Investigation of Pb(II) binding to pectin in *Arabidopsis thaliana*

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Phytochelatins and glutathione are known as the most common peptides responsible for binding with toxic metals in plants, but recently the role of other bioligands, such as proteins, organic acids, flavonoids and oligosaccharides, has received renewed attention. Size-exclusion chromatography (SEC) coupled to ICP-MS and electrospray MS were applied for the analysis of lead species synthesised by *Arabidopsis thaliana*, a model genetic plant. Lead was found to be accumulated mainly in roots (9.6 ± 0.1 μg of Pb per g of the dry mass) and showed good affinity to galacturonic acid, the main component of two pectin domains homogalacturonan and rhamnogalacturonan I. The results demonstrate the potential application of SEC-ICP-MS and SEC-ESI-MS as a complementary and efficient approach to study toxic heavy metals in biological systems.

Introduction

Size-exclusion chromatography coupled to an isotope specific mass spectrometer (SEC-ICP-MS) is considered as an important approach for screening of metal complexes in biological samples and is frequently used for investigation and isolation of metal complexes extracted from plant tissue prior to ESI-MS identification.[1–4] Such a procedure provides indirect proof that organic compounds found in the collected fractions are involved in metal binding. Despite the plethora of molecular data available on the phytochelatin (PC) complexes with cadmium, remarkably little is known on the mechanism of lead deactivation due to the low stability of metal complexes.[5–8] The low stability of the $Pb(GSH)_2$ complex with a mercapto-bond, in contrast to the one with cadmium, found by electrospray MS experiments enabled the proposition that Pb(II) has a much lower capability of induction of PCs than Cd(II).[9]

An interesting insight was provided thanks to electron spectroscopic imaging on the cellular and sub-cellular level, which offers an alternative means of identifying and quantifying the element's presence with 50–100 nm resolution. It was found that the majority of the lead was deposited in, or adjacent to, the cell wall as particles with sharp and angular edges[10] near the plasmodesmata and, more rarely, in vacuoles.[11–13] The concept was confirmed by X-ray spectroscopy, which proved the presence of Pb–O binding within the pectic and lingo-cellulosic structures in roots.[14] Although the SEC-ICP-MS method was used to provide proof that pectin polysaccharides are cross-linked to each other by a borate diol diester[15] and to study lead affinity to rhamnogalacturonan II released from grape berry cell walls into wine,[16] in fruits and vegetables,[17] the presence of lead complexes in plants from the *Brassicaceae* family has never been studied due to the extraction problems of poorly dissolving cellulose, hemicellulose and pectin. In such a case enzymatic extraction or hydrolysis of cell wall components and the separation of small oligosaccharides is recommended for initial experiments.[18–20] The purification of released oligosaccharides is usually carried out by gel and ion exchange chromatography[15] prior to its molecular characterization by NMR[21,22] or ESI-MS.[23,24] On-line ESI-MS detection of polysaccharides and their components extracted from plants was carried out only for gel-permeation chromatography,[25] although size-exclusion coupled to NMR has already been reported as a successful approach.[26] It was found and confirmed by the analysis of the genome of *Arabidopsis thaliana* that plants are capable of synthesising homogalacturonan (HG) and rhamnogalacturonan I (RGI), which are quantitatively the most important domains of pectin in the primary cell wall.[27] It was also reported that divalent ions like Cu^{2+} and Pb^{2+} show good affinity to HG in spite of the degree of acetylation, in contrast to calcium.[25,28,29]

The aim of our work was to investigate, on the molecular level, the response of a model genetic plant (*Arabidopsis thaliana*) to lead stress and to characterize the involvement of cell wall components in lead deactivation by SEC-ICP-MS and SEC-ESI-MS.

Experimental

Instrumentation

Chromatographic separations were performed using a Model HP 1100 gradient HPLC pump (Agilent Technologies, Waldbronn, Germany) as the sample delivery system. An Agilent Model 7500a ICP mass spectrometer (Tokyo, Japan) was used for quantification of the metal content in plants and for on-line metal monitoring in HPLC eluate.

