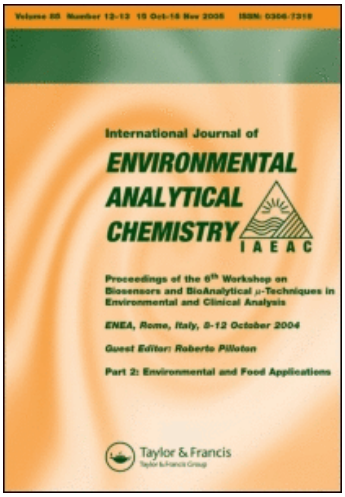

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E. Vassileva[a]; J. Deshusses[b]; D. Robin[c]; M. Martin[c]; W. Haerdi[c]
[a] Faculty of Chemistry, University of Sofia, Bulgaria [b] Department of biochemistry, University of Geneva, Switzerland [c] Department of Inorganic, Analytical and Applied Chemistry, University of Geneva, Geneve 4, Switzerland

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THE ROLE OF PROTEINS IN THE FIXATION OF HEAVY METALS ON OAK LEAVES

E. VASSILEVA[1], J. DESHUSSES[2], D. ROBIN[3], M. MARTIN[3]*, and W. HAERDI[3]

[1]Faculty of Chemistry, University of Sofia, Bulgaria. [2]Department of biochemistry, University of Geneva, Switzerland. [3]Department of Inorganic, Analytical and Applied Chemistry, University of Geneva, 30 quai Ernest-Ansermet, CH-1211 Geneve 4, Switzerland

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Dry oak leaves exhibit the property of accumulating metals when exposed to atmospheric pollution and thus can be used as bioindicators of metal pollution. In this paper the role of proteins, one of the components of leaves involved in the metal fixation, has been investigated.

Proteins and protein-metal complexes were extracted purified, separated and concentrated by means of ion exchange chromatography and electophoresis. The results revealed that three different types of proteins having different molecular weights are responsible for metal fixation.

KEY WORDS: Bioindicator, heavy metals, proteins, plants, oak leaves

INTRODUCTION

The oak leaves have shown by Robin *et al.*[1,2] to be interesting bioindicators for metals such as cadmium, zinc and lead. These leaves, when dry, accumulate heavy metals and bind to them strongly. However, when green, they do not accumulate these metals[3]. A direct correlation between the atmosphere metal fall out and the concentration of metals in the leaves was found, thus allowing calculations of metal fall out in a given site from the metal concentration measured in the oak leaves. This method was used to establish metal fallout map in the vicinity of an incineration plant.

Metal fixation by other plants and their use as bioindicators has been described in the literature[4,5]. The strong fixation of metals by plants is due to the tight interaction with the organic functional groups present in then plants[6–8]. For instance in the case of algae, amino, carboxylic, imidizole of histidine, oxygen and nitrogen bonds of peptides interact, in complexation reactions, with metals.

The interaction between the carboxylate oxygen and the sulphates with metals is of the electrostatic type[9].The mechanism of metal fixation by plants is still not fully elucidated yet, despite many studies on the bioaccumulation.

For better understanding of the mechanism of metal accumulation by dry leaves, it would be interesting to investigate which class of natural compounds present in these

Dedicated to Professor D. Klockow on the occasion of his 60th birthday.

* Corresponding author.

leaves may act as metal complexants. In this paper, the role of leaf protein as metal complexant has been investigated.

EXPERIMENTAL

Treatment of oak leaves

Oak leaves sampling. Oak leaves are collected and treated as described in the literature[1,2].

Extraction of leaf proteins. 10 g of dried oak leaves were added to a solution containing 10 ml of 0.1 M Tris-HCl buffer (pH = 8.6) and 0.1 M NaCl placed in a mortar. 0.1 ml of PMSF (phenyl methyl sulphonyl fluoride) were added to the mixture and the leaf tissues were ground with a pestle. The suspension was cooled to 0°C, left to stand at this temperature for 24 hrs, centrifuged for 1 h (4000 x g) at 4°C and the supernatant was stored at 4°C.

