Biochemistry and Biophysics Reports 3 (2015) 68–75

Contents lists available at ScienceDirect

Biochemistry and Biophysics Reports

journal homepage: www.elsevier.com/locate/bbrep

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

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ARTICLE INFO

Article history:
Received 16 June 2015
Received in revised form
18 July 2015
Accepted 22 July 2015
Available online 23 July 2015

Keywords:
Cerebrospinal fluid
Dog
High-abundant protein depletion
Shotgun proteomics
Orbitrap
Mass spectrometry

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

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http://dx.doi.org/10.1016/j.bbrep.2015.07.013

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.

In human CSF depletion studies, there have been reports of volumes from 65 μL up to 3 mL or even more [34,41–43,54]. From a dog, about 1 mL CSF per 5 kg body weight can safely be removed [55]. Based on this, 400 μL dog CSF was chosen as a reasonable volume to work with and still be able to use all methods in replicate.

The CSF samples were dried in a SpeedVac system until complete dryness and were then re-suspended in the buffer that was included in each depletion kit. The smallest buffer volumes suggested by the manufacturer were used. Each depletion columns were run in four technical replicates, according to the manufacturer's instructions. The two antibody based columns were reusable and therefore a stripping buffer was included in those columns. The ProteoExtract® and ProteaPrep columns were single use columns and thus there was no stripping buffer included. Therefore a stripping buffer was prepared using the dilution buffer from Agilent with an addition of 2% SDS. After collection of the flow through fraction, the bound proteins were eluted, either with the included stripping buffer or the 2% SDS buffer. The fractions were split in two and one was acetone precipitated and the precipitate was dried. The other half was completely dried in a SpeedVac system.

For the quantification of the protein concentration, an in-house validated method (Dot it Spot it protein assay kit, http://dot-it-spot-it.com, Maple Stone AB, Uppsala, Sweden) was used. The method has been thoroughly described by Berglund et al. [56].

2.4. Sample processing

2.4.1. In-solution tryptic digestion

An aliquot of 400 μL dog CSF sample corresponding to approximately 120 μg total protein was dried down in a SpeedVac system and re-suspended in 50 μL digestion buffer (8 M urea and 0.4 M NH_4HCO_3). 5 μL of 45 mM DTT was added and the sample was kept at 50 °C for 15 min to reduce the proteins. To irreversibly carbamidomethylate the cysteines, 5 μL of 100 mM IAA was added, followed by 15 min incubation at room temperature in darkness. After the incubation 290 μL 0.4 M NH_4HCO_3 was added to dilute the urea to ~1 M. Trypsin (~4% w/w) was added and the sample was incubated over night at 37 °C. A volume of 35 μL corresponding to ~12 μg protein of the tryptically digested sample was desalted on a ZipTip® C18 column (Merck Millipore). This aliquot was completely dried in a SpeedVac system and was then re-suspended in 20 μL 0.1% FA. The tip was activated by 5 × 10 μL of 100% ACN and equilibrated with 5 × 10 μL of 0.1% FA. Then the sample was coupled to the matrix by 30 repeated cycles of 10 μL sample loading. The tip was then washed with 5 × 10 μL 0.1% FA. Finally the sample was eluted in 10 μL 80% ACN, 0.1% FA by 15 cycles of aspirating and dispensing. This was done twice and then the sample was completely dried in a SpeedVac system. The peptides were re-suspended in 20 μL of 0.1% FA in Milli-Q water before they were analyzed on a nanoLC-LTQ-Orbitrap mass spectrometer.

