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# Coupling SEC and IEF to SXRF for Metallation Analysis of SOD1 Enzyme Isolated from the Human Central Nervous System

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**Abstract.** Copper-zinc superoxide dismutase enzyme (SOD1) is one of the 16,227 expressed proteins in the central nervous system (CNS). Abnormal metallation and aggregation of SOD1 are suspected to play a role in amyotrophic lateral sclerosis (ALS) and Parkinson's disease, but data describing SOD1 metal occupancy in human tissues have not previously been reported. The analysis of metals in metalloproteins under native conditions remains a difficult exercise, especially when the protein is present at physiological levels in a complex tissue. In this work, we developed a methodological workflow that allows synchrotron X-ray fluorescence (SXRF) analysis of metalloenzymes isolated from small amounts of tissue. This protocol allowed us to characterize the metallation of endogenous SOD1 in the human CNS from only 20 mg of post-mortem tissue. In a first 2D-chromatography step, a soluble protein extract is prepared from post-mortem CNS tissues and then separated according to molecular weight using Size Exclusion Chromatography (SEC), and then according to isoelectric point by native isoelectric focusing (IEF) in gel. The processed samples were analyzed by SXRF in the microprobe hutch of the Hard X-ray Micro/Nano-Probe beamline P06 at PETRA III (DESY) in Hamburg (Germany). In total, we analyzed post-mortem CNS tissue from 30 individuals (17 without neurological disease and 13 ALS cases). The Cu/Zn atomic ratio in active SOD1 from control cases was  $1.12 \pm 0.28$  (mean  $\pm$  sd) and did not differ between CNS regions or individuals. To our knowledge, this is the first report of a ratio value close to the theoretical value of 1 in active SOD1 isolated from human tissue. In addition to its excellent detection limit ( $\sim 0.1 \mu\text{g/g}$ ), SXRF also offers excellent repeatability (Cu/Zn ratio in SOD1 standard =  $0.93 \pm 0.01$ ,  $n = 9$ ). Furthermore, our protocol preserves both the metallation and the activity of SOD1, and reduces proteome complexity by 97.8%. 2D chromatography enriches SOD1 99-fold compared to IEF alone, and also improves the accuracy and precision of the measurements (mean Cu/Zn =  $1.12$  vs  $2.39$ , sd =  $0.28$  vs  $1.48$ ). Using this SXRF-based method we revealed that SOD1 metallation is altered in ALS. This method is applicable to other metalloproteins when only small amounts of tissue are available and excellent sensitivity and reproducibility are required.

