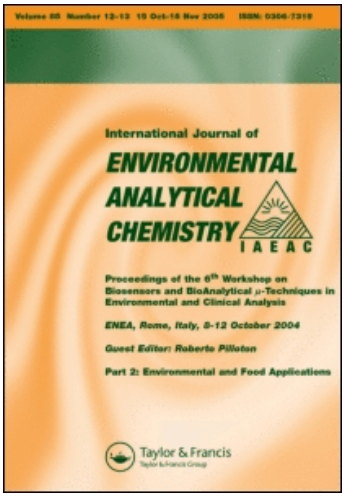

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Danuta Baralkiewicz[a]; Małgorzata Kozka[a]; Piotr Kachlicki[b]; Aneta Piechalak[c]; Barbara Tomaszewska[c]
[a] Faculty of Chemistry, Department of Trace Elements Analysis by Spectroscopic Method, Adam Mickiewicz University, Poznań, Poland [b] Institute of Plant Genetics, Polish Academy of Sciences, Poznań, Poland [c] Faculty of Biology, Department of Biochemistry, Adam Mickiewicz University, Poznań, Poland

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Danuta Baralkiewicz[a]*, Małgorzata Kozka[a], Piotr Kachlicki[b], Aneta Piechalak[c] and Barbara Tomaszewska[c]

[a]*Faculty of Chemistry, Department of Trace Elements Analysis by Spectroscopic Method, Adam Mickiewicz University, Poznań, Poland;* [b]*Institute of Plant Genetics, Polish Academy of Sciences, Poznań, Poland;* [c]*Faculty of Biology, Department of Biochemistry, Adam Mickiewicz University, Poznań, Poland*

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Plants exposed to heavy metals activate a detoxification system capable of chelating and transporting these harmful ions to vacuoles. Phytochelatins – low molecular weight oligopeptides containing thiols such as glutathione and cystein – have been reported to play a very important role in this respect. High performance liquid chromatography coupled to the electospray ion trap mass spectrometer (HPLC-ESI-IT-MS) was used for identification of phytochelatins induced by Cd^{2+} and Pb^{2+} in roots, stems and leaves of pea (*Pisum sativum* L.) and yellow lupin (*Lupinus luteus* L.). This approach enabled unambiguous identification of phytochelatins in plant tissues and detection of phytochelatins and homophytochelatins in reduced as well as in oxidised form. Significant differences were detected in phytochelatin relative amounts and profiles in different parts of plants treated with heavy metals. Roots of both plant species contained mainly reduced phytochelatins, reduced and oxidised forms of these peptides were observed in stems in similar amounts, whereas only the oxidised phytochelatins were present in leaves.

Keywords: phytochelatins; HPLC-ESI-MS; ion trap; *Pisum sativum Lupinus luteus*

1. Introduction

Pollution of soil with heavy metals represents a major environmental hazard to human health. Cadmium (Cd) and lead (Pb) are widespread heavy metal pollutants released into the environment by human activities including agriculture, mining, smelting, sludge disposal and industry [1]. The presence of Pb^{2+} and Cd^{2+} as well as ions of other metals in the environment leads to a number of disturbances in many metabolic processes in plants. Inhibition of growth is a major symptom and it is associated with a number of morphological, physiological and biochemical events [2–5]. The strong affinity of Pb and Cd ions to sulfhydryl (SH), carboxyl (COOH) and amine (NH_2) groups may explain the high toxicity of these metals. Plants defence against heavy metals involves the activation of a range of various mechanisms resulting in the modification of ion mobility within the rhizosphere, active exclusion of heavy metals from cells or synthesis of bioligands [6].

*Corresponding author. Email: danutaba@amu.edu.pl

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The synthesis of phytochelatins (PCs) is one of the main and best characterised metal detoxification mechanisms in plant cells [7–9]. PCs are synthesised in an enzymatic reaction in which glutathione and its homologs are the substrates. The resulting peptides chelate metal ions, so they can be transported into vacuoles as low-molecular complexes (LMW-HM). The last step of the detoxification process is the release of heavy metals there and formation of solid complexes with organic acids or long-chained PCs and S^{2-} [6,10].

