected and evaluated with respect to efficiency, repeatability and the number of detected proteins prior to and after depletion. Two of the columns were antibody-based spin columns, while the other two were gravity columns based on either recombinant proteins or an affinity ligand. Another goal of this study was to determine how much of the dog CSF proteome that could be revealed using state-of-the-art shotgun proteomics, based on high-resolution mass spectrometry in combination with the preparation strategies used in the study. We here present the largest number of proteins in dog CSF that have been published to date.

2. Experimental

2.1. Chemicals and reagents

Acetonitrile (ACN), acetone, formic acid (FA), acetic acid (HAc), methanol (MeOH) and sodium chloride (NaCl) were purchased from Merck (Darmstadt, Germany). Ammonium bicarbonate (NH_4HCO_3), urea, sodium dodecyl sulfate (SDS), dithiothreitol (DTT) and iodoacetamide (IAA) were obtained from Sigma-Aldrich (St. Louis, MO, USA). For the tryptic digestion, trypsin (sequence-grade bovine pancreas 1418475, Roche diagnostic, Basel, Switzerland) was used. XT sample loading buffer and XT MOPS buffer were acquired from BioRad Laboratories (Hercules, A, USA). Ultrapure water was prepared by Milli-Q water purification system (Millipore, Bedford, MA, USA).

2.2. Cerebrospinal fluid sample

The dog CSF was collected through lumbar puncture from a beagle that had to be euthanized at the Swedish National Veterinary Institute (SVA), Uppsala. A total of 9.5 mL dog CSF sample was collected and centrifuged at 2000 × *g* for 10 min at 4 °C to remove any cells. The supernatant was collected and the sample was divided into 400 μL aliquots and stored at −80 °C until use. The sample was clear without any visual signs of blood contamination. The owner had given permission to collect the sample and to use the sample in research projects. The Swedish Board of Agriculture confirmed that no additional ethical permission was required for performing this study.

2.3. Method optimization – high-abundant protein depletion

The high-abundant depletion columns were chosen due to their different technical solutions to specifically immobilize proteins, see Table 1. Seppro® Rat Spin Column (Sigma-Aldrich, St. Louis, MO, USA), based on chicken IgY antibodies [52,53] and Multiple Affinity Removal Spin Cartridge – Human 14 (MARS-Hu14) (Agilent Technologies, Waldbronn, Germany) based on rabbit polyclonal antibodies and affibodies were re-usable spin columns. The other two columns were single use gravity columns. The ProteoExtract® (Calbiochem, Merck Millipore, Darmstadt, Germany) column uses an affinity ligand (not Cibacron based) to remove albumin and Protein A for the removal of IgG. The ProteaPrep (Protea Biosciences, Morgantown, USA) column uses recombinant proteins for the albumin and IgG depletion.

The volume of plasma/serum that the different columns could handle according to the kit instructions varied between 8–60 μL.

Table 1
A summary of different parameters for the high-abundant protein depletion columns that were evaluated in the study.

Product name	ProteoExtract® albumin/IgG removal (Cat. no. 122642)	Seppro® rat spin column (Cat. no. SEP110)	ProteaPrep albumin and IgG depletion sample prep (Cat. no. SP-240)	Multiple affinity removal spin cartridge – human 14 (MARS-Hu14) (Product no. 5188-6560)
Targeted proteins	Albumin and IgG	Albumin, IgG, fibrinogen, transferrin, IgM, haptoglobin, alpha1-antitrypsin	Albumin and IgG	Albumin, IgG, antitrypsin,IgA, transferrin, haptoglobin, fibrinogen, alpha2-macroglobulin, alpha1-acid glycoprotein, IgM, Apolipo-protein AI, apolipoprotein AII, complement C3 and transthyretin
Developed for	Human serum/plasma	Rat serum/plasma	Human serum/plasma	Human serum/plasma
Matrix	Affinity matix	IgY antibodies	Recombinant protein	IgG and affibodies
Recommended total protein amount (μg)[a]	~1300–3900	~975–1300	~65–650	~520–650
CSF protein amount (μg)	~120	~120	~120	~120
Dilution buffer used (μL)	300	500	400	200
Re-usable	No	Yes	No	Yes

[a] Calculated with an approximated total protein content of 65 μg/μL and the volume that the manufacturer recommended.