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Detection of Zn-containing proteins in slug (Genus Arion) tissue using laser ablation ICP-MS after separation by gel electrophoresis

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ABSTRACT

Assessing the inventory of biological systems in respect to metal species is a growing area of life science research called metallomics. Slugs are of special interest as monitor organisms for environmental contaminations. Laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) was applied to map the distribution of total Zn in a section of a slug sample and to detect Zn-containing proteins after one-dimensional separation by gel electrophoresis (Blue Native PAGE). Interestingly, by far the largest fraction of protein bound Zn was explained by three sharp and prominent bands at 75, 100 and 150 kDa. Analysis of tryptic digests of selected bands using MALDI-TOF-MS and public databases failed to identify proteins within the Zn bands what may be due to coverage gaps concerning the species *arion ater*. Three non-Zn containing bands could be assigned to proteins known from other mollusc species.

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1. Introduction

Laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS), due to its sensitivity and isotopic specificity, is the technique of choice for fast and direct detection of metals and metalloids at trace level in biological tissues. The amount of ablated material, and hence sensitivity, is directly dependent on the laser spot size and the laser energy per spot area. Quantitative imaging LA-ICP-MS methods have been established for the distribution of metals and non-metals in sections of biological tissue [1–4].

The study of metalloproteins and the detection and quantification of proteins on the basis of heteroelements are of increasing importance in biochemical, toxicological, and pharmacological sciences. Investigations on metal-containing proteins require sensitive analytical techniques for metal detection and also for protein identification [5–8]. LA-ICP-MS has been applied as a fast screening technique for protein bands and spots separated by 1D and 2D gel electrophoresis [9] allowing the detection of metal-containing (e.g., Zn and Cu) proteins, for example in brain proteins or mitochondria [10–12]. The metallation states of superoxide dismutase (SOD) were assessed in bovine liver [13], bovine erythrocytes and in human isoforms [15], or the binding of Zn to alcohol dehydrogenase [14]. The technique was also applied for sensitive detection of selenoproteins such as formate dehydrogenase in *Escherichia coli*, unidentified species *Desulfococcus multivorans* [16], and creatine kinase, glyceral-3-phosphate dehydrogenase or enolase in African catfish [17].

Slugs have been used as environmental monitors for metal contaminations in several studies and there is some interest in increasing sensitivity and validity by the analysis of appropriate body parts instead of homogenates of entire animals. Furthermore, understanding the metabolism, of metals, including their incorporation into proteins might precise the conclusions drawn from field studies [18].

The aim of the present work was to investigate slug tissues with respect to Zn-containing proteins after protein separation by gel electrophoresis.

2. Experimental

2.1. Instrumentation

For detection of Zn-containing proteins in 1D gels from extracts of slug samples and for imaging mass spectrometry a sector field ICP-MS spectrometer (ICP-SFMS), Element I, from Thermo Fisher Scientific and a CETAC LSX 200 laser ablation system were used. The analytical procedures were described in previous papers [19,20]. The optimized experimental parameters for analyzing the gels are summarized in Table 1.

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Table 1
Experimental parameters of LA-ICP-MS.

ICP-MS		LA	
Rf power, W	1200	Wavelength, nm	266
Cooling gas flow rate, L min^{-1}	18	Laser power density, W cm^{-2}	3×10^9
Auxiliary gas flow rate, L min^{-1}	1	Laser energy per pulse, mJ	6
Carrier gas flow rate, L min^{-1}	1.2	Repetition frequency, Hz	20
Mass resolution ($m/\Delta m$)	4400	Spot diameter, μm	50
Number of runs (pass)	150 (1)		

For identification of the proteins gel bands of interest were cut out and the proteins were digested in-gel with trypsin (Promega, Mannheim, Germany). MALDI-TOF-MS measurements of tryptic digests were performed with a Voyager-DETM STR instrument (Applied Biosystems). The pulsed nitrogen laser was operated at 337 nm, and ions were directly desorbed from the sample surface into the time-of-flight mass analyser. A solution of α-cyano-4-hydroxycinnamic acid (LaserBio Labs, Sophia Antipolis Cedex, France) in acetonitrile: 0.1% TFA in water (2:1) was used as matrix solution. 0.7 μL of matrix solution and 0.7 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 3000 (BioRad, Marnes-la-Coquette, France).