The ESI-MS detection Model LC-MSD 1100 (Agilent Technologies, Wilmington, NC, USA) with a quadrupole

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mass analyser was used for complementary SEC-ESI-MS experiments. The ESI-MS spectra for SEC were registered thanks to supplementation of the eluate with a methanol stream supplied by a PerkinElmer HPLC pump (Model LC-95, PerkinElmer, Norwalk, CT, USA) using a T-connector placed between the chromatographic column and the capillary of the ESI source; it was found to be necessary for ensuring efficient ionisation.

Chemicals and materials

All reagents used were of analytical grade purchased from Sigma–Aldrich (Sigma–Aldrich, Buchs, Switzerland). The acetonitrile (ACN) of HPLC gradient grade was used for RPLC separation method (LabScan Analytical Science, Dublin, Ireland).

Plants of *Arabidopsis thaliana* L. Columbia 4 (NASC, N 933) were cultivated in continuously aerated hydroponics culture with Hoagland's solution nutrient which was renewed weekly. Five week old plants were divided into seven groups and exposed over 14 days to 0.0 (control), 5.0, 12.5, 25.0, 50.0, 75.0 and 100.0 μM of Pb(II) in the form of nitrate salt. After 14 days of Pb^{2+} treatment plants were collected and washed with MQ-water (Millipore Elix 3, Millipore, Saint-Quentin, France). Leaves and roots were separated from each other, immediately frozen and lyophilised.

Sample preparation

A weighted sample of 40 mg of freeze-dried material, obtained by lyophilizator Model Alpha 1-2 LD (Christ, Osterode, Germany), was frozen in liquid nitrogen (−196 °C) to break cell walls. Subsequently, 20 mg of pure quartz sand was added and sample was ground with mortar and pestle until a homogenous powder was formed.

Homogenized sample was sequentially extracted with 2 mL portions of sonicated buffers as already described in detail.[30]

ICP-MS detection

The determination of the total amount of metal in plant extracts was carried out by ICP-MS using 10 ng mL^{-1} of Y as an internal standard for analysed samples and the standard solutions used for instrument calibration. The residue after extraction of plant tissue was analysed after lyophilization and mineralization with nitric acid. The calibration graphs were obtained for lead concentration within the range 0.1–100.0 μg L^{-1} for three lead isotopes ^{206}Pb, ^{207}Pb and ^{208}Pb. The limit of detection (LOD) was calculated for standard deviations (3.3 SD_{blank}/slope of calibration graph) of 10 measurements obtained for the blank and it was found to be 0.5–0.6 μg L^{-1}.

Size exclusion chromatography

Superdex Peptide HR 10/30 column 300 × 10 mm (Pharmacia Biotech, Uppsala, Sweden) with an exclusion limit of 20 kDa was applied for separation of extracted compounds. The cleaning stage was followed by the procedure described in the previous paper.[30] 2 mM of NaCl was added to Tris buffer as a non-complexing salt in order to suppress ion exchange on the stationary phase. The isotopes monitored were: ^{206}Pb, ^{207}Pb, ^{208}Pb, ^{10}B, ^{11}B, ^{43}Ca and ^{44}Ca to observe changes of isotopic profiles and to control the isotopic ratio for each metal in case of interference.

SEC-ESI-MS experiments

ESI-MS spectra for SEC eluate were acquired in the range 50–1500 u using 100 ms dwell time and 0.2 u step size. The ion spray voltage of 4000 V was applied for acquisition of negative ions recommended for acidic organic compounds. The orifice potential was established for sucrose as 80 V, offering the best signal intensity for quasi-molecular ion and its partial fragmentation.

Results and discussion

ICP-MS monitoring of *Arabidopsis thaliana* reaction to lead stress exposure

The efficiency of lead translocation from roots to leaves in the plant was examined by ICP-MS after mineralization of three representative samples. It was found that only 0.9% of the total amount of Pb was accumulated in leaves and, as a consequence, roots were selected as a further investigation object. The maximum ability of the plant to uptake metal ions (9.6 ± 0.1 μg of Pb per g of the dry mass) was found for a concentration of 50 μM of Pb(II) with significant drop of the total lead amount in plant tissue for higher concentrations of metal in hydroponics (4.1 ± 0.1 mg of Pb per g of the dry mass for 100 μM of Pb^{2+}). The lead distribution on a cellular level was investigated for plants treated with 50 μM of Pb^{2+}, and Table 1 presents the results obtained.