PCs have the general formula of (γGlu–Cys)*n*-Gly (with $n = 2$–11) and are synthesised by the transpeptidation of γGlu–Cys moieties of glutathione (GSH) onto another GSH molecule or the enzymatic elongation of the PC peptide catalysed by PC synthase [11,12]. Isoforms of PCs, called homo-PCs are observed in pea (*Pisum sativum*) plants. These peptides are derived from homoglutathione and they differ from PCs in substitution of β-Ala for the C-terminal Gly [13–15]. It is known that plants lacking enzymes involved in the GSH synthesis [16] or deficient in the PC synthase activity [17] are hypersensitive to Cd.

The classical methods for determination of GSH and PCs in plants are high performance liquid chromatographic (HPLC) separations with either the post-column derivatisation of the thiol groups with DTNB (5,5-dithio*bis*-(2-nitrobenzoic acid)) [14,15,18] or pre-column derivatisation of free –SH groups with monobromobimane (mBrB) [19–21]. These methods are not perfect, the most important limitation is the lack of available PCs standards and impossibility to determine the oxidised forms of glutathione and PCs and their isoforms. The coupling of HPLC with electrospray (ESI) ionisation mass spectrometer (MS) enables the analysis of high molecular weight molecules such as peptides and proteins [22–24]. The HPLC-ESI-MS/MS technique may be successfully used to the identification of both reduced and oxidised forms of thiol compounds. The quadrupole ion trap (IT) functions both as an ion store and as a mass spectrometer [22]. The greatest advantage of the ion trap technique lies in the possibility of performing sequential MS^n experiments and reveal complex fragmentation patterns of the studied compounds. Ions with a particular m/z may be selectively isolated in the trap and fragmentation of these isolated precursor ions can then be induced by collision induced dissociation (CID) experiments. The isolation and fragmentation steps can be repeated a number of times which is only limited by the trapping efficiency of the instrument. This technique allows a precise determination and identification of the studied ion structure [22].

2. Experimental

2.1 *Instrumentation and chemicals*

Chromatographic separations were performed on C_{18} column (150 mm × 2.1 mm), (Alltima, Alltech). An Agilent 1100 HPLC pump (Palo Alto, CA, USA) was used as the solvent delivery system and injections were made using a Rheodyne valve with a 20 µL injection loop. MS^n characterisation of peptides was achieved using an Esquire 3000 (Bruker Daltonics, Bremen, Germany) ion trap mass spectrometer with the electrospray ion source. A Biofuge Fresco (Heraeus Instruments, Germany) refrigerated ultracentrifuge was used for the separation of the supernatant from plant tissues.

Analytical grade reagents and deionised, ultrafiltered water were used throughout. Sulfosalicylic acid (SSA) was prepared in deionised water as 5% solution for sample extraction. Acetonitrile (Fluka, MS grade), water (MiliQ) and formic acid were used for the HPLC eluents.

2.2 *Plant material and sample preparation*

Seeds of pea (*Pisum sativum* L.) cv. Kwestor and yellow lupin (*Lupinus luteus* L.) cv. Juno were purchased from Przebędowo Plant Breeding. Seeds were soaked in water for 4 h and germinated in the dark for 3 days at 24°C. After that, the seedlings were cultivated hydroponically in Hoagland solution with metal addition. Pea and lupin plants were incubated with 0.5 mM $Pb(NO_3)_2$ (Pb), 0.1 mM $CdCl_2$ (Cd) and 0.1 mM $Pb(NO_3)_2$ + 0.1 mM $CdCl_2$ (CdPb). The plants were grown under controlled conditions for 96 h with 16/8 day/night cycle (light intensity 70 $\mu m\, m^{-2}\, s^{-1}$). Collected samples were separated, rinsed for 10 min in 10 mM $CaCl_2$ and deionised water, frozen in liquid nitrogen and stored at −80°C until analysis. Plant material (250 mg fresh weight for each sample) was ground with 5% SSA in ratio 1 : 2 using mortar and pestle (in ice) immediately before the HPLC experiments. The homogenate was centrifuged twice for 10 min at 10,000 *g* at 4°C, filtered through 0.45 μm PTFE syringe filters (Whatman) and kept at 0°C until the injection to the LC-MS system.

