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High resolution mass spectrometric brain proteomics by MALDI-FTICR-MS combined with determination of P, S, Cu, Zn and Fe by LA-ICP-MS

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Abstract

The combination of atomic and molecular mass spectrometric methods was applied for characterization and identification of several human proteins from Alzheimer's diseased brain. A brain protein mixture was separated by two-dimensional (2D) gel electrophoresis and the protein spots were fast screened by microlocal analysis using LA-ICP-MS (laser ablation inductively coupled plasma mass spectrometry) in respect to phosphorus, sulfur, copper, zinc and iron content. Five selected protein spots in 2D gel containing these elements were investigated after tryptic digestion by matrix assisted laser desorption ionization Fourier transform ion cyclotron resonance mass spectrometry (MALDI-FTICR-MS). Than element concentrations (P, Cu, Zn and Fe) were determined in three identified human brain proteins by LA-ICP-MS in the 2D gel. Results of structure analysis of human brain proteins by MALDI-FTICR-MS were combined with those of the direct determination of phosphorus, copper, zinc and iron concentrations in protein spots with LA-ICP-MS. From the results of atomic and molecular mass spectrometric techniques the human brain proteins were characterized in respect to their structure, sequence, phosphorylation state and metal content as well.

Keywords: Brain proteomics; LA-ICP-MS; MALDI-FTICR-MS; Metal-containing protein; 2D gel electrophoresis

In 1906 Alzheimer used a silver impregnation technique to demonstrate the presence of abnormal tangle-like structure (NFT: neurofibrillary tangles) in the brain of a patient with dementia. In 1963 Kidd and Terry independently demonstrated that the NFTs consist of dense perinuclear aggregates of a unique nature paired helical filaments (PHFs) [1]. In the following years the structural composition was studied and proteins were identified in many laboratories [2–4]. The main component of the PHFs found in NFTs of Alzheimer's disease brain was identified as the microtubule-associated protein tau in an abnormal phosphorylated state [5,6]. As a result of these studies the hyperphosphorylation of the microtubule-associated tau protein has been recognized as a key process leading to protein aggregation and resulting in neurofibrillary degeneration [7]. Phosphorylation is one of the most important modifications and is of crucial relevance for many physiological as well as pathophysiological processes such as in carcinogenesis and neurodegenerative disease [8–12]. Structure determination of investigated proteins is required in addition to analyze phosphorylation for the determination of post-translational modifications such as glycosylation or fatty acylation. Furthermore metal ions play an important role because these are utilized by biological systems in fundamental processes such as signaling, gene expression, and catalysis [13,14]. Therefore information on metal-containing proteins will comprise the identities of individual metal species and their concentrations in biological systems. Mass spectrometers with electrospray ionization – ESI and matrix assisted laser desorption ionization – MALDI as soft ionization techniques allow the identification of proteins [15,16]. Whereas MALDI and ESI mass spectrometry cannot provide direct quantitative determinations of phosphorus and metal concentration in biomolecules, ICP-MS (inductively

