ELSEVIER

Journal of Chromatography A, 803 (1998) 315–320

JOURNAL OF CHROMATOGRAPHY A

Short communication

Separation of respective species of phytochelatins and their desglycyl peptides (class III metallothioneins) and the precursors glutathione and γ-glutamylcysteine with capillary zone electrophoresis

Hiroki Kubota, Kyoko Sato, Takashi Yamada, Tamio Maitani*

National Institute of Health Sciences, Kamiyoga 1-18-1, Setagaya, Tokyo 158, Japan

Received 5 August 1997; received in revised form 24 November 1997; accepted 28 November 1997

Abstract

By exposure to excess heavy metals, plants induce phytochelatins [PCs; $(\gamma\text{-Glu–Cys})_n\text{–Gly}$, $n \geq 2$] and their desglycyl peptides (hereafter, both are referred to as class III metallothioneins, CIIIMTs). To analyze respective CIIIMT species induced by cadmium along with glutathione (GSH) and γ-Glu–Cys (γEC) that may be precursors of CIIIMT, HPCE was applied. EDTA and ABD-F (4-aminosulfonyl-7-fluoro-2,1,3-benzoxadiazole, a thiol (SH)-reacting agent) were added to the supernatant fraction of root cultures of *Rubia tinctorum* L., and the solution was applied to HPCE apparatus [capillary zone electrophoresis, borate buffer (pH 8.5)], and the SH group was monitored at 380 nm. GSH, γEC, and respective CIIIMT species were separated well, and the analysis was performed within 6 min. Consequently, HPCE was found to be very useful for CIIIMT analysis. © 1998 Elsevier Science B.V.

Keywords: Phytochelatins; Peptides; Metallothioneins; Glutathione; Glutamylcysteine; Proteins

1. Introduction

Phytochelatins [PCs; $(\gamma\text{-Glu–Cys})_n\text{–Gly}$, $n \geq 2$] are induced in plants through exposure to excess heavy metals. The role of PCs is proposed in metal detoxification and metal homeostasis [1]. Recently, therefore, PCs have also been used as indicators of forest decline caused by heavy metal pollution [2,3].

Although the structures of PCs are conservative in a wide range of plants, we have shown by post-column derivatization HPLC and LC–MS that the desglycyl peptides of PCs are also induced when large amounts of PCs are induced by cadmium (Cd) in root cultures of *Rubia tinctorum* L. [4]. PCs and desglycyl PCs are classified as class III metallothioneins [5], and therefore both together are referred to as CIIIMTs hereafter.

To clarify the induction mechanism of the desglycyl PCs, the determination of glutathione (GSH) and γ-Glu–Cys (γEC), which may be precursors of PCs [6], is essential. However, the separation of these two compounds is poor on the post-column derivatization HPLC analysis under the conditions used for PC determination [7].

Capillary zone electrophoresis (CZE) has been applied successfully to the separation of various peptides [8,9]. Therefore, we applied HPCE to determine the two compounds and respective

*Corresponding author

0021-9673/98/$19.00
PII S0021-9673(97)01229-6

CIIIMT species simultaneously. EDTA and ABD-F (4-aminosulfonyl-7-fluoro-2,1,3-benzoxadiazole, a thiol (SH)-reacting agent) [10] were added to the sample solution to prevent interference of Cd bound to CIIIMTs, and to detect the SH group of GSH, γEC, and respective CIIIMT species at 380 nm, respectively.

2. Experimental

2.1. Reagents

Cadmium chloride hemipentahydrate and reduced glutathione were purchased from Wako (Osaka, Japan). γEC was obtained from Kohjin (Tokyo, Japan). EDTA disodium salt was purchased from Dojindo Labs. (Kumamoto, Japan). Other chemicals were of reagent grade or the highest grade commercially available. Ultrapure water (≥18 MΩ cm) prepared with a Milli-Q SP Reagent Water System (Millipore, Bedford, MA, USA) was used as water throughout the experiment.

