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TECHNICAL NOTE

Molecular mass spectrometric identification of superoxide dismutase in the liver of mice *Mus musculus* and *Mus spretus* using a metallomics analytical approach

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Abstract This paper reports the identification and quantification of superoxide dismutase in the liver of *Mus musculus* and *Mus spretus* mice using a metallomics analytical approach. The approach consisted of using orthogonal chromatographic systems coupled to ICP–MS and UV detectors. Size-exclusion fractionation of the cytosolic extracts was followed by anion-exchange chromatographic separation of Cu- and Zn-containing species. After purification then tryptic digestion, Cu- and Zn-containing superoxide dismutase was identified by nESI-QqTOF. The MS–MS spectra of doubly charged peptides, with the Mascot searching engine, were used to obtain the sequence of the protein.

Keywords Mus musculus · ICP–MS · Metallomics · Metals · Size-exclusion chromatography · Superoxide dismutase

Introduction

Mus musculus mice are classical inbred laboratory species whose genome is already known. They can be used as model organisms for free-living mice *Mus spretus* because of the close genetic homology between them [1]. The *Mus spretus* mice can be used as bioindicators in pollution monitoring and environmental conservation and, in this sense, biochemical markers have been measured in free-living mice (*Mus spretus*) from Doñana National Park (DNP, UNESCO Reserve of the Biosphere, Ramsar Site and World Heritage Site North of Guadalquivir River Estuary in Huelva SW Spain) [2].

In this context, the possibilities of a metallomic approach have been considered for characterization of metal-linking proteins in *Mus musculus* associated with the biological response against environmental issues [3, 4].

Superoxide dismutases (SODs) catalyze the dismutation of superoxide radicals into oxygen and hydrogen peroxide. For this reason, they are important antioxidant defenses in almost all cells exposed to oxygen, protecting them against superoxide toxicity. SOD cofactored with Cu and Zn is an erythrocuprein [5]. The Cu–Zn enzyme is a homodimer with a molecular mass of 32,500 Da. The ligands of the copper and zinc are six histidine and one aspartate side chains in which one histidine is shared between the two metals [6]. Because SOD can be induced by exposure to many pollutants we consider the availability of the analytical approach described in this paper of great interest for measurement of this enzyme to enable comparison between both mice. SODs have previously been identified by GE–LA–ICP–MS, AE–ICP–MS, and SEC–ICP–MS [7–11].

In this paper we report the identification and quantification of SOD in the liver of the *Mus musculus* and *Mus spretus* mice captured in polluted and non-polluted areas of Doñana National Park.

Experimental

Reagents and materials

Standards used for molecular mass calibration of the size-exclusion chromatography column and mobile

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phase have been used previously and are described elsewhere [12].

Amicon Ultra centrifugal filters (containing a 3,000-Da membrane of regenerated cellulose) were from Millipore (Billerica, MA, USA). Trypsin (EC 3.4.21.4) TPCK was obtained from Sigma–Aldrich (Steinheim, Germany) and urea, iodoacetamide, and dithiotreithol from Bio-Rad (Madrid, Spain).

Instrumentation

Instruments for cryogenic homogenization, element detection by ICP–MS, and SEC has been used previously and are described elsewhere [4, 12].

To avoid high sample consumption, an SEC analytical column was first used to monitor the metal-binding molecules by ICP–MS. This column was a Superdex-75 of 10 mm×300 mm (13 μm) (GE Healthcare, Uppsala, Sweden) with an exclusion limit of 100 kDa and with an effective separation range from 3 to 70 kDa. The AEC column used (7.5 mm×75 mm, 10 μm; Waters, Milford, MA, USA) was mounted in an Agilent 1100 liquid chromatograph with a 50-μL sample loop and was used to purify the fractions collected from preparative SEC.

A HiTrap desalting column (GE Healthcare) was used to remove salts from the fractions collected by SEC.

Mass spectrometry measurements were performed on a nano-electrospray ionization tandem mass spectrometer (API Qstar XL Hybrid system; Applied Biosystems, Foster City, CA, USA).