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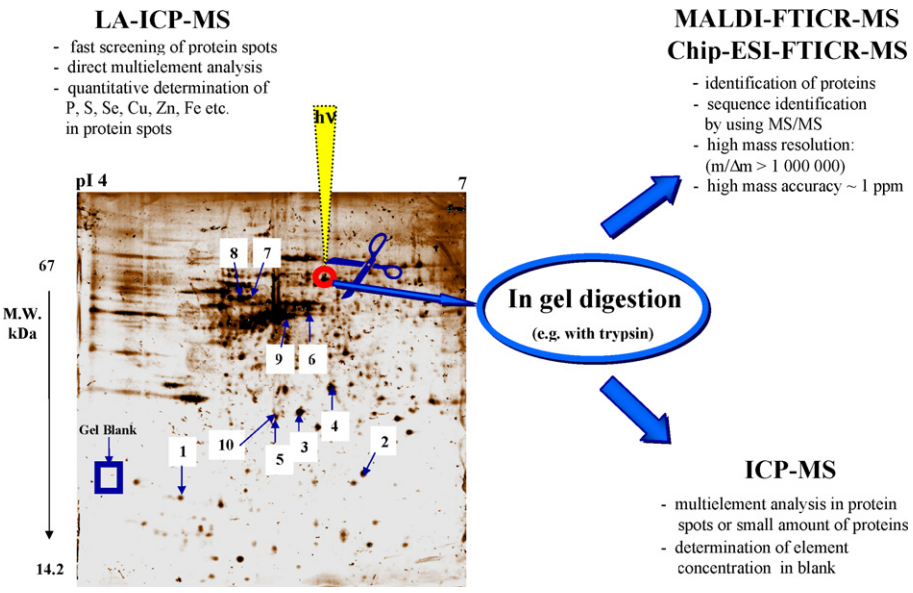

Fig. 1. Mass spectrometric techniques which were used for protein analysis in 2D gels of Alzheimer's disease brain proteins in the mass range of ~100–10 kDa within pH 4–7 using immobilized gel strip; staining was performed with silver staining.

coupled plasma mass spectrometry) was applied, e.g., for phosphorus determination in intact protein samples, or tryptic protein digests after HNO_3 treatment [17–19].

Laser ablation ICP-MS (LA-ICP-MS) as a powerful inorganic mass spectrometric technique for fast multielement determination at major, minor, trace and ultratrace concentration level in any samples and has been introduced to a microlocal analysis of proteins in gels after separation by electrophoresis [20–27] and was used as fast qualitative screening technique (finger printing) of phosphorus, sulfur and metal containing proteins in brain proteins [20,24] or in mitochondria [26,27]. The main problem in determination of phosphorus and metal concentration in protein spots is the quantification of analytical data which was solved by on-line solution based calibration [23] or using the known sulfur content in protein for internal standardization [24]. In a first application of combination LA-ICP-MS together with MALDI-FTICR-MS to the multi-phosphorylated tau protein as a target protein in Alzheimer's disease, an average phosphorus content of ca. 20% was determined [23]. In Fig. 1 applied mass spectrometric techniques for the analysis of proteins and some figures of merit of FTICR-MS, LA-ICP-MS and ICP-MS are summarized. These mass spectrometric techniques were used to obtain the results presented in this paper. The gel shown in Fig. 1 was utilized for screening of protein spots by LA-ICP-MS for the elements of interest. An identical gel prepared under the same conditions was applied for identification of the same proteins using MALDI-FTICR-MS.

In the present study, high resolution MALDI-FTICR-MS is used as a powerful tool for the molecular identification of proteins from Alzheimer's diseased brain after two-dimensional (2D) gel electrophoresis and tryptic digestion. The results of structure analysis by MALDI mass spectrometry were combined with those of microlocal analysis by LA-ICP-MS for the simultaneous quantitative determination of phosphorus, copper, zinc and iron in small protein spots in 2D gels.