2.3 *LC-MS conditions*

Mixtures of two solvents A (99.5% H_2O, 0.5% formic acid) and B (95% acetonitrile, 4.5% H_2O, 0.5% formic acid) were used for peptides elution from LC column. Chromatographic separation was performed with the following programme: 0–25 min from 99.8 to 83% of A, 25–27 min down to 45% of A, 27–32 min isocratic at 45% of A, 32–33 min linear gradient up to 99.8% of A. The flow rate of mobile phase was 0.2 mL min^{-1}. Aliquots of 20 μL of sample were injected into LC system.

The settings of the mass spectrometer were: ESI source voltage 4 kV, nebulisation with nitrogen at 30 psi, dry gas flow 9 L min^{-1}, gas temperature 310°C, collision energy set to 1 V and ramped within 40–200% of this value. The ion number accumulated in the trap was set to 10,000 and the maximum accumulation time was 200 ms. According to results of preliminary experiments spectra were recorded in the mass range m/z 50–1200 for the root samples. Positive ions corresponding to the respective m/z of oxidised and reduced PCs and hPCs were monitored in the targeted mode in the stem and leaf samples.

2.4 *Evaluation of relative quantities of phytochelatins in parts of pea and lupin plants*

Quantities of the studied phytochelatins in parts of plants of both species were evaluated by comparison of the chromatographic peak areas obtained for the respective protonated molecule ions $[M + H]^+$ (along with $[M + 2H]^{2+}$ for PC_4) in relation to these obtained for PC_2 in roots of pea or lupin. Each evaluation is a mean value from three separate samples obtained from plants incubated with both Cd^{2+} and Pb^{2+}.

3. Results and discussion

The on-line analysis of PCs by HPLC-ESI-MS is still a subject of research [23,24]. A direct analysis of phytochelatins is a challenging task due to their low concentration in plant samples and the presence of matrix components that affect the ionisation yield.

In our previous studies [18,25] total concentrations of phytochelatins in pea and lupin samples were determined by HPLC with their post-column derivatisation. Identification of respective PCs and hPCs was not possible due to the lack of standards and could be only

tentatively performed according to the retention times of the recorded peaks. The use of an analytical technique capable of detecting compounds specifically, for example mass spectrometry, is therefore required.

In this study, identification of phytochelatins in plant extracts was carried out with LC/MS^n system equipped with an IT analyser. In the positive ion mode, protonated molecule ions $[M+H]^+$ were recorded. Phytochelatins and homophytochelatins were detected in the samples extracted from pea roots as protonated molecule ions. PC_2, PC_3, PC_4, hPC_2, hPC_3 and hPC_4 appeared at m/z 540, 772, 1004, 554, 786 and 1018, respectively (Figure 1). However, in the case of PC_4 double charged ions $[M+2H]^{2+}$ at m/z 502 were