2.4.2. SDS-PAGE and in-gel tryptic digestion

The protein pellets were re-suspended in 12.5 μL of XT Sample Loading Buffer (BioRad Laboratories) together with 27.5 μL Milli-Q water and the samples were shaken until the pellets had been dissolved. A volume of 5 μL of 45 mM DTT was added and samples were heated for 5 min at 95 °C. Samples were cooled to room temperature and 5 μL of 100 mM IAA was added and the tubes were incubated in darkness for 15 min. Untreated CSF samples were re-suspended in twice the volume. A volume of 25 μL with approximately 10–15 μg of protein was loaded into each lane of an 18-well, 4–12% Bis–Tris Criterion XT Precast Gel (BioRad Laboratories). The electrophoresis was run at 200 V constant for 60 min (starting current 165–175 mA/gel, final current 60–70 mA/gel) in XT MOPS running buffer. Finally, the gels were stained by Coomassie blue R-250 (BioRad Laboratories) according to manufacturer's instructions and scanned with an Epson scanner (Epson perfection 4990 photo). The gel lanes were cut in three pieces but the effect of cutting the gel lanes in 10 pieces was also investigated. All gel pieces were placed in separate test tubes. The gel slices were divided into smaller pieces (~1 mm^3) and destained by washing in 25 mM NH_4HCO_3 and 100% ACN twice or until sufficient color had been removed. Then the slices were vacuum centrifuged in a SpeedVac system for 15 min, 10 mM DTT was added and the samples were incubated at 50 °C for 1 h and this was followed by 1 h incubation in 50 mM IAA at room temperature in darkness. Once again, the slices were washed in 25 mM NH_4HCO_3 and 100% ACN and dried in SpeedVac for 15 min. Tryptic digestion was done for 1 h at room temperature with 12.5 ng/μL trypsin dissolved in 25 mM NH_4HCO_3. After the 1 h incubation, 25 mM NH_4HCO_3 was added to completely cover the gel bands and the incubation proceeded overnight in darkness at 37 °C. The solution was transferred to a new test tube and the gel slices were covered with a solution containing 60% ACN and 5% FA and sonicated for 5 min. Then the solution was transferred to the same test tube as the previous fraction and the samples were completely dried in a SpeedVac system. The peptides were re-suspended in 20 μL of 0.1% FA in Milli-Q water before they were analyzed on a nanoLC-LTQ-Orbitrap mass spectrometer.

2.5. NanoLC-LTQ-Orbitrap-MS/MS analysis

An EASY-nLC II system (ThermoFischer Scientific) was used for the on-line Nano-LC separations. 5 μL of the sample was loaded onto a pre-column (EASY-Column, 2 cm, inner diameter 100 μm, 5 μm, C18-A1, ThermoFischer Scientific) at a maximum pressure of 280 bar. The peptides were then eluted onto to an EASY-column, 10 cm, inner diameter 75 μm, 3 μm, C18-A2 (ThermoFischer Scientific), which was used for the separation. The separation was performed at a flow rate of 200 nL/min using mobile phase A (Milli-Q water with 0.1% FA) and B (ACN with 0.1% FA). A 2-step 90 min gradient, 2% B up 50% B in 75 min followed by wash step of 100% B for 15 min was used. The EASY-nLC II system was connected to a LTQ Orbitrap Velos Pro ETD mass spectrometer (ThermoFischer Scientific) equipped with a nano-flex ion source. The spray voltage was set to 2.0 kV. The instrument was controlled through Tune 2.6.0 and Xcalibur 2.1. The LTQ Orbitrap Velos Pro ETD was operated in data dependent mode to automatically switch between high-resolution mass spectrum and low resolution in the LTQ. The survey scan was preformed from m/z 400–2000 at 100,000 resolution and the 10 most abundant ion peaks were CID fragmented for each full scan cycle. The mass window for precursor ion selection was set to 1.9 Th. Screening was done for charge state +2, +3 and +4 and the dynamic exclusion was set to 30 s. Normalized collision energy of 35%, activation time of 10 ms and activation q of 0.25 were set for MS/MS. The fragments were scan at “normal scan rate” in the low pressure cell of the ion trap and detected with a secondary electron multiplier.

