

CE–ICP–SFMS for the Detection of S and Zn in *Aeromonas Hydrophila* Zn- β -Lactamase

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Aeromonads are microorganisms that can cause both human and animal infections and is the known source of hospital-acquired infection. Some *Aeromonas* strains produce metallo- β -lactamase enzymes, which are at the origin of β -lactam resistance in members of this genus. The metallo- β -lactamases are clinically relevant because of their ability to hydrolyse carbapenem antibiotics, and they also represent a relevant investigational model for studying molecular class B β -lactamases because of their unique enzymological behaviour.^{1,2} The *Aeromonas hydrophila* metallo- β -lactamase contains Zn as enzymatic cofactor.^{3–5} In this work, Zn bound to the metallo- β -lactamase is separated from free Zn ions by capillary electrophoresis (CE) and the elements Zn and S are detected and quantified simultaneously by use of an inductively coupled plasma-sector field mass spectrometer (ICP-SFMS) operated at medium mass resolution. The goal of this investigation is to develop a method that allows a fast and accurate determination of the Zn/protein ratio. This ratio can be calculated from the Zn/S ratio. The quantification method is limited to proteins where the sulphur stoichiometry is known. The *A. hydrophila* Zn- β -lactamase investigated in this work is known to contain 4 methionines and 1 cysteine. For proteins of which the primary structure is unknown, additional structural information is necessary. In this work *A. hydrophila* Zn- β -lactamase is being used as proof of concept. Optimum conditions for CE, ICP-SFMS and their hyphenation were investigated. The use of the sodium salt of phytic acid as buffer additive is discussed into more detail.

Introduction

In recent decades, the efficiency of antibiotics has strongly deteriorated because of the increasing presence of resistant bacterial strains. This resistance is often caused by the presence of β -lactamases that hydrolyse the β -lactam ring of penicillin-like antibiotics.^{1,2} Investigation of β -lactamases can help the battle against these resistance phenomena. On the basis of their primary structure, β -lactamases are divided into four classes. Classes A, C and D have serine in their 'active site' while class B are metallo- β -lactamases.^{3–5} Class B β -lactamases use bivalent metal ions (usually Zn²⁺) as enzymatic cofactors. The metallo- β -lactamase enzymes are clinically relevant because of their ability to hydrolyse carbapenem antibiotics.⁶ These

antibiotics are used extensively because they are hydrolysed much slower by the large group of serine- β -lactamases. To date, no useful clinical inhibitors are known for the class B β -lactamases. Therefore, it is important to get as much information as possible on the hydrolysis mechanism of these enzymes. In spite of a certain degree of consensus about this mechanism, conflicting data concerning the role of the Zn ions are found in the literature.⁷ An improved understanding of the role of the metal in metallo- β -lactamases and, therefore, a better understanding of the mechanism by which these enzymes operate, requires an easy and fast way to determine the amount of metal bound to the protein. As a result of the small quantity of protein available and the small

amount of bound metal ions, an extremely sensitive detection method is required. Moreover, the metalloenzymes are present in an environment that also contains free metal ions. Therefore, the use of a separation method which allows separation of the metalloenzymes from free metal ions is also necessary. A frequently used analytical approach for the detection of metal complexes in biological matrices is the coupling of a high-resolution separation technique with an inductively coupled plasma-mass spectrometer (ICP-MS) as an element-specific and very sensitive detector (detection limits down to pg/L).^{8–10} Recently, capillary electrophoresis (CE) has become a useful tool in elemental speciation.^{11–13} The advantages of CE include high separation efficiency, low

sample requirements, low operating costs and rapid analysis. The key to the successful coupling of CE with ICP-MS is the interface; the closing of the electrical circuit from CE, and optimized nebulization and analyte introduction efficiencies are major requirements. The interface introduced by Schaumlöffel and Prange in 1999 (commercialized by CETAC, Omaha, Nebraska, USA^{14,15}) is based on a microconcentric nebulizer, operated in the free aspiration mode. It provides an electrical connection for stable electrophoretic separations and adapts the flow-rate of the electroosmotic flow (EOF) inside the CE capillary (~0.1–0.9 $\mu\text{L}/\text{min}$) to the spontaneous sample uptake of the nebulizer (~6–12 $\mu\text{L}/\text{min}$) for efficient transport of the analytes into the plasma. The interface design solves the problem of suction by the nebulizer affecting the CE separation, allowing scientists to focus exclusively on optimization of the CE

separations without having to take into account the disturbances resulting from hyphenation.

