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Proteomic analysis of subarachnoid hemorrhage – liquid-phase isoelectric focusing in complex protein sample

Joanna Hajduk[1], Bartosz Sokół[2], Agata Swiatly[1], Jan Matysiak[1], Piotr Nowicki[1], Ewa Garbiec[1], Norbert Wąsik[2], Roman Jankowski[2], Zenon J. Kokot[1]

[1] Department of Inorganic and Analytical Chemistry, Poznan University of Medical Sciences, Poland
[2] Department of Neurosurgery, Poznan University of Medical Sciences, Poland

ABSTRACT

Aim. The aim of this study was to present the proteomic approach based on liquid phase isoelectric focusing fractionation coupled to nLC-MALDI-TOF/TOF-MS/MS analysis to characterize cerebrospinal fluid from control patients and those suffering from subarachnoid hemorrhage. The new perspective in characterization of this brain neuropathology is in constant demand to point a valuable panel of indicators which could improve the treatment outcome.

Material and Methods. The cerebrospinal fluid samples were applied to a commercial liquid phase isoelectric focusing apparatus and separated into 10 fractions by pI. Further, the untargeted mass spectrometry investigations were performed with data dependent acquisition mode for full-scan MS analysis with subsequent MS/MS fragmentation using nLC-MALDI-TOF/TOF-MS/MS.

Results. In total, the detection of 1664 and 2187 unique tryptic peptides provided biological evidence for 134 and 271 proteins in control and subarachnoid hemorrhage sample, respectively. The interpretation of liquid phase separation was performed by intersection analysis of two items between groups of ten fractions. The cumulative intersection exploration revealed the highest concentration of the detected components in the middle fractions of the focusing chamber, whereas the gradual dilution appeared on its extreme.

Conclusions. The employed strategy ensured overall screening of investigated material presenting the proteins abundance in the current state of analysis. Few proteins such as proenkephalin A, peroxiredoxin-6, cathepsin B, thrombospondin-1, glial fibrillary acidic protein and α-spectrin were recognized as potential indicators, according to literature, pointing the possibility for monitoring in further studies as panel of valuable biomarkers.

Keywords: MALDI mass spectrometry, proteomic strategies, protein separation, cerebrospinal fluid, subarachnoid hemorrhage.

Introduction

Cerebrospinal fluid (CSF) surrounds the central nervous system including the ventricular system of the brain, spinal canal and subarachnoid space cranial cavity. It is produced by choroid plexus of the brain cells, lining cells and microglia [1]. The main functions of the CSF are the protection of the brain and spinal cord from the mechanical injuries, compensation of the intracranial pressure and transfer of the humoral information. Analysis of this fluid may access the state of the environment in human central nervous system (CNS) [2].

Aneurysmal subarachnoid hemorrhage (SAH) belongs to cerebrovascular diseases and contributes to 6–8% of all cerebral stroke events. It is caused by the extravasation of blood into the sub-arachnoid space [3]. The pathophysiological process of cellular and molecu-

lar changes following SAH are still not fully characterized [4]. Therefore, the proteomic examination of CSF may contribute to detection of potential cerebral indicators, which will lead to better prediction of patients deterioration and treatment outcome [3, 5–6]. Currently performed proteomic analyses are mostly based on mass spectrometry (MS) with application of soft ionization methods: MALDI (matrix-assisted laser desorption/ionization) and ESI (electrospray ionization). Due to significant specificity and sensitivity, these techniques are commonly used in numerous clinical studies e.g. monitoring of selected compounds [7]. However, direct analysis of complex biological samples is associated with problems of analyte suppression and high dynamic range in protein content [8, 9]. Thus, the preparation steps including separation and concentration of analyzed sample are often required before MS analysis.

Cerebrospinal fluid suffers protein dynamic range problems with the small number of proteins constituting the sample. Preparation techniques are important in order to identify the largest number of constituents and thus appropriate biomarker within sample. One of the widely used method is two dimensional gel electrophoresis (2-DE), which combines two independent separation processes: due to isoelectric point and molecular weight of proteins [10]. Its compatibility with mass spectrometry techniques is defined as bottom-up strategy in clinical proteomic research [11].

An alternative approach to gel electrophoresis in modern proteomics is fractionation based on gel free liquid phase isoelectric focusing (LP-IEF) [12], where the protein charge is changing until its pI is reached. By adding carrier ampholytes [13], smooth and relatively stable pH gradient with greater buffering capacity could be achieved. Moreover, the main advantage of gel free technology is associated with the possibility of protein recovery in the liquid phase [14]. Therefore, this methodology becomes an attractive tool as a separation technique prior to the nano-LC/MS analysis, widely applied into various biological samples like a human erythroleukemia cell line [15], ovarian carcinoma cells [16–17] or brain tissue [18]. Regarding to our study, LP-IEF in combination with gel electrophoresis was reported to be an important tool for identifying low abundant proteins in human cerebrospinal fluid and membrane proteins in frontal cortex [19].

Aim

The aim of this study was to present method for proteomic analysis of CSF for screening analysis of SAH. We used LP-IEF combined with nLC-MALDI-TOF/TOF mass spectrometry to characterize the protein content of analyzed cases with and without SAH. The usefulness of gel free strategy was estimated by intersection analysis.

Material and Methods

Study cases

The research project has been approved by the Regional Ethics Committee of the Poznan University of Medical Sciences (decision No. 503/15). The SAH case was admitted due to the subarachnoid hemorrhage. A computed tomography (CT) scan demonstrated intracerebral hemorrhage of the left frontal lobe and intraventricular bleeding causing mass effect (**Figure 1**). Digital subtraction angiography demonstrated ruptured anterior communicating artery aneurysm which was coiled. The patient required insertion of external ventricular drainage during which 10 mL of cerebrospinal fluid was collected for the analysis. The material was characterized: blood stained with opalescent transparency; white cell count (WCC) – 118/μL; red blood cell count (RBC) – 20000/μL; protein concentration – 1944 mg/dL. The blood in the ventricles was gradually absorbed and patient`s condition improved. After 14th day the patient was woken up and the ventricular drainage was removed. Control case was admitted with six months history of gait disturbance, urinary incontinence and mental decline. The patient was diagnosed with the normal pressure hydrocephalus (**Figure 2**) and the programmable ventriculo-peritoneal shunt (Sophy Mini SM8 valve, Sophysa) was inserted where 10 mL of cerebrospinal fluid was collected. The material was characterized: colorless with clear transparency; white cell count (WCC) – 2/μL; red blood cell count (RBC) – 320/μL; protein concentration – 166 mg/dL. The neurological condition of the case gradually improved after the operation. Immediately after collection of cerebrospinal fluid, the material was centrifuged at 3000 rpm for 10 min and stored at -80°C until analyzed.