It should be pointed out that only 1% of lead accumulated in the roots was extracted with Tris buffer pH 7.5 which was intended to release water soluble metal complexes from cytosol and cell organelles. It shows the insignificance of phytochelatins in the lead deactivation mechanism of plants in contrast to cadmium.[30] Owing to the low yield of lead obtained from driselase leaching (2%) and SDS extraction at pH 7.5 (14%), further molecular characterisation of driselase and SDS extracts was difficult to perform. In the next step 100 mM CH_3COONH_4 (pH 4.6) was used to extract pectin components due to partial hydrolysis of borate esters ($pK \approx 4.8$).[18] The recovery of lead achieved 40% and further investigation of this fraction was carried by SEC-ICP/ESI-MS.

SEC-ICP-MS and SEC-ESI-MS characteristics of ammonium acetate extract

The ^{208}Pb profile of the chromatogram obtained for last extract presented in Fig. 1(A,a) consists of three peaks. The

Table 1 Lead concentrations in individual extracts obtained from roots of *Arabidopsis thaliana* treated with 50 μM of Pb^{2+} ($n = 5$)

Extracting medium	Amount of lead in individual extracts/μg g^{-1}	Total amount of lead (%)
10 mM Tris-HCl, pH 7.4	0.10 ± 0.06	1
2% Driselase in 10 mM Tris-HCl, pH 7.4	0.19 ± 0.02	2
1% SDS in 10 mM Tris-HCl, pH 7.4	1.34 ± 0.09	14
100 mM CH_3COONH_4, pH 4.6	3.84 ± 0.10	40
Residue after leaching	4.13 ± 0.11	43

addition of $Pb(NO_3)_2$ to the extract obtained from the control group of plants was responsible for the presence of a small tailing peak (t_R 18 min, Fig. 1(A,b)), which can be considered as proof that lead eluted in first two peaks is covalently bond to bioligands synthesised by plants. The chromatogram obtained for ^{43}Ca and ^{11}B consisted of two and three peaks, respectively, which were co-eluting with lead compounds (Fig. 1(B,a,b)). The presence of boron in the first peak ($\sim$10 kDa) could indicate the existence of borate esters responsible for linking of RG-II. However, the other two peaks might indicate that partial degradation of RG-II took place. Boric acid and metal salts dissolved in acetate buffer were injected on to the column. The retention times varied in the range 18–30 min, depending on the ion concentrations and the degree of column contamination. It was impossible to estimate retention times for non-bonding metals and boric acid. The plant extract was acidified with hydrochloric acid (0.1 M), incubated for 16 h and injected on to the column. Only one peak after 17.8 min was observed for each element, which could indicate that non-bound ions are eluted in the third peak. Taking into account the fact that lead was eluted mainly as a high molecular complex, as opposed to calcium, and that the height of peaks obtained for calcium in the control and exposed group of plants was only 2.0 and 1.4 times lower (Fig. 1(B,a,b)), it can be expected that lead and calcium are bound with different compounds. However, it should be confirmed using molecule specific electrospray MS.

