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Preparative Native Continuous Polyacrylamide Gel Electrophoresis (PNC-PAGE): An Efficient Method for Isolating Cadmium Cofactors in Biological Systems

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ABSTRACT

A new electrophoretic method [*p*reparative *n*ative *c*ontinuous *p*oly*a*crylamide *g*el *e*lectrophoresis (PNC-PAGE)] was developed to isolate cadmium cofactor-containing proteins in biological systems. For this purpose *Arabidopsis* cytosol was subjected to gel permeation chromatography (GPC) and high molecular mass cadmium proteins (MW $\approx$ 200 kDa) in a GPC fraction of this plant were isolated by PNC-PAGE. Furthermore plant cytosol was directly separated by this method. The cadmium concentrations in all GPC and PAGE fractions were

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determined by GF-AAS using matrix modifier. As electrophoresis buffer of the PAGE method a Tris–HCl buffer (20 mM Tris, 1 mM NaN_3, pH 10.00) was used. The gel (degree of polymerization of the polyacrylamide: 4%; gel length: 4 cm) was polymerized for 69 hr. By these procedures it was revealed that the high molecular mass cadmium proteins of *Arabidopsis* can be detected in quantitative amounts by using PNC-PAGE. It could be shown that the chemical structure of the cadmium proteins did not change under these PAGE conditions. PNC-PAGE is supposed to be an efficient method for isolating other metal cofactors such as Zn, Cu, Ni, Pd, Co, Fe, Mn, Pt, Cr, and Mo and might be suitable for subsequent structural determinations of metalloproteins present in PAGE fractions.

Key Words: Cadmium cofactors; *Arabidopsis*; PNC-PAGE.

INTRODUCTION

Protein and enzyme research play an important role in today's scientific discussions at questions of the relationships between structure and function of proteins and enzymes in biological systems.[1] About one-third of all known proteins contain metal cofactors and the vast majority of these function as essential metalloenzymes.[2] Cofactors are required to perform the biological activity of many proteins[3] and the presence of a cofactor often stabilizes native protein molecules.[4] The specific characteristics of metal ions, like e.g., Fe, Ni, Cu, Mn, or Mo in particular, as cofactors of proteins and enzymes are of biochemical relevance, because they play a key role in enzymatic catalytic process.[5] The qualitative determinations of these cofactors and the analysis of the metal cofactor/protein stoichiometries represent an important step when metal enzymes and metalloproteins are classified.[6]

Because of difficult and time consuming isolations and cleaning procedures enzyme samples are often available in small amounts only.[5] For the elucidation of the structures of certain metal proteins present in organisms larger quantities of these compounds have to be isolated in biological matrices by applying analytical methods such as extraction, gel permeation chromatography (GPC) and *p*reparative *n*ative *c*ontinuous *p*oly*a*crylamide *g*el *e*lectrophoresis (PNC-PAGE).[7] By these analytical processes proteins and enzymes must not be denatured because otherwise the function of a certain biomolecule can not be assessed. For those reasons preparative and native analytical methods for separating proteins and enzymes in biological samples are developed.

For example, an off-line coupling of GPC and PNC-PAGE was used to isolate high molecular mass cadmium proteins in different vegetable foodstuffs.[7] Plant cytosols of these matrices were subjected to GPC and

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the cadmium elution maxima were detected in the range of about 200 kDa. The cadmium proteins with molecular mass 200 kDa were further separated by a PNC-PAGE method. The results obtained from the electrophoretic separation of the high molecular mass cadmium proteins had to be regarded as semiquantitative because the cadmium concentrations detected in the respective PAGE fractions were very small and not reproducible.[7]

Therefore, a new preparative native continuous PAGE method was developed for the quantitative analysis of high molecular mass cadmium proteins in plants. Exemplary, this analytical system was used for separating protein molecules in cytosol samples of the model plant *Arabidopsis thaliana*.

EXPERIMENTAL

Gel Permeation Chromatography

Plant cytosol of *Arabidopsis* was separated by gel permeation chromatography. All parameters and experimental conditions of the GPC are pointed out in a study of Alt, Weber et al.[8] A Sephacryl S-400 Superfine (HR) column was calibrated with 4 high molecular weight globular proteins (aldolase 158 kDa, catalase 232 kDa, ferritin 449 kDa, and thyroglobuline 669 kDa, high molecular weight gel filtration calibration kit, Amersham Pharmacia). Gel permeation chromatography system (Amersham Pharmacia, Freiburg, Germany): Peristaltic pump (LKB pump P-1), one channel UV monitor (Uvicord S II, Pharmacia), automatic fraction collector (Recorder REC 102, LKB). Parameters: Column length: 700 mm; column diameter: 30 mm; buffer: Tris–HCl 20 mmol/L, NaN_3 1 mmol/L, pH 8.00; flow rate: 13.8 mL/hr; gel volume: 500 mL; fraction volume: 8.6 mL; detection wavelength: 254 nm.[8] Temperature of the separation system: 4°C; sample volume of the cytosol sample: 5 mL. The size-range of the GPC method used was about 20–8000 kDa for globular proteins. The cadmium concentrations of the resulted GPC fractions were analyzed by a GF-AAS method.

