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Investigation of Cu-, Zn- and Fe-containing human brain proteins using isotopic-enriched tracers by LA-ICP-MS and MALDI-FT-ICR-MS

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Abstract

Identification of metal-containing proteins and determination of Cu, Fe, Zn concentration in very small protein volumes is of increasing importance in protein research. Proteins containing metal ions were analyzed directly and simultaneously in separated protein spots in two-dimensional gels (2D gels) by laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) as an element mass spectrometric technique. In order to study the formation of proteins containing Cu, Zn and Fe in a human brain sample, isotopic-enriched tracers (^{54}Fe, ^{65}Cu and ^{67}Zn) were doped to two-dimensional gels of separated Alzheimer-diseased brain proteins after two-dimensional (2D) gel electrophoresis. The protein spots were screened systematically by LA-ICP-MS with respect to these metal ion intensities. ^{54}Fe/^{56}Fe, ^{65}Cu/^{63}Cu and ^{67}Zn/^{64}Zn isotope ratios in metal-containing proteins were measured directly by LA-ICP-MS. The isotope ratio measurements obtained by LA-ICP-MS indicate certain protein spots with a natural isotope composition of Cu, Zn and/or Fe. These proteins already contained the metal investigated in the original proteins and are stable enough to survive the reducing conditions during gel electrophoresis. On the other hand, proteins with a changed isotope ratio of metals in comparison to the isotope ratio in nature demonstrate the accumulation of tracers within the protein complexes during the tracer experiments in 2D gels. The identification of singular protein spots from Alzheimer-diseased brain separated by 2D gel electrophoresis was attempted by biopolymer mass spectrometry using MALDI-FTICR-MS after excision from the 2D gel and tryptic digestion.

Keywords: LA-ICP-MS; MALDI-FTICR-MS; Metal-containing brain proteins; Tracer experiments

1. Introduction

Metals play an important and essential role as cofactors of proteins in biological systems (e.g., in single cells or cell organelles). The absence or a deficit of essential metals (such as Fe, Cu, Se, Zn) in proteins results in deficiency diseases, but these metals can also catalyze cytotoxic reactions [1,2]. The investigation of metal-containing proteins is a new and challenging task in the proteomics field including the protein identification and the determination of the metal concentration, which requires sensitive analytical techniques and powerful equipment. Apart from the determination of phosphorus, the quantitative determination of zinc, copper and iron in brain proteins is of special interest for studying neurodegenerative diseases, e.g., Alzheimer's and Parkinson's disease, etc. [3,4]. Furthermore, metal-binding proteins containing essential trace elements such as Fe, Cu, Ni and Zn, which are coordinated, for example, by sulfur ligands, sometimes possess catalytic properties (metalloenzymes) [5].

Whereas for the multielement analysis of small protein solutions inductively coupled plasma mass spectrometry

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(ICP-MS) is the method of choice [6], laser ablation ICP-MS (LA-ICP-MS) is being used more and more frequently as a powerful microlocal element analytical technique for the fast and direct determination of metal concentrations at trace level in biological and medical materials (plants or tissues) and nowadays also in protein research [3,7–11]. LA-ICP-MS is used as a fast screening technique for protein spots in gels separated by 2D gel electrophoresis with respect to selected metals (and phosphorus) [10,12]. The quantitative element determination in separated proteins spots in 2D gels is performed using sulfur as the internal standard element or by solution-based calibration as described in the literature [4]. This technique is applicable for sulfur-containing proteins if their structure (molecular weight and the number of cystein residues) is known. Therefore, an identification and structure determination of investigated biopolymers is required, which is made possible by biopolymer mass spectrometry. Mass spectrometric techniques that employ soft ionization (electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI)) permit the identification of large biomolecules [9,12–14]. While MALDI and ESI mass spectrometry can be used for the identification of proteins and how the metals are bonded in proteins, these techniques cannot provide direct quantitative determinations of metals.

The development of MALDI-FTICR mass spectrometry has recently enabled a breakthrough in the high-resolution mass spectrometric structure analysis of proteins [15–17]; in combination with 2D gel electrophoresis, the high (sub-ppm) mass determination accuracy and isotopic fine structure obtained by FTICR-MS provides particular advantages for the identification of proteins even at low abundance. Therefore, a combination of molecular and atomic mass spectrometry is advantageous for the characterization of metal-containing proteins.

In the present study, metal-containing proteins were investigated by tracer experiments in which isotopic-enriched tracers (^{54}Fe, ^{65}Cu and ^{67}Zn) were doped to Alzheimer-diseased brain sample after separation by 2D gel electrophoresis. Tracer experiments using enriched stable isotopes include the measurement of ^{54}Fe/^{56}Fe, ^{65}Cu/^{63}Cu and ^{67}Zn/^{64}Zn isotope ratios in metal-containing proteins separated using 2D gel electrophoresis by microlocal analysis with LA-ICP-MS, which was used as a direct microlocal analytical technique for fast screening of small protein spots in two-dimensional gels with respect to the presence of metal.

The combination of LA-ICP-MS with high-resolution MALDI-FTICR-MS is already used for the molecular identification and quantification of Fe, Cu und Zn element concentrations in proteins [3]. By applying isotope dilution analysis an absolute quantitative determination of metal content in proteins is possible. In this study, the capability of proteins to bind metals will be investigated by tracer experiments.

The aim of this work is the study of the metal-containing proteins (especially with copper, iron and zinc ions) in Alzheimer brain sample via tracer experiments.