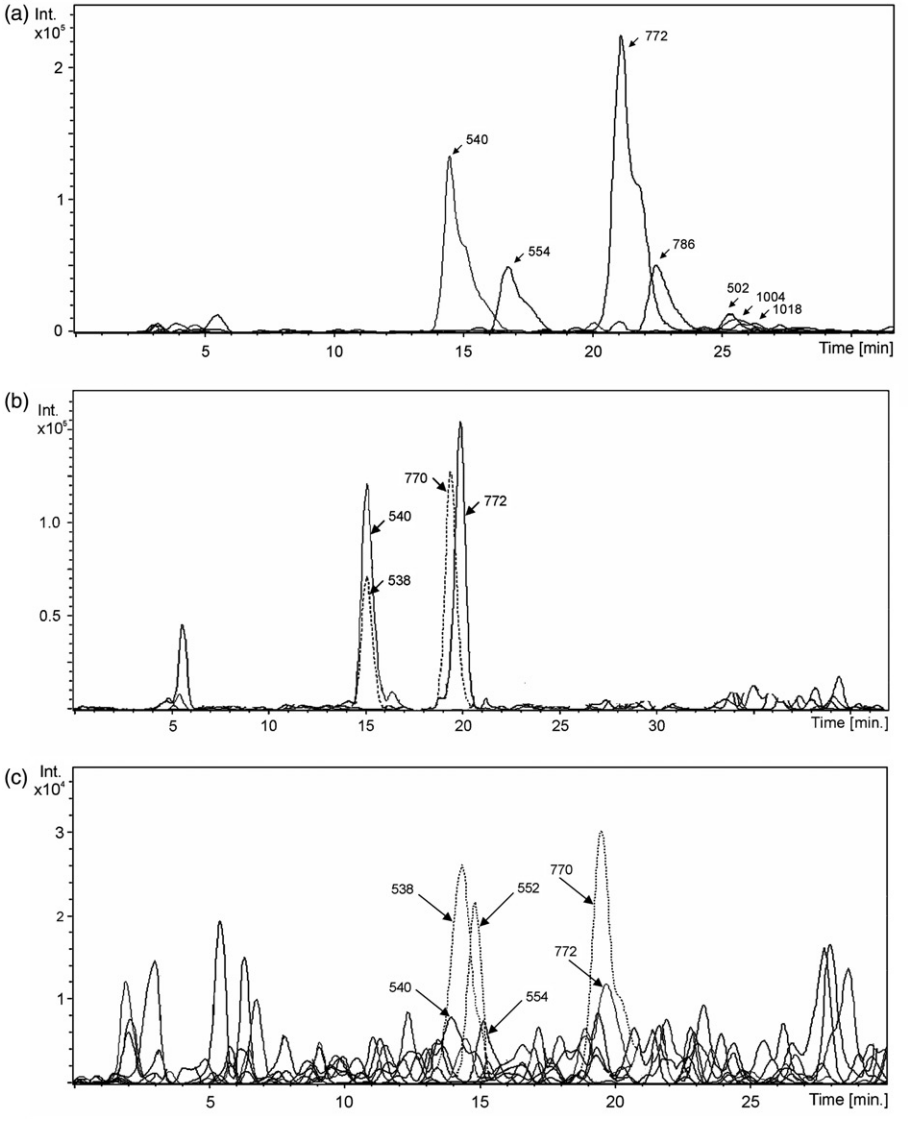

Figure 1. Single ion chromatograms of phytochelatins from parts of pea plants treated with Cd^{++} and Pb^{++} ions: roots (a), stems (b) and leaves (c) obtained using $HPLC/MS^n$ system.