Sample Preparation. Dried oak leaves ground into powder were added to a solution containing 1 g/l of the target metal ion in such way that the leaves/solution ratio was 1:50 (w/v). This mixture is agitated for few hours and centrifuged. The supernatant solution was discarded. The residue was washed several times with distilled water to eliminate the unfixed metal ions. The metal bound was extracted with concentrated nitric acid.

Metal and protein analyses. The total protein concentration in the leaf extracts were determined using the procedure described by Bradford.[10] Bovine serum albumin was used as standard. Metal analysis in the samples were performed using flameless graphite furnace atomic absorption spectrometry (FAAS).

Separation by ion exchange chromatography

Chromatographic methods. 0.5 ml capacity column packed with DEAE-DE52 (diethylaminomethyl cellulose) was used. The proteins were eluted using a linear gradient of NaCl (0–0.5 M) solution buffered with 0.025 M Tris-HCl buffer solution (pH = 7.5). The total elution volume was 150 ml. Fractions of 1,5 ml were collected at the outlet of the column by means of a fraction collector.

Analysis of various protein fractions. Proteins were estimated by measuring absorbances at 280 and 254 nm. Metal analysis were done by means of FAAS, after nitric digestion.

Purification and preconcentration of proteins

Purification by ion exchange chromatography. A glass column packed with DEAE–DE52 anion resin (0.8 ml) was used for purifying proteins. The outlet of the column was connected to a peristaltic pump to obtain a constant flow rate of 10 ml/hr. The protein extracts were diluted four times (to lower the ionic strength) prior

chromatographic separation. 0.4 M NaCl was used for elution. Separations were performed at room temperature.

Concentration and analyses of proteins. The purified protein fractions that were rich in proteins were analysed directly whereas the other fractions were concentrated by lyophilisation.

Electrophoresis on acrylamide gel

A solution of the purified protein (0.9 ml) was placed in a 2 ml centrifugation tube and 0.1 ml of trichloracetic acid 72% (w/w) were added to precipitate the proteins. The mixture was centrifuged for 10 min at 14000 g, and the supernatant decanted immediately. The precipitate was washed with demineralised water and dissolved in sample buffer.[11] 1 ml of this solution was heated to 80°C in a thermostat for 10 min, cooled in an ice bath, and centrifuged for 10 mins at 14000 g to remove insoluble material.

Electrophoretic separations were carried out using polyacrylamide slab gel (slab gel Mini Protean, Bio-Rad). In order to separate small molecular weight proteins, a fairly high concentration of polyacrylamide (12% w/v) was employed. Freshly prepared gels were used. Gels were prepared using fixed proportion of acrylamide to cross linking agent N, N-methylene-bisacrylamide (0.8%) in the absence of SDS[10].

A 5 cm long acrylamide gel (12%) was used as the resolving gel and it was not padded with a stacking gel to avoid any significant variations in pH which could result in the release bound metal.

To monitor the migration front, a sample buffer consisting of 0.188 M Tris buffer, bromophenol blue and glycerine were added to the samples.

The separation of the proteins in the precipitate was achieved by applying a potential of 200 V for about 50 mins and using the Tris-glycine buffer as the electrophoresis running buffer.

Detection. The electrophoretically separated proteins were detected using Coomassie brillant blue stain. Silver staining was performed as described by Oakley[12] and adapted by Morrisey. The metals bound to proteins were determined by FAAS.

To determine metal content, unstained gels after electrophoresis were fractionated transversely into 2 mm slices using a home made razor blade slicer. Each slice was dissolved in 2 ml hot concentrated nitric acid (65%) solution and the metal concentration determined by FAAS.

RESULTS AND DISCUSSION

Metal and protein contents in leaf extracts

A few percent of proteins can be extracted from green leaves. The percentage of this in dried leaves, collected after 150 days exposure on the tress, rises to 0.2–0.3%.