2.2. Separation of proteins by one-dimensional gel electrophoresis

Slug (species *arion ater*) naturally occurring at the area of Research Center Juelich, Germany were collected, kept in separate air permeable transparent boxes at 25–30 °C room temperature onto lettuce leafs. Organs were dissected from the slugs, homogenized using an ultrasonic probe and extracted. For Blue native polyacryl gel electrophoresis (BN-PAGE) the proteins were extracted with ultrapure water (Millipore water system, 18.2 MΩ cm), whilst for SDS-Page they were extracted with 7 M urea in a Tris–HCl solution with a pH of 7.5 (Sigma, Deisenhofen, Germany). Gel electrophoresis of the extracts, separating proteins according to their molecular weight, was performed in a Mini-Protein-Chamber V100 (Biostep GmbH, Jahnsdorf, Germany). The gels were stained with colloidal Coomassie, which was obtained by mixing 0.1% (m/v) brilliant blue R (Sigma, Deisenhofen, Germany), a solution of 25% (v/v) methanol (Fluka, Seelze, Germany) and 10% (v/v) acidic acid (Fluka, Seelze, Germany) in ultrapure water. Afterwards, the background of the gels was destained with 25% (v/v) methanol.

For the LA-ICP-MS measurements, the gels were dried on filter paper (chromatography paper 3MM CHR, Whatman, VWR, Fontenay-sous-Bois, France) by using a gel dryer (Model 583 Gel Dryer, BioRad, Marnes-la-Coquette, France).

3. Results and discussion

The Zn distribution within a section of a slug measured by imaging LA-ICP-MS is illustrated in Fig. 1, whilst details of imaging studies were described elsewhere [4]. In line with ICP-MS measurements of acidic digests of dissected organs and body parts [21]. Highest Zn levels were detected in the digestive gland and the dorsal skin, medium Zn levels in the mucous ventral skin (foot) and low Zn in all other parts.

To investigate the binding of zinc to proteins, extracts of the foot skin and the digestive gland were analyzed separated by native one dimensional gel electrophoresis containing two parallel lanes of each sample (see Fig. 2). LA-ICP-MS was used to analyze pieces of

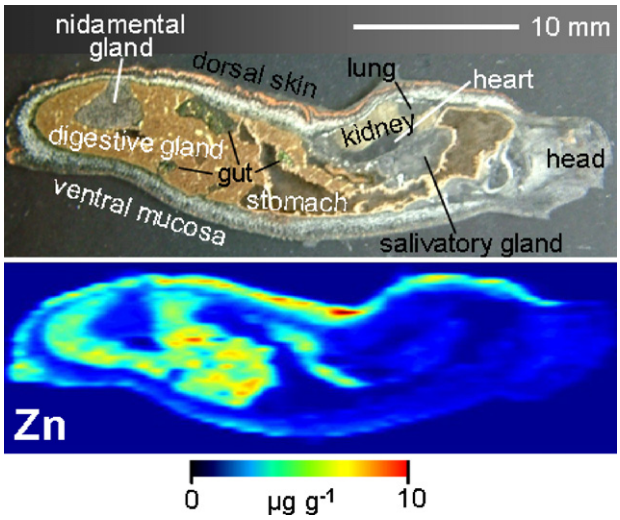

Fig. 1. Section of a slug, optical image and Zn map measured by LA-ICP-MS.

dried gel by line scans and in the imaging mode. No Zn was detected in the 1D gel lane of the belly skin, whereas three sharp peaks corresponding to Zn-containing proteins were found for the digestive gland. The transient signals of the zinc isotopes ^{64}Zn and ^{67}Zn detected in the gel-pieces A and B at molecular weights of 100 kDa, 150 kDa and 75 kDa, respectively, listed by decreasing abundance, are shown in Fig. 3. The corresponding $^{64}Zn^+$ ion image of piece A is shown. All Zn bands showed a similar fronting effect. In the present setting using an anionic detergent and proteins migrating towards the anode this fronting could be explained by a small fraction of the same protein being only partially occupied by Zn^{2+} whilst the larger fraction of identical Zn stoichiometry is virtually saturated. As no Zn-proteins were detected in the foot skin, the Zn should be free there, or present as non-proteinaceous species. This finding was somewhat surprising as we had expected a high Zn affinity of the cysteine rich mucins which are major constituent of the mucus at the slug foot.