1. Experimental

1.1. MALDI-FTICR-MS instrumentation and measurements

MALDI-FTICR-MS measurements on protein samples after separation by 2D gel electrophoresis and subsequent tryptic in gel digestion were performed with a Bruker APEX II FTICR mass spectrometer equipped with an actively shielded 7 T superconducting magnet, a cylindrical infinity ICR analyzer cell, and an external Scout 100 fully automated X–Y target stage MALDI source with pulsed collision gas (Bruker Daltronic, Bremen, Germany). The pulsed nitrogen laser is operated at 337 nm, and ions are directly desorbed into a hexapole ion guide situated one millimeter from the laser target. The device for pulsing collision gas in direct proximity to the laser target provides cooling of the ions, which have a kinetic energy spread of several electron volts when produced by the MALDI process. These ions are trapped in the hexapole where positive potentials at the laser target and at the extraction plate help to trap ions along the longitudinal axis. After a predefined trapping time the voltage of the extraction plate is reversed and the trapped ions are extracted for transmission to the ICR cell. Accumulation of ions from multiple laser shots in the hexapole before mass spectrometric analysis increases sensitivity [12,28]. Ions generated by 10–20 laser shots were accumulated in the hexapole for 0.5–1 s at 30 V and extracted at −15 V into the analyzer cell. A 100 mg mL^{-1} solution of 2,5-dihydroxybenzoic acid (DHB; Aldrich, Steinheim, Germany) in acetonitril: 0.1% TFA in water (2:1) was used as the matrix. 0.5 μL matrix solution and 0.5 μL of sample solution were mixed on the stainless-steel MALDI sample target and allowed to dry. Calibration was

performed with a standard peptide mixture with a *m/z* range of approximately 5000.

1.2. LA-ICP-MS instrumentation and measurement protocol

A double-focusing sector-field ICP-MS (ICP-SFMS, ELEMENT, Finnigan MAT, Bremen, Germany) coupled with a commercial laser ablation system LSX 200 (CETAC LSX 200, Cetac Technologies, Omaha, NE, USA) was used for element determination of P, S, Cu, Zn and Fe via microlocal analysis in selected protein spots in 2D gels. In order to separate isobaric interferences on analyte ions by isobaric molecular ions all LA-ICP-MS measurements were performed at a mass resolution $m/\Delta m$ of 4400. The background intensity of $^{31}P^+$, $^{32}S^+$, $^{63}Cu^+$, $^{56}Fe^+$ and $^{64}Zn^+$ was determined after digestion of a small cut of the blank gel with HNO_3 and measurement using ICP-SFMS. Experimental details of optimization of experimental parameters and the quantification procedure are described in a previous work [24].

From all the determined protein spots in 2D gel five proteins were selected as model proteins, because they contained all the essential elements of interest (S, P, Zn, Cu, Fe). Sulphur was used as internal standard for the LA-ICP-MS measurements.

1.3. Samples and sample preparation

Human brain samples from patients with Alzheimer's disease were analyzed for to phosphorus, sulfur, copper, iron and zinc content by LA-ICP-MS after 2D gel electrophoresis directly and by ICP-MS after digestion in solution. To isolate the soluble and membrane proteins from Alzheimer's brain samples a 60 g peace of brain was separated and a buffer, containing NaCl, Tris–HCl buffer (Merck, Darmstadt, Germany) and protease inhibitors (e.g., aprotinin, antipain, leupeptin, pepstatin, from Sigma, Deisenhofen, Germany), was added and the mixture was ultra centrifuged. The pellet was resuspended in the buffer, including also Triton X-100 (Sigma, Deisenhofen, Germany), shaken und centrifuged. An acetone precipitation for removing the buffer and salts was done with the supernatant containing the membrane proteins. The separation of the proteins was done by using 2D gel electrophoresis. Proteins were digested with trypsin (Promega, Mannheim, Germany) and analyzed by MALDI-FTICR-MS as previously described [14].

1.4. Protein separation by 2D gel electrophoresis

The 2D gel electrophoresis separation of human brain samples was performed as described by Baykut and Jertz [29]. Isoelectric focusing (IEF) in the first step was performed on Immobilized DryStrip gels (Immobilized pH Gradient Strip) with pH 4–7 and 3–10 using a Multiphor horizontal electrophoresis system (Amersham Bioscience, Uppsala, Sweden). The second dimensional was carried out with a Bio-Rad Protean IIxi vertical electrophoresis system using 12% SDS-PAGE gels of 1.5 mm thickness. All protein separations were performed in duplicate by using the selected separated proteins in parallel from one gel spot for analysis by MALDI-FTICR-MS and the second gel for P, S, Cu, Fe and Zn determinations directly by LA-ICP-MS. After 2D gel separation the gel was dried for several days for the determination by LA-ICP-MS.