2.2. Tissue culture in Cd-containing medium

Root cultures used were those reported previously [11]. Cd (1 m*M*) and GSH (2 m*M*) were added to the culture medium, and the root cultures were maintained for 5 days, then washed with water, and stored at −80°C.

2.3. Postcolumn derivatization HPLC analysis

PCs and desglycyl PCs were analyzed according to the method of Grill et al. [7] with some modifications reported previously [4].

2.4. HPCE analysis with ABD-F

A 0.2-g portion of the root cultures was milled in two volumes of 0.1% $NaBH_4$ aqueous solution (400 μl) with an agate motor. After centrifugation (11 000 *g*, 10 min), the supernatant fraction was obtained (approximately 500 μl). CZE was performed using a P/ACE 5500 system with System Gold software (Beckman Instruments, Fullerton, CA, USA). An untreated fused-silica capillary of 57 cm (detection window at 50 cm)×75 μm I.D. (Beckman) was housed in a cartridge which allowed liquid cooling. The capillary was prepared by flushing the column 5 min each with 0.1 *M* HCl and 0.1 *M* NaOH solutions alternatively twice, and with water for 1 min, and then rinsed with the buffer solution for 1 min. Between the sample analyses, the capillary was successively rinsed with 0.1 *M* NaOH (5 min), water (1 min), and the buffer (1 min). To 20 μl of the supernatant fraction of CIIIMT-containing root cultures, 10 m*M* (in general) EDTA (5 μl) and 20 m*M* ABD-F (20 μl) were added, and the solution was reacted at 50°C for 15 min. Pressure (0.5 p.s.i.) injection for 5 s was used, and HPCE run was performed with 100 m*M* borate buffer (pH 8.5 in general) (voltage, 30 kV; temperature, 25°C), and the SH-containing compounds were detected at 380 nm with diode array detector (Beckman).

2.5. Metal determination

Portions of about 0.1 g of the root cultures were digested with mixed acid (HNO_3–$HClO_4$, 5:1), and the solution was diluted with water to 3 ml. Metal concentrations were determined by inductively coupled plasma atomic emission spectrometry (ICP-AES) (ICAP-61, Thermo-Jarrell Ash, Waltham, MA, USA).

3. Results and discussion

3.1. Effect of EDTA

CIIIMTs induced by Cd are bound with Cd through the SH group [11]. Accordingly, ABD-F may not react with SH group of CIIIMTs in the supernatant fraction. To prevent the interference of Cd (and other metals), therefore, EDTA was added to the supernatant fraction. Fig. 1 shows the effects of EDTA on the electropherogram. Assignments were performed with the respective isolated species [4]. The peak areas of $(\gamma EC)_3G$, $(\gamma EC)_3$, $(\gamma EC)_4G$, $(\gamma EC)_4$, and $(\gamma EC)_5G$ increased with the EDTA concentration until 10 m*M*, and those of $(\gamma EC)_2G$ and $(\gamma EC)_2$ increased until 2 m*M*, while those of GSH and γEC remained almost constant regardless of the presence of EDTA.