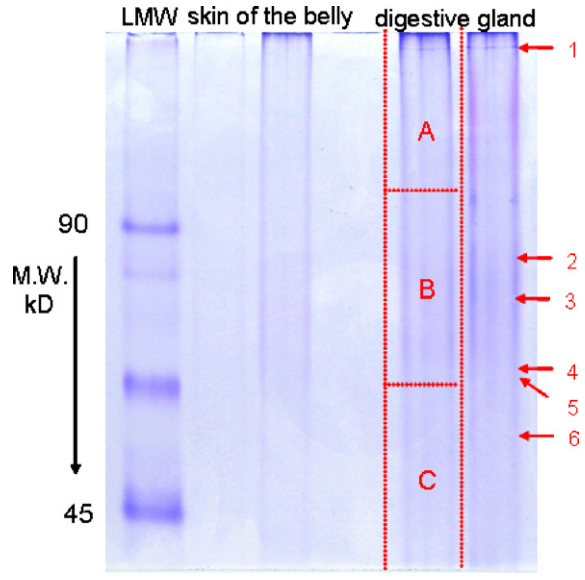

Fig. 2. Blue Native (BN)-PAGE of extracts from homogenates of slug body parts stained with colloidal Coomassie. Stained bands visible by optical inspection that were cut out for tryptic digests and MALDI-TOF-MS are indicated by red arrows. The second parallel lane was cut into the pieces A, B and C, dried and further analyzed by LA-ICP-MS. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

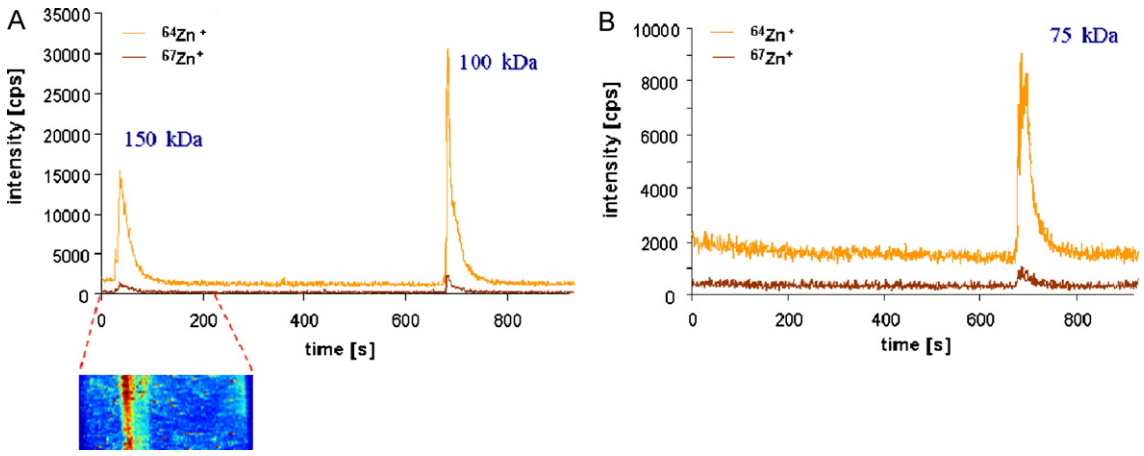

Fig. 3. Zn-containing proteins detected in part A of the gel shown in Fig. 2 at molecular weights of 150 kDa and 100 kDa and in part B at 75 kDa using LA-ICP-MS in line scan modus. LA-ICP-MS in the imaging modus yielded the inserted image, showing a band without horizontal deformation and a small fronting effect.