2. Results and discussion

2.1. Separation and isolation of proteins by 2D gel electrophoresis and detection of P, S, Cu, Fe, Zn and Cu in protein spots

In Fig. 1 a graph of a gel of human brain proteins (Alzheimer's disease) with different protein spots well separated by 2D gel electrophoresis is shown. At the standard conditions of isoelectric focusing and separation (see Section 1), the major and medium-abundant proteins are well resolved within the immobilized pH gradients 4–7. For comparative characterization, the major protein spots were excised from the gel using standard techniques and the gel-immobilized protein digested with trypsin, followed by extraction and MALDI mass spectrometric analysis of the resulting peptides. Mass spectrometric proteome analysis using MALDI-FTICR-MS provided unequivocal identification of proteins from all of the peptide mixtures analyzed by using Swissprot database and/or related databases [30]. Several areas from these 2D gels were employed as references and gel blanks for elemental determination by LA-ICP-MS, as described before [24]. In selected protein spots (marked by 6–10) all elements of interest (S, P, Fe, Cu and Zn) were detected using LA-ICP-MS. By qualitative analysis of protein spots 1–5 only in spot 5 sulfur and phosphorus were found. The detection limits measured on blank gel by LA-ICP-MS in comparison to ICP-MS are summarized in Table 1.

2.2. Identification of proteins by high resolution FT-ICR-mass spectrometry

The methods for the identification and characterization of phosphorylated proteins by mass spectrometry generally include the following analytical procedure: (i) degradation of the phosphoprotein into small peptides by specific enzymatic treatment; (ii) separation of the phosphorylated peptides, with or without purification by metal-ion-affinity enrichment (IMAC: ion metal affinity chromatography); (iii) differential peptide mapping before and after alkaline phosphatase treatment; (iv) mass spectrometric identification of phosphorylation sites using several fragmentation approaches (including MS/MS). The development of FT-ICR mass spectrometry has recently enabled a breakthrough in the high resolution mass spectrometric structure analysis of phosphorylated proteins using MALDI

Table 1
Comparison of detection limits ($\mu g\,g^{-1}$) in protein spots in 2D gel of human brain by LA-ICP-MS and ICP-MS after digestion

Method	S	P	Fe	Cu	Zn
LA-ICP-MS	150	0.6	3.5	15	236
ICP-MS	0.48	0.0021	0.01	0.023	0.65

Table 2
Combination of element analysis using LA-ICP-MS and protein identification by MALDI-FTICR-MS

Spot	Protein	MW (kDa (exp))	MW (kDa)	Cystein residues	Element concentration[a] (mg g^{-1})					Phosphorylation state
					P	S	Fe	Cu	Zn	
6	Creatine kinase B-type	~43	42.62	16	0.027	8.7	0.31	1.05	1.61	1
8	ATP synthase	~56	56.54	14	0.041	7.2	0.62	0.64	1.3	3
9	Mutant β-actin	~42	42.14	24	0.041	9.2	0.55	1.46	0.92	4

[a] Relative standard deviation (R.S.D.) of measured element concentration is about 30%.

ionization [6,13]. In combination with 2D gel electrophoresis the high (sub-ppm) mass determination accuracy and isotopic fine structure by FTICR-MS provide particular advantages for the identification of phosphorylated proteins with medium and low abundance [6]. The partial primary structure of selected proteins could be directly identified after the tryptic digestion by MALDI-FTICR-MS.

Results of mass spectrometric analysis of protein spots in 2D gels MALDI-FTICR-MS are summarized in Table 2. The MALDI-FTICR mass spectrum and the sequence of, e.g., the protein of spot 8 is shown in Fig. 2. In Fig. 3 the MALDI-FTICR-MS of spot 6, identified as Creatine kinase B-type, is shown. This protein has a molecular weight of 42.62 kDa and was identified by database search [31] with a high score of 125.