more abundant than the $[M+H]^+$ ions (Figure 1a). In agreement with our previous results [18,25] no hPCs were detected in any sample obtained from lupin tissues. In addition, there were detected oxidised forms of all PCs as a result of internal disulfide bridge formation. These forms had molecular weights 2 units lower and the chromatographic properties almost identical to their reduced counterparts (Figures 1b and 1c). No PCs were detected in extracts from the control plants whereas all of them were present in Cd, Pb and CdPb samples. Nevertheless, for both plant species the intensity of the respective ions differed depending on the plant part and heavy metal ions used. The amounts of PCs in stem and leaf extracts were much lower than these observed in roots (Table 1) and in these samples there was observed an $[M+H]^+$ ion at m/z 613 corresponding to GSSG – the oxidised form of the glutathione dimer coupled with the disulfide bond. This compound was present in large amounts, exceeding the amount of PCs by two orders of magnitude and chromatograms of its $[M+H]^+$ ion are not shown at the Figure 1. Synthesis of PCs and hPCs in roots was the highest in plants incubated with Cd, which is in agreement with our previous results [18]. The oxidised forms of PCs were also found in root extracts but their ion intensities were 100 times lower than these of the reduced forms (Table 1). Both forms of PCs were observed at a similar level in stem extracts. The PCs ion intensities in stems were the highest in extracts from the CdPb treated plants. In the leaf extracts phytochelatins were detected only in Cd and CdPb samples and the ion intensities of the oxidised forms of PCs were much higher than these of the reduced forms which were found only in trace amounts. Quantitative analysis of PCs in the plant tissues using MS methods encounters serious problems. The most important ones arise from the lack of standards of these compounds and the presence of the matrix of numerous other compounds present in tissues and influencing the ionisation efficiency. The matrix effect is diminished to some extent using the HPLC separation of analytes prior to the MS measurements. In any case, the amounts of individual PCs in different parts of plants presented in the Table 1 are only relative and no conclusions regarding comparison of absolute amounts of these peptides between plant species may be made. Quantitative analysis of PCs using former techniques [14,19,20] brings better results in this case.

Due to the extremely low amounts of PCs and hPCs present in samples from stems and leaves of pea plants the HPLC/MS^n experiments had to be performed to analyse these peptides properly. The analyses were conducted in the targeted mode – with monitoring of only m/z corresponding to the PC peptides. It may be seen (Figure 1) that all these peptides were well separated and the corresponding protonated molecule ions could be identified using the MS^n spectra. In the IT analyser, it was possible to perform multiple stage CID MS/MS experiments from the protonated molecule ions. The MS^n fragmentation in the ion trap provided information sufficient for correct structural identification (Figure 2). The characteristic and expected fragments corresponding to cleavage of peptide bonds have been found as shown at Figure 2.

Figure 3 shows the mass spectra produced by the fragmentation of molecular ions $[M+H]^+$ at m/z 540 characteristic for PC_2 and at m/z 538 which corresponds to the oxidised form of PC_2. The fragments of PC_2 can be observed at m/z 411 (–Glu), 336 (–Glu and –Gly) and 308 which corresponds to GSH and results from the loss of the γGlu–Cys group. The same fragmentation can be observed in the oxidised form of PC_2 but the fragment masses are lower by 2 units, which suggests the internal disulfide bridge formation. Figure 4 presents the fragmentation mass spectra of hPC_2 (reduced and oxidised form) which are similar to the PCs spectra. The main difference is in the loss of

Table 1. Relative contents of phytochelatins in different parts of pea and yellow lupine plants treated with a solution containing both Cd^{++} and Pb^{++} ions.

Compound	m/z of ion $[M+H]^+$	*Pisum sativum*			*Lupinus luteus*		
		Roots	Stems	Leaves	Roots	Stems	Leaves
PC_2 reduced	540	100[a]	28.3 ± 3.2	Trace	100[a]	22.4 ± 3.4	Trace
PC_2 oxidised	538	1.4 ± 0.6	21.8 ± 2.3	3.8 ± 1.3	0.9 ± 0.4	21.2 ± 2.9	4.9 ± 1.3
hPC_2 reduced	554	37.1 ± 3.6	6.4 ± 1.8	Trace	*	*	*
hPC_2 oxidised	552	0.6 ± 0.3	7.1 ± 2.4	2.5 ± 1.1	*	*	*
PC_3 reduced	772	196 ± 12	36.4 ± 4.1	0.9 ± 0.4	124 ± 11	28.2 ± 3.1	Trace
PC_3 oxidised	770	3.2 ± 1.1	31.2 ± 2.4	5.3 ± 1.7	2.4 ± 0.9	26.4 ± 2.3	3.7 ± 0.8
hPC_3 reduced	786	61.3 ± 7.4	12.7 ± 3.1	n.d.	*	*	*
hPC_3 oxidised	784	0.9 ± 0.3	9.8 ± 1.5	Trace	*	*	*
PC_4 reduced	1004** (502)	22.3 ± 5.6	0.6 ± 0.3	n.d.	29.1 ± 4.1	0.6 ± 0.2	n.d.
PC_4 oxidised	1002** (501)	Trace	Trace	Trace	Trace	Trace	Trace
hPC_4 reduced	1018** (509)	6.2 ± 0.9	n.d.	n.d.	*	*	*
hPC_4 oxidised	1016** (508)	n.d.	Trace	n.d.	*	*	*