2.6. Data analysis

For protein identification, Proteome Discoverer version 1.4.1.14 (ThermoFischer Scientific) was used and searches were performed using Sequest HT. The searches were done against a dog reference proteome without isoforms (taxonomy 9615) that was downloaded from www.uniprot.org (2013-08-23). The reference proteome contained 29209 sequences. The parameters for the search were set to: fixed modifications: carbamidomethyl (C), variable modifications: deamidated (N, Q) and oxidation (M), precursor

mass tolerance: 10 ppm, fragment mass tolerance: 0.6 Da and maximum two missed cleavage sites. The S/N threshold was set to 1.5. The search results were validated using the Percolator algorithm and an FDR of 5%. A minimum of 2 unique peptides per protein was applied.

3. Results and discussion

3.1. Evaluation of the selected depletion strategies for dog CSF

3.1.1. Removal of target proteins

The aim of this study was to evaluate the performances of four strategies to remove abundant proteins from canine CSF, as potential preparation steps in front of high-resolution mass spectrometry. Since there are no depletion columns available for body fluids from dog, it is important to establish if commercial depletion columns developed for humans or rat could be functional alternatives. Some characteristics of the methods are summarized in Table 1. All four columns were easy to use. The two antibody-based columns, Seppro® rat and MARS-Hu14 were rather quick (20–30 min) to run while the two columns based on gravity (ProteoExtract® and ProteaPrep) had longer runtimes (1–2 h). An amount of 400 µL dog CSF sample, corresponding to ~120 µg protein, was depleted in each run from four technical replicates. After depletion, approximately 15–40 µg of total protein remained in the samples, indicating that substantial portions (67–88%) of the proteins had been removed. The flow through and the bound fractions from each preparation were loaded on SDS-PAGE (Fig. 1A and B). Due to detergents and high salt concentrations in the bound fractions, acetone precipitation was introduced as a cleaning step. The SDS-PAGE indicated good repeatability between the technical replicates. All columns could, to variable extent, deplete at least albumin (66 kDa) (Fig. 1A and B). Albumin was most efficiently removed by the MARS-Hu14 and the Seppro® rat columns, and least efficiently by the ProteoExtract® column. Transferrin (80 kDa) is seen in the bound fractions for Seppro® rat and MARS-Hu14. Bands at 150 kDa give an indication of that intact IgG have been depleted.

The SDS-PAGE lanes of three of the four technical replicates were divided into three fractions, digested by trypsin and thereafter analyzed by LC–MS/MS. In Table 2A, the four different depletion columns are listed together with their target proteins and the corresponding Peptide Spectrum Matches (PSMs) for all technical replicates of the flow through and bound fractions. In spectral counting, the number of identified PSMs for a certain protein is assumed to give its relative abundance [57,58]. Here, we considered this parameter a good general estimation of the relative abundances in the samples under study. Immunoglobulins are difficult to handle, because there is limited information about dog immunoglobulins in the UniProt database.

For MARS-Hu14, 11 out of 14 target proteins were detected. Of the 11 detected proteins, 9 had higher PSM values in the bound fractions than in the flow through fractions. The large differences in PSMs between the flow through and bound fractions also indicate efficient removal of albumin, transthyretin and apolipoprotein AI. Both MARS-Hu14 and Seppro® rat showed higher protein scores for transferrin in the flow through fractions than in the bound fractions, which supports what was seen on SDS-PAGE. For Seppro® rat all targeted proteins were found, and albumin, haptoglobin and alpha-1-antitrypsin showed higher or similar PSMs in the bound fraction. Both ProteaPrep and ProteoExtract® were solely constructed to capture human albumin and IgG and both of them capture the corresponding canine proteins, but not as efficiently as the two antibody-based columns. ProteoExtract® is the only column that shows higher PSM numbers for albumin in the flow through fraction, which is in line with what was seen on SDS-PAGE. Table 2B lists the findings correlated to the total number of protein fragments identified as belonging to immunoglobulins. In total 27 protein fragments were found, searching all MS runs. The two antibody-based columns performed best of the tested columns with 12 out of 27 possible protein fragments found in the bound fraction, but only 3 for Seppro® rat and none for MARS-Hu14 in the flow through fraction. ProteoExtract® also showed 12 protein fragments in the bound fraction, but there were also 7 found in the flow through fraction.