Table 2
Proteins identified from tryptic digests of 1D BN-PAGE gel-bands obtained from extracts of the digestive gland of the slug *arion ater* (see Fig. 2).

Gel band	Protein	M.W./kD	Taxonomy
2	Actin-2	41.7	*Schistosoma mansoni* (Blood fluke)
3	Actin	41.3	*Lumbricus rubellus* (Humus earthworm)
4	Ras-related protein Rab-1A	22.8	*Lymnaea stagnalis* (Great pond snail)

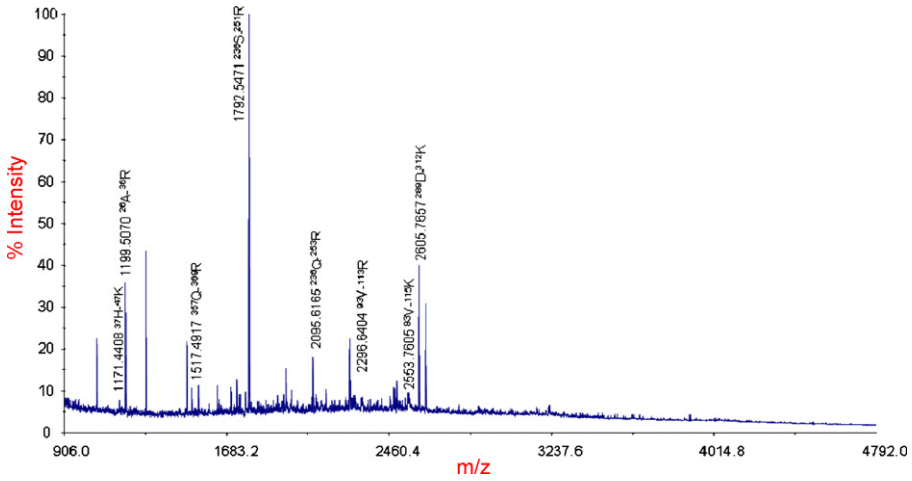

Fig. 4. MALDI-TOF-MS spectrum of gel band 3 (see Fig. 2) identified as actin.

For identification of zinc-containing and zinc-free proteins separated by 1D BN PAGE, MALDI-TOF-MS of tryptic digests was employed. Three proteins could be identified by database search (www.matrixscience.com). The identified proteins are summarized in Table 2. The MALDI-TOF-MS spectrum of gel band 3 with the identified peptides indicated is shown in Fig. 4. Unfortunately, none of the three Zn-containing proteins could be identified. These proteins might not be known in the database used for identification. In this context it seems remarkable that an Entrez database query (in November 2010) yielded only two protein sequences known for *arion ater*: cytochrome oxidase and NADH dehydrogenase whilst Expasy and Mascot files were empty. All the more it is surprising that three proteins could by identified here with large sequence coverage, each previously known from a different mollusc with only lymnea stagnalis beeing a gastropod, the two other being worms. The apparent molecular weights quite well represent the double of the predicted (e.g., ≈80 kDa and 41.7 for band No. 2) which can be explained by dimerization.

Rab-1A was reported to be involved into secretory mechanisms which seem plausible in the digestive gland. It will be a forthcoming task of a next era of genomic and proteomic research to enlarge our knowledge on DNA and protein sequences from a small set of laboratory model organisms such as rattus norvegicus, mus musculus, homo sapiens, cenorhabdidis elegans, drosophila melanogaster, arabidopsis, saccharomyces, escherichia, etc., to a broader collection representing a larger fraction of our environment.

4. Conclusions

This paper demonstrates the potential of LA-ICP-MS regarding a two-step metallomic approach of (1) identification of substructures of interest by quantitatively mapping the total content of elements; (2) element speciation in extracts from substructures using parallel separations for ICP-MS and organic mass spectrometric structure identification. An unbeatable advantage of ICP-MS detection is quantitative response and sub-femtomolar

sensitivity, regardless of the protein structure. In this context, the high sensitivity of LA-ICP-MS allows also the detection of low abundant proteins invisible after Coomassie Blue staining.

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