Isoelectric focusing fractionation

Prior the fractionation, the filter membrane Amicon Ultra 100K (Millipore, Bedford, MA) devices were used for ultrafiltration procedure to cut off the high molecular weight proteins, according to the manufacturer's instructions [20]. Firstly, the membrane was rinsed with deionized water, then 2000 μL of cerebrospinal fluid was centrifuged at 5400 × g for 30 min. The collected filtrate was mixed with n-octylglucoside to a concentra-

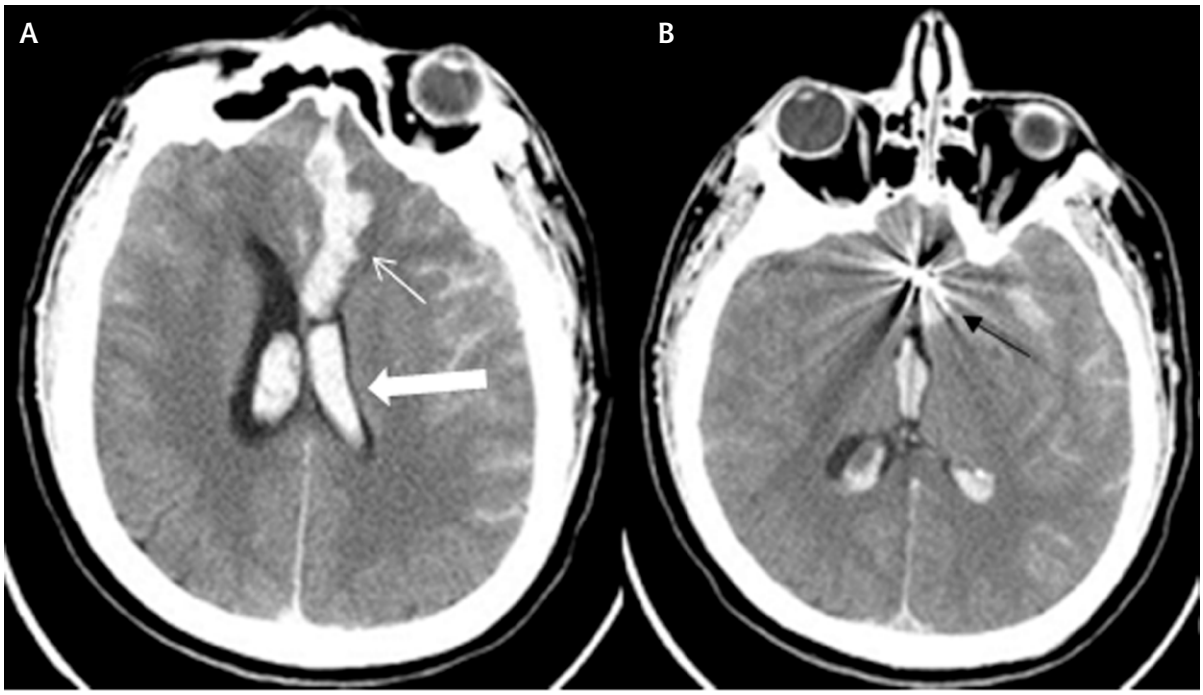

Figure 1. A computed tomography (CT) scan of the patient with subarachnoid hemorrhage on admission demonstrating: (A) intracerebral hemorrhage (white thin arrow) with intraventricular extension (white thick arrow); (B) coiled aneurysm of anterior communicating artery and the blood in fourth ventricle (black arrow)

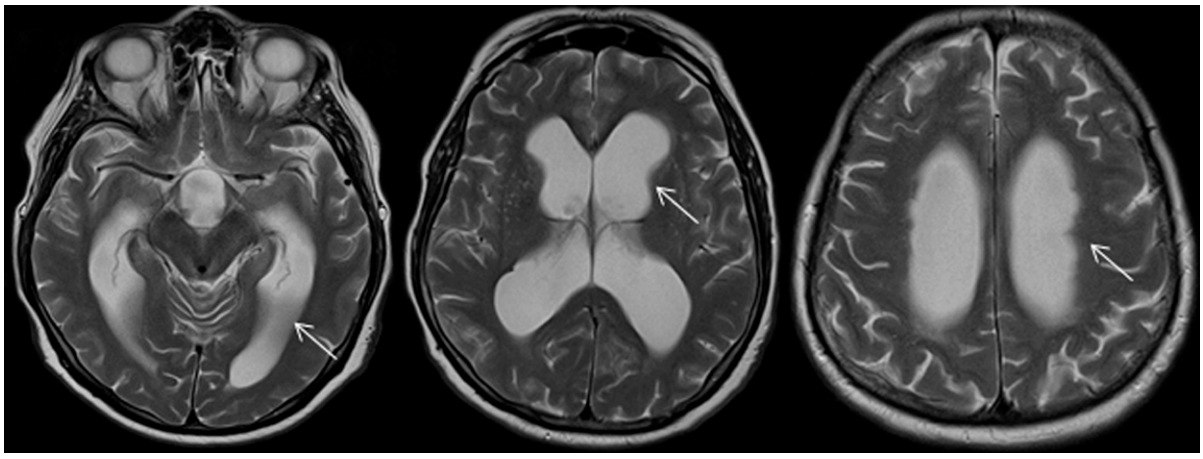

Figure 2. A magnetic resonance imaging (MRI) of the control patient with normal pressure hydrocephalus demonstrating dilated ventricles (white thin arrow)