2. Experimental section

2.1. LA-ICP-MS instrumentation

A double-focusing sector-field ICP-MS (ICP-SFMS, ELEMENT, Thermo Electron Corporation, Bremen, Germany) coupled with a powerful laser ablation system Ablascop (Bioptic, Berlin, Germany) was used for the microlocal analysis of metals in proteins separated in 2D gels. The laser ablation of protein spots was performed with a frequency-quintupled Nd:YAG laser (wavelength: 213 nm, repetition frequency: 20 Hz, spot diameter: 50 μm; laser power density: 3×10^9 W cm^{-2}). The ablated material was transported by argon as a carrier gas into the inductively coupled plasma (ICP). The ions formed in the ICP were extracted in the sector-field mass spectrometer and separated according to their mass-to-charge and energy-to-charge ratios. In order to separate interfering molecular ions from atomic ions Cu^+, Zn^+ and Fe^+, all LA-ICP-SFMS measurements were performed at the medium mass resolution $m/\Delta m$ of 4400. Possible interferences of atomic and molecular ions for measurements of $^{54}Fe^+$, $^{65}Cu^+$ and $^{67}Zn^+$ ion intensities by LA-ICP-SFMS and the required mass resolution are summarized in Table 1.

The ICP torch was shielded with a grounded platinum electrode (GuardElectrode^{TM}, Thermo Electron Corporation). For optimization of ICP-MS parameters, a single gas flow solution-based procedure was applied using an ultrasonic nebulizer (USN, CETAC Technologies Inc., Omaha, NE, USA), described elsewhere [4]. Using this arrangement, simultaneous optimization of the nebulizer gas flow rate for the USN and the carrier gas flow rate for the transport of laser-ablated material in ICP is performed. The experimental parameters of LA-ICP-MS were optimized with respect to the maximal ion intensity of $^{63}Cu^+$ using a 1 μg L^{-1} copper solution introduced by the USN, which is coupled on-line to the laser ablation chamber. Maximal ion

Table 1
Possible interferences and required mass resolution for measurements of $^{54}Fe^+$, $^{65}Cu^+$ and $^{67}Zn^+$ by LA-ICP-SFMS

Isotope	Possible interference	Mass (u)	Required mass resolution ($m/\Delta m$)
$^{54}Fe^+$		53.9396	–
	$^{54}Cr^+$	53.9389	73900
	$^{38}Ar^{16}O^+$	53.9576	2992
	$^{40}Ar^{14}N^+$	53.9654	2088
$^{65}Cu^+$		64.9278	–
	$^{40}Ar^{25}Mg^+$	64.9482	3197
	$^{32}S^{33}S^+$	64.9613	1939
	$^{49}Ti^{16}O^+$	64.9509	2808
$^{67}Zn^+$		66.9271	–
	$^{51}V^{16}O^+$	66.9389	5698
	$^{40}Ar^{27}Al^+$	66.9439	3986
	$^{134}Xe^{2+}$	66.9527	2619
	$^{35}Cl^{16}O^{16}O^+$	66.9587	2122

intensity was observed at a carrier gas flow rate of 1 L min^{-1} for the transport of ablated material to the ICP-MS and an optimal mixing of nebulized standard solutions and laser-ablated solid sample directly in the ablation chamber is possible. The background concentration of Cu, Fe and Zn was determined directly in the gel blank by LA-ICP-SFMS. The optimized experimental parameters of LA-ICP-SFMS measurements are summarized in Table 2.

Table 2
Optimized experimental parameters of LA-ICP-MS

ICP-SFMS	
Rf power	1200
Cooling gas flow rate (L min^{-1})	18
Auxiliary gas flow rate (L min^{-1})	1
Carrier (nebulizer) gas flow rate (L min^{-1})	1.2
Mass resolution ($m/\Delta m$)	4400
Number of runs (pass)	150 (1)
Analysis time per spot (s)	90
Laser ablation system	Ablascope
Wavelength (nm)	213
Laser power density (W cm^{-2})	3×10^9
Laser energy per pulse (mJ)	6
Repetition frequency (Hz)	20
Spot diameter (μm)	50

2.2. Isotope ratio measurements in protein spots by LA-ICP-MS

Three cuts in the two-dimensional gel were selected for the LA-ICP-SFMS measurements (see Fig. 1). The separated protein spots were screened directly using LA-ICP-MS at medium mass resolution ($m/\Delta m = 4400$) at a mass-to-charge ratio of 54, 56, 63, 65, 64 and 67 with respect to the occurrence of Fe, Cu and Zn. Isotope ratio measurements were performed by measuring transient signals of ion intensities for $^{63}Cu^+$, $^{65}Cu^+$, $^{64}Zn^+$, $^{67}Zn^+$, $^{54}Fe^+$ and $^{56}Fe^+$ in the protein spots and calculating the isotope ratios of the three essential metals investigated considering the background signals in the gel blank as a result of possible contamination during chemical procedures, especially during silver staining. Several protein spots containing Cu, Zn and/or Fe were selected for further studies by MALDI-FTICR-MS.