Ion exchange chromatography of leaf extracts.

Only extracts of oak leaves naturally exposed to metal fallout from an incineration plant were investigated 60 to 70% of the metals accumulated on the leaves were extracted by

two successive extractions. Ion exchange chromatographic separation indicates that about 25% of the metals are bound to proteins.

Copper, cadmium and protein concentrations in the various fractions are shown in Figure 1. The three observed peaks suggest that three different types of proteins are present in the leaves.

Control experiments showed that free metal ions are not retained by the column and only the metals bound to proteins are retained. Fairly high absorbances observed at 280 nm may be due to the presence of proteins rich in aromatic amino acids.

The metal concentration to UV absorbance ratios at two wawelenghs (280 and 254 nm) in the three protein fractions were found to be as follows:

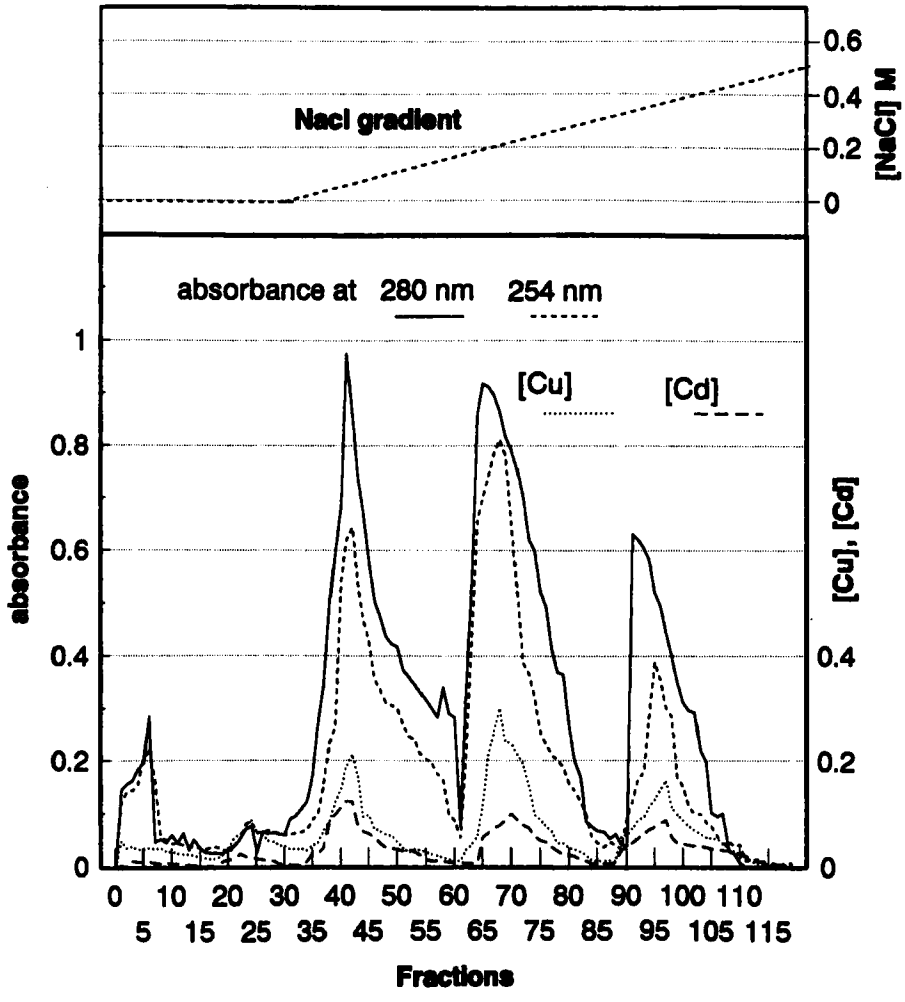

Figure 1 Separation of proteins bound to metal ions (Cu and Cd) in oak leaves by ion exchange chromatography. Concentration in μg/g. Conditions used:
Extracts of oak leaves concentrated by lyophilisation were used; volume of the column: 0.5 ml; volume of fractions collected: 1.5 ml; elution in Tris-HCi 25 mM pH 7.5, with a linear gradient of NaCl from 0 to 0.5 M.