tion of 0.1% and with ampholyte (40%, pH range 3–10 isodalt, Bio-Lyte, Biorad) to a concentration of 2.5%. The 500 µg of CSF protein was loaded into the Micro-Rotofor chamber (Bio-Rad Laboratories, Hercules, CA, USA) for focusing without further treatment. Constant power (1 W) was applied. Initial voltage was approximately 80 V and a plateau of 600 V was reached after 2 h. Ten separate fractions for each investigated case were rapidly harvested and purified using ReadyPrep 2-D (Bio-Rad Laboratories, Hercules, CA, USA) cleanup kit in accordance to manufacturer's instruction [21]. The precipitated proteins were resolved in 30% ACN/0.1% TFA solution and subjected to MALDI-TOF/TOF-MS/MS analysis acquiring gel free and SDS-PAGE strategies.

Gel electrophoresis

Additionally, the IEF fractions from SAH case were separated using SDS-PAGE. The 20 µL of sample mixture was loaded on a 14% Tris-glycine–SDS-PAGE gel and the process was carried out at 200 V for 40 min. Gel separation was performed on a Mini-Protean Tetra Cell (Bio-Rad Laboratories, Hercules, CA, USA) and was continued until the blue bromophenol front reached the bottom of the gels. The gel was stained with Coomas-

sie Brilliant Blue G250 and the background was washed out by use of 9% acetic acid.

In-gel and In-solution digestion

All in-solution CSF fractions obtained from MicroRotofor were subjected to digestion protocol according to the procedure modified from Pierce In-Solution Tryptic Digestion Kit. While the most intense proteins bands in the mass range from 20 to 37 kDa of Coomassie stained gel were processed with in-gel digestion according to adopted Shevchenko et al. protocol [22], the digested peptides were extracted from the gel by incubation in 50 µL of 50% ACN/ 0.1% TFA solution.

MALDI-TOF/TOF-MS/MS protein identification

The digested peptides were subjected to nano-LC analysis. The system consisted of EASY nano-LC II (Bruker Daltonics) and fraction collector Proteineer-fc II (Bruker Daltonics). Firstly, loaded peptides were concentrated on a trap column NS–MP-10 BioSphere C18 5 µm particle size, 120-Å pore size, 100 µm inner diameter, 20 mm length (NanoSeparations, Nieuwkoop, Netherlands), then separated on a Acclaim PepMap 100 column C18, 3 µm, 100 Å, 75 µm × 150 mm (Thermo Scientific, Sunnyvale, CA, USA) by linear gradient of water (mobile phase A) and 90% ACN (mobile phase B), both containing 0.05% TFA. The gradient elution method was: 2–50% B in 96 min. The flow rate was maintained at 300 nL min-1 and the injection volume was 6 µL. In total, 384 fractions of each separated fraction were automatically mixed with matrix solution and spotted onto a AnchorChipTM target (Bruker Daltonics). Per fraction 80 nl of eluent was mixed with 420 nl matrix solution. Matrix solution was generated by mixing: 748 µL of 95:5 (v/v) acetonitrile: 0.1% TFA, 36 µL of saturated solution of HCCA in 90:10 (v/v) acetonitrile:0.1% TFA, 8µL of 10% TFA and 8µL of 100 mM ammonium phosphate monobasic. The system was controlled using HyStar 3.2 software (Bruker Daltonics). Afterwards, the tandem mass spectrometry analysis was performed using the MALDI-ToF/ToF UltrafleXtreme instrument equipped with a SmartBeam II laser (Bruker Daltonics). Typical instrument setting for MS mode was as follows: ion source 1, 25.09 kV; ion source 2, 22.59 kV; lens voltage, 7.89 kV; pulsed ion extraction time, 120 ns; matrix suppression mass cut off, m/z 700. All spectra were acquired by accumulating 4000 shots from 40 non-overlapping positions with a repetition rate of the pulsed laser of 2 kHz. By routine, a standard peptide calibration mixture in the mass range of 700–3500 Da (Bruker Daltonics) was analyzed for external calibration of the mass spectrometer. The MS/MS mode for protein identification was applied with the following setting: ion source 1, 7.50 kV; ion source 2, 6.75 kV; lens, 3.50 kV; reflectron 1, 29.50 kV; reflectron 2, 14.00; lift 1, 19.00 kV; lift 2, 3.00 kV, pulsed ion extraction time, 80 ns; fragments only. Precursors with a signal-to-noise ratio above 10 were automatically subjected to MS/MS analysis. The maximum number of MS/MS per fraction was set to 20. The control of the instrument, data acquisition, processing and evaluation was performed using the following software platforms: flexControl 3.4, FlexAnalysis 3.4 and WARP-LC 1.3 and ProteinScape 3.1 (Bruker Daltonics). The MS/MS spectra were processed with ProteinScape 3.0 platform by searching the SwissProt database with Mascot 2.4.0 search engine (Matrix Science, London, UK). The LC-MALDI results were filtered to a false discovery rate at a peptide-spectrum match level of less than 1% based on decoy counts and only proteins with at least one unique peptide were included. The LC-MALDI data were used with the significant threshold of $p > 0.05$ set by the search engine. The general protein search parameters were included: trypsin and semi trypsin digestion, 1 and 2 missed cleavages, peptide precursor mass tolerance: 35 ppm; fragment mass tolerance: 0.7 Da; peptide charge: +1; monoisotopic mass; carbamidomethylation of cysteine as fixed modification; oxidation and dioxidation of methionine as variable modification. The results were compiled into one protein list.