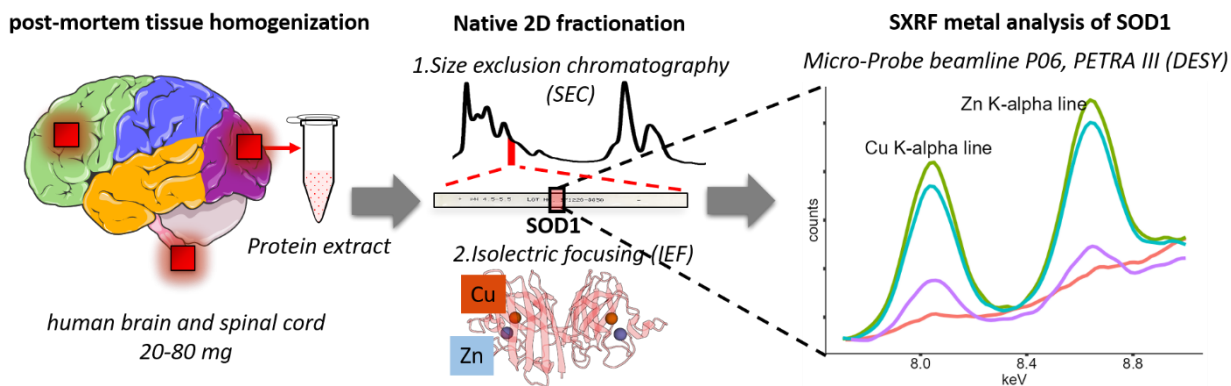
## INTRODUCTION

Metal cofactors are involved in many essential biological functions and it is estimated that about half of all enzymes require a metal for their function<sup>1</sup>. In addition, dysregulation of metal and metalloprotein homeostasis is found in neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis (ALS), a fatal disease characterized by the loss of motor neurons. In the case of Parkinson's disease and ALS, aggregation of SOD1 and abnormal binding of metal cofactors Cu and Zn are suspected to play a key role in the toxic gain of function eventually leading to the death of motor neurons<sup>2</sup>. One of the challenges of studying metals bound to metalloproteins is to ensure that their affinity is not disturbed by sample preparation or analysis conditions. On the other hand, another difficulty lies in the study of metalloproteins expressed at an endogenous basal level, without any overexpression system. It is then necessary to purify the metalloprotein of interest to avoid the possible contribution of other metalloproteins while using an analytical technique with an excellent detection limit, especially when the amount of biological material is limited. Synchrotron X-ray fluorescence analysis is the technique of choice, with a typical detection limit of 0.1  $\mu\text{g/g}$  for metals<sup>3</sup>. To address these issues, we developed a two-dimensional separative method that combines SEC and IEF with SXRF analysis and applied it to the measurement of SOD1 metallation isolated from the human central nervous system. We verified that this method maintains SOD1 in its native and active state, with substantial enrichment where confounding metalloproteins are negligible. Coupled with SXRF, this method is capable of quantifying SOD1 metallation in its native state, from a very small amount (20 mg) of human brain tissue or post-mortem spinal cord<sup>4</sup>. In total, we analyzed post-mortem CNS tissue from 30 individuals (17 without neurological disease and 13 ALS cases) at beamline P06 at PETRAIII DESY (Hamburg). We found a Cu/Zn ratio of 1.12  $\pm$  0.28 in control cases while this ratio is altered in ALS cases.

## MATERIALS AND METHODS

### Tissue, sample preparation and native 2D fractionation

Fresh post-mortem CNS tissues were obtained from the Sydney Brain Bank (Sydney, Australia) and King's College Brain Bank (London, UK). Tissues were obtained from 17 control and 13 ALS cases (7 familial and 6 sporadic) and from six CNS regions: anterior cingulate cortex (ACC), substantia nigra (SN), occipital cortex (OCx), locus coeruleus (LC), ventral spinal cord (vSpC), and dorsal spinal cord (dSpC). A graphic of the analytical steps is presented in Fig. 1.



**FIGURE 1.** Schematic workflow of the analytical process for the metallation measurement of SOD1 from human CNS tissues.

The first step is the preparation of a protein extract. Briefly, the tissues (stored at  $-80^{\circ}\text{C}$ , 20-80 mg hydrated) were homogenized at  $4^{\circ}\text{C}$  under non-denaturing conditions and using ultra-pure chemicals to avoid trace element contamination<sup>5</sup>. This soluble protein extract was then processed by sequential fractionation where the proteins were first separated according to their molecular weight (SEC step) and then according to their net charge defined by their isoelectric point (pI, IEF step). SEC was performed using a Superdex 75 Increase 10/300 GL column and an Äkta

chromatography system cooled at 4°C, using 100 mM ammonium acetate (pH 7.4) as eluant. Elution peak of SOD1 was identified with standard of SOD1 from human erythrocytes (Sigma Aldrich S9636), confirmed by colorimetric enzymatic test (NBT test) and protein mass spectrometry. After SEC, the protein fraction containing SOD1 (500 µL, ~20 µg/mL) was deposited on a native IEF gel where the proteins are separated according to their charge. Fractions were stored at -20°C until further analysis. IEF was performed under native condition and using IPG (immobilized pH gradient) strips and Protean IEF Cell (Bio-Rad)<sup>6</sup>. The exact position of SOD1 on the gel was determined by NBT test. IEF strips were run in duplicate, the first for NBT staining (may introduces trace element contamination) within the second is kept unstained to measure Cu and Zn at SOD1 location. Details of the method are published in a recent paper<sup>4</sup>. The IEF strips were dried in a laminar flow hood and kept free of airborne metallic contaminants until the SXRF analysis.

## SXRF analysis

The SXRF analyses were performed on the Hard X-ray Micro/Nano-Probe beamline P06 at PETRA III (DESY synchrotron, Hamburg, Germany)<sup>7</sup>. SXRF analysis were performed using a 12 keV X-ray beam at a photon flux of  $1 \times 10^{12}$  ph/s. The beam was collimated to 0.5 (H) x 1 (V) mm<sup>2</sup> by crossed slits in order to cover the complete SOD1 area within the IEF gel. The samples were scanned in air at room temperature over a length of 1 to 4 mm in a sequence of 5 to 10 steps, with 10-30 s dwell time per step. The X-ray fluorescence signal was detected with a silicon drift detector (Vortex EM, Hitachi High-Tech), calibrated on an Xspress3 Mini 125. Normalization to the incoming X-ray flux was applied via the photon flux measurement using Canberra PD300-500CB PIPS detectors behind the slits. The X-ray yields of Cu and Zn were calculated via in-house code using reference materials with a known Cu and Zn concentration in the range of 2-12 ng/cm<sup>2</sup>. We also used a solution of human SOD1 from erythrocytes as a reference. This SOD1 solution was previously analyzed for Cu and Zn by particle-induced X-ray emission (PIXE) using Micromatter™ XRF calibration standards at AIFIRA facility of the LP2IB (formerly CENBG, Gradignan, France). SXRF data treatment was performed using PyMca 5.5.0 taking into account scatter, sum and escape peaks and using STRIP background correction. Samples were analyzed in triplicates, Cu and Zn concentrations were used to calculate mean Cu/Zn atomic ratio.