In this work, the determination of the stoichiometric amount of Zn bound per protein molecule (via monitoring of S) of the *A. hydrophila* Zn- β -lactamase (amino acid sequence known) using CE-ICP-SFMS, is discussed. The *A. hydrophila* AE036 metallo- β -lactamase is known to contain two metal ion binding sites. The first Zn^{2+} ion is bound with a picomolar dissociation constant and binding of a second Zn^{2+} ion results in non-competitive inhibition. The most challenging problem is to find an adequate CE separation buffer to make sure that (1) the *A. hydrophila* metallo- β -lactamase is not retained in the CE capillary as a result of electrostatic interactions between capillary surface and protein, and (2) no Zn is removed from the enzyme. The metalloenzyme is separated from free Zn^{2+} by CE and the elements Zn and S are detected

Figure 1: CE-ICP-SFMS set-up.

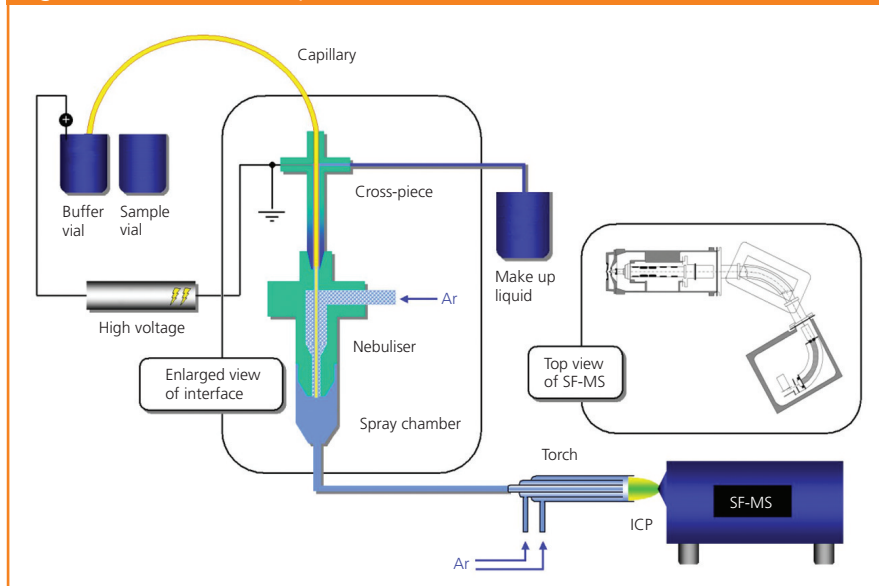


Table 1: Characteristics of the *A. hydrophila* Zn-beta-lactamase and of the solution investigated.

Molecular Mass	25,189 kDa
Isoelectrical point (pI)	7.99
Amino acid residues	227
S-containing amino acids	5
Zn binding sites	2
Concentration	4.94 g/L
Buffer	15 mmol/L sodium cacodylate pH 6.5
pH stability range	6.5–8.5

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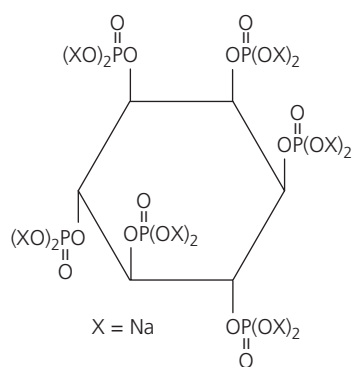
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Figure 2: Dodeciasodium salt of phytic acid.

simultaneously using ICP-SFMS operated at medium mass resolution ($R = 3000$).