The SEC-ESI-MS analysis was carried out in the scanning mode and two major ions were selected for reconstruction of chromatograms and further discussion: at m/z 191 observed in extracts of control and the exposed to lead group of plants; at m/z 237, which was not observed in the extract of the control group. The reconstructed chromatograms consisted of three and two peaks, respectively (Fig. 1(C,a,b)). The mass spectra registered for the second peak consisted of six main signals (Fig. 2(2)). The most intense peaks observed at m/z 385 and 367 ($-H_2O$) corresponding to disaccharide consisted of galacturonic acid (GalA) and methylated GalA residue (MeGalA). Signals registered at m/z 543 and 561 enlarged with one residue of galacturonic acid (176 u) and signals at m/z 191 and 209 received after loss of 176 u, allow us to propose the sequence of the extracted oligosaccharide as a MeGal · GalA · GalA, which corresponds to homogalacturonan. The presence of a signal at m/z 237 can be attributed to specific fragmentation in the electrospray ion source. However, acetylation was more probable because this ion was dominant in the mass spectrum obtained for the apex of the first peak (Fig. 2(1)) and other signals corresponding to components of acetylated rhamnogalacturonan I were also found at m/z 719, 737 and 779 at t_R 16 min (not shown). However, higher molecular mass was rather expected at such a short elution time (10.9 min). The multiple charge of the compound typical for an ESI source had also to be excluded. Careful investigation of the mass spectrum allowed us to find signals (indicated as 919.0 in Fig. 2(1)), corresponding (with 82% probability) to the isotopic pattern of two lead ions. Additionally, the signal at m/z 716 was found to correspond to one atom of lead but the correlation of isotopic pattern was only 56%. Such a low agreement of isotopic profile and low molecular mass of adduct observed

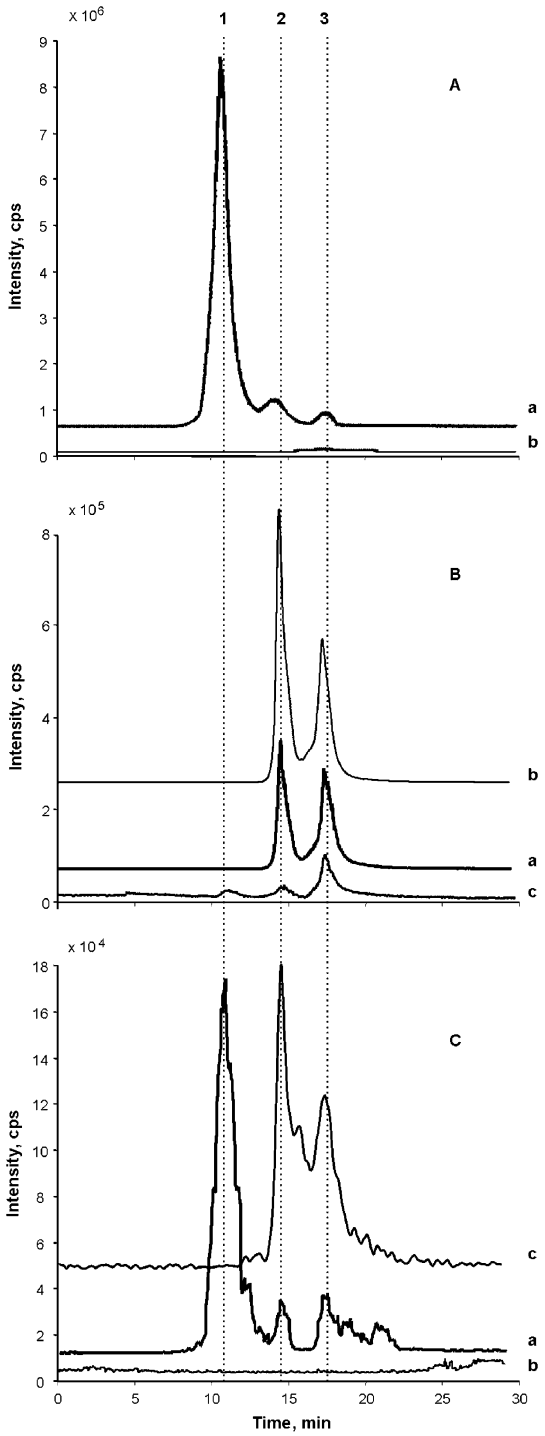

Fig. 1 Size exclusion chromatograms obtained for ammonium acetate extracts of control group of plants (line b) and exposed to lead obtained by ICP-MS detection of: (A,a), ^{208}Pb, (B,a), ^{43}Ca and (B,c), ^{11}B. Reconstructed SEC-ESI-MS chromatograms for selected ions: m/z 237 for control group of plants (C,b) and exposed to lead (C,a) and m/z 191 for the control group of plants (C,c).

on the mass spectrum can be caused by a low intensity of ions due to its fragmentation in the ESI ion source.[9] Identified signals on the mass spectrum correspond to partially degraded homogalacturonan (HG), which was reported to show good