In conclusion, particularly the two antibody-based columns were very capable of removing canine proteins. A few of the target proteins could not be detected, but none of these are very abundant in dog CSF. MARS-Hu14 was demonstrated to efficiently deplete the targeted proteins from dog CSF. However, the drawback was that this column removed many additional proteins, as seen both on SDS-PAGE (Fig. 1A) and in LC–MS/MS analysis (Table 3).

3.1.2. Protein identification and investigation of repeatability

The total number of identified proteins prior to and after depletion was determined and compared. To estimate the repeatability, the CV of the total number of proteins for the 3 technical replicates from the different preparations was calculated and the total number of proteins identified in all experiments was determined (Table 3). The fractions from Seppro® rat and ProteoExtract® gave approximately the same average number of detected proteins, but Seppro® rat showed better repeatability compared to ProteoExtract®. MARS-Hu14 provided repeatable results, but gave the lowest number of detected proteins in this study. When comparing the CV-values, all columns had similar or even better repeatability than the non-depleted CSF and both Seppro® rat and ProteoExtract® gave higher total number of proteins. The bound fractions showed similar repeatability with around 55% of the detected proteins found in all technical replicates, but the total number of proteins varied. Importantly, it was concluded that the evaluated depletion strategies do not

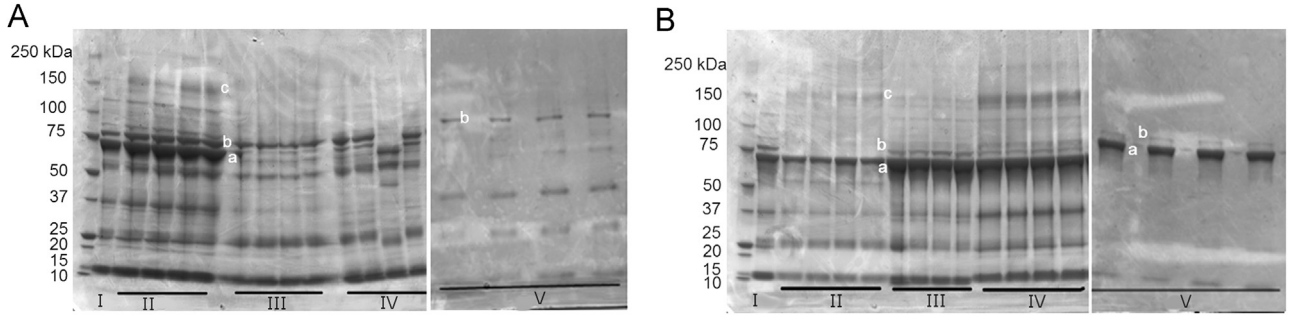

Fig. 1. (A) SDS-PAGE of the flow through fractions and (B) of the bound fractions of the dog CSF sample. I: Non-depleted CSF, II: ProteoExtract®, III: Seppro® rat, IV: ProteaPrep, V: MARS-Hu14. Lower-case letters represent protein bands for (a): albumin (~66 kDa), (b): transferrin (~80 kDa) and (c): IgG (~150 kDa).

Table 2A
A comparison of PSMs of the proteins that the different depletion columns were designed to capture (except immunoglobulins). Both the flow through (FT) and the bound (B) fractions are listed for the three technical replicates.