Experimental

CE system: An Agilent 3D Capillary Electrophoresis system is used at 20 °C, containing bare fused-silica capillaries with a total length of 70 cm, an inner diameter of 75 μm and an outer diameter of 360 μm . A voltage of 20 kV is applied to limit the current generated in the CE capillary to 60 μA . The sample is injected hydrodynamically at 500 mbar·s (50 mbar for 10 s), which corresponds to a sample volume of 55.5 nL. The capillary is rinsed for 30 min with 1 mol/L KOH before use and subsequently for 30 min with milli-Q water. Before each run, the capillary is rinsed for 0.5 min with 1 mol/L KOH and then for 4 min with the buffer to obtain a reproducible silica surface. Under these conditions, the repeatability of the migration time can be reduced to 4% relative standard deviation (RSD). The CE system is also equipped with a UV detector allowing simultaneous recording of the CE-UV and CE-ICP-SFMS electropherograms. The effective length of the capillary for the UV detection is 21.6 cm (inlet to detector).

ICP-SFMS system: An ICP-SFMS is used as an element-specific detector (ELEMENT, Finnigan MAT, Bremen, Germany). The ICP-SFMS is operated in the medium mass resolution mode ($R = 3000$) to eliminate the spectral interference on the $^{32}\text{S}^+$ signal caused by $^{16}\text{O}^{2+}$. The instrument is fitted with a guard electrode mounted between the ICP torch and the RF coil to achieve the highest sensitivity possible.¹⁶ The signals of $^{32}\text{S}^+$ and $^{64}\text{Zn}^+$ were monitored in the peak hopping mode using a dwell time of 450 ms per nuclide.

CE-ICP-MS interface: The interface (CEI-100, CETAC) is based on a modified

microconcentric nebulizer¹⁷ (MCN-100) with a special, low dead volume spray chamber. The nebulizer works in the self-aspiration mode at a flow-rate of around 12 $\mu\text{L}/\text{min}$. A make-up liquid (same buffer as used for the separation) provides the electrical connection and adapts the flow-rate of the EOF inside the CE capillary to the spontaneous sample uptake of the nebulizer. Figure 1 shows the CE-ICP-SFMS set-up.

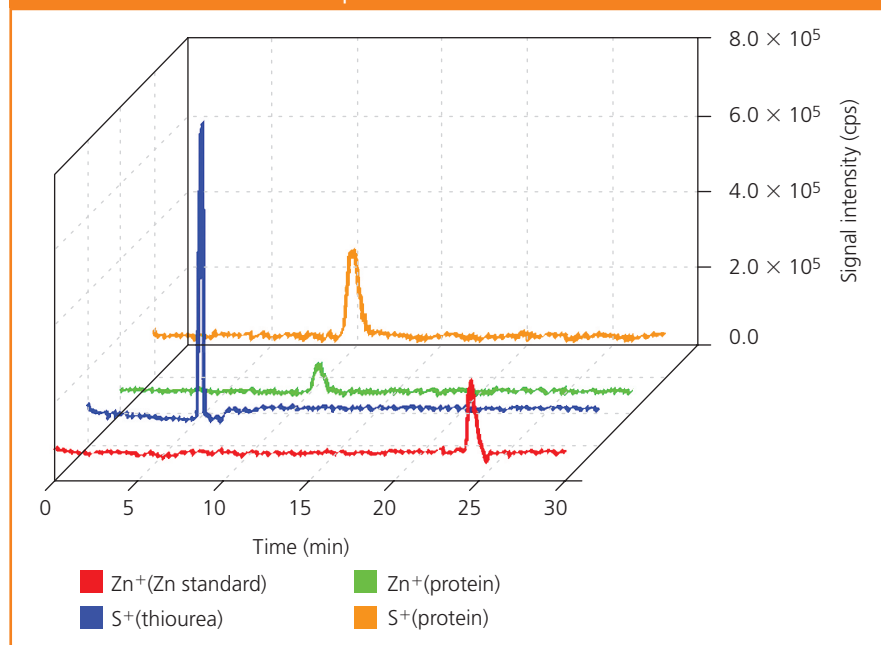
Chemicals and materials: *A. hydrophila* AE036 metallo- β -lactamase was overexpressed and purified as described previously.¹⁸ The enzyme solution was prepared in 15 mmol/L sodium cacodylate buffer (pH 6.5). Table 1 shows the characteristics of the *A. hydrophila* metallo- β -lactamase AE036 used in the experiments. The *A. hydrophila* Zn- β -lactamase was stored at -20 °C. Dodeciasodium salt hydrate of phytic acid [sodium salt of myoinositol hexakis-(dihydrogen phosphate)] was purchased from Aldrich Chemical Company Inc. (Milwaukee, Wisconsin, USA).