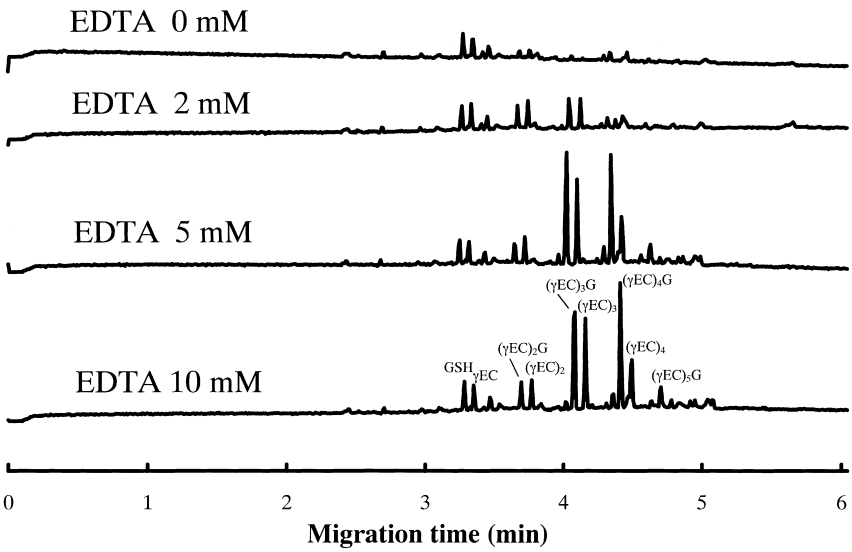

Fig. 1. Dependency of peak areas of GSH, γEC, and CIIIMT species on the concentration of EDTA added.

The above findings may be interpreted as follows: metals bound to GSH and γEC, if any, are replaced easily by ABD-F without EDTA, while EDTA is required to remove metals bound to CIIIMTs. The fact that larger CIIIMT species require a higher concentration of EDTA is consistent with the view that, in larger PC molecules, Cd is sequestered tightly into a $Cd(SCys)_4$ structure [12].

In Fig. 1, EDTA concentration was sufficient at 10 m*M* under the sample conditions. EDTA was added to prevent the interference of Cd (and other metals) bound to CIIIMTs. Therefore, the required concentration may be mainly dependent on the Cd concentration in the supernatant fraction.

As described in Section 2.4, a 20-μl portion of the supernatant fraction was mixed with a 5-μl portion of EDTA solution. The Cd content analyzed by ICP-AES was a 0.04 μmol/20 μl supernatant fraction. Therefore, when 10 m*M* EDTA solution was added, EDTA:Cd is 5:4 in molar basis. Namely, the required molar content of EDTA was near the total Cd content. This result may indicate that most Cd ions should be chelated before ABD-F (an SH-reacting agent) reacts with the CIIIMT molecule. When both free Cd and CIIIMT-bound Cd are present, EDTA may react first with free Cd and then CIIIMT-bound Cd. Therefore, an EDTA content near the total Cd content may be required to remove Cd from the CIIIMT molecule.

3.2. Molar ratio of ABD-F to the SH group

In this study, ABD-F that was developed as a fluorescence reagent for the SH group was used as a colorimetric reagent for the SH group. In general situations, the content of CIIIMT or the SH group is not known before analysis. When fluorescence detection is available, a sufficiently high ABD-F/SH ratio is readily attainable, because the SH content in sample solution could be much lower. For example, Imai and Toyo'oka proposed an analytical procedure with an ABD-F/SH ratio of 100 [13]. On the other hand, ABD-F/SH ratio is not so high in colorimetry because of the sensitivity at 380 nm and the solubility of ABD-F. Therefore, it is necessary to ascertain the sufficienct amount of ABD-F to be added.

Fig. 2 shows the dependency of peak area on the ABD-F concentration. At least 10 m*M* ABD-F was necessary, which corresponded to an ABD-F/SH ratio of about 7, based on the calibration curves for GSH and γEC (data not shown). An ABD-F concentration of higher than 20 m*M* was impracticable because of its solubility in water. In the actual analysis by colorimetry, a retrial with a half dose of