Notes: [a]All amounts of PCs and hPCs are evaluated in relation to the amount of the reduced PC_2 in roots of the respective species taken as 100%; *hPCs are not synthesised in lupine plants [18]; **Ions $[M+2H]^{2+}$ at m/z in parentheses are also created for PC_4 and hPC_4; n.d. – not detected; trace – parent ion is present at the corresponding retention time, but no mass spectra recorded.

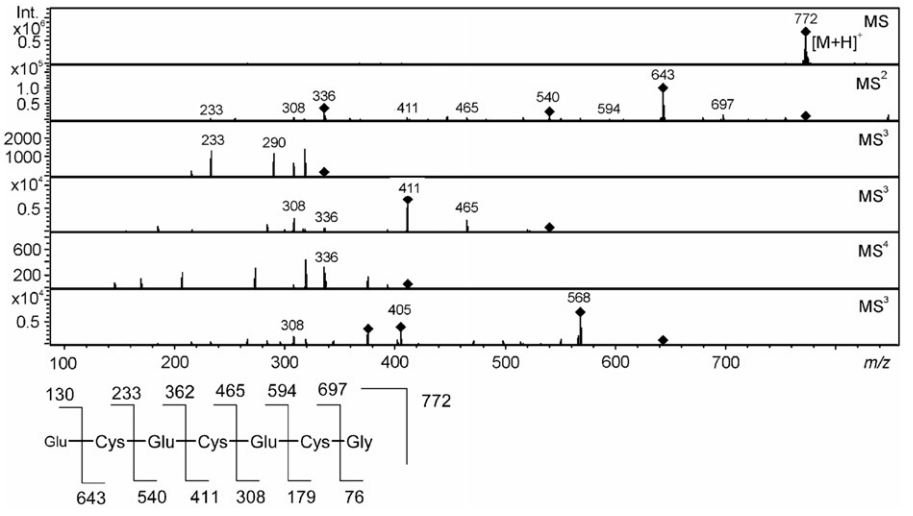

Figure 2. CID MS^n spectra of PC3 at m/z 772. The inserted scheme shows the origin of major ions in the spectra.