Whereas the selected separated proteins in gels were analyzed (e.g., spot 8 – ATP synthase or spot 9 – mutant β-actin) with molecular weight of 56.54 or 42.14 kDa, respectively, it was not possible to identify the protein spots 7 and 10. This may be explained by the modification of human brain proteins by several processes, e.g., phosphorylation or metallization. Furthermore, only a very low amount of protein in these spots in the 2D gel was present. More detailed studies are requested for identification of these interesting phosphorus and metal-containing proteins.

2.3. Determination of element concentrations in protein spots after separation of 2D gel electrophoresis by LA-ICP-SFMS

LA-ICP-MS is a promising surface analytical technique for the direct analysis of protein spots in a 2D gel, whereby fast and quasi-simultaneously P, S, Cu, Fe, Zn and other elements in proteins can be detected. Due to relative high background of elements studied in gel the blank in these experiments, determination element concentration in protein spots was sometimes difficult [24]. The limits of detection (LOD) for phosphorus and iron were determined with 0.6 and 3.5 μg/g, respectively (see Table 1).

In selected proteins a multielement analysis via transient signals measured by LA-ICP-MS (single point microlocal analysis with 500 laser shots) was performed. Fig. 4 compares the ion

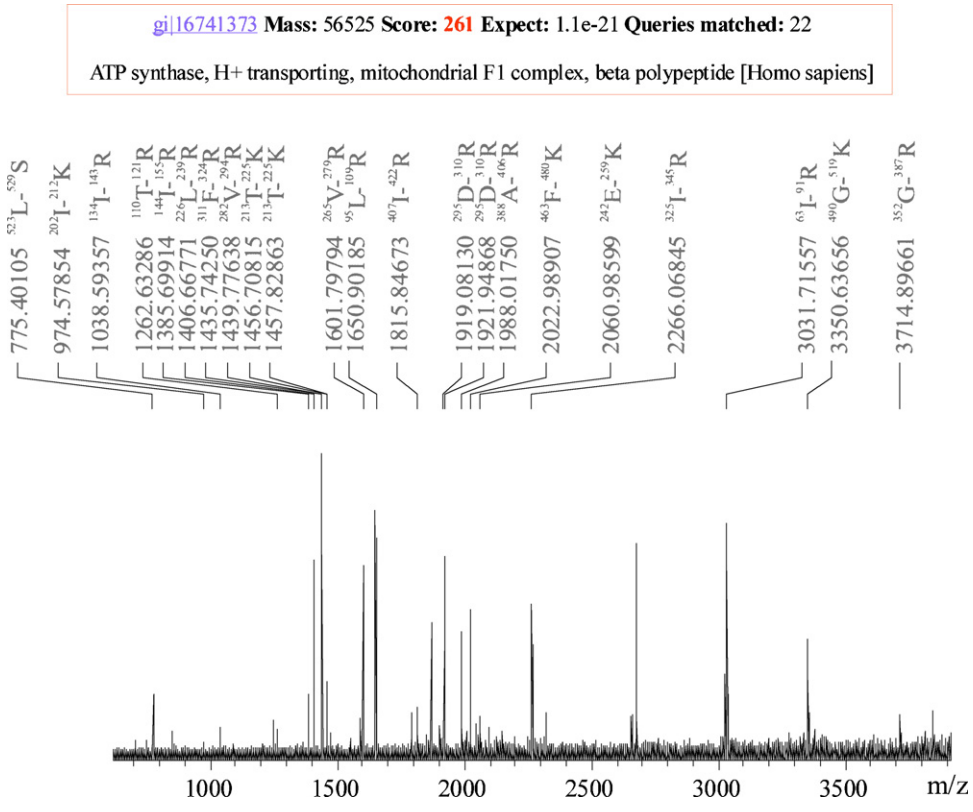

Fig. 2. MALDI-FTICR-MS of protein spot 8 (ATP synthase) with the identified tryptic peptides and the result of the database search.