Fractions	1	2	3		1	2	3
Cu/Abs_{280}	6.81	5.38	4.11	Cu/Abs_{254}	4.00	3.29	2.13
Cd/Abs_{280}	8.58	13.20	9.04	Cd/Abs_{254}	5.05	9.04	3.23

It is apparent that these ratios are different for the various fractions suggesting that the proteins present in these fractions have different structures, could have different affinities or could be contaminated by subtances of non proteic nature.

In order to get further insight on the nature of these proteins, electrophoretic measurements were carried out with these fractions. Owing to the low protein concentration in the sample no conclusive results could be obtained. Consequently, we used another approach to solve this problem. The proteins present in the leaves as a whole were preconcentrated by lyophilization and then separated by gel electrophoresis.

Analyses of leaf extracts by gel electrophoresis

Denaturating conditions were inapplicable since they result in the release of the bound metal. Metal-protein complexes were then separated in the absence of SDS[13], to retain native structure.

Untreated oak leaves sample (not enriched with metal) were then analysed. Two identical gel were run on one, proteins were revealed with silver stains (Figure 2). While in the other, slab was cut into slices and cadmium and copper concentrations determined by FAAS (Figure 3).

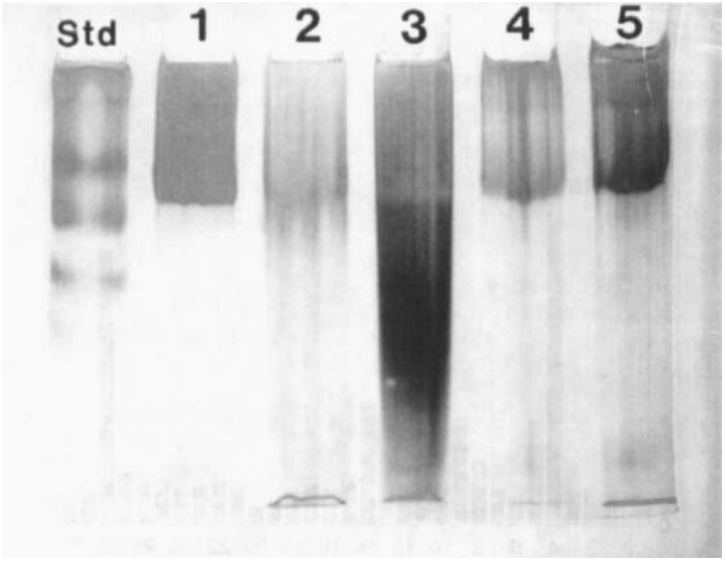

Figure 2 Typical results obtained for separation of proteins by electrophoresis under non denaturing conditions. Silver stains used for detection of protein.
Track 1 and 4: preconcentrated fraction of copper enriched leaf extracts (50 and 70 μg of proteins)
Track 2 and 3: crude cadmium enriched leaf extracts (30 and 70 μg of proteins)
Track 5: preconcentrated fraction of cadmium enriched leaf extracts (20 μg of proteins).