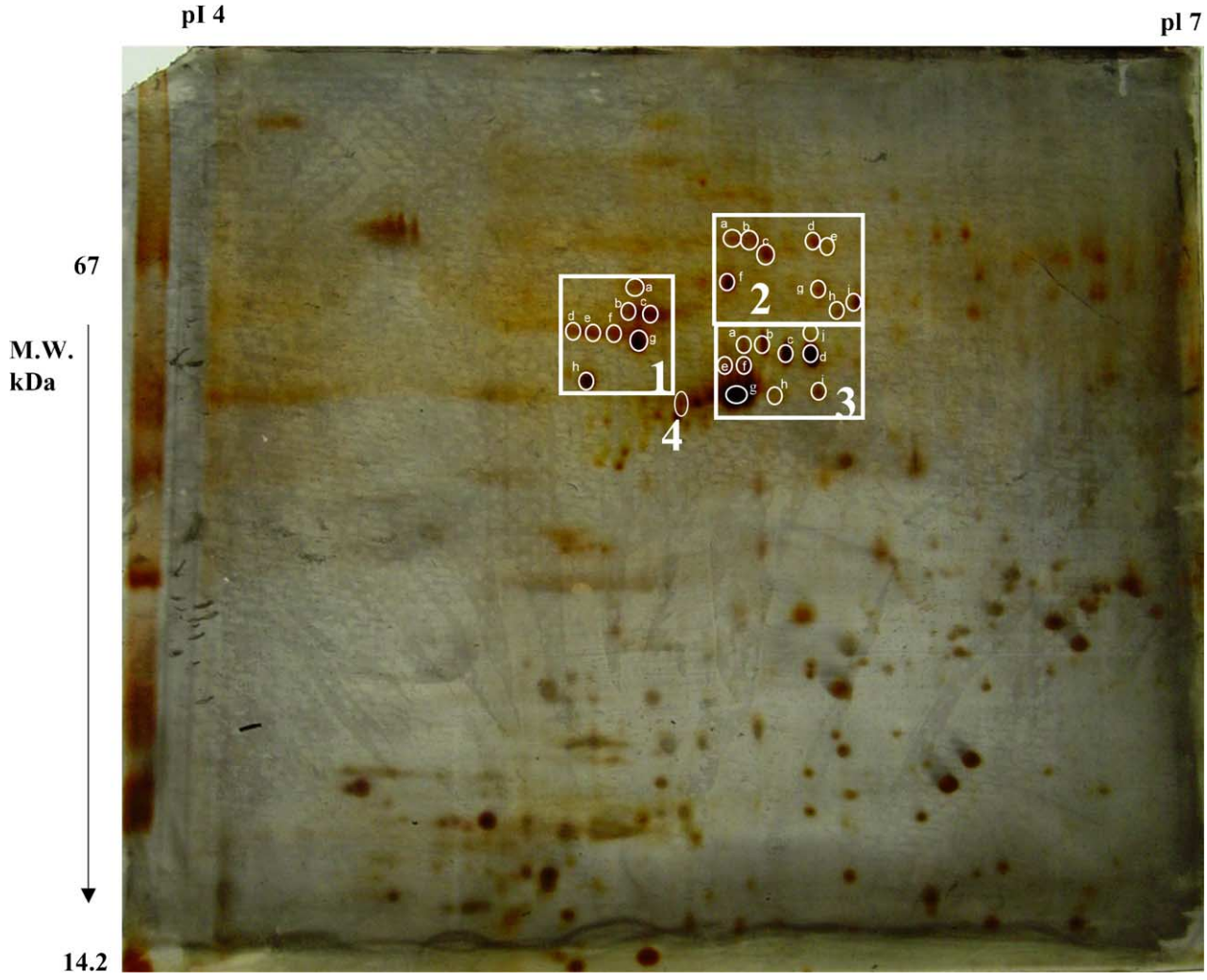


Fig. 1. Two-dimensional gel of Alzheimer-diseased brain doped with enriched ^{54}Fe, ^{65}Cu and ^{67}Zn isotopes after 2D gel electrophoresis.

2.3. MALDI-FT-ICR-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 instrument equipped with an actively shielded 7 T superconducting magnet, a cylindrical infinity ICR analyzer cell, and an external MALDI ion source. A detailed description of this instrumentation has been given elsewhere [16]. The MALDI source with pulsed nitrogen laser is operated at 337 nm, and ions are directly desorbed into a hexapole ion guide while being cooled during formation using Ar as the collision gas. Ions generated by 10 laser shots were accumulated in the hexapole at 15 V and extracted at 7 V into the analyzer cell. A 100 mg/mL solution of 2,5-dihydroxybenzoic acid (DHB, Aldrich, Germany) in acetonitrile:0.1% trifluoroacetic acid in water (2:1) was used as the matrix. A volume of 0.5 μL of matrix solution and 0.5 μL of sample solution were mixed on the stainless steel MALDI sample target and allowed to dry.

2.4. Tracers and reagents

Enriched ^{54}Fe, ^{67}Zn and ^{65}Cu isotope tracer compounds (of Russian origin) with isotope abundances of 89.2, 39.8 and 96.4%, respectively, were used for tracer experiments. Solutions of highly enriched isotopic tracer were prepared via the digestion of solid enriched isotope tracer compounds using subboiled HNO_3 of supragrade purity from Merck (Darmstadt, Germany) and dilution with deionized water. The concentrations of enriched tracer used are summarized in Table 3. Copper standard stock solution of natural isotope composition for optimization procedures was obtained from Merck (Darmstadt, Germany). For all dilutions, deionized Milli-Q water (18 MΩ) was obtained from a Millipore Milli-Q-Plus water purifier.

2.5. Samples and sample preparation

To isolate the soluble and membrane proteins from Alzheimer brain samples, a 60 g piece of brain was separated and a buffer solution, 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 ultracentrifuged. The pellet was resuspended in the buffer solution, also including Triton X-100 (Sigma, Deisenhofen, Germany) shaken and centrifuged. An acetone precipitation for removing the buffer and salts was performed with the supernatant containing the membrane proteins.

Table 3
Isotope abundances of enriched isotope tracer compounds in comparison to abundance in nature and composition of tracer solution doped with enriched isotopes for tracer experiment

Isotope	Isotope abundance of enriched tracer (%)	Concentration of enriched tracer (μg/g)	Isotope abundance in nature (%)
$^{54}Fe_2O_3$	89.2	4.2	5.8
^{67}ZnO	39.8	4.5	4.1
^{65}Cu	96.4	4.5	69.2

2.6. Protein separation by two-dimensional gel electrophoresis of an Alzheimer-diseased brain sample and doping of gel with enriched isotope tracer before silver staining

Protein mixtures from Alzheimer-diseased brain were separated in a first step by isoelectric focusing separating the proteins according to their isoelectric point. The separation of the complex constituents was performed by a second dimension (SDS-PAGE) to separate the proteins according to their molecular weight in a polyacrylamide gel. The protein separation of the protein mixture by gel electrophoresis was performed in duplicate (one gel for LA-ICP-MS measurements and the other for MALDI-FTICR-MS studies).