Statistical analysis

The interpretation of isoelectric focusing separation was performed by intersection analysis of two items between groups of ten fractions, which contained measured masses of peptides obtained by nLC-MALDI-TOF/TOF-MS/MS. The 45 selections of interaction (as a result of combination of two items choose ten fractions) were showed as an upper triangular image plot. For clarity, the symmetrical lower triangular part was presented as a blackened area. The cumulative intersection analysis was made in both left and right directions starting from the fifth fraction, as a point of injection of the sample. We define $m \in \{1,2,...10\}-\{5\}$, as a number of fraction and $n = 5$ (fifth fraction). Therefore, Lm a result for m fractions can be presented using following notations:

$$\bigcap_{n=5}^{n-m} = L_m$$

for the left direction and

$$\bigcap_{n=5}^{m-n} = L_m$$

for right one. Furthermore, the membership of set was created by using of all peptides included by search engine to identify two proteins: peroxiredoxin-6 and cathepsin B, which were described as potential indicators for this brain pathology and had enough number of peptide to analyze. For all steps, MATLAB v.8.1.0.604 software with the Bioinformatics Toolbox was used.

Results

Gel-free LC-MS/MS approach

Using gel free approach consisting of the LP-IEF set and nLC-MALDI-TOF/TOF-MS/MS, the CSF digested peptides derived from patient with and without SAH were analyzed. The data dependent acquisition (DDA) mode was acquired, where full-scan MS analysis was performed and subsequently MS/MS fragmentation on a defined number of the most intense ions. The approach is valuable in the untargeted investigation of samples with no hypothesis about which parent ions should be fragmented as a priority. The identified proteins and peptides from the different fractions were collected into compilation list. In total, 1664 and 2187 unique tryptic peptides provided biological evidence for 134 and 271 proteins in control and SAH sample, respectively (**Supplementary material 1 and 2**). Among these the characteristic of high abundant components of CSF were acknowledged. Due to high dynamic range between analyzed samples, in CSF with SAH up to 177 unique proteins were recognized. The data obtained from particular IFE fractions of SAH specimen were compared. The highest identification number was found in fraction 5, where from 167 detected proteins as many as 67 were unique. Further, the fraction 4, 8 and 7 also gave valuable contribution to proteomic characterization of the analyzed case, whereas fraction 1, 2 and 10 presented poor description in protein content. Analyzing the specificity of separation process, it was observed that many common species were overlapping with the neighboring fractions. The pI of detected proteins were varying from 3.9 to 16.4. Thus, in overall distribution of biomolecules in artificial pH gradient (**Figure 3**), we were able to observe higher repletion of proteins with pI 5–6 in IEF fraction 3, 4 and 5, whereas the proteins with pI 8–9 were more abundant in the last fractions. The interaction analysis of masses identified by MALDI-TOF/TOF-MS/MS between two selected fractions was shown as the upper triangular image plot

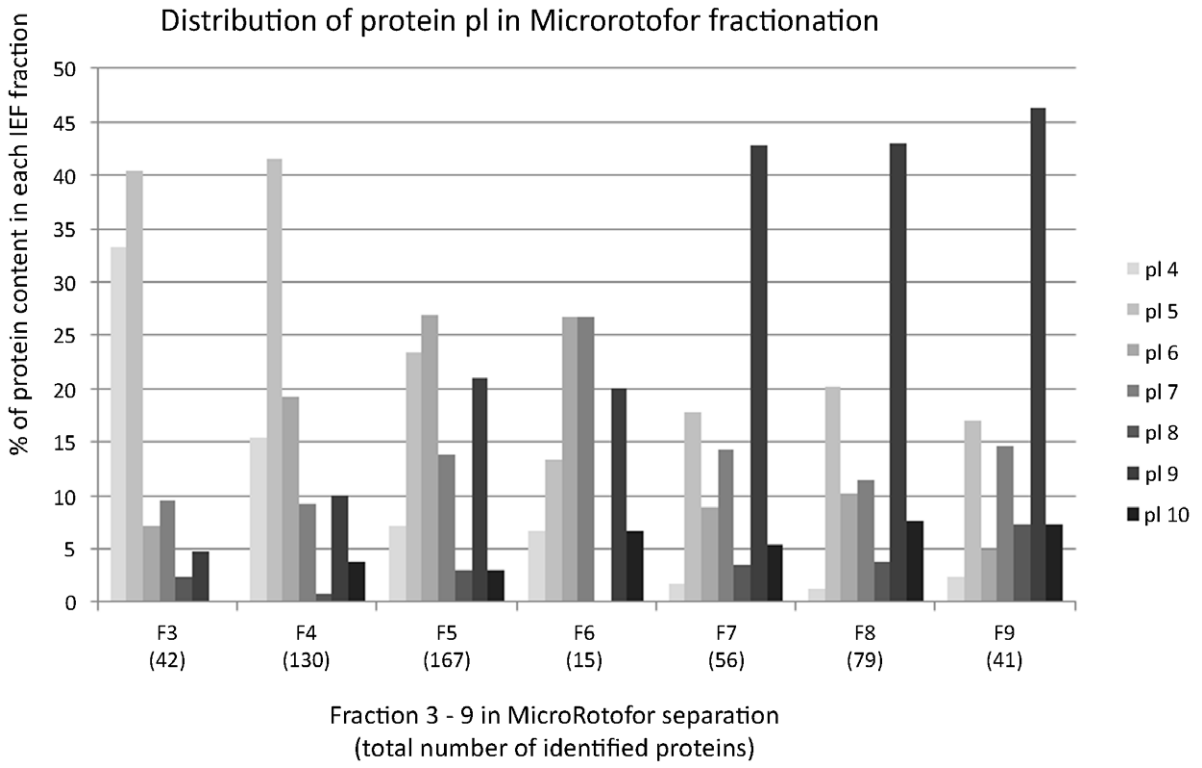

Figure 3. A bar graph represents the distribution of protein isoelectric point (pI) in Microrotofor separation between fractions 3 to 9. On the horizontal axis the investigated fractions and identified per each proteins with the percentage content according to its pI