Results and Discussion

Buffer compounds: To perform the separation of the *A. hydrophila* metallo- β -lactamase from free Zn by CE, it was necessary to find an adequate CE buffer system. This turned out to be a challenging problem, mainly because of the surface interaction of the (rather large) metallo- β -lactamase with the inner surface of the

bare fused-silica capillary at a pH around the pI of the protein. First, the experiments were performed with UV detection and, later on, were extended to ICP-SFMS detection. In a first attempt to separate the metalloprotein from the free Zn, a boric acid buffer (pH 8.5) was used. At a pH > pI the protein is negatively charged. The use of buffer concentrations of 50 mmol/L to 500 mmol/L boric acid did not result in sharp CE-UV signals for the protein, indicating that, although the protein is slightly negatively charged, there is still adsorption onto the fused-silica capillary wall, even at the highest ionic strength (500 mmol/L) investigated.

The addition of the sodium salt of phytic acid to the separation buffer has already allowed the analysis of several basic proteins (pIs > 9) by CE.^{19–21} Some very basic proteins that are totally adsorbed onto the capillary fused-silica surface in the presence of buffer only, can be eluted as sharp signals when this polyanionic species is included in the running electrolyte. Phytic acid (Figure 2) contains six phosphate groups with pKa values ranging from 1.9 to 9.5 giving this molecule a polyanionic character over a wide pH range.²² This compound is non-toxic and readily available. The addition of small amounts of the sodium salt of phytic acid to the separation buffer resulted in sharp CE-UV signals for the *A. hydrophila* Zn- β -lactamase, most likely the result of an ion-pairing mechanism between this polyanion

Figure 3: Electropherogram of thiourea, *A. hydrophila* Zn- β -lactamase and Zn standard under the conditions specified.

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and basic residues of the protein, such as lysine and arginine. For reasons of conciseness, we will apply the term 'phytic acid' instead of 'the sodium salt of phytic acid' in the remainder of the text.

Preliminary buffer compound optimization: A rotatable central composite design (with four centre points) was applied to obtain the optimum combination of both buffer compounds.^{23–25} To avoid blockage of the microconcentric nebulizer of the CEI-100 interface, the concentration of both buffer compounds must be limited. Therefore, preliminary optimization of the separation buffer was performed with concentrations ranging from 1–10 mmol/L for phytic acid and from 1–25 mmol/L for boric acid. All buffers were adjusted to a pH of 8.2 using acetic acid. The CE–UV signal for the enzyme and the CE–ICP–SFMS signals for $^{32}\text{S}^+$ and $^{64}\text{Zn}^+$ from the metallo- β -lactamase were recorded simultaneously. The peak height was taken as response for the rotatable central composite design. For the UV detection, the sharpest signal of the metallo- β -lactamase was achieved in the presence of a higher concentration (≥ 50 mmol/L) of phytic acid. Practically, Joule heating occurring in the CE capillary limits the use of high concentrations of phytic acid. From the response surface it was clear that for detection with ICP–SFMS, the buffer combination providing maximum peak heights for the $^{32}\text{S}^+$ and $^{64}\text{Zn}^+$ signals consisted of 16.6 mmol/L boric acid and 6 mmol/L phytic acid. This combination was used in all subsequent experiments.