## RESULTS

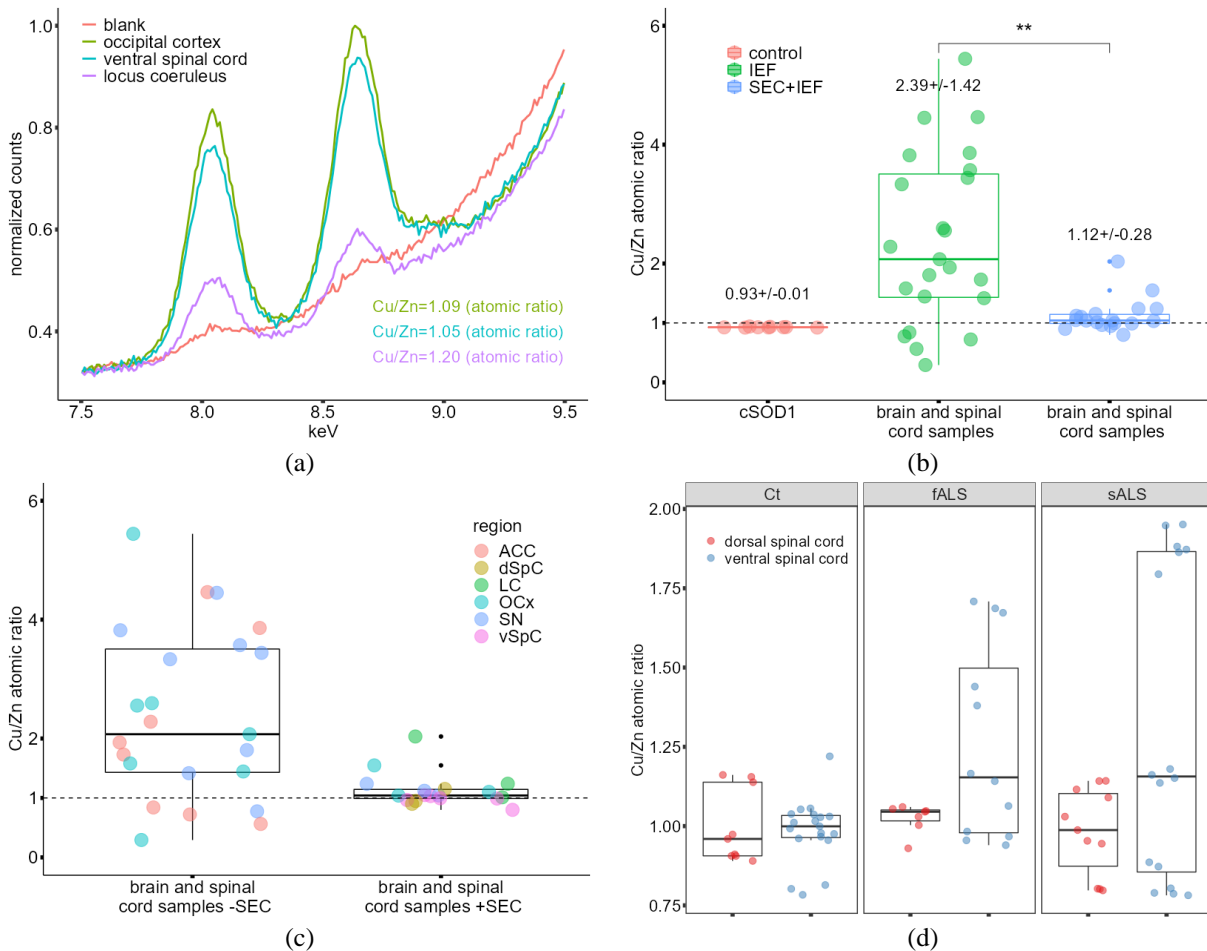
One of the challenges is the low relative abundance of SOD1 among more than 16,000 proteins expressed in the CNS. Our 2D protein fractionation protocol preserves the conformation and Cu/Zn ratio of SOD1 and strongly enriches SOD1 which is the most abundant protein after SEC + IEF, reducing the complexity of the protein extract by almost 98% and making the contribution of other metalloproteins negligible<sup>4</sup>. For this study, we analyzed a total of 30 individuals (17 healthy individuals and 13 ALS cases) and 6 CNS regions (Table 1).