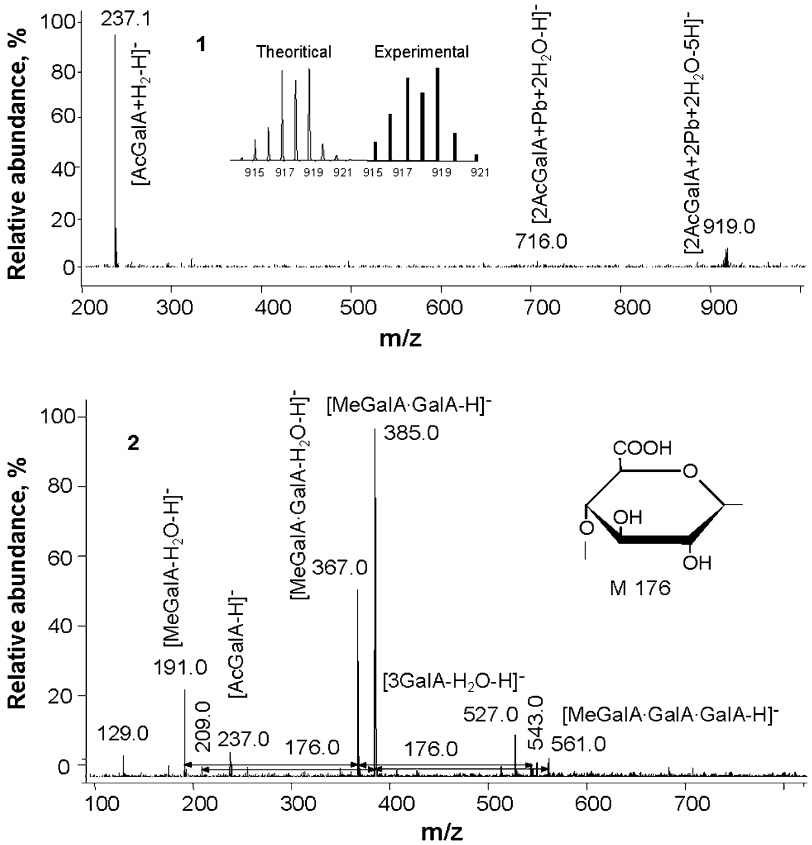

Fig. 2 Mass spectra taken in the apex of the selected peaks from the chromatogram presented in Fig. 1(C). The number in the inset of the mass spectrum corresponds to the chromatographic peak number in Fig. 1. The comparison of theoretical and experimental isotopic pattern for Pb(II) adduct is presented in the inset of mass spectra.

affinity to both Ca^{2+} and Pb^{2+}.[28,29] However, lead was found to elute mainly with AcGalA. It can be explained by the high affinity of Pb^{2+} to hydroxyl and carboxylic groups, which changes insignificantly with DAc in contrast to Ca^{2+}. Calcium was observed in the second and the third peaks with non-acetylated polymers of GalA and corresponded to eluted and non-covalently bond ions, respectively.

It was impossible to estimate the level of acetylation of HG due to the non-specific extraction procedure, but a high affinity of lead to acetylated galacturonic acid, in contrast to calcium, is evident.

Conclusions

Size exclusion chromatography coupled with ICP and ESI mass spectrometry have been demonstrated to be an attractive analytical set of tools for the detection and careful investigation of selected pectin components in *Arabidopsis thaliana* exposed to Pb^{2+} stress. The ICP-MS detection of eluate enables demonstration of the affinity of ligands to metal and ESI-MS completes its identification. The SEC-ESI-MS method was found to be an attractive and successful tool to show good affinity of lead to acetylated HG in contrast to calcium.