Description	Accession	Replicate 1 (FT)	Replicate 2 (FT)	Replicate 3 (FT)	Replicate 1 (B)	Replicate 2 (B)	Replicate 3 (B)
ProteoExtract®							
Serum albumin	F2Z4Q6	3392	2930	3595	1702	1746	2976
Seppro® rat							
Serum albumin	F2Z4Q6	1360	434	877	3177	3225	3089
Transferrin	J9P430	2201	2039	1866	284	309	455
Haptoglobin	P19006	20	2	6	121	123	118
Alpha 1-antitrypsin	A1ILJ0	98	89	81	89	63	68
Fibrinogen beta and gamma chain	F1PGS2 F1P8G0	2			2		
ProteaPrep							
Serum albumin	F2Z4Q6	683	687	868	1243	2849	1111
MARS-Hu14							
Serum albumin	F2Z4Q6	496	321	263	6981	4913	3900
Alpha 1-antitrypsin	A1ILJ0	30	29	49	102	123	63
Transferrin	J9P430	1411	1315	1260	899	461	352
Haptoglobin	P19006	29	34	33	145	171	160
Alpha-2-macroglobulin	F6UME0	11	4	7	87	120	110
Apolipoprotein A-I	F1PDJ5	14	13	10	185	191	178
C3	F1PIX8	367	395	433	120	130	120
Transthyretin	E2R5U8	41	22	51	375	246	313
Fibrinogen	Not present						
Alpha1-acid glycoprotein	Not present						
Apolipo protein AII	Not present						

Table 2B
In total, 27 protein fragments possibly related to immunoglobulins were found, searching all MS runs. The table lists the number of fragments found in the flow through and bound fractions for each depletion column. Note that the different depletion columns were design to capture different number of immunoglobulins.

	Flow through fraction (number of protein fragment detected)	Bound fraction (number of protein fragment detected)
ProteoExtract®		
IgG	7	12
Seppro® rat		
IgG and IgM	3	12
ProteaPrep		
IgG	6	4
MARS-Hu14		
IgA, IgG and IgM	None	12

Table 3
Total number of detected proteins and the repeatability of the different columns evaluated in the study. The numbers are based on 3 technical replicates of the flow through and bound fractions for 4 different depletion columns and non-depleted sample.

Preparation	Average number of detected proteins	CV, number of detected proteins (%)	Total number of unique proteins in all replicates	Number of proteins detected in all replicates
Non-depleted	167	21	211	111
ProteoExtract® FT	199	17	265	134
Seppro® rat FT	205	9	258	156
ProteaPrep FT	167	10	209	123
MARS-Hu14 FT	151	10	184	118
ProteoExtract® Bound	102	20	125	67
Seppro® rat Bound	104	4	136	76
ProteaPrep Bound	93	25	118	64
MARS-Hu14 Bound	72	9	87	51

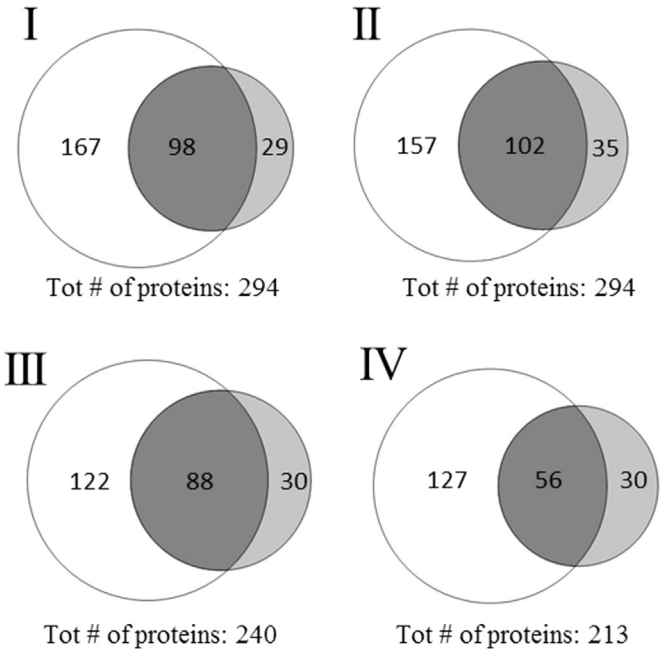

Fig. 2. Number of proteins found in the mass spectrometry runs of flow through and bound fractions from four different depletion columns. I: ProteoExtract®, II: Seppro® rat, III: ProteaPrep, IV: MARS-Hu14. The white field indicates proteins found in the flow through fraction and the light gray area is proteins detected in the bound fraction. The intersection represents proteins found in both fractions. All numbers are based on three merged replicates.