In order to investigate metal-binding proteins, the 2D gel was placed (after separation of the proteins by two-dimensional gel electrophoresis and before silver staining) in 400 mL Milli-Q-Plus water spiked with ^{54}Fe, ^{65}Cu, and ^{64}Zn tracer for 10 h. The final concentrations of enriched ^{54}Fe, ^{65}Cu, and ^{64}Zn isotope tracer in the aqueous solution are summarized in Table 3. Proteins were stained by using a formaldehyde solution containing silver nitrate (Sigma, Deisenhofen, Germany), and a formaldehyde sodium carbonate solution. All protein separations were performed in duplicate. Human brain samples from patients with Alzheimer's disease were analyzed directly with respect to copper, iron and zinc content and ^{54}Fe/^{56}Fe, ^{65}Cu/^{63}Cu and ^{67}Zn/^{64}Zn isotope ratios were measured by LA-ICP-MS after 2D gel electrophoresis by LA-ICP-MS. Selected protein spots containing Cu, Zn and Fe were cut, in the second gel, digested with trypsin (Promega, Mannheim, Germany) and analyzed by MALDI-FTICR-MS, as previously described [16].

An overview of the experimental procedure including the tracer experiment using enriched isotope spikes and the application of LA-ICP-MS and MALDI-FTICR-MS is given in Fig. 2.

3. Results and discussion

3.1. Separation of proteins from Alzheimer-diseased brain by two-dimensional gel electrophoresis and screening of protein spots by LA-ICP-SFMS

Alzheimer brain proteins were separated by 2D gel electrophoresis as described in the experimental part. Protein spots were stained with silver and subjected to systematic screening for various elements (Cu, Zn and Fe) in cuts 1–3 (Fig. 1) by a newly developed screening analytical technique

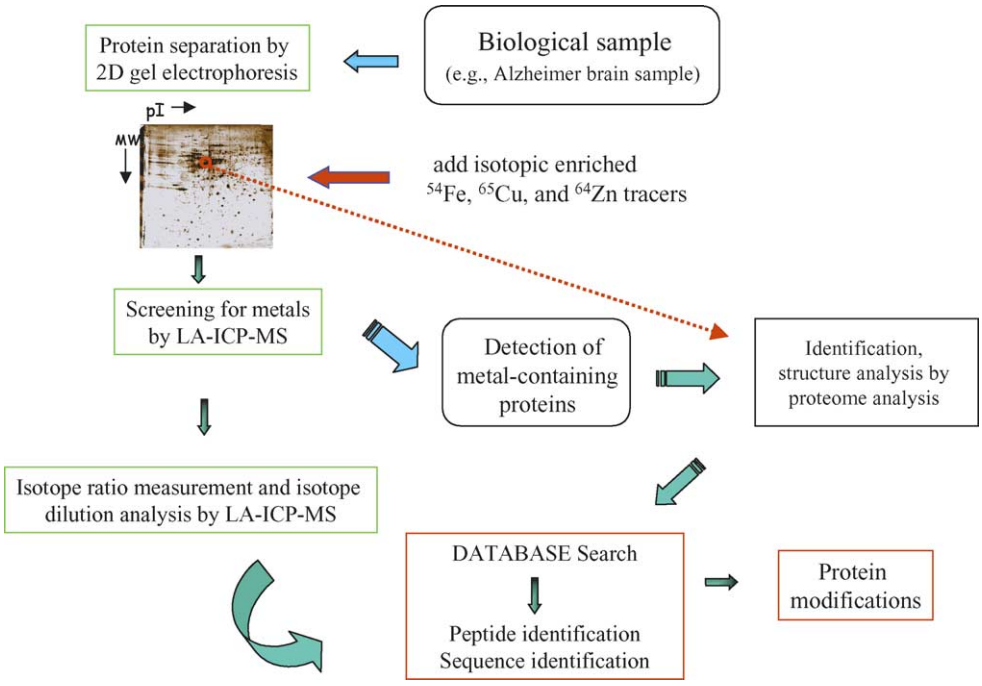


Fig. 2. Schematic of tracer experiment using enriched ^{54}Fe, ^{65}Cu and ^{67}Zn isotopes.

using LA-ICP-SFMS [10]. An advantage of LA-ICP-SFMS as a microlocal analytical technique is that the screening of separated protein spots with respect to the analytes of interest in protein spots on a two-dimensional gel can be performed in just 1 h. LA-ICP-MS thus allows a fast detection of Cu, Zn and Fe with natural isotopic patterns or an accumulation of isotope-enriched tracers within the proteins. In several protein spots (spot numbers: 2c, 2d, 2h, 3b, 3e, 3g, 3h), none of the elements of interest were detected or relatively low ion intensities (close to the background signal) were observed. In a few protein spots were remarkable ion intensities of the analytes measured (see Figs. 3–6). The qualitative results of the protein spot screening technique are summarized in Table 4. For example, in protein spot 1a, all metals (Cu, Zn and Fe) were detected simultaneously. A relatively high ion intensity of Cu together with the occurrence of Zn was also observed in spot 1d. In several proteins only selected elements are presented (e.g., in protein spot 3f: Fe).