Electrophoretic Separation

Samples of *Arabidopsis* cytosol were either directly separated by a preparative native continuous PAGE method or first chromatographed on Sephacryl S-400 HR. Native method means that the metal cofactor-containing proteins to be separated must not be denatured nor that a metal cofactor is dissociated during the electrophoretic run. Continuous method refers to the electrophoresis buffer used in PNC-PAGE. Only one type of buffer with the

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same pH and composition, a Tris–HCl-buffer (pH 10.00; 20 mM Tris; 1 mM NaN_3) is applied to the anode chamber, cathode chamber and gel column of the Model 491 Prep Cell from Bio Rad.[9] Peripheral tools are PowerPAC 1000 (5 W constant, 8 hr), Model EP-1 Econo Pump (1 mL/min, 5 mL/fraction, 80 fractions, 80 mL prerun volume, 480 mL total volume), Model 2110 (fraction collector), Model EM-1 Econo UV Monitor (AUFS 1.0; $\lambda = 254$ nm), Model 1327 Econo Recorder (100 mV; 6 cm/hr), Buffer Recirculation Pump (95 mL/min), gel column (28 mm inner diameter), all Bio Rad. Degree of polymerization of the polyacrylamide: 4%. Gel length: 4 cm.

To prepare 40 mL of a gel (4%) the following chemicals were used: 32 mL purest water, 4 mL Tris–HCl stock solution (200 mmol/L Tris, 10 mmol/L NaN_3, pH 10.00), 4 mL acrylamide/bis stock solution (40%), 20 μL TEMED, 200 μL APS. Before usage all chemicals were adapted to room temperature, APS solution (10%) and gels were renewed at each trial. The gel solution was loaded with 3 mL 2-propanol and after a polymerization of 60 min the gel surface was rinsed with 8 × 4 mL electrophoresis buffer (pH 10.00; 20 mM Tris; 1 mM NaN_3). Then the surface was covered with 4 mL electrophoresis buffer (20 mmol/L Tris, 1 mmol/L NaN_3, pH 10.00).

For a total of 69 hr the gel was given time to be polymerized at room temperature before the electrophoretic prerun was started. As eluent a Tris–HCl buffer (20 mM Tris, 1 mM NaN_3, pH 8.00) was used. The separation system was cooled in a refrigerator at 4°C. Before the electrophoretic separation started the PAGE system was equilibrated and stabilized for 80 min. After 75 min of the electrophoretic prerun a cooled mixture of the cytosol sample (2.7 mL) or a respective GPC fraction (2.7 mL) and glycerol (0.3 mL) were carefully layered under the upper electrophoresis buffer on the gel surface.

Cadmium Determination

Cadmium concentrations in the resulted GPC and PAGE fractions were determined by use of GF-AAS (Perkin Elmer SIMAA 6000; Software: AAWinLab 2.50, Auto-Sampler: PE AS 72). All fractions were analyzed for cadmium without digestion using Pd–Mg matrix modifier. Small graphite tubes with L'vov platform were applied. So-called "end cap tubes" were inserted in a transverse heated graphite atomizer of the SIMAA 6000. By this procedure a higher sensitivity of the detection method was achieved as compared to measurements with standard graphite tubes.

Parameters: Resonance line: 228.8 nm; sample volume: 40 μL; modifier volume: 5 μL; Cd was analyzed in a linear mode with calculated intercept. Graphite furnace temperature program: Temperature (°C) (110; 130 drying);

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(500 matrix separation); (1500 atomizing); (2450 cleaning); ramp time (s) (1; 15; 10; 0; 1); hold time (40; 40; 20; 5; 3); all necessary parameters were read in the computer program. Cadmium concentrations in each fraction were determined 3-fold.