introduce more variances than what was detected when performing the experiments on non-depleted dog CSF.

Fig. 2 shows Venn diagrams with a combination of proteins found in the flow through and bound fractions for each depletion column. For all columns, a rather large number of proteins were detected in the bound fractions, but most of those proteins were also found in the flow through fractions. There are many reports of non-specific binding related to protein depletion [41,59–61] and the low PSM-values for many of the proteins in the bound fractions support that. Recently, a shotgun study on dog proteins was published, where three different depletion columns developed to deplete human albumin and IgG were tested. The authors found a lot of non-specifically bound

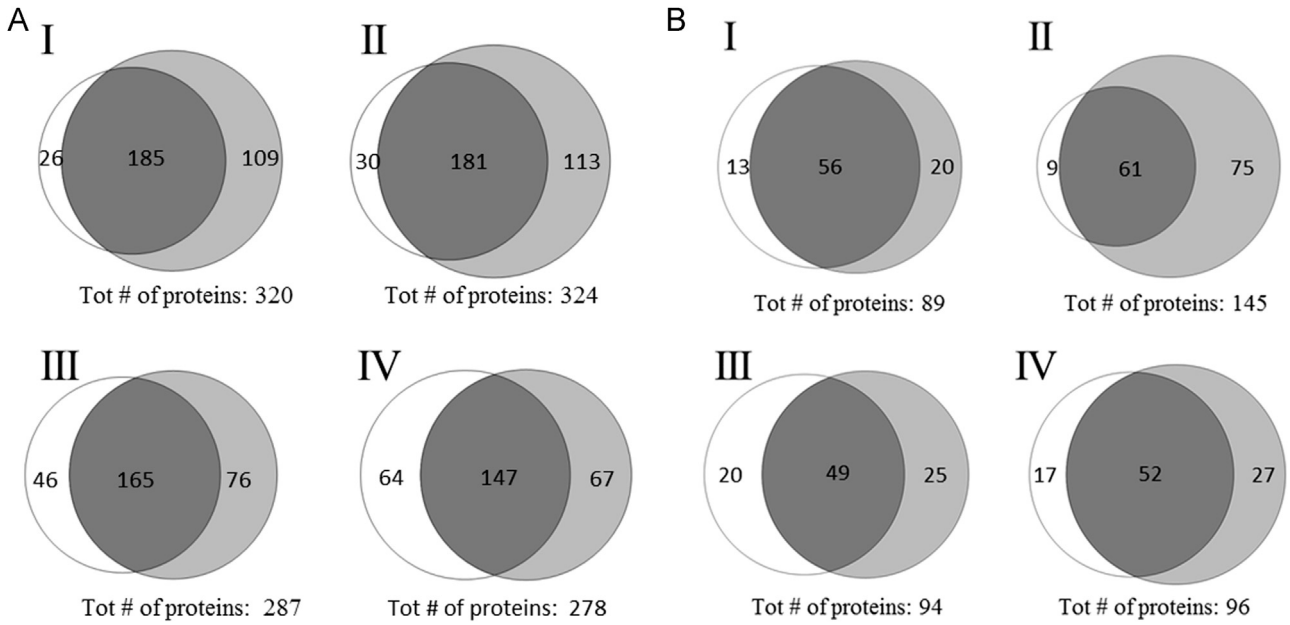

Fig. 3. A comparison of the number of proteins detected in non-depleted dog CSF samples versus CSF prepared with four different depletion columns (flow through and bound fractions). (A) All proteins detected in the samples and (B) proteins in the gel fractions of size 55–75 kDa. The white field shows proteins only found in non-depleted fraction and the light gray area represents proteins detected in the samples depleted by I: ProteoExtract®, II: Seppro® rat, III: ProteaPrep and IV: MARS-Hu14. All numbers are based on three merged replicates.