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References

1 V. Vacchina, R. Łobiński, M. Oven and M. H. Zenk, *J. Anal. At. Spectrom.*, 2000, **15**, 529.
2 E. Moreli, B. H. Gruz, S. Somovigo and G. Scarano, *Plant Sci.*, 2002, **163**, 807.
3 D. Schaumlöffel, L. Ouerdane, B. Bouyssiere and R. Łobiński, *J. Anal. At. Spectrom.*, 2003, **18**, 120.
4 V. Vacchina, S. Mari, P. Czernic, L. Marquès, K. Pianelli, D. Schaumlöffel, M. Lebrun and R. Łobiński, *Anal. Chem.*, 2003, **75**, 2740.
5 J. Meija, M. Montes-Bayón, J. A. Caruso and A. Sanz-Medel, *Trends Anal. Chem.*, 2006, **25**, 44.
6 G. Scarano and E. Morelli, *BioMetals*, 2002, **15**, 145.
7 M. Gupta, U. N. Rai, R. D. Tripathi and P. Chandra, *Chemosphere*, 1995, **30**, 2011.
8 A. Piechalak, B. Tomaszewska, D. Baralkiewicz and A. Malecka, *Phytochemistry*, 2002, **60**, 153.
9 K. Połeć-Pawlak, R. Ruzik and E. Lipiec, *Talanta*, 2007, **72**, 1564.
10 X. Yang, Y. Feng, Z. He and P. J. Stoffella, *J. Trace Elem. Med. Biol.*, 2005, **18**, 339.
11 M. D. Jarvis and D. W. M. Leung, *Environ. Exp. Bot.*, 2002, **48**, 21.
12 M. Wierzbicka, E. Przedpelska, R. Ruzik, L. Ourdane, K. Polec-Pawlak, M. Jarosz, J. Szpunar and A. Szakiel, *Protoplasma*, in the press.

13 M. D. Jarvis and D. W. M. Leung, *Environ. Exp. Bot.*, 2002, **48**, 21.
14 M. Marmiroli, G. Antonioli, E. Maestri and N. Marmiroli, *Environ. Pollut.*, 2005, **134**, 217.
15 T. Ishii and T. Matsunaga, *Phytochemistry*, 2001, **57**, 969.
16 J. Szpunar, P. Pellerin, A. Makarov, T. Doco, P. Williams, B. Medina and R. Łobiński, *J. Anal. At. Spectrom.*, 1998, **13**, 749.
17 J. Szpunar, P. Pellerin, A. Makarov, T. Doco, P. Williams and R. Łobiński, *J. Anal. At. Spectrom.*, 1999, **14**, 639.
18 T. Ishii and T. Matsunaga, *Carbohydr. Res.*, 1996, **284**, 1.
19 S. Kaneko, T. Ishii and T. Matsunaga, *Phytochemistry*, 1997, **44**, 243.
20 S. L. Gardner, M. M. Burrell and S. C. Fry, *Phytochemistry*, 2002, **60**, 241.
21 S. Pérez, M. A. Rodríguez-Carvajal and T. Doco, *Biochemie*, 2003, **85**, 109.
22 T. Ishii and S. Kaneko, *Phytochemistry*, 1998, **49**, 1195.
23 A. M. Striegel, *Carbohydr. Polym.*, 1997, **34**, 267.
24 A. M. Striegel, J. D. Timpa, P. Piotrowiak and R. B. Cole, *Int. J. Mass Spectrom. Ion Processes*, 1997, **162**, 45.
25 M. J. Deery, E. Stimson and C. G. Chapell, *Rapid Commun. Mass Spectrom.*, 2001, **15**, 2273.
26 T. G. Neiss and H. N. Cheng, *ACS Symp. Ser.*, 2003, **834**, 382.
27 R. M. Perrin, A. E. de Rocher, M. Bar-Peled, W. Zeng, L. Norambuena, A. Orellana, N. V. Raikher and K. Keegstra, *Science*, 1999, **284**, 1976.
28 M.-Ch. Ralet, M.-J. Crépeau, H.-Ch. Buchholt and J.-Ch. Thibault, *Biochem. Eng. J.*, 2003, **16**, 191.
29 V. M. Dronnet, C. M. Renard, M. A. Axelos and J.-Ch. Thibault, *Carbohydr. Polym.*, 1997, **34**, 73.
30 K. Połeć-Pawlak, R. Ruzik, K. Abramski, M. Ciurzyńska and H. Gawrońska, *Anal. Chim. Acta*, 2005, **540**, 61.