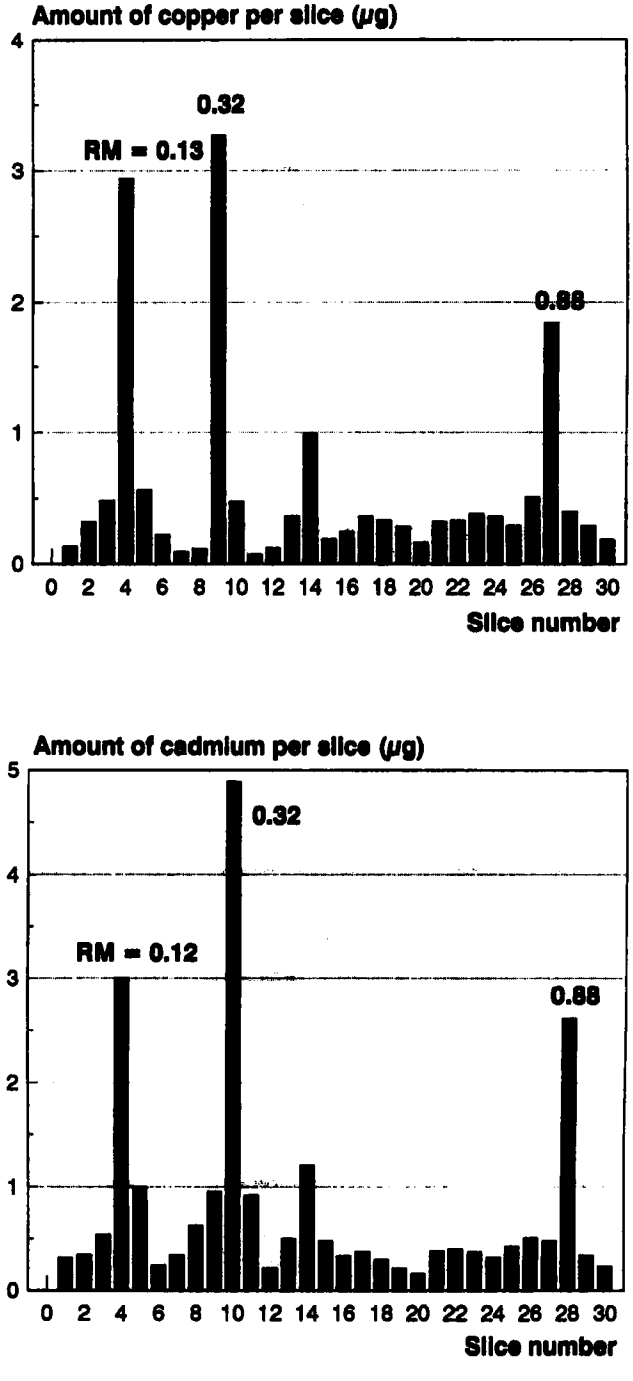

Figure 3 Cadmium and copper contents in a gel divided into 30 slices, after separation of by electrophoresis. Oak leaves exposed naturally to metal fallout were used.

Copper and cadmium accumulated into three bands at relative migrations of 0.12 0.32 0.88 respectively.

Accumulation of metals in three bands was observed at similar position as observed on the stained gel. Thus, again at least 3 types of proteins are responsible for the bioaccumulation of heavy metals on oak leaves.

Electrophoresis of the same samples under denaturing conditions were carried out to determine the molecular weight of the proteins present in the extracts. Relative migrations were used to as indicators for the presence of proteins.

These RMs correspond to the following molecular weights (PM):

RM	0.98	0.74	0.61	0.47	0.33	0.21
PM	12800	18600	27000	36300	49000	60225

At present, it is not possible to make correlation between the proteins loaded with metals as seen by non-denaturing electrophoresis and the bands observed in denaturing conditions. Further work with more refined techniques should be performed in order to characterize the nature of the protein-metal complexes.

CONCLUSIONS

The chromatographic as well as the electrophoretic analyses of oak leaves indicate that at least three types of proteins having different molecular weights that are responsible for the accumulation of metals.

These proteins could not be identified, but it is unlikely that the metal complexes observed are of the metal-thioneine type[13], from the difference in electrophoretic behaviour.

Proteins seem then to play an important role in metal fixation in the case of oak leaves. Although the oak leaves exposed to metal fall out contain low amounts of proteins (ca. 0.2%), they bind about 25 to 30% of the total cadmium, the remainder being fixed to other organic compounds. Further investigation must be carried out to define the role of other classes of compounds responsible of the binding of copper or cadmium. The best candidates would then be polysaccharides or glycolipides.

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