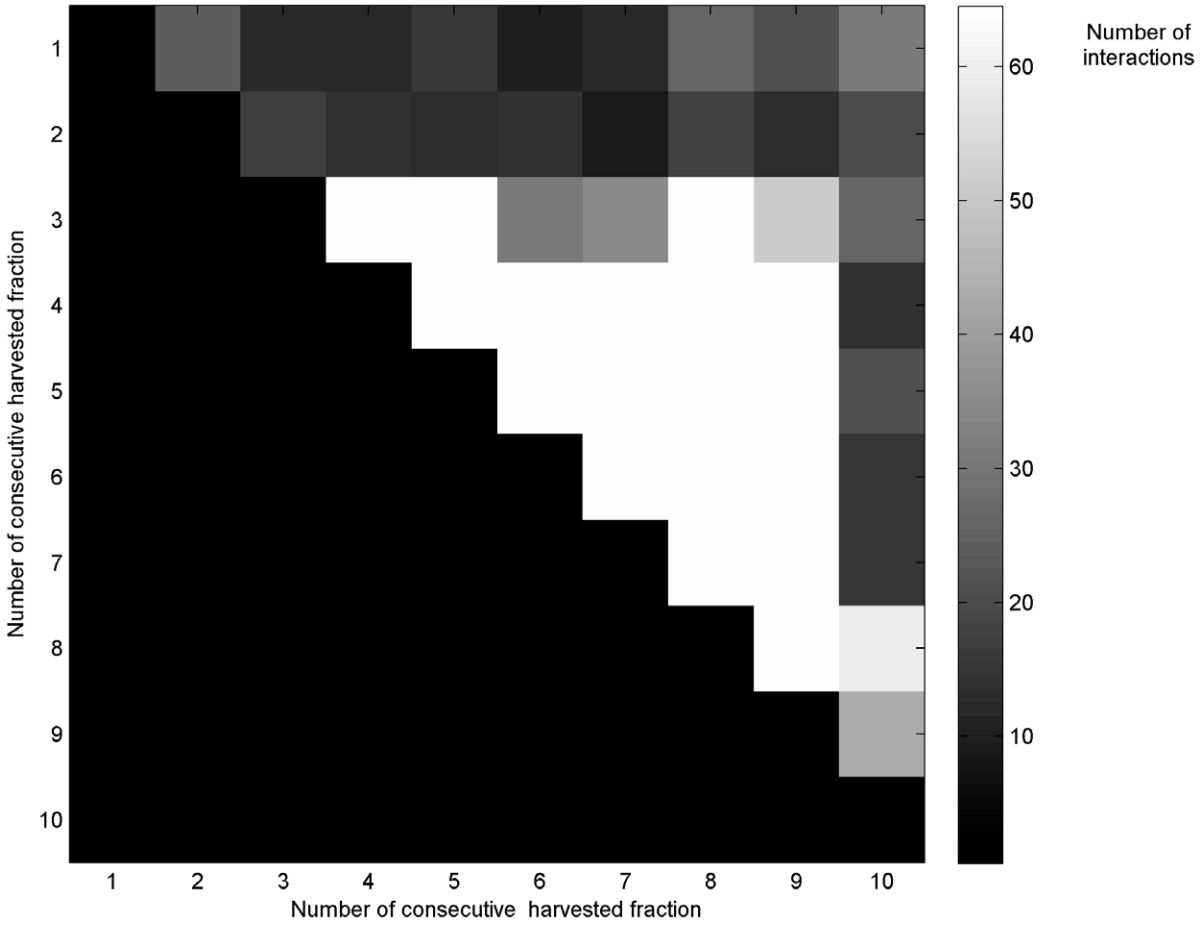

Figure 4. Upper triangular image plot of cumulative intersection analysis of two items in group of ten fractions. For clarity symmetrical lower triangular part was presented as a blackened area