Table 4
LA-ICP-MS results of tracer experiments in selected protein spots

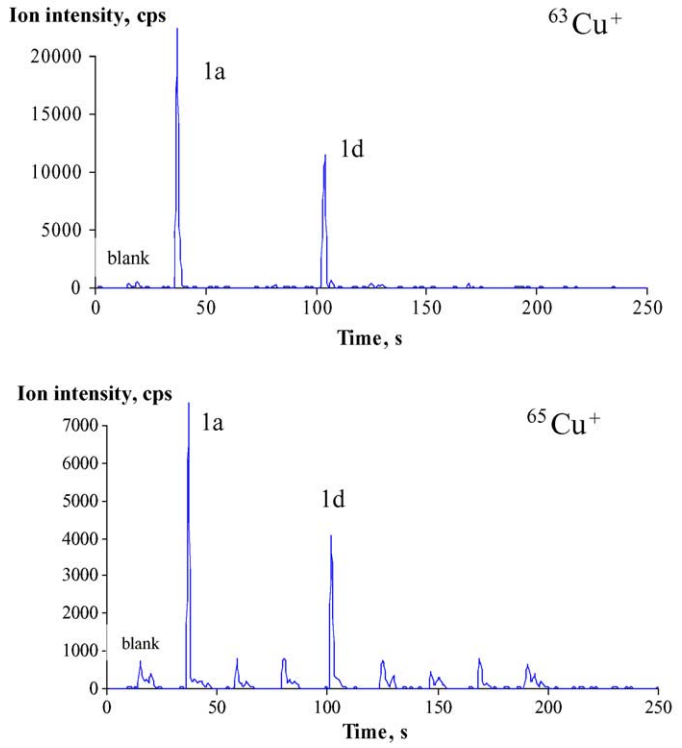
Protein spots	Fe	Cu	Zn
1a	$+_{nat}$	$++_{nat}$	$++_{nat}$
1d	$+_{enr}$	$++_{nat}$	$+_{nat}$
1f	$+_{enr}$	−	$+_{enr}$
2a	$+_{enr}$	$++_{nat}$	$+_{nat}$
2b	$+_{enr}$	$+_{nat}$	$++_{nat}$
2e	$+_{enr}$	−	$+_{enr}$
2f	$+_{enr}$	$++_{nat}$	$++_{nat}$
2j	$+_{enr}$	−	$+_{nat}$
3a	−	$+_{nat}$	$++_{nat}$
3f	$+_{enr}$	−	−

($+_{nat}$) protein contains metal with natural isotopic composition; ($+_{enr}$) proteins contains metal with enriched tracer; (−) metal was not detected; (++) relative high ion intensity.

A main advantage of the screening procedure by LA-ICP-MS is that the time required for the structure analysis of all proteins can be reduced significantly by a preselection of protein spots containing metals of interest. After a qualitative survey analysis several protein spots from Alzheimer-diseased brain containing Cu, Zn and Fe were analyzed with respect to ^{54}Fe/^{56}Fe, ^{65}Cu/^{63}Cu and ^{67}Zn/^{64}Zn isotope ratios. If possible, the structure and sequence of selected metal-containing proteins were determined by MALDI-FTICR-MS.

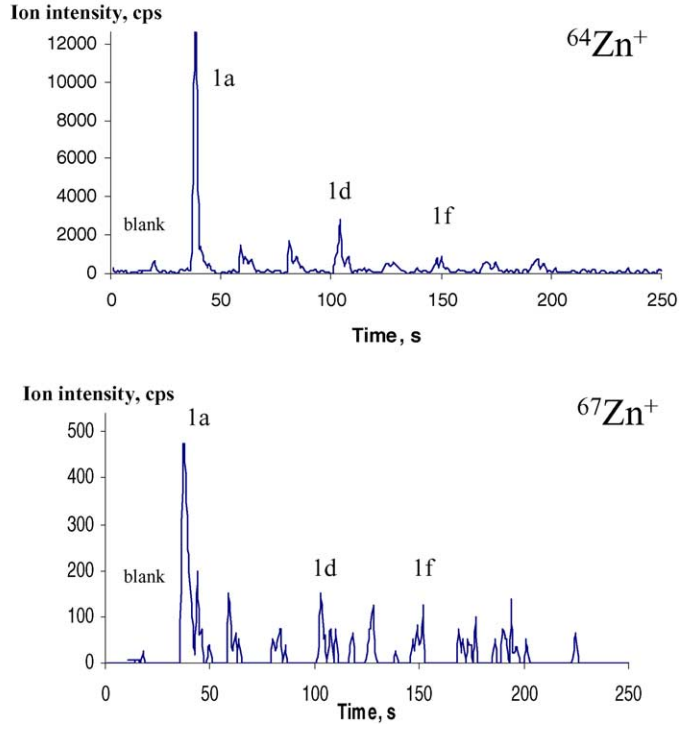
3.2. Isotope ratio measurements by LA-ICP-MS

Fig. 3 shows the transient signals of $^{63}Cu^+$ and $^{65}Cu^+$ in protein spots of Alzheimer-diseased brain sample after tracer experiments measured by LA-ICP-SFMS. Significant ion intensities were detected in protein spots 1a and 1d. An interesting experimental result is that for both protein spots an agreement was found of the measured ^{65}Cu/^{63}Cu isotope ratio (considering a relative standard deviation of about 5% for isotope ratio measurements using LA-ICP-MS for microlocal analysis) of 0.414 (protein spot 1a) and 0.413 (protein spot 1d) with the table value of ^{65}Cu/^{63}Cu = 0.446. This result is evidence that both the copper-containing proteins are stable enough to survive reducing conditions during gel electrophoresis. In all other proteins only the background signal was measured. In the case of copper, in none of the Cu containing proteins investigated was a protein found with a ^{65}Cu/^{63}Cu isotope ratio higher than the isotope ratio observed in nature of 0.446. The tracer experiments demonstrated that the separated proteins studied do not