proteins in the bound fraction and concluded not to recommend the use of depletion columns for dog studies [59]. In the present study, we investigated both the flow through and bound fractions, and from our experience, it can be very limiting to judge the different depletion columns, solely based on proteins found in the bound fraction. In many studies, there will be a gain from using the depletion columns if additional proteins can be identified in the sample. It would, however, not be advisable to use depletion columns in quantitative studies.

In Fig. 3A, a comparison of the three technical replicates of non-depleted samples and all the different fractions (flow through and bound) of each depletion column is presented. Once again, Seppro® rat and ProteoExtract® were the best performing columns with a total of ~320 proteins detected. As compared to the non-depleted preparation, 113 and 109 unique proteins were detected, respectively, which is ~35% of the total number of detected proteins in those combinations. The low number of extra proteins for both ProteaPrep and MARS-14 is eye-catching, demonstrating that protein depletion of dog CSF sample results in a substantial loss of proteins using these columns. One major reason for using depletion strategies is to enhance the detection of proteins of similar sizes as the dominating ones, in this case albumin. The SDS-PAGE were divided in three fractions by cutting one narrow band ~55–75 kDa and two larger fractions containing proteins >75 kDa and <55 kDa, respectively. Fig. 3B shows the number of proteins found in depleted versus non-depleted 55–75 kDa fractions. For all evaluated columns, there were more proteins detected in the depleted 55–75 kDa fractions than in the untreated fraction. The result is especially pronounced for Seppro® rat.

Altogether, our results demonstrate that high-abundant protein depletion can be fulfilled with all the tested columns, but with varying efficiency. Seppro® rat and ProteoExtract® performed much better regarding the total number of detected proteins compared to the two other evaluated columns. Seppro® rat was the best performing column. In particular, the detection of proteins of sizes around 65 kDa was enhanced using this column. If also cost is considered and less repeatability could be accepted, ProteoExtract® is an alternative.

3.2. Additional fractionation of the SDS-PAGE as a tool for increased protein detection

A pilot study was set up to get an indication of to what extent more fractionation of the SDS-PAGE lanes would improve the total protein output from this sample. Based on the results from Section 3.1, the gel lanes of the untreated, Seppro® rat and ProteoExtract® depleted samples were divided in 10 fractions each. As expected, the number of proteins increased dramatically compared to the fractionation in three regions (Table 3). Most proteins, 724, were detected for the non-depleted sample. Seppro® rat gave 516 proteins and ProteoExtract® 496 proteins. Importantly, Seppro® rat added 87 unique proteins compared to the non-depleted sample and ProteoExtract® add 54 proteins. There has to be more replicates run to establish the fact that the non-depleted sample gave the highest number of proteins. However, we consider this beyond the scope of this pilot study. In this study, 400 μL CSF sample was used in the depletion step which is a volume that can be used clinically. In human studies a lot more CSF sample have been used, e.g. Schutzer et al. [16], that used 18 mL of pooled CSF for the depletion step or Guldbrandsen et al. [18] that used 3 mL CSF for the depletion followed by a fractionation of the gel in 83 fractions. Those studies resulted in many more detected proteins but with much larger volumes and extensive fractionation. The objective of this study was to try to get an easy way of preparing the dog CSF samples that could also be used for larger studies. Extensive fractionation will make it very labor-intensive to run large studies and notably, the size of dogs can also be a limiting factor. Using all approaches presented here, the study resulted in a total of 983 detected proteins, applying Sequest, with at least two peptides, see Supplementary Data, Table S1. The table includes 801 previously uncharacterized dog proteins based on homology studies to other species, which means that only 182 of the detected proteins had been manually annotated in the UniProt database. This is the largest reported number of detected proteins in CSF from a single dog and is considerably larger than the 36 proteins that were reported in the dog CSF study done by Nakamura et al. [47].