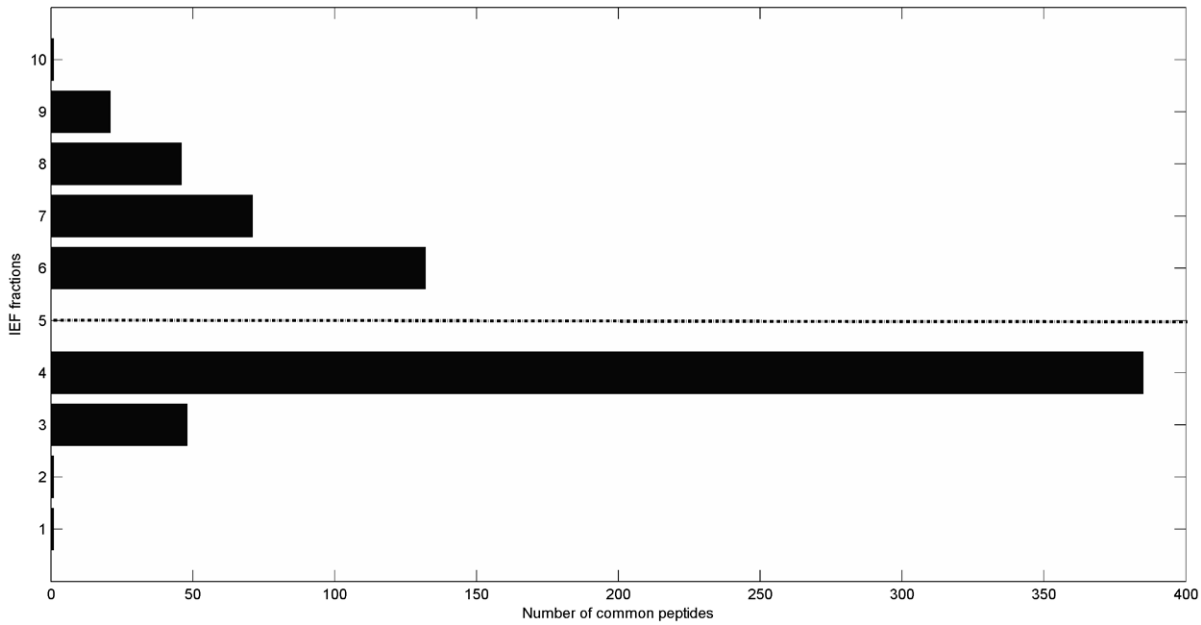

Figure 5. Cumulative intersection analysis for all measured compounds

(**Figure 4**). The intersection analysis of two items in group of ten fractions resulted in 45 compared areas. The performed analysis indicated a large number of common compounds in the adjacent fractions, especially true for the fractions in the center. Moreover, it was noticed that larger distance between the fractions ensured the identification of more unique elements. The best separation performance was obtained between pairs of 1–6 and 2–7. The highest concentration of the detected components was observed in the middle fractions, whereas the gradual dilution appeared on the extremes of the MicroRotofor chamber. The dilution process occurred better in the left direction from the center. This effect is clearly seen in **Figure 5**, which shows the cumulative intersection of the fractions starting from the fraction number 5 in the decreasing direction on the left and increasing on the right. Regarding to the quantity, the fractions are not arranged symmetrically with respect to a fraction number 5. The total number of all detected compounds (included peptides) were the highest in fraction 4 (2995) and 5 (3667). Although, some of their measured masses were presented also in other fractions (**Figure 6**), which is confirmation for dilution phenomenon. The **Figure 6** shows the membership of the peptides masses used for identification of chosen proteins: cathepsin B and peroxiredoxin-6. The measured m/z of enzyme protease cathepsin B were additionally observed in fraction 2, 3, 7 and 8. With regard to protein peroxiredoxin-6 a similar conclusion was proposed. The characteristic masses assigned to this protein have been detected in the whole range of analyzed fractions.

Gel-based LC-MS/MS approach

The most intense protein bands in the mass range from 20 to 37 kD from SDS-PAGE analysis were excised from the gel and subjected into in-gel digestion procedure (**Supplementary material 3**). The common identified proteins in both strategies (SDS-PAGE vs gel free) were compared with respect to percentage of sequence coverage and number of identified tryptic peptides (**Table 1**). We observed that approximately 80% of the analyzed proteins was better characterized using gel free strategy, thus we concluded that this methodology is valuable in screening analysis of various biological samples.

Discussion

In our study we implemented the LP-IEF based on distribution of current via the electrolytes system gradually increasing pH from anode to cathode. The principle underlies in placement of proteins and others components between both electrodes where the pH is equal with the isoelectric point of analyzed molecules. However, it is difficult to predict or achieve the optimal length of focusing run. Moreover, the separation of all components in pH gradients should be perceived more as a quasi-equilibrium process [23]. The Rotofor

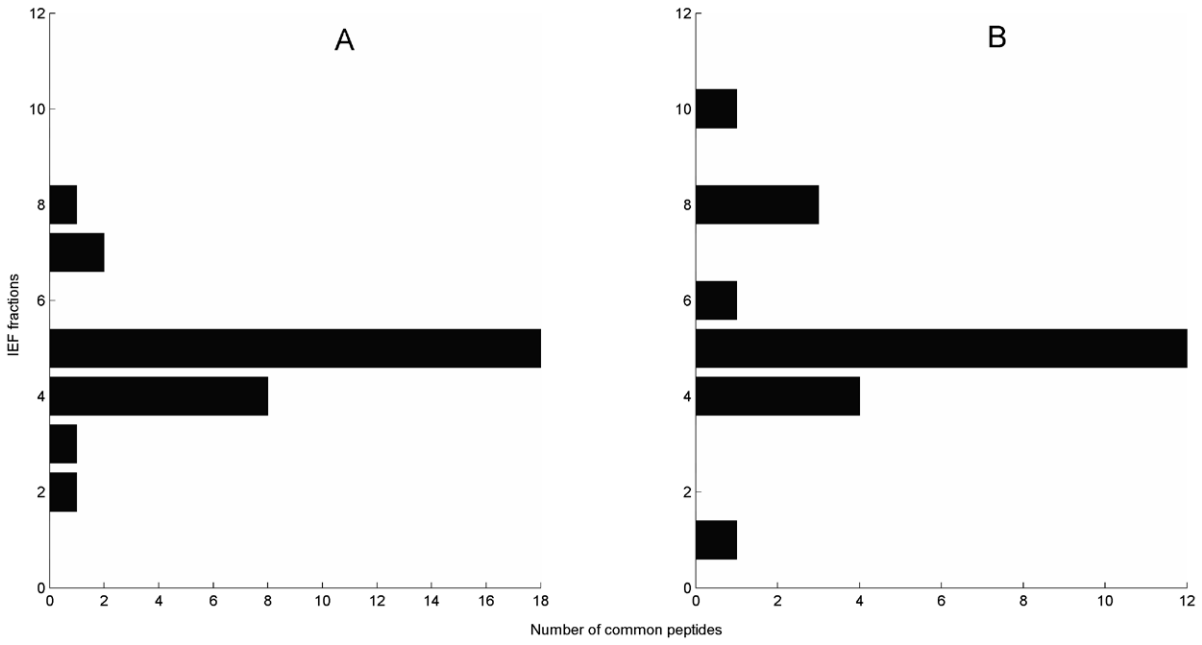

Figure 6. The membership of the detected peptides used for identification of protein cathepsin B (A) and peroxiredoxin-6 (B) in particular IEF fractions

Table 1. The common proteins and peptides identified in cerebrospinal fluid with subarachnoid hemorrhage using MALDI gel free and SDS-PAGE approach. The comparison based on number of identified tryptic peptides and obtained sequence coverage (SC)