Procedures

Animals and sample preparation

Five *Mus musculus* mice (inbred BALB/c strain) weighing between 22 and 24 g from Charles River Laboratory (Spain) were used for this experiment. Mice of seven weeks of age were fed ad libitum with water and conventional pellets for five days with the purpose of acclimatization. The feed has been used previously and is described elsewhere [4]. Mice were handled as previously described. Five livers were pooled and the cytosolic extracts were prepared as previously described [12].

Size exclusion chromatography coupled to inductively coupled plasma mass spectrometry and UV

The extract was analyzed by analytical size-exclusion chromatography coupled to ICP–MS as described in detail elsewhere [12].

After SEC separation the Cu and Zn-containing fraction was collected and loaded on to a HiTrap desalting column to remove salts. The mobile phase used for desalting was ammonia solution (pH 8) at 4 mL min^{-1} [13]. Finally, the fraction excluded from the desalting column was collected and lyophilized. The lyophilizate was dissolved in 100 μL Milli-Q water. Quality control of the SEC–ICP–MS system to overcome problems related to contamination, loss, and stability of species has been described elsewhere [14].

Anion-exchange chromatography of the SEC fraction

Purification of SEC fraction containing Cu and Zn was achieved by use of a gradient from 2 to 200 mmol L^{-1} ammonium acetate buffer. The operating conditions are described elsewhere [15]. After analysis, buffer A was switched for 15 min in order to re-equilibrate the column before the next injection. A total volume of 500 μL of the AEC fraction previously isolated was pipetted into the reservoir of an Amicon centrifugal filter device containing a 3,000-Da membrane. The reservoir, inserted into a vial, was sealed with a cap and the assembly was then centrifuged at 13,000×*g* for 45 min at 4 °C. With the reservoir placed upside down in a new vial, centrifugation at 1,000×*g* was performed for 10 min at 4 °C to transfer the concentrate to the vial. Different concentrates were pooled and the centrifugal filtration procedure was carried out to 10-fold sample volume preconcentration.

Tryptic digestion and nano-electrospray Q-TOF analysis

The lyophilized fraction was re-dissolved in 150 μL of a solution containing 6 mol L^{-1} urea and 100 mmol L^{-1} ammonium bicarbonate (pH 8.3). An aliquot of 50 μL of this solution was treated with 5 μL 200 mmol L^{-1} DTT in 100 mmol L^{-1} ammonium bicarbonate to reduce the disulfide bonds in the proteins. After 1 h at room temperature, 20 μL of 200 mmol L^{-1} iodoacetamide (IAA) was added to alkylate the thiol groups of the proteins and after 1 h at room temperature 20 μL of 200 mmol L^{-1} DTT was added to consume any unreacted IAA. Finally, the sample was dissolved in 450 μL water to reduce the urea concentration to ~0.6 mol L^{-1} to retain the activity of trypsin. Trypsin solution (100 μL, 0.2 mg mL^{-1}) was then added and the digestion was carried out for 15 h at 37 °C. The reaction was stopped by the addition of 10 μL acetic acid (glacial) [16].

The TOF mass analyzer was calibrated immediately before sample analysis using renin as standard. ESI–TOFMS data acquisition was performed in positive-ion mode and mass spectra of the peptides were acquired in the *m/z* range 450–1500. The ion-spray potential, electron-multiplier potential, curtain gas, and declustering potential were set to 1500 V, 2200 V, 20 psi and 90 V, respectively. Data analysis was performed by use of Analyst QS software (Applied Biosystems).

After recording the mass spectra, doubly charged peptide ions were selected, and MS–MS spectra were obtained with a collision energy of 45 V. Peptide sequences were then searched by database (NCBI) using the Mascot search engine.