A gene ontology (www.geneontology.org) annotation was performed with the support of the Proteome Discoverer software.

Approximately 46% of the CSF proteins in dog presented in our study are classified as plasma membrane, cell surface or extracellular proteins which is higher than reported for humans. A similar comparison for cytoplasm and nucleus give a more overlapping pattern with about 10% for both compartments in both species. For the dog, there is also a large quantity of proteins with no annotation, which is probably due to the limited information about the dog proteome to date. The results from the annotations are presented in Figs. S1 (cellular components), S2 (molecular functions) and S3 (biological processes). The results are in line with what have been presented in earlier studies on human and mouse CSF [11,16]. Among the detected proteins, there were many that have been reported as brain specific in human and mouse CSF studies [22,62,63], e.g. tubulin alpha-1A chain, microtubule-associated protein 6, neuron-specific enolase, brain acid soluble protein 1, ubiquitin carboxyl-terminal hydrolase isozyme L1, fructose-bisphosphate aldolase C, 14-3-3 proteins, ephrin type-A receptor 4, neurexin-3-alpha, major prion protein, glial fibrillary acidic protein, myelin basic protein, creatine kinase B-type and brain-specific angiogenesis inhibitor 2. The fact that many of the proteins have been reported in human CSF studies shows that there is a potential to do comparative studies based on the methods presented here.

Extensive fractionation showed to be a very good tool to get at better coverage of the dog proteome. One drawback is that it is a labor-intensive approach. A more efficient method to reach a specified population of proteins could be depletion of a selected high-abundant protein in combination with SDS-PAGE, followed by cutting out a small band. In this study, Seppro® rat gave a dramatic improvement of the coverage of proteins of similar size as albumin. This could e.g. be used when selecting good peptide candidates for targeted proteomic approaches.

4. Conclusions

In this study, it was concluded that it is possible to use commercially available depletion columns, primarily developed for human or rat plasma/serum samples, to prepare dog CSF samples with an improved coverage of the dog CSF proteome. Even if all methods could deplete CSF samples, there were large differences between the methods. Seppro® rat was in our hands the overall best performing depletion column with high numbers of detected proteins and good repeatability. Seppro® was also a very good method, compared to the others, to get a better detection of proteins of similar size as albumin. Gel fractionation proved to be an effective approach to increase the number of detected proteins. However, it has to be considered that increasing the number of fractions adds substantial more work effort and time required for the sample preparation, LC–MS/MS experiments and data analysis. Altogether, we here demonstrate that the combination of high-abundant protein depletion, gel fractionation, in-solution and in-gel tryptic digestion followed by analyses with nanoLC-Orbitrap MS/MS, allows for a comprehensive map of the dog CSF proteome with a much larger coverage than published before. In total 983 proteins, were detected using all different preparations of the investigated dog CSF sample. Several of the detected proteins have previously been reported as brain specific in human and mouse, which shows that the methods applied in this study also could be used in comparative studies between dog and humans/mouse.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Funding Sources

This work was supported by grants from the Swedish Research Council (Grant 621-2011-4423) and Science for Life Laboratory. The funding sources had no involvement in the preparation or submission of the article.

Acknowledgment

The authors would like to thank Dr. Ingrid Ljungvall, Department of Clinical Sciences, Swedish University of Agricultural Sciences for providing us with dog CSF sample. Swedish Research Council 621-2011-4423 is acknowledged. We are also grateful for the possibility to have long-term storage of the raw data by Bioinformatics Infrastructure for Life Sciences (BILS).

Appendix A. Suplementary Information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2015.07.013.

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