RESULTS AND DISCUSSIONS

An *Arabidopsis* cytosol sample was electrophoretically separated by PNC-PAGE. The UV absorption profile received showed two significant UV peaks in the range of PAGE fraction 21–25. Also in the elution range from fraction 6–20 a clear UV absorption was detected. The determination of cadmium in PAGE fractions 1–77 using GF-AAS revealed that there are cadmium concentrations up to about 2 ppb Cd analyzed in fraction 9–20. Then a cadmium peak with a concentration maximum of about 10 ppb Cd in fraction 24 followed. The results for Cd are illustrated in Fig. 1. The total amount of Cd recovered in the PAGE fractions added up about 250 ng Cd.

The cytosol fraction of this plant contained 97 ppb Cd according to an absolute amount of 262 ng Cd present in the cytosol sample (2.7 mL) to be separated by PNC-PAGE. The ratio of the cadmium amount (250 ng)

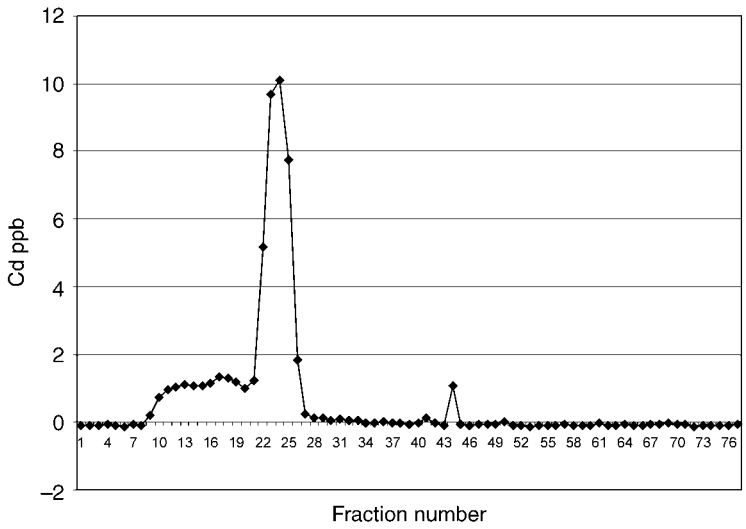

Figure 1. Cadmium elution profile after electrophoretic separation of *Arabidopsis* cytosol using PNC-PAGE. Determination of Cd in PAGE fractions in μg/L was carried out by use of GF-AAS.

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recovered in the PAGE fractions and the cadmium available in the respective plant cytosol sample (262 ng Cd) results in a cadmium recovery rate of about 95% after PNC-PAGE. Consequently, nearly the whole cadmium present in *Arabidopsis* cytosol is eluted after separation by a PAGE method. Therefore, the results for cadmium obtained by this method have to be regarded as quantitative.

A PAGE fraction containing the highest cadmium concentration (fraction 24, Fig. 1) was further separated by a GPC method to determine the molecular mass of the cadmium species present in this PAGE fraction. As a result cadmium eluted in a single peak in the high molecular mass range with an elution maximum of about 200 kDa after GPC. This result means that the cadmium proteins of *Arabidopsis* are chemically stable when separated by PNC-PAGE. Furthermore quantitative amounts of the high molecular mass cadmium proteins present in *Arabidopsis* cytosol are eluted in a few fractions by PNC-PAGE.

A cytosol sample of *Arabidopsis* was subjected to GPC and cadmium was determined in the different GPC fractions. High and low molecular mass cadmium species eluted over a broad molecular mass range in GPC. The most important cadmium species with a very low UV absorption were detected to be high molecular mass cadmium proteins having a molecular mass of about 200 kDa. A sample (2.7 mL) of the cadmium protein fraction with the highest cadmium concentration was directly separated by PNC-PAGE.

The electropherogram of this PAGE trial showed an extreme low UV absorption around fraction 23 and 24. PAGE fractions 1 to 77 were analyzed for Cd using GF-AAS. As result in Fig. 2 it is presented that cadmium was eluted in a single peak with a maximum cadmium concentration of about 0.5 ppb Cd detected in PAGE fraction 24. These results were reproduced. PAGE experiments revealed that the high molecular mass cadmium proteins of *Arabidopsis* present in a GPC fraction can be eluted in detectable amounts by PNC-PAGE. From the results obtained by the PAGE and GPC examinations it can be concluded that the chemical structure of the cadmium proteins of this plant did not change during the electrophoretic run.

This is an important result because data on the functions of metalloproteins in biological organisms mainly depend on the structural determination of the native forms of metal cofactor-containing proteins isolated by electrophoretic and chromatographic methods.