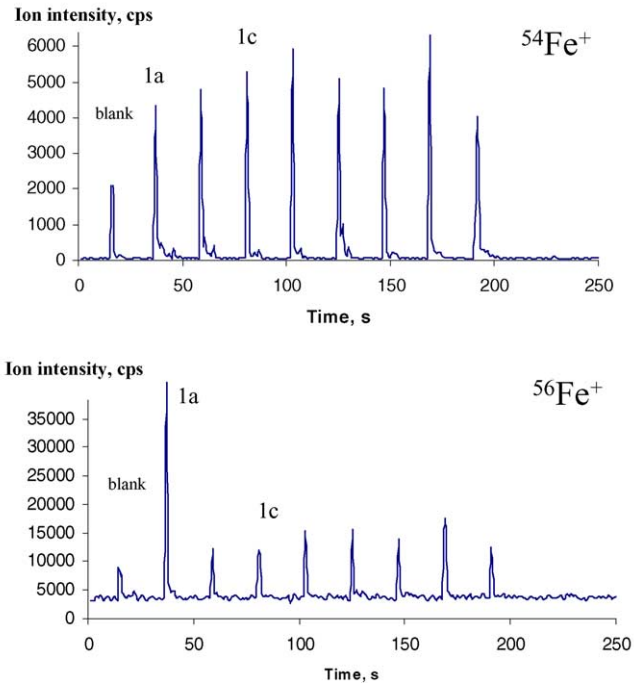
	$^{65}Cu/^{63}Cu$
protein 1a	0.414 ± 0.038
protein 1d	0.413 ± 0.054
table value	0.446
enriched tracer	26.78

Fig. 3. Transient signals of $^{63}Cu^+$ and $^{65}Cu^+$ in protein spots of Alzheimer-diseased brain sample after tracer experiment measured by LA-ICP-SFMS at mass resolution $m/\Delta m = 4400$. (Table of ^{65}Cu /^{63}Cu isotope ratios determined in protein spots 1a and 1d in comparison to isotope ratio in nature and in enriched tracer measured by LA-ICP-SFMS.)



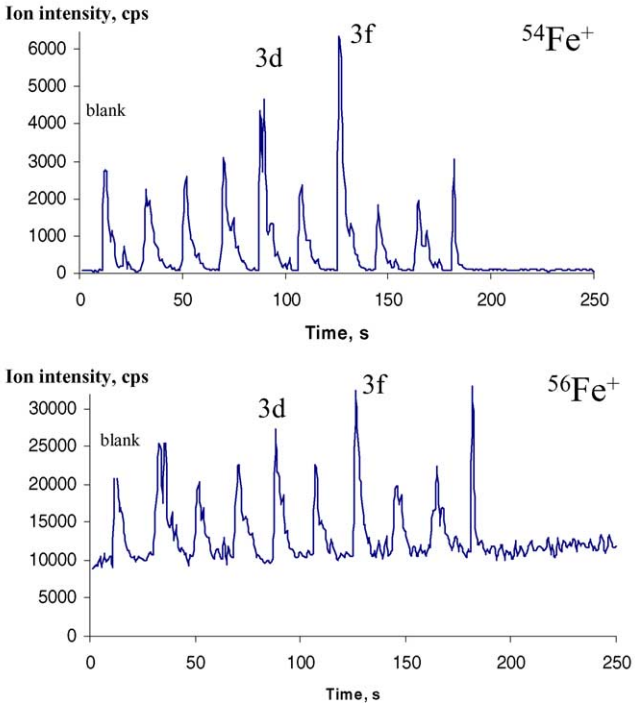
	$^{67}Zn/^{64}Zn$
protein 1a	0.083 ± 0.010
protein 1d	0.099 ± 0.013
protein 1f	0.411 ± 0.184
table value	0.085
enriched tracer	0.661

Fig. 4. Transient signals of $^{64}Zn^+$ and $^{67}Zn^+$ in protein spots of Alzheimer-diseased brain sample after tracer experiment measured by LA-ICP-SFMS at mass resolution $m/\Delta m = 4400$. (Table of ^{67}Zn /^{64}Zn isotope ratios determined in protein spots 1a, 1d and 1f in comparison to isotope ratio in nature and in enriched tracer measured by LA-ICP-SFMS.)



	$^{54}Fe/^{56}Fe$
protein 1a	0.105 ± 0.012
protein 1c	0.441 ± 0.097
table value	0.063
enriched tracer	8.259

Fig. 5. Transient signals of $^{54}Fe^+$ and $^{56}Fe^+$ in protein spots of Alzheimer-diseased brain sample after tracer experiment measured by LA-ICP-SFMS at mass resolution $m/\Delta m = 4400$. (Table of $^{54}Fe/^{56}Fe$ isotope ratios determined in protein spots 1a and 1c in comparison to isotope ratio in nature and in enriched tracer measured by LA-ICP-SFMS.)



	$^{54}Fe/^{56}Fe$
protein 3f	0.196 ± 0.050
protein 3d	0.173 ± 0.060
table value	0.063
enriched tracer	8.259

Fig. 6. Transient signals of $^{54}Fe^+$ and $^{56}Fe^+$ in protein spots of Alzheimer-diseased brain sample after tracer experiment measured by LA-ICP-SFMS at mass resolution $m/\Delta m = 4400$. (Table of $^{54}Fe/^{56}Fe$ isotope ratios determined in protein spots 3f and 3d in comparison to isotope ratio in nature and in enriched tracer measured by LA-ICP-SFMS.)