Results and discussion

Orthogonal chromatographic analysis of liver from *Mus musculus*

Figure 1 (inset) shows the chromatographic profile obtained from the liver of the mouse *Mus musculus* using preparative SEC coupled to ICP–MS. In this chromatogram we can observe a peak that contains Cu and Zn with a molecular mass of 32 kDa (calculated by injection of mass calibration standards in SEC). This peak was desalted, lyophilized, and analyzed by AEC as described in the previous section. The resulting chromatogram is shown in Fig. 1. As can be inferred from this figure, small quantities of impurities are present in SEC fractions but they were easily eliminated by collecting the predominant fraction containing Cu and Zn. After lyophilization the fraction was submitted to tryptic digestion for identification by nESI-qQ-TOF. The collection was repeated several times up to a final volume of 1.5 mL.

Identification of SOD in the liver of *Mus musculus* by nESI-qQ-TOF

Figure 2 shows the mass spectrum of the peptides obtained after the tryptic digestion. It is apparent the sensitivity was not very high, because of the complex sample matrix, which has also been reported by other authors [17]. For protein sequencing, the doubly charged peptide ions of *m*/*z* 584.3 and 684.4 were fragmented.

The data obtained by nano-ESI–MS and nano-ESI–MS–MS were introduced into the Mascot database for protein identification. The sequences obtained in each case gave a single, highly significant hit for the same protein, referred to as superoxide dismutase (SwissProt accession no. P08228) with sequence coverage of approximately 51%. The nominal mass of the protein is 16,104 Da because it has a subunit structure (homodimer).

Quantification of SOD in the liver of *Mus musculus* and *Mus spretus* mice by AEC–ICP–MS

For quantification of SOD a commercial standard of this protein was used in the AEC–ICP–MS system monitoring the Cu signal. The calibration curve was constructed from 0.25 to 4 mg L^{-1} SOD. The recovery of the procedure