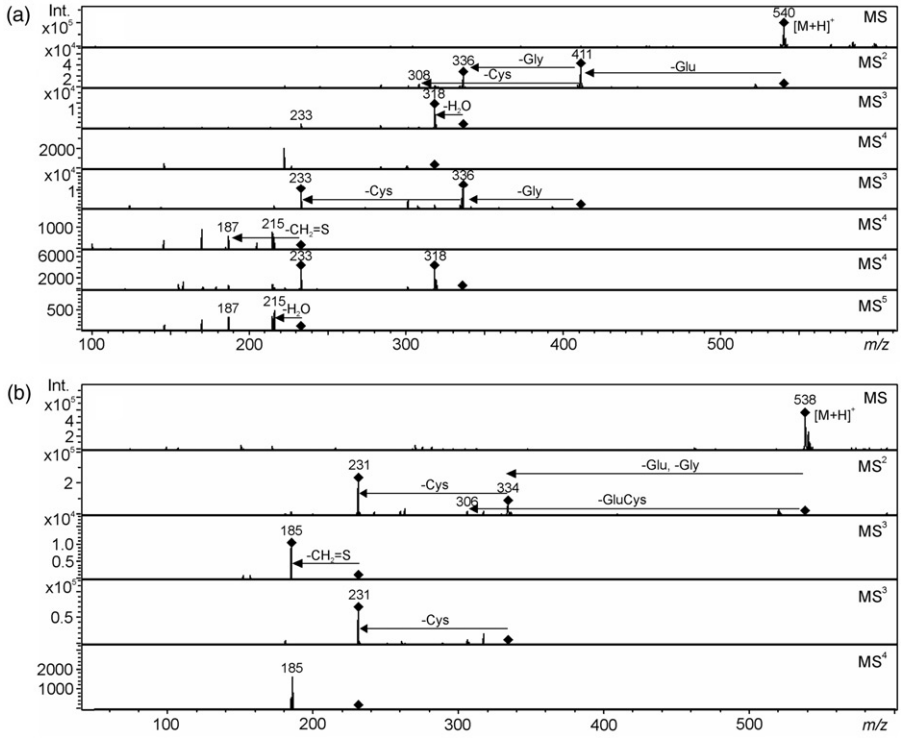

Figure 3. CID MS^n spectra of PC_2 $[M+H]^+$ at m/z 540 (a) and oxidised form of PC_2 $[M+H]^+$ at m/z 538 (b).

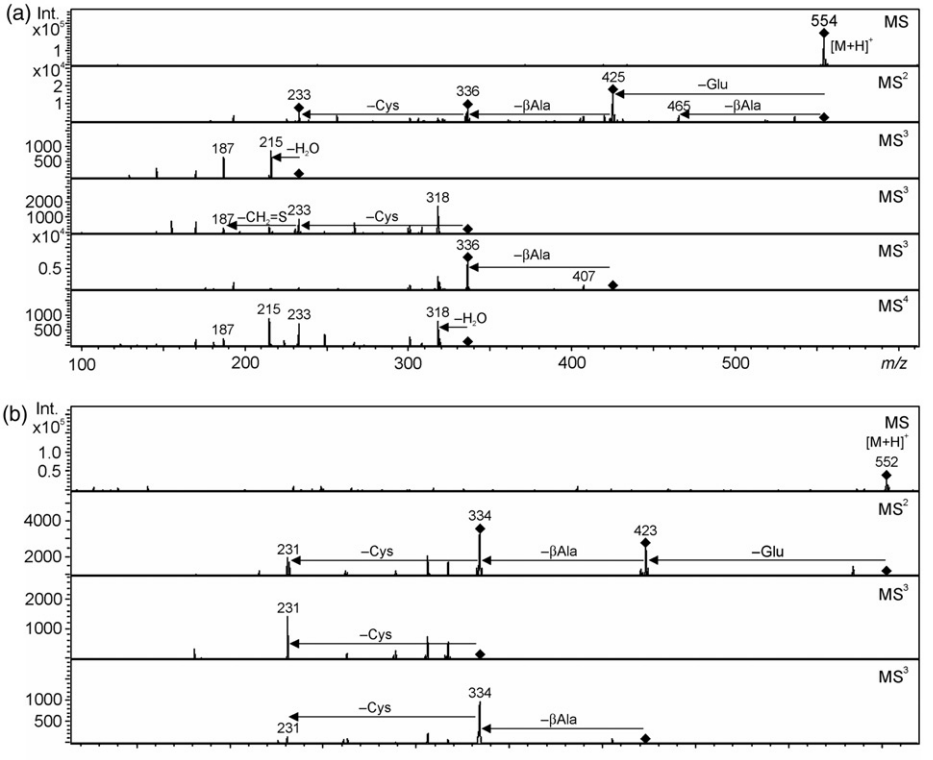

Figure 4. CID MS^n spectra of hPC_2 $[M+H]^+$ at m/z 554 (a) and oxidised form of hPC_2 $[M+H]^+$ at m/z 552 (b).