No.	Accession	Protein name	MW [kDa]	Peptides SDS-PAGE	Peptides gel free	SC [%] SDS-PAGE	SC [%] gel free
1	APOA1_HUMAN	Apolipoprotein A-I	30.8	6	19	33.7	61.8
2	B2MG_HUMAN	Beta-2-microglobulin	13.7	4	19	46.2	63.9
3	PMGE_HUMAN	Bisphosphoglycerate mutase	30.0	2	6	16.2	32.4
4	CAH1_HUMAN	Carbonic anhydrase 1	28.9	66	54	80.1	86.2
5	CAH2_HUMAN	Carbonic anhydrase 2	29.2	28	30	68.8	68.5
6	CAH3_HUMAN	Carbonic anhydrase 3	29.5	5	7	42.7	41.9
7	COTL1_HUMAN	Coactosin-like protein	15.9	2	3	27.5	35.2
8	COF1_HUMAN	Cofilin-1	18.5	2	10	28.9	66.3
9	DOPD_HUMAN	D-dopachrome decarboxylase	12.7	3	3	28.0	30.5
10	HEM2_HUMAN	Delta-aminolevulinic acid dehydratase	36.3	2	3	12.1	16.1
11	NPC2_HUMAN	Epididymal secretory protein E1	16.6	2	2	17.9	25.8
12	BLVRB_HUMAN	Flavin reductase (NADPH)	22.1	19	15	60.2	63.6
13	GSTO1_HUMAN	Glutathione S-transferase omega-1	27.5	7	10	34.9	33.2
14	G3P_HUMAN	Glyceraldehyde-3-phosphate dehydrogenase	36.0	10	9	40.6	34.3
15	HEMO_HUMAN	Hemopexin	51.6	2	17	8.7	46.1
16	IGHA2_HUMAN	Ig alpha-2 chain C region	36.5	2	11	7.9	42.1
17	IGHG1_HUMAN	Ig gamma-1 chain C region	36.1	4	26	24.2	61.8
18	IGKC_HUMAN	Ig kappa chain C region	11.6	6	7	80.2	80.2
19	LAC3_HUMAN	Ig lambda-3 chain C regions	11.2	4	3	55.7	46.2
20	IBP7_HUMAN	Insulin-like growth factor-binding protein 7	29.1	3	12	21.6	55.3
21	KLK6_HUMAN	Kallikrein-6	26.8	3	5	23.4	33.2
22	LXN_HUMAN	Latexin	25.7	2	2	9.5	12.2
23	PPAC_HUMAN	Low molecular weight phosphotyrosine protein phosphatase	18.0	3	4	35.4	43.7
24	TIMP1_HUMAN	Metalloproteinase inhibitor 1	23.2	3	9	15.5	67.1
25	PPIA_HUMAN	Peptidyl-prolyl cis-trans isomerase A	18.0	3	19	24.8	70.9
26	PRDX2_HUMAN	Peroxiredoxin-2	21.9	15	14	58.1	76.8
27	PRDX6_HUMAN	Peroxiredoxin-6	25.0	9	14	47.8	67.4
28	PEBP1_HUMAN	Phosphatidylethanolamine-binding protein 1	21.0	3	14	34.8	79.7
29	PTGDS_HUMAN	Prostaglandin-H2 D-isomerase	21.0	7	22	46.8	74.7
30	PARK7_HUMAN	Protein DJ-1	19.9	4	17	30.7	65.1
31	PNPH_HUMAN	Purine nucleoside phosphorylase	32.1	12	12	40.1	50.9
32	TRFE_HUMAN	Serotransferrin	77.0	2	67	3.9	69.8
33	ALBU_HUMAN	Serum albumin	69.3	11	126	18.1	85.7
34	SODC_HUMAN	Superoxide dismutase [Cu-Zn]	15.9	4	9	38.3	64.9
35	TTHY_HUMAN	Transthyretin	15.9	38	18	86.4	69.4
36	TPIS_HUMAN	Triosephosphate isomerase	30.8	13	15	65.7	71.0

separation has no barriers and it is common that the same proteins can be found in several focused fractions, causing the fractionation less sufficient [24]. In our study the effectiveness of the IEF fractionation was assessed with combination of direct MALDI analysis. For MS analysis the computer-controlled data dependent acquisition mode related to ion abundance levels in analyzed sample was used. The ions selection for MS/MS analysis is associated with the width of the chromatographic peaks or with the concentration of peptides comprised in complex mixtures. In biological fluids the number of peptides co-eluting can considerably exceed the number of ions subjected for MS/MS acquisition. Therefore, the data acquisition can be biased against the low abundance signals that corresponded to proteins at low concentration. Also if the ionization technique favors certain peptide features, then ions section is determined towards those ions. Thus, more concentrated proteins will be selected, reflecting the abundance level in sample as the peptide hits and spectral count correlated to protein abundance [25]. Regarding to our results, the identified proteins were presented mostly in higher concentration as neither immunodepletion methods nor combination with additional chro-

Table 2. The identified proteins with potential value as biomarkers in cerebrospinal fluid after subarachnoid hemorrhage using gel free nLC-MALDI-TOF/TOF-MS/MS mass spectrometry approach