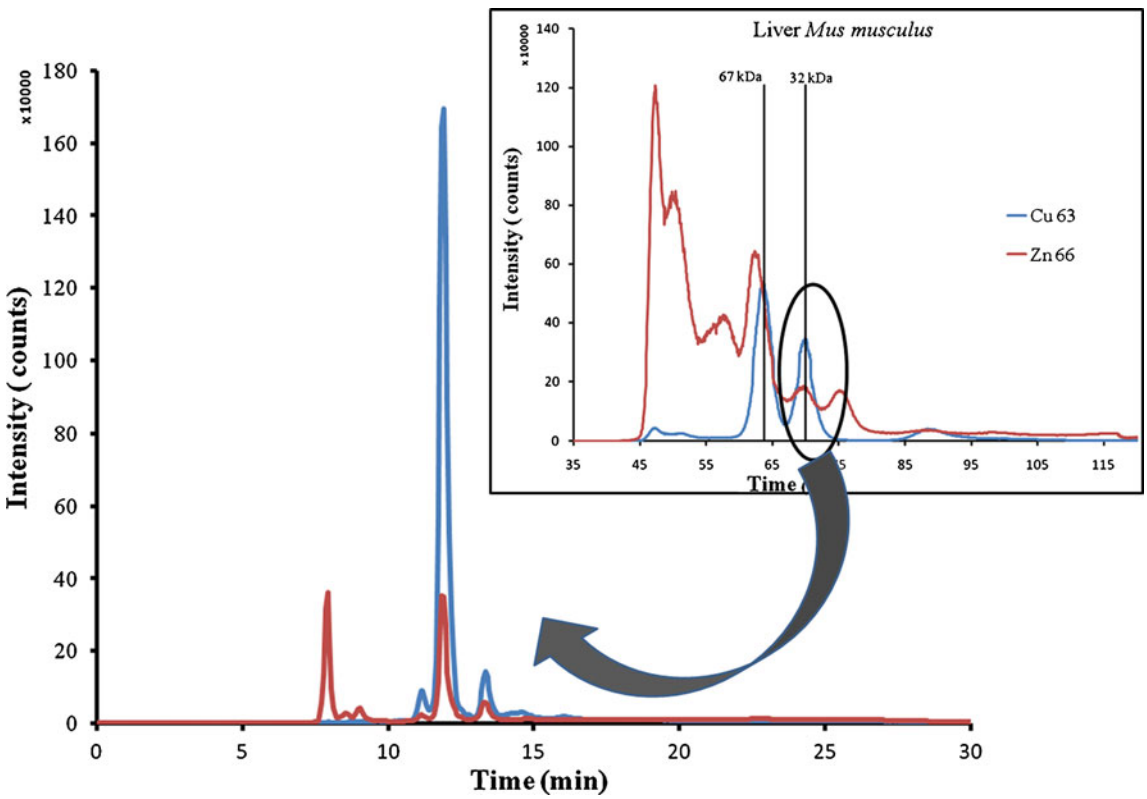

Fig. 1 AEC–UV–ICPMS chromatogram of the Cu and Zn-containing fraction collected by SEC. The *inset* shows SEC the fraction collected with the preparative column (MW 32 kDa)