**TABLE 1.** Cases and sample description.

Cases	IEF + SXRF	SEC + IEF + SXRF
Healthy Individuals	8	9
Amyotrophic Lateral Sclerosis (ALS)		13
<i>Familial Cases (fALS)</i>		7
<i>Sporadic Cases (sALS)</i>		6
CNS regions analyzed	anterior cingular cortex, occipital cortex, substantia nigra, locus coeruleus, dorsal spinal cord, and ventral spinal cord	

This SEC + IEF + SXRF protocol allowed the measurement of the Cu/Zn ratio of SOD1 in all tissues except the spinal cord of one individual where the amount of Cu and Zn was below the detection limit. Representative SXRF spectra of tissues from healthy individuals treated with SEC + IEF + SXRF and the corresponding Cu/Zn ratios are shown in Fig.2a. These values are close to the expected theoretical value of 1. In the case of a control ("blank") IPG strip, the

K-alpha X-ray emission lines of Cu and Zn are below the limit of quantification. The quality and reproducibility of the assays were routinely checked using a positive control of human SOD1 treated with SEC + IEF + SXRF (Fig.2b, cSOD1) and for which the Cu/Zn ratio is  $0.93 \pm 0.01$  ( $n = 9$ ). Tissues from healthy individuals treated only with IEF + SXRF show a higher Cu/Zn ratio ( $2.39 \pm 1.42$ ,  $n = 23$ ) and are significantly different from that of the positive control. On the contrary, tissues from healthy individuals treated with SEC + IEF + SXRF show a Cu/Zn ratio of  $1.12 \pm 0.28$  ( $n=18$ ). The SEC step improves both the accuracy and precision of the measurements (5-fold improvement in the SD). To our knowledge, this is the first time that a Cu/Zn ratio close to 1 is reported in active SOD1 from human CNS, most of previous studies reported Cu deficiency and Cu/Zn ratio ranging from 0.11 to 0.33 (discussed in Roudeau et al.<sup>4</sup>). We observed little variability in the Cu/Zn ratio of SOD1 in the different CNS regions studied in healthy individuals, showing no statistically significant difference (Fig.1c). The situation is different in ALS cases. We observed extreme values and an increase in the Cu/Zn ratio of SOD1 in the ventral spinal cord - the degenerating part of the spinal cord in ALS - while this ratio is close to 1 in the dorsal spinal cord<sup>8</sup>. The Cu/Zn ratio of SOD1 in the ventral spinal cord is higher in 42% of ALS patients, confirming that a defect in SOD1 metallation is associated with the disease. It should be noted that the SXRF analysis allowed short acquisition times, typically 3 to 5 min per region of interest at the Hard X-ray Micro/Nano-Probe beamline P06 at PETRA III, and thus the analysis of a large number of samples in a short time.



**FIGURE 2.** (a) Representative SXRF spectra of SOD1 from three CNS regions (occipital cortex, ventral spinal cord, and locus coeruleus) processed by SEC + IEF. (b) Cu/Zn ratios (mean  $\pm$  SD) of active SOD1 from CNS tissues of healthy individuals, purified by IEF alone or by SEC + IEF, compared with SOD1 positive control (cSOD1) processed by SEC + IEF. (c) Cu/Zn ratios from (b) but color-coded according to the CNS region: anterior cingular cortex (ACC), dorsal spinal cord (dSpC), locus coeruleus (LC), occipital cortex (OCx), substantia nigra (SN), and ventral spinal cord (vSpC). (d) Cu/Zn ratios of SOD1 isolated from dorsal or ventral spinal cord from healthy individuals, familial cases and sporadic cases of ALS (fALS, sALS).

## CONCLUSION

Coupling SEC and IEF to the excellent sensitivity of SXRF enabled for the first time the measurement of metals bound to SOD1 in its native state and from a minimal amount (~20 mg) of human post-mortem CNS tissue. Moreover, it does not alter the conformation or metal binding of SOD1 and provides a high enrichment factor, SOD1 being the most abundant protein at the last separation step. The Cu/Zn ratio of active SOD1 ( $1.12 \pm 0.28$ ) was found to be very close from the theoretical expected value of 1 in brain and spinal cord tissue from healthy individuals while it is altered in the ventral spinal cord of ALS patients. This methodological workflow applied to SOD1 also offers the advantage of being adaptable to the study of other metalloproteins.

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