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Journal of Chromatography A, 732 (1996) 390–393

JOURNAL OF CHROMATOGRAPHY A

Short communication

Rapid quantification of the nitrification inhibitor dicyandiamide in soil samples, nutrient media and bacterial cell-free extracts

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Received 18 July 1995; revised 13 November 1995; accepted 17 November 1995

Abstract

An analysis system based on high-performance liquid chromatography (HPLC) was developed for the quantification of dicyandiamide (DCD) present in different forms of aqueous solutions and cell-free extracts. This novel method is simple, rapid and sensitive with a quantification limit as low as 0.5 ng/μl. Separation was carried out on an H^+-cation-exchange column using 0.025 *M* H_2SO_4 as eluent. This method overcomes problems inherent to the standard colorimetric method, such as interference of proteins, urea or buffers.

Keywords: Soil; Dicyandiamide

1. Introduction

Dicyandiamide (DCD), or cyanoguanidine, is produced on a large scale from nitrochalk via the intermediate compound cyanamide in a two-step process [1]. In addition to its various industrial applications its potential as a nitrification inhibitor is of high importance in agriculture [2–5]. DCD is applied in combination with organic and inorganic nitrogen fertilizers like prilled urea, dung and ammonia compounds. Its nitrification inhibiting potential depends on soil pH, soil structure, humidity, temperature, time and method of fertilization and the rate of inorganic and microbial degradation [6–11]. For optimization of the application of DCD in agriculture and horticulture the degradation of DCD, as well as uptake by plants and loss by leaching need to be studied [9–16]. For such studies the development of an accurate and sensitive assay is necessary. Separation on a cation-exchange column combined with UV detection provides the basis for a highly reproducible and sensitive method for DCD quantification.

So far, this method was applied in the following investigations. (1) Isolation of DCD degrading soil bacteria by enrichment cultures. For this purpose bacteria were extracted from soil and cultivated in minimal medium containing DCD as single nitrogen source. DCD degrading strains were isolated, and their growth rate and degradation activity determined under pure culture conditions [15]. (2) With one of these strains physiological [15] and enzymatical studies in cell-free extracts [14] were carried out for further investigation of the DCD degradation. (3) Genomic DNA fragments from DCD degrading soil bacteria were cloned in *Escherichia coli*; subsequent-

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0021-9673/96/$15.00
SSDI 0021-9673(95)01283-4

ly, these clones were screened for DCD degrading activity. All these investigations required the development and application of the new DCD quantification method described in this study.

2. Experimental

2.1. Chemicals and solutions

All chemicals were of analytical grade, unless otherwise noted. Deionized water was used for nutrient media and aqueous extracts.

Bacteria were grown in the following nutrient media:

DCD medium [17] containing (per litre) 10.25 g $Na_2HPO_4 \cdot 12H_2O$; 5.2 g KH_2PO_4; 5.5 g glucose·H_2O; 1.6 g DCD; 0.4 g $MgSO_4 \cdot 7H_2O$, 40 mg $CaCl_2 \cdot 2H_2O$, supplemented with solutions of $ZnCl_2$ 10 μl; $FeSO_4$ 10 μl; $MnSO_4$ 5 μl; $CoCl_2$ 5 μl, $MoPO_4$ 5 μl and $CuSO_4$ 1 μl (salt concentration 10 g/l each). This medium was modified by adding penicillin V (50 mg/l), actidion (50 mg/l) and 0.1% Tween-80 (puriss.) for special applications.

Minimum medium M9 [18] was made up from the following four solutions: (1) 20 g glucose·H_2O in 500 ml H_2O; (2) 2.5 g $MgSO_4 \cdot 7H_2O$ in 100 ml H_2O; (3) 0.2 g $CaCl_2 \cdot 2H_2O$ in 100 ml H_2O and (4) 35 g $Na_2HPO_4 \cdot 2H_2O$; 15 g KH_2PO_4; 2.5 g NaCl; 5 g NH_4Cl in 500 ml H_2O. After sterilization (120°C, 20 min) 100 ml of solutions (1) and (4) and 10 ml of solutions (2) and (3) were mixed with 750 ml H_2O.

Complex medium (TY) contained 8 g bacteriological peptone (US Biochemical 20048), 5 g yeast extract (Merck 3753) and 2.5 g NaCl per 1000 ml H_2O.

Soil extracts were prepared by shaking (2 min, end-over-end, 60 rpm) 10 g (fresh weight) soil with 90 ml 1:4 concentrated Ringer solution (Merck 15525) or 90 ml NaCl solution (0.9%).

2.2. Preparation of samples for DCD quantification

Soil extracts, bacterial cultures, culture filtrates and other aqueous solutions were centrifuged in 1.5-ml disposable tubes for 10 min at 15 000 *g* and 4°C. From a 1-ml sample 600 μl of the supernatant were transferred to a fresh autosampler tube to avoid resuspension of the pellet. Protein-rich samples like cell-free extracts had to be deproteinized. For the cation H^+-column separation precipitation with 5% trichloroacetic acid (TCA) and centrifugation for 15 min at 15 000 *g* and 4°C gave the best results. Samples were kept in 1.5 ml polypropylene tubes at 4°C.

Standard solutions were prepared as similar to the respective sample as possible, using nutrient media, soil extracts and cell-free extracts as matrix for the DCD solutions.