Table 5
$^{65}Cu/^{63}Cu$ isotope ratios in selected protein spots measured by LA-ICP-MS

Spot	$^{65}Cu/^{63}Cu$
1a	0.414 ± 0.038
1d	0.413 ± 0.054
2a	0.390 ± 0.058
2b	0.485 ± 0.051
2f	0.364 ± 0.083
Table value	0.446

accumulate Cu after gel electrophoresis. Table 5 compares the measured $^{65}Cu/^{63}Cu$ isotope ratios in copper-containing proteins with the table value in nature. It should be noted that the $^{65}Cu/^{63}Cu$, $^{67}Zn/^{64}Zn$ and $^{54}Fe/^{56}Fe$ isotope ratios measured in the doped 2D blank gel are significantly higher in comparison to the isotope ratio in nature due to a contamination of the gel with enriched tracer solution. So the $^{65}Cu/^{63}Cu$, $^{67}Zn/^{64}Zn$ and $^{54}Fe/^{56}Fe$ isotope ratios in the gel blank (cut 1) was measured with 9.3 ± 2.8, 0.28 ± 0.09 and 0.27 ± 0.08, respectively. On the other hand, protein spots with natural isotope ratios were in some cases observed (e.g., in case of copper). It could be concluded that no contamination with enriched spikes like as observed for the gel blank occurs in case of protein spots, because also proteins with natural isotope pattern of metals were found.

Together with copper in protein spots 1a and 1d, also Zn with a natural $^{67}Zn/^{64}Zn$ isotope ratio of 0.083 and 0.099, respectively, (table value: $^{67}Zn/^{64}Zn = 0.085$) was measured at significantly higher ion intensity in spot 1a (see Fig. 4). For example, the $^{67}Zn/^{64}Zn$ isotope ratio in protein spot 1a was measured as 0.083, which is very close to the isotope ratio in nature. Simultaneously with Cu and Zn, also Fe of natural isotopic composition ($^{54}Fe/^{56}Fe$: 0.063) was found in protein spot 1a. In protein spot 1f, a small transient signal for $^{67}Zn^+$ was found, whereby the isotope ratio measurement results in $^{67}Zn/^{64}Zn = 0.411$. This zinc isotope ratio, which is nearly five-fold higher than the isotope ratio in nature, could be evidence for accumulation of isotopic-enriched ^{67}Zn tracer within the protein. The measured $^{67}Zn/^{64}Zn$ isotope ratios in zinc-containing proteins with the table value in nature and the blank value of the gel are summarized in Fig. 7. By isotope ratio measurements, zinc-containing proteins with natural isotope composition were found in spots 1a, 1d, 2a, 2b and 2f. In contrast, during tracer experiments proteins containing enriched Zn isotope were also detected (e.g., spots 1f and 2e). By isotope ratio measurements, a clear distinction can be made between zinc-containing proteins (stable zinc complexes), which survive reducing conditions of gel electrophoresis, and proteins, which accumulate isotopic-enriched tracers after gel electrophoresis. In analogy, the $^{54}Fe/^{56}Fe$ isotope ratio was found to be somewhat higher than the table value of 0.063, e.g., in protein spot 3d measured by LA-ICP-MS (see Fig. 6). Transient signals higher than the blank value were also found for ^{54}Fe in protein spots 1c and 3f (see Figs. 5 and 6), which results in a $^{54}Fe/^{56}Fe$ isotope ratio of 0.411 and 0.196, respectively. For these proteins, it can be assumed that the accumulation of ^{54}Fe tracer proteins occurs during tracer experiments. Isotope ratio measurements of $^{67}Zn/^{64}Zn$, $^{65}Cu/^{63}Cu$ and $^{54}Fe/^{56}Fe$ in protein spots by LA-ICP-SFMS, where an enrichment of the spiked tracer isotope is found, can give us information on proteins which like to bond to zinc, copper or iron (after separation by gel electrophoresis and before silver staining), e.g., protein 1f and 2e for Zn (see Fig. 7) or protein 1f and 2a for Fe (see Fig. 8). A possible contamination of gel during the silver staining (especially Cu impurities were detected in the staining solutions) is considered by measuring the gel blank and does not significantly change the result of the isotope ratio measurements.

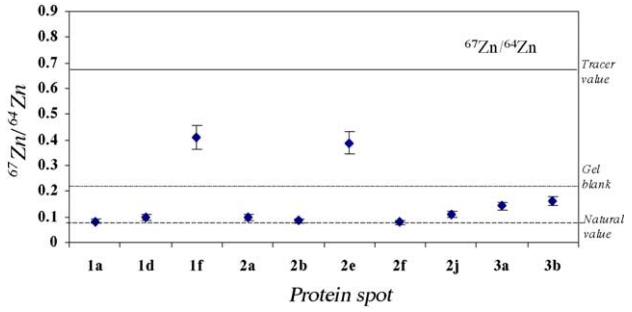


Fig. 7. $^{67}Zn/^{64}Zn$ isotope ratios in selected protein spots of Alzheimer-diseased brain sample measured by LA-ICP-SFMS. ($^{67}Zn/^{64}Zn$ (in nature) 0.085 and $^{67}Zn/^{64}Zn$ (isotope-enriched tracer) 0.67.)

3.3. Identification of proteins by high-resolution MALDI-FTICR-MS

The methods for the identification and characterization of proteins by mass spectrometry generally include the degradation of the protein into small peptides by enzymatic treatment. The complete primary structure of selected proteins could be directly identified after tryptic digestion by MALDI-FTICR-MS [9,18]. Whereas Cu, Zn and Fe in protein spots were detected by LA-ICP-SFMS, which is a very sensitive atomic spectrometric method, the identification of protein

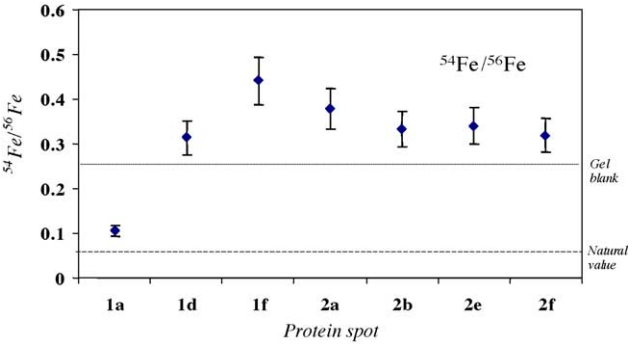


Fig. 8. $^{54}Fe/^{56}Fe$ isotope ratios in selected protein spots of Alzheimer-diseased brain sample measured by LA-ICP-SFMS. ($^{54}Fe/^{56}Fe$ (in nature) 0.063 and $^{54}Fe/^{56}Fe$ (isotope-enriched tracer) 8.26, the isotope ratio of enriched tracer is not shown in the figure.)