Influence of phytic acid on S and Zn signals: External calibration was used to investigate the influence of the presence of phytic acid on the $^{32}\text{S}^+$ and $^{64}\text{Zn}^+$ ICP–SFMS signals. The sulphur compound thiourea was selected as sulphur standard. It was preferred over SO_4^{2-} because it has a shorter migration time under the conditions applied, and it does not give rise to significant memory effects developing from adsorption onto the capillary walls. The $^{64}\text{Zn}^+$ ion had to be stabilized by complexation with a strong ligand for the Zn to stay in solution at pH 8.2. Phytic acid was used as a buffer compound and at the same time as a complexing agent. It was concluded that the Zn–phytic acid complex is negatively charged because its migration time is longer than that of the Zn- β -lactamase. Figure 3 shows the electropherogram of the *A. hydrophila* Zn- β -lactamase, thiourea and Zn standard under the conditions specified.

Repeatability of the peak area for the *A. hydrophila* Zn- β -lactamase was 16% for the $^{32}\text{S}^+$ signal, 22% for the $^{64}\text{Zn}^+$ signal and 12% for the UV signal ($n = 5$). For the thiourea standard ($n = 6$) and the Zn standard ($n = 3$), repeatability of the peak area was 12% for the $^{32}\text{S}^+$ signal, 8% for the UV signal and 7% for the $^{64}\text{Zn}^+$ signal. The comparison of UV and ICP–SFMS detection indicates that the main source of this imprecision lies in the electrophoresis (including sample injection), but that the CE–ICP–SFMS set-up, however, is an additional source of imprecision. To eliminate nebulization instabilities and to obtain a better precision for the peak area, an internal standard will be used in future investigations. With the optimized separation buffer (16.6 mmol/L boric acid and 6 mmol/L phytic acid at a pH of 8.2), the recovery of sulphur from *A. hydrophila* Zn- β -lactamase was $89 \pm 17\%$ (95% confidence interval). This indicates that no adsorption of the *A. hydrophila* Zn- β -lactamase onto the capillary wall occurs. The recovery of Zn, however, was only $58 \pm 15\%$. This is most likely caused by a matrix-induced difference in detector sensitivity between the Zn- β -lactamase and the external Zn standard and not because of loss of Zn caused by the presence of phytic acid. This hypothesis is supported by the fact that in some instances, Zn can be added to the buffer as an ion-pairing cation to result in protein–positive ion–phytic acid complexes for the separation of basic peptides and proteins (because not all proteins show the ion-pairing effect with phytic acid).^{21,26} Therefore, it would be very unlikely for the phytic acid to remove the rather strongly bound Zn from the Zn- β -lactamase. In fact, it is more likely that the presence of phytic acid in the separation buffer would keep the free Zn and the protein together, because the free $^{64}\text{Zn}^+$ ions in the protein sample can bind to phytic acid molecules before these phytic acid molecules bind to the Zn- β -lactamase. However, in contrast with the results obtained, this would result in a higher Zn recovery. To clarify the low Zn recovery, separation buffers in the absence of phytic acid will be investigated. Use of a borax buffer has already been investigated and despite tailing of the signal, good results were obtained for the sulphur measurements.

To eliminate the problems of matrix-induced detector sensitivity differences between standards and samples, isotope dilution (ID) will be used in the near future for calibration purposes. The use of ID

eliminates all matrix effects because isotope ratios are measured. This technique has previously been used for the characterization and quantification of metallothioneins.^{27,28} Metallothioneins are cysteine-rich proteins of low molecular mass (6–7 kDa) that occur in animal and human cells with origins in such organs as the liver, the kidney or the brain. This study will attempt to extend CE-ICP-IDMS to proteins with higher molecular weight and containing much less sulphur and metal atoms compared with metallothioneins.

Conclusions

This study indicates that for the fast and accurate determination of the stoichiometric ratio Zn/S for *A. hydrophila* Zn-β-lactamase, a hyphenated analytical technique based on both electrophoretic and mass spectrometric methods shows considerable potential. The application of external calibration seemed to be most attractive as a routine technique for Zn quantification in *A. hydrophila* Zn-β-lactamase, but gave rise to problems. These problems were most likely the result of matrix-induced differences in detector sensitivity between standard and sample. For this reason, we will switch to isotope dilution to determine the stoichiometric Zn/S ratio of Zn-β-lactamase in future work.

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