2.3. HPLC-based DCD assay

The following HPLC system was used: Bio-Rad 1350 soft-start pump; Bio-Rad 1306 UV-monitor or ISCO S500 absorbance detector, detection wavelength 210 nm; Bio-Rad autosampler AS 100, cutout injection with 20-μl injection loop, overfill 60 μl, filling speed 0.5 ml/min, flushing volume 1 ml, Bio-Rad column heater at 65°C. Recording and computing of data was carried out with Bio-Rad HRLC 800 software run on an IBM PC (PS2/60).

Separation was carried out on a prepacked cation H^+-column (30×4.6 mm I.D.), usually used as a guard column. This is an Animex hydrogen form column with a divinylbenzene (DVB) styrene matrix, particle size 9 μm, cross-linkage 9%, operation pH-range 1–3. The separation conditions were as follows: isocratic elution at a flow-rate of 0.6 ml/min using 0.025 *M* H_2SO_4 as mobile phase. The column was heated to 65°C. One run takes about 3 min.

Regeneration, necessary only after more than 100 runs of cell-free extracts, was carried out by flushing with several column volumes of eluent containing 50% methanol. The methanol content was changed following a linear gradient program over 10 min. For re-equilibration the column was flushed with eluent for 2 h, although a stable baseline was already reached after 15 min.

3. Results and discussion

The combination of separation on a cation H^+-column and UV detection facilitated optimization of the chromatographic system with regard to develop-

ment of a highly sensitive and selective DCD quantification method. Due to detection based upon absorption at 210 nm only very few sample components other than DCD show any signal. Substances which gave signals could be clearly separated from DCD using 0.025 *M* H_2SO_4 as eluent. Therefore addition of BSA or urea did not affect the measurement. At eluent concentrations lower than 0.025 *M* DCD gives rise to double peaks. These double peaks may derive from hydration of DCD in an acid environment [1]. At the acid concentrations used, probably all DCD is hydrated before separation.

The postulated intermediates of DCD degradation such as guanylurea, guanidine, urea or cyanamide [12], which have to be expected in soil samples and culture liquids, did not interfere with the DCD measurement. At a column temperature of 65°C and a flow-rate of 0.6 ml/min DCD was eluted after 1.6 min giving rise to a single distinct peak (Fig. 1).

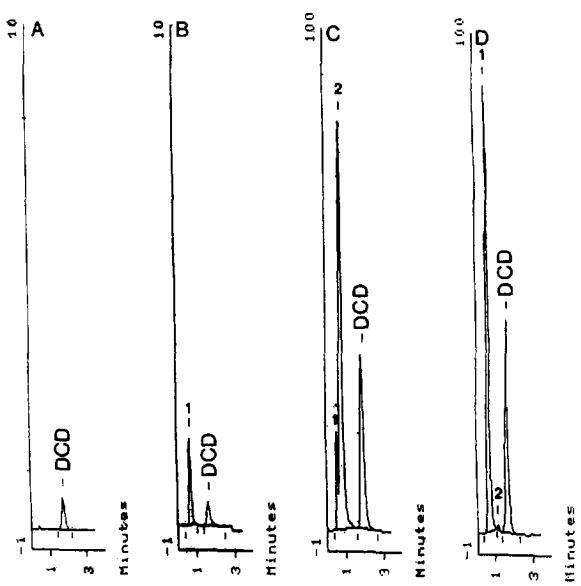

Fig. 1. HPLC chromatograms of two separations near the detection limit representing: (A) 1 ng DCD/μl in aqueous solution; (B) 1 ng DCD/μl in an aqueous soil extract; (C) 500 μM DCD in DCD medium supplemented with thiamine (1 m*M*) and IPTG (isopropyl-β-D-thiogalactopyranoside, 10 m*M*); (D) 500 μM DCD in a deproteinated cell-free extract of *E. coli*, as used in degradation studies. Separation on a cation H^+-column (30×4.6 mm I.D.), 65°C, elution 0.6 ml/min 0.025 *M* H_2SO_4, UV detection at 210 nm. Peaks: 1, unretained substances; 2, undetermined substances not interfering with DCD.

The separation and detection system was highly reproducible for DCD amounts between 0.5 and 50 ng/μl. Calibration was done by injection of different concentrations of DCD dissolved in nutrient medium. A typical equation for the standard line calculated from peak area (pixels on screen) of five repetitive injections of 20 μl each was: $y=15.50x+2.05$ (y=pixels on screen, $x=\mu$g DCD/ml; $r_{val}=0.999$). To avoid peak broadening due to overloading of the small column (30×4.6 mm I.D.) the injection volume was set to 20 μl, using cutout injection. After more than 5000 analytical runs the retention time decreased to 1.4 min, probably due to a decrease of free interactive protons on the stationary phase. The peak symmetry of 1.4 measured at 10% of peak height did not change when the retention time decreased. The DCD peak still was well separated from any other peak.

After analysing protein-free or low-protein samples, such as nutrient media (i.e., DCD, M9 and TY) or soil extracts, there was no need to wash the column with organic solvents. Only after more than 100 analyses of TCA-deproteinized cell-free extracts, the column had to be cleaned by flushing with several volumes of eluent containing 50% methanol. Based on all the data obtained so far, this system can be regarded as suitable for the detection and quantification of DCD in aqueous samples, minimal or complex nutrient media and TCA precipitated bacterial cell-free extracts.

Acknowledgments

We would like to thank the 'Bund-Bundesländer-kooperation' for financial support.

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