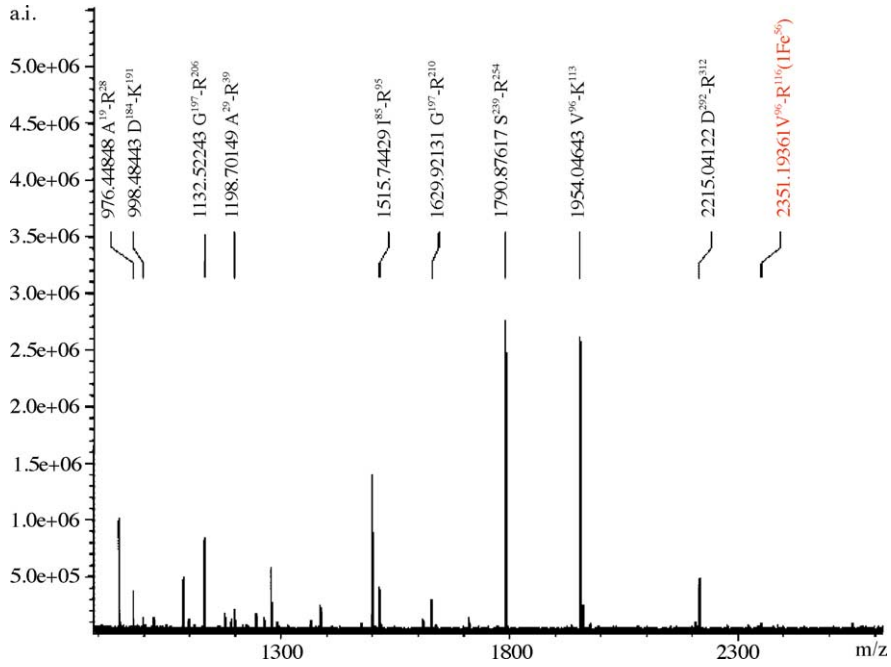


Fig. 9. MALDI-FTICR mass spectrum of spot 3f (actin, cytoplasmic 1 (β-actin)) with identified Fe-containing peptides (Alzheimer-diseased brain sample).

spots 1h, 1f and 3d by MALDI-FTICR-MS is very difficult due to extremely low amounts of protein. In larger protein spots marked by silver staining (1h, 1g, 3g, 3c, 3d), no remarkable ion intensities of metal ions were measured (see Table 4).

The results of mass spectrometric analysis of protein spots in 2D gels are summarized in Table 6. For example, spot 1e was identified as ATP synthase β subunit with a molecular mass of 56.5 kDa, spot 1h was identified as γ enolase with a molecular mass of 44.2 kDa and spot 3d was identified as glial fibrillary acidic protein, astrocyte with a molecular mass of 49.8 kDa. A mass spectrum of the identified protein spot 3f is shown in Fig. 9. Spot 3f was identified as actin, cytoplasmic 1 (β-actin) with a molecular weight of 41.7 kDa. Iron was found on one histidine containing peptide (V^{61}–R^{116}). The existence of iron in this protein was shown by a ^{56}Fe containing peak on $m/z = 2351$ in the mass spectra (see Fig. 9). The corresponding ^{54}Fe, ^{57}Fe and ^{58}Fe containing peaks could not be detected in this mass spectra due to low abundance of these isotopes.

Table 6
Identified protein spots by MALDI-FTICR-MS

Spot	Protein	MW (kDa) (exp.)	MW (kDa) (theor.)
1e	ATP synthase β subunit	∼56	56.5
1h	γ-enolase	∼44	44.2
3d	Glial fibrillary acidic protein	∼50	49.8
3e	Actin, cytoplasmic 2 (γ-actin)	∼41	41.8
3f	Actin, cytoplasmic 1 (β-actin)	∼41	41.7
3g	Glial fibrillary acidic protein, astrocyte (GFAP)	∼50	49.4
4	Actin, cytoplasmic 2 (γ-actin)	∼40	41.8

In an additional study, the tracer experiment was repeated using enriched isotope tracer solution with a 10 times higher concentration of these isotope spikes and the identified protein spots in Table 6 were screened by LA-ICP-MS. As a result of this experiment, a higher contamination of gel blank with enriched isotope solution was observed but no metals were detected in these proteins.

Future work is focused on the development of MALDI-FTICR-MS in order to identify very small amounts of metal-containing proteins and to apply isotope detection analysis for the quantification of metal content. The tracer experiments will be extended to cell lysis directly to study the stability of metal-containing proteins during 2D gel electrophoresis.

4. Conclusions

In the present study, tracer experiments using enriched stable isotopes after gel electrophoresis were performed in order to study metal-containing proteins. The protein spots in two-dimensional gels were screened with respect to ^{67}Zn/^{64}Zn, ^{65}Cu/^{63}Cu and ^{54}Fe/^{56}Fe isotope ratios by LA-ICP-SFMS as the microlocal analytical technique. LA-ICP-SFMS represents a powerful tool for the detection of metal-containing proteins in Alzheimer-diseased brain after separation by 2D gel electrophoresis and determination of isotope ratios of metals in selected proteins. In this way, it was possible to

demonstrate the existence of Cu- and Zn-containing proteins, which survive reducing conditions during 2D gel electrophoresis and the accumulation of enriched isotope tracer. Future work will focus on improving the screening technique using a laser ablation system with better lateral resolution and the development of quantification procedures for the determination of metal content via isotope dilution analysis.

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