Accession	Protein name	Score	SC%	m/z meas.	Peptide Sequence
PRDX6_HUMAN	Peroxiredoxin-6	813.62	67.40	2098.1237	M.PGGLLLGDVAPNFEANTTVGR.I R.FHDFLGDSWGILFSHPR.D
				2030.9957	R.DFTPVCTTELGR.A K.LIALSIDSVEDHLAWSK.D
				1395.6609	K.DINAYNCEEPTEK.L
				1897.0140	K.LPFPIIDDR.N
				1582.6731	R.ELAILLGMLDPAEKDEK.G + Oxidation
				1085.6053	R.ELAILLGMLDPAEKDEK.G
				1900.9822	R.VVFVFGPDKK.L
				1884.9907	K.LSILYPATTGR.N
				1135.6600	R.NFDEILR.V
				1191.6760	K.DGDSVMVLPTIPEEEAK.K
				906.4737	R.VVFVFGPDK.K
				1829.8861	R.VATPVDWK.D
				1007.5473	
				915.4934	
PENK_HUMAN	Proenkephalin A	294.4	14.2	2222.0680	R.LVRPADINFLACVMECEGK.L
				1853.8109	R.PADINFLACVMECEGK.L
				2125.1529	K.ELLQLSKPELPQDGTSTLR.E
				1528.8011	L.SKPELPQDGTSTLR.E
TSP1_HUMAN	Thrombospondin-1	74.11	2.80	2195.0619	R.IPESGGDNSVFDIFELTGAAR.K
				1394.7346	R.FVFGTTPEDILR.N
GFAP_HUMAN	Glial fibrillary acidic protein	139.3	10.9	1098.6294	K.ALAAELNQLR.A R.DNLAQDLATVR.Q
				1215.6248	R.LEAENNLAAYR.Q
				1263.6353	
SPTA1_HUMAN	Spectrin alpha chain, erythrocyte	129.9	2.2	1468.8693	Y.GRDLQGVQNLLKK.H
				1667.7788	K.HEALENDFAVHETR.V
CATB_HUMAN	Cathepsin B	1413.4	54.9	2102.0655	R.SRPSFHPLSDELVNYVNK.R
				1858.9369	R.PSFHPLSDELVNYVNK.R
				1527.8217	R.LCGTFLGGPKPPQR.V
				982.4931	R.VMFTEDLK.L
				1855.9301	R.VMFTEDLKLPASFDAR.E
				1839.9336	R.VMFTEDLKLPASFDAR.E
				1286.6291	R.EQWPQCPTIK.E
				2172.9245	R.DQGSCGSCWAFGAVEAISDR.I K.GLVSGGLYESHVGCR.P
				1590.7879	K.GLVSGGLYESHVGCRPY.S
				1850.8756	K.ICEPGYSPTYK.Q
				1314.6047	K.HYGYNSYSVSNSEK.D
				1634.7256	K.NGPVEGAFSVYSDFLLYK.S
				2005.9942	K.SGVYQHVTGEMMGGH.A K.SGVYQHVTGEMMGGHAIR.I K.
				1589.6918	SGVYQHVTGEMMGGHAIR.I N.SWNTDWGDNGFFK.I R.
				1945.9297	GQDHCGIESEVVAGIPR.T
				1929.9390	K.LPASFDAR.E
				1573.6741	
				1823.8596	
				876.4665	

matographic columns (i.e. weak cation exchange chromatography) were applied to overcome the problem of protein dynamic range concentration. Moreover, keeping in mind that blood contamination directly disturbs the detailed proteome investigation; our goal was to show the current state of the protein composition during the SAH. The extensive data obtained from MALDI analysis contribute much to overall screening of the analyzed material, pointing the protein abundance of in this brain pathology. However, not all of the collected MS/MS spectra were correctly assigned to sequence database. The reason could be seeing that in complex mixtures the majority of confidently identified peptides are based on tryptic ends [26]. Thus, limits of this approach can be related to digestion process which increased sample complexity [27].

In the recent studies few reports were published describing SAH as a neuropathology complication that significantly increased mortalities of the cases. Despite rapid hospitalization, the treatment outcome is difficult to predict. Therefore, the biomarkers of SAH are needed to improve knowledge about this condition and to

monitor all changes occurring in the central nervous system. From our protein identification list we were able to recognize proteins which were already recognized as potential indicators by other groups (**Table 2**). Proenkephalin A, a stable precursor fragment of the encephalin, was detected in our study based on four peptides. It was reported that high level of plasma proenkephalin A was associated with poor clinical outcome of aneurysmal SAH and might carry predictive value for 6-month mortality [4, 28]. Further, the glutathione S-transferase P (GSTP1) and peroxiredoxin-6 (PRDX6) significant increases were observed in extracellular microdialysate of stroke patients [29]. By using MALDI proteomic approach we were able to identify the peroxiredoxin-6 with 67.4% sequence coverage. Additionally, the protein cathepsin B was also recognized with high sequence coverage on the level of 54.9%. Interestingly, it was highlighted by Yu et al. that cathepsin B/D was up-regulated in the neurons of rat cortexes after SAH [30]. The time course investigation reveals the expression of cathepsin B/D peaked at 48 h suggesting that these proteases may be released into neurone cytoplasm after SAH, where the lysosomal iron overload may lead to the activation of the apoptotic signaling. Moreover, three other potential indicators of early brain injury such as thrombospondin-1, glial fibrillary acidic protein and α-spectrin were identified. So far they were not analyzed in the CSF of patients with subarachnoid hemorrhage [31]. The thrombospondin-1 is a glycoprotein known to take part in hemostasis and angiogenesis. The increased expression was observed in experimental intracerebral hemorrhage. Consequently, the higher plasma level was also found to be related with clinical severity and long-term prognosis [32]. Further, the glial fibrillary acidic protein (GFAP) a brain specific biomarker is seeking to have diagnostic potential and prognostic value as two experimental studies pointed to higher GFAP levels in serum of patients with greater SAH severity and poorer patient outcomes [33–34]. Lastly, the a-II spectrin breakdown products (SBDPs) released from degenerating neurons, were also proposed and identified in higher level in the CSF with SAH [31].

Perspectives

The proteomic approach based on liquid phase isoelectric focusing fractionation combined with nLC-MALDI-TOF/TOF-MS/MS analysis was proposed to characterize CSF with SAH. The cumulative intersection analysis of in-solution sample separation revealed the highest concentration of the detected components in the middle fractions of the focusing chamber with gradual dilution on its extreme. Thus, rather pointing to the MicroRotofor utility as a tool for concentration of complex protein sample than selectively fractionation technique. The employed strategy ensured overall screening of investigated material presenting the proteins abundance in the current state of analysis. Few proteins such as proenkephalin A, peroxiredoxin-6, cathepsin B, thrombospondin-1, glial fibrillary acidic protein and α-spectrin were recognized as potential indicators, according to literature, pointing to the possibility for their monitoring in further studies as panel of valuable biomarkers. Nevertheless, the main limitation is connected with difficulties in availability of the cerebrospinal fluid in routine collection. Further examination should be conducted in term of quantitative analysis of the proposed proteins with inclusion of different time points during the SAH event.

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Conflict of interest statement
The authors declare no conflict of interest.

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Correspondence address:
Prof. Zenon J. Kokot, PhD
Department of Inorganic and Analytical Chemistry
Poznan University of Medical Sciences
6 Grunwaldzka Street, 60-780 Poznan, Poland
phone: +48 61 854 66 10, fax: +48 61 854 66 09
email: zkokot@ump.edu.pl