βAla group. The shown similarities of the retention times and m/z values of the oxidised and reduced forms of phytochelatins are a major reason for the difficulties in making a proper analysis of these peptides using HPLC of these compounds with the post-column derivatisation.

There is increasing information concerning accumulation of the oxidised forms of SH containing peptides taking part in the detoxification processes in plants. Such oxidised forms of peptides were detected in *Brassica juncea* [26] and *Rauwolfia serpentina* [8] and it was suggested to be the effect of transitions of As(III)/As(V). Moreover, in recent years several articles indicated the participation of PCs in antioxidation system and scavenging the reactive oxygen species (ROS) as superoxide anion radical $O_2^{-\bullet}$ and hydrogen peroxide H_2O_2. The heavy metals as many other biotic and abiotic factors initiate the oxidative stress [27,28]. It has been demonstrated that Cd and Pb cause ROS generation and contribute to the occurrence of the oxidative stress conditions in plant cells [28,29]. Plant cells protect themselves against the disadvantageous influence of heavy metals by activating the mechanisms that alleviate the cellular oxidative damage by scavenging the ROS species. These mechanisms involve low-molecular weight antioxidants such as glutathione (GSH), phenolics, ascorbate and enzymatic antioxidants, such as superoxide dismutases, catalase, glutathione reductase or ascorbate peroxidases [28]. It is supposed that GSH and peptides derived from it form the first line of defence against the heavy metal-mediated free radical formation. The prolonged exposure of plants to this stress

increases the activity of enzymatic antioxidants [30]. Tsuji *et al*. [31] reported that PCs synthesised in marine micro algae *Dunaliella tertiolecta* pretreated with Zn protected cells against the oxidative stress caused by hydrogen peroxide or paraquat. The PC_3 exhibited in vitro a scavenging activity towards H_2O_2 and $O_2^{-\bullet}$ higher than glutathione or ascorbate. Also Morelli and Scarano [32] found that some PCs were present in extracts from metal-free cells of the marine diatom *Phaeodactylum tricornutum* and they suggested that these PCs represented the oxidised forms.

PCs have generally $(\gamma\text{-Glu–Cys})_n$ Gly structure and therefore contain cysteine moieties with free SH groups in abundance. It has been reported that both glutathione, and metallothioneins (MTs) display antioxidant activities [33,34]. This implies that PCs may also act as antioxidants in the same manner as GSH and MTs in the cells. Our earlier studies [14,18,25] showed that large amounts of lead and cadmium were accumulated in the roots of pea and lupin plants and only minute amounts of metals were transported to the aboveground parts of plants. This suggests that probably the main function of phytochelatins in the roots of *Pisum sativum* and *Lupinus luteus* is the binding and sequestration of heavy metals. The relatively high level of oxidised form of PCs in leaves indicated that the high ROS level generated during photosynthesis irreversibly oxidised cysteinyl thiols both in GSH and phytochelatins to protect plants from the oxidative stress.

This hypothesis confirms reports that not only heavy metals can induce PC synthesis. Gamma radiation [35] and heat shock [36] also increased the formation of PCs in roots and leaves of plants. The authors suggest that PCs might have some other important roles in addition to the heavy metal detoxification. However, the mechanism of PCs participation in mitigation of oxidative stress is yet unclear.

4. Conclusion

Electrospray mass spectrometry allows the identification of phytochelatins in extracts from roots, stems and leaves of pea and lupin plants. Ion trap enables the determination of the amino acid sequence of PCs and unambiguous identification of these thiol compounds. The sensitivity of the HPLC-ESI-IT-MS method allowed the determination of phytochelatins in stems and leaves both in reduced and oxidised form which were not detected in these samples using HPLC with the post-column derivatisation method.

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