P12277 **Mass:** 42617 **Score:** 125 **Expect:** 1.9e-08 **Queries matched:** 12
(KCRB_HUMAN) Creatine kinase B-type (EC 2.7.3.2) (Creatine kinase, B chain)

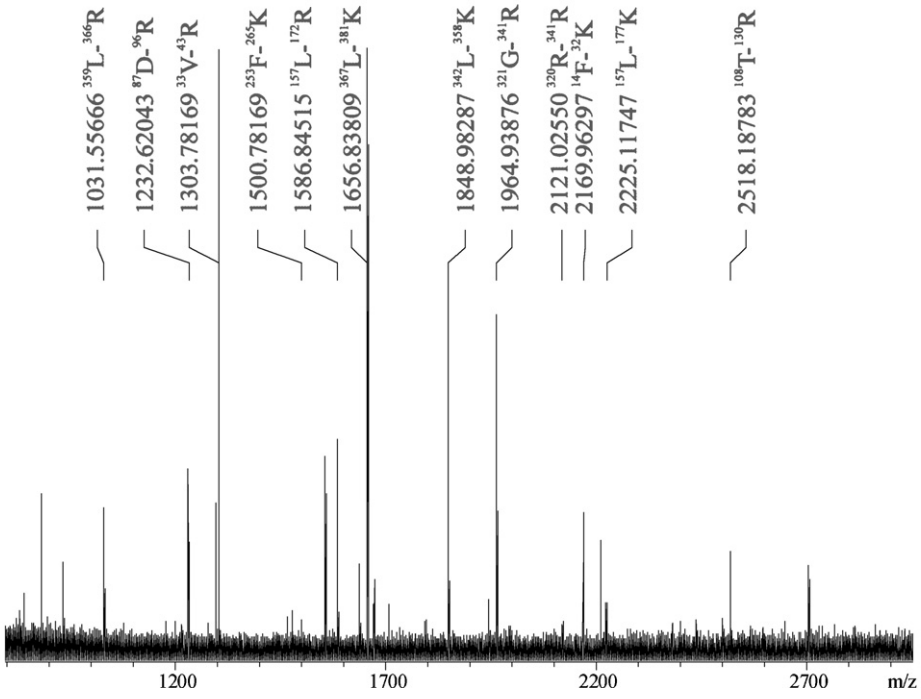

Fig. 3. MALDI-FTICR-MS of protein spot 6 (creatine kinase B-type) with the identified tryptic peptides and the result of the database search.

intensities $^{31}P^+$, $^{32}S^+$, $^{63}Cu^+$, $^{56}Fe^+$and $^{64}Zn^+$in an Alzheimer's relevant protein (spot 8). The protein in spot 8 shows relatively high signal intensity for sulfur, iron and zinc and was identified as ATP synthase. The MALDI-FTICR mass spectrum and the identified peptides of the protein of spot 8 is shown in Fig. 2, respectively. The identification of proteins (spot 8) performed by database search (mascot database for peptide mass finger printing [31]) is given in Fig. 2. The phosphorus content was calculated to be 0.041 mg g^{-1} (phosphorylation – 3). In protein (spot 6), which was identified as a creatine kinase β chain with molecular weight of 42.91 kDa, a smaller content of P and Fe in comparison to spot 8 was found. The phosphorus concentration was determined to be 0.027 mg g^{-1} (phosphorylation – 1). In spot 9, which was identified as mutant β-actin with a molecular weight of 42.14 kDa, contains phosphorus with a concentration of 0.041 mg g^{-1} (phosphorylation – 4). The results the of element concentration in the three protein spot 6, 8 and 9 are summarized in Table 2. In Fig. 5 the transient signals of $^{56}Fe^+$ measured in selected protein spots and blank are shown.