CONCLUSION AND FUTURE TRENDS

In this study a new PNC-PAGE method to separate cadmium cofactor-containing proteins in biological systems is presented. As example, high molecular mass cadmium proteins (MW $\approx$ 200 kDa) were isolated by this

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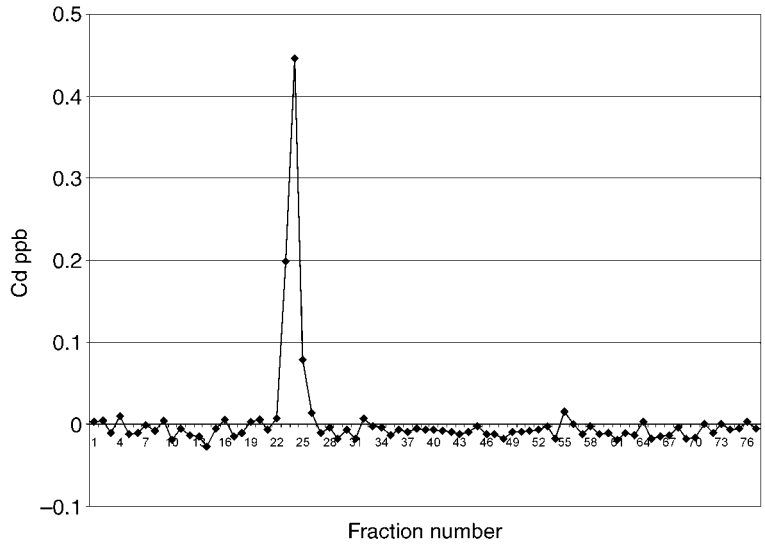

Figure 2. Cadmium elution profile after electrophoretic separation of a GPC fraction containing high molecular mass cadmium proteins (MW $\approx$ 200 kDa) of *Arabidopsis*. Determination of Cd in PAGE fractions in μg/L was carried out by use of GF-AAS.

method using *Arabidopsis* cytosol as sample. The cytosol sample was either directly separated by PNC-PAGE or subjected to GPC before applying this electrophoretic system. It was shown that PNC-PAGE is a very efficient method for separating high molecular mass cadmium proteins in plants. These compounds eluted in a single peak separated from the base line in the resulted electropherogram after the PAGE experiments.

It would be interesting to identify the isolated cadmium compounds and to elucidate the exact chemical structure of the high molecular mass cadmium proteins of the investigated plant. For this purpose MALDI-TOF-MS can be applied for an identification of the cadmium proteins present in the respective PAGE fractions. Also element species other than cadmium binding forms can be isolated by PNC-PAGE. For example, proteins containing metal cofactors such as Zn, Cu, Ni, Pd, Co, Fe, Mn, Pt, Cr, and Mo could be analyzed in PAGE fractions of plants using inductively coupled plasma mass spectrometry (ICP-MS) or total reflection x-ray fluorescence spectrometry (TXRF) as detection methods. Total reflection x-ray fluorescence spectrometry[10] is well suited to the quantitative determination of metals in proteins and enzymes.

There are different methods to determine the structures of cofactor-containing proteins in biological systems. For example, 3-D structures of protein cofactor complexes are elucidated by x-ray crystallography.[11] The

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availability of well ordered three-dimensional crystals is a prerequisite to obtain detailed knowledge on the structure of sized biological macromolecules detected by high resolution x-ray crystallography.[12]

In the future other biomatrices should be analyzed by PNC-PAGE. Metal proteins and metalloenzymes present in cytosol samples of human matrices and animal matrices as well as cytosols of microorganisms can be separated by GPC. Metal cofactor-containing proteins of these matrices which are eluted in the high molecular (>10 kDa) or low molecular (<10 kDa) mass range by this chromatographic method could be further isolated by PNC-PAGE. Using NMR spectroscopy as detection method it might be possible to elucidate the structures of high molecular and low molecular mass metal cofactor-containing proteins in PAGE fractions of biological samples.

NMR spectroscopy is used for three-dimensional structure determination of proteins and nucleic acids at atomic resolution but also structural, thermodynamic, and kinetic aspects of interactions between proteins and other components can be measured directly in solution applying this method. Solution NMR spectra have been recorded for structures with molecular weights up to 870,000 Da.[13]

In aqueous extracts of medicinal plants the binding forms of essential elements were analyzed because there is only little knowledge about the potential influence of metals on the pharmacological effects of natural drugs obtained from these organism. It was revealed that high amounts of metals Mn and Zn are present as low molecular weight species (<5000 Da) in the investigated plant extracts.[14] For isolating pharmacologically active compounds in phytosystems PNC-PAGE is supposed to play an important role.

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