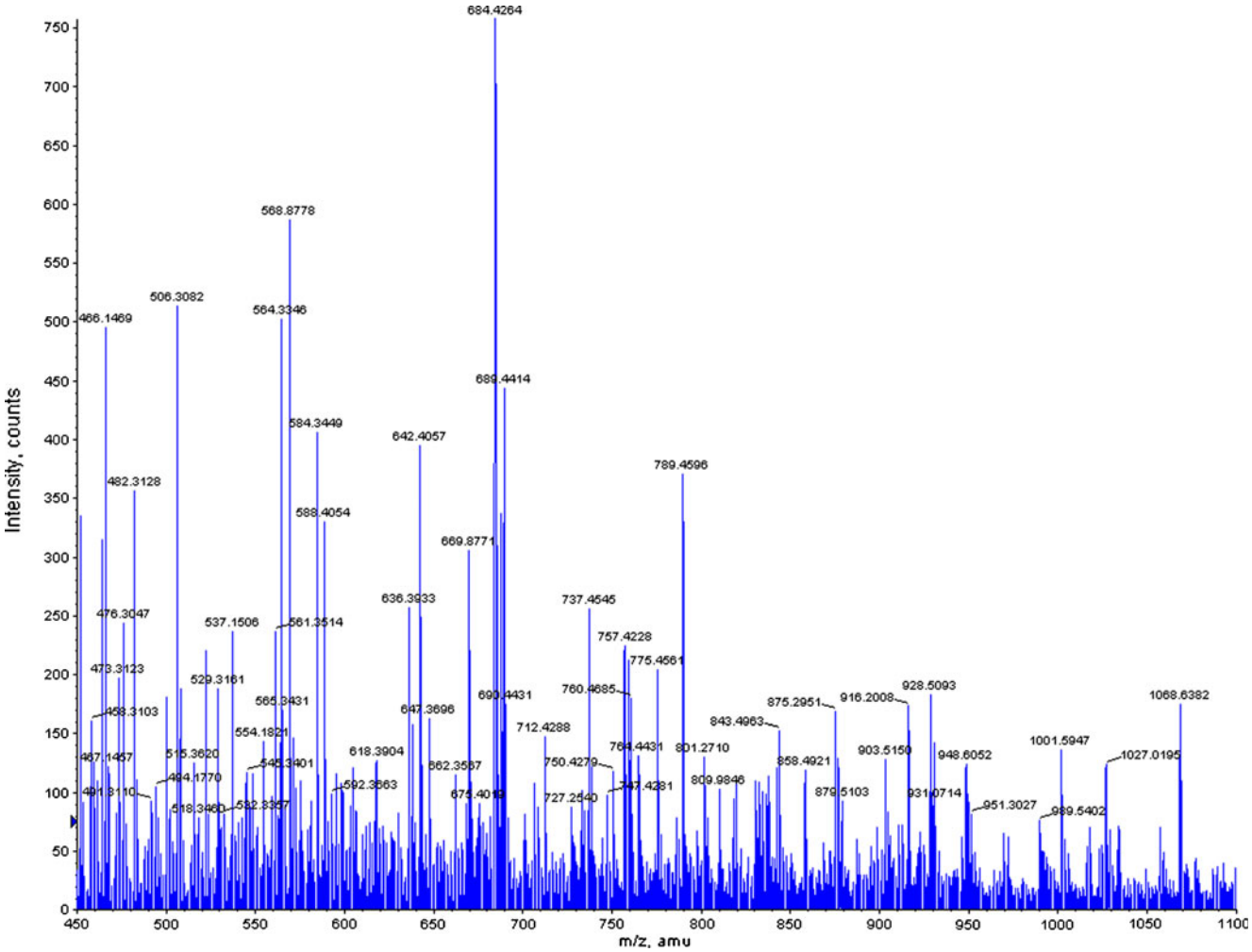

Fig. 2 Mass spectrum obtained by nano-ESI-MS of the fraction collected by AEC

calculated with the SOD standard was 75%. The concentration of the protein in the liver SEC fraction was determined by external calibration to be 1.44 mg L^{-1}, which corresponds to a concentration of 52 ng g^{-1} in the liver fraction of *Mus musculus*. The relative standard deviation (%RSD) from ten sequential injections of SOD at 2 mg L^{-1} was 2%.

The same procedure was applied to the liver of the free-living mice *Mus spretus* captured in two areas of Doñana National Park, the first in a non-polluted part of the park (Lucio del Palacio, LDP) and the second in an area affected by agricultural and industrial activity (Rocina stream, ROC). The calculated concentrations were 82 ng g^{-1} and 28 ng g^{-1} in the liver of *Mus spretus* from ROC and LDP, respectively. Thus the concentration of SOD is higher in the liver of the free living mice *Mus spretus* captured in ROC whereas the concentration in *Mus spretus* from LDP is approximately one third lower. The concentration of SOD in liver from laboratory mouse *Mus musculus* is similar to that for *Mus spretus* from the non-contaminated area (LDP). This fact can be related to the upregulation of SOD in the mice from polluted areas of Doñana which supports the feasibility of *Mus spretus* as bioindicator of contamination on the basis of its biological response established by the proposed metallomic approach.

Determination of the Cu: Zn ratio in the AEC–ICP–MS fraction identified as SOD

The ratio of Cu:Zn in the putative SOD from the AEC–ICP–MS fraction was determined by external calibration. The same calibration curve described in the previous heading was used to quantify the total concentration of Cu and Zn in the protein and, consequently, the ratio of the metals. A concentration from 0.25 to 4 mg L^{-1} SOD corresponds to 1.54×10^{-8} and 2.46×10^{-7} mol L^{-1} for Cu and Zn, respectively. The resulting concentrations in the AEC–ICP–MS peak are 9.54×10^{-8} and 9.17×10^{-8} mol L^{-1} for Cu and Zn, respectively, which confirms the expected

Cu:Zn ratio of 1:1 in the protein, in agreement with theoretical data. This fact gives additional support to the identification of SOD in the livers of the mice.

Conclusions

The proposed metallomic approach based on the use of orthogonal chromatographic systems, namely SEC and AEC, with ICP–MS detection enabled the separation of Cu,Zn–SOD present in the liver of mice *Mus musculus* and *Mus spretus*. In addition, the complementary use of desalting procedures and ultrafiltration enabled isolation of a final protein fraction with a purity suitable for identification and quantification by molecular mass spectrometry. Further experiments based on determination of the Cu:Zn ratio in the AEC–ICP–MS fraction using the SOD commercial standard confirmed the identity of the Cu,Zn–SOD present in the liver of mice. This paper reports, for the first time, identification of superoxide dismutase in the liver of the mouse *Mus musculus* using the above described metallomics analytical approach. The development of this new approach is very useful for study of the metallome of laboratory mice *Mus musculus* as model organisms to be compared with free-living mice *Mus spretus* that are used as bioindicators in pollution monitoring and environmental conservation.

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