In the present study we described the multielement determination in separated protein spots of 2D gels by LA-ICP-MS as a microlocal analytical technique. The combination of high resolution MALDI-FTICR-MS and LA-ICP-MS represents a powerful tool for the identification of proteins from Alzheimer's diseases and determination of element concentration of P, Cu, Zn, and Fe in these proteins. The complementary data from

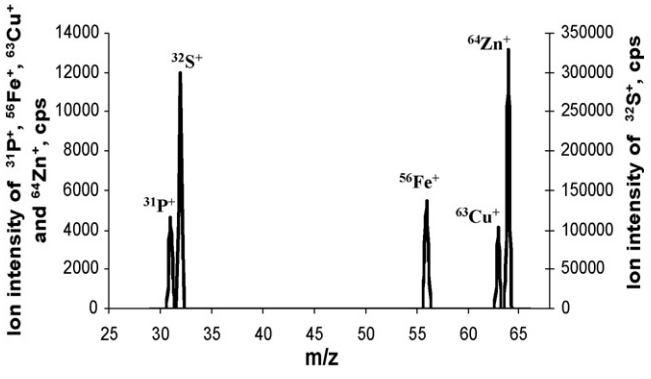

Fig. 4. Transient signals of $^{31}P^+$, $^{32}S^+$, $^{63}Cu^+$, $^{56}Fe^+$ and $^{64}Zn^+$ measured in protein spot 8.

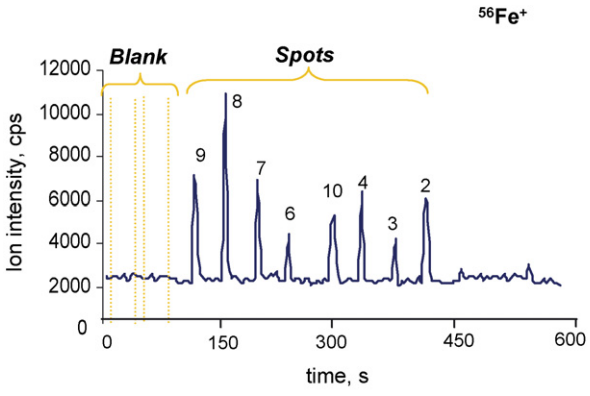

Fig. 5. Transient signals of $^{56}Fe^+$ measured in selected protein spots and blank.

both methods are particularly valuable in the case of multiple modifications where no other technique will provide corresponding molecular information. The phosphorus content, the phosphorylation degree and metal concentration was determined in selected protein spots with detection limits in the $\mu g\,g^{-1}$ range (Zn: 236 $\mu g\,g^{-1}$; S: 150 $\mu g\,g^{-1}$; Cu: 15 $\mu g\,g^{-1}$; Fe: 3.5 $\mu g\,g^{-1}$) and below (P: 0.6 $\mu g\,g^{-1}$).

Future work will focus on the application of developed on-line calibration for determination of metal concentration in protein spots by on-line isotope dilution technique using a micro-concentric nebulizer coupled to the laser ablation chamber as proposed for the analysis of biological reference materials [32] and of development of near field LA-ICP-MS with a lateral resolution in the sub-μm range for direct quantitative determination of element distribution on single cells and cell organelles [33].

3. Conclusion

Phosphorus and metal-containing proteins were detected in a short analysis time using LA-ICP-MS with a cooled laser ablation chamber. The combination of high-resolution MALDI-FTICR-MS and LA-ICP-MS represents a powerful tool for the identification of phosphorylated and metal-containing human brain proteins and determination of phosphorus and metal content in selected proteins. The complementary data from the two methods are particularly valuable in the case of multiple modifications where no other currently available technique will provide corresponding molecular information. The most important problem for the determination of phosphorus and other elements in the gel is possible contaminations during sample preparation or by staining.

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