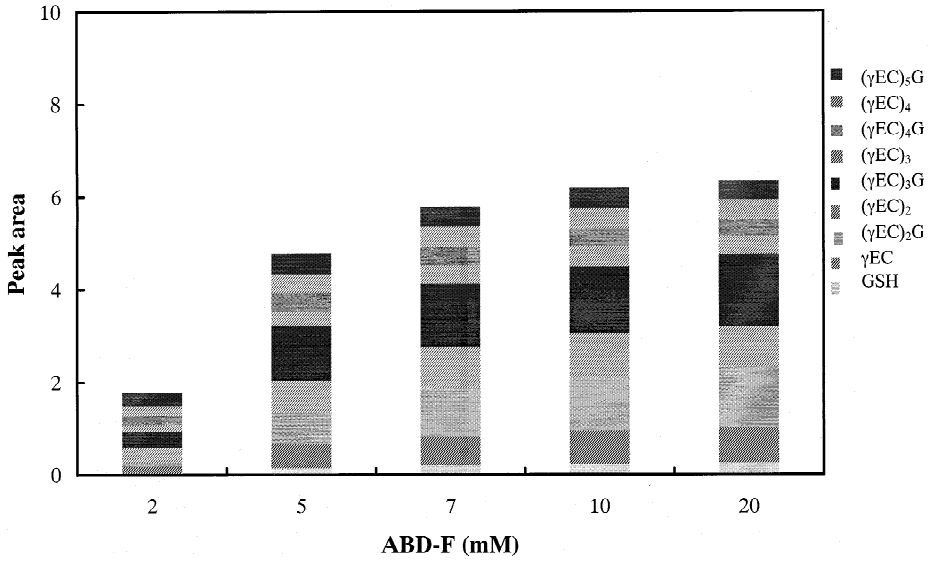

Fig. 2. Dependency of peak areas of GSH, γEC, and CIIIMT species on the concentration of ABD-F added.

one sample may be necessary for a series of similar samples to ascertain the sufficiency of ABD-F concentration.

Imai and Toyo'oka recommended a reaction time of 5 min [13]. Under our conditions, however, a reaction time of 5 min was insufficient, and 15 min was necessary because of the higher concentration of the SH group.

3.3. Effect of running buffer pH

Fig. 3 shows the effect of running buffer pH on the peak areas and migration times of GSH, γEC, and CIIIMT species. At pH 7.0, the peak areas were small and the separation of the compounds was not good (data not shown). Although the separation became better and the migration times were short at

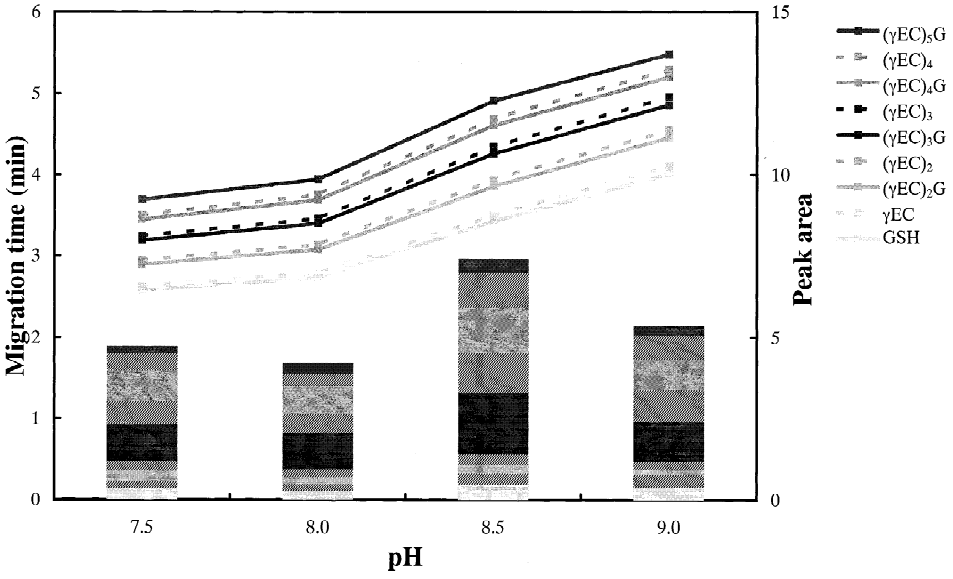

Fig. 3. Effect of running buffer pH on peak area and migration time of GSH, γEC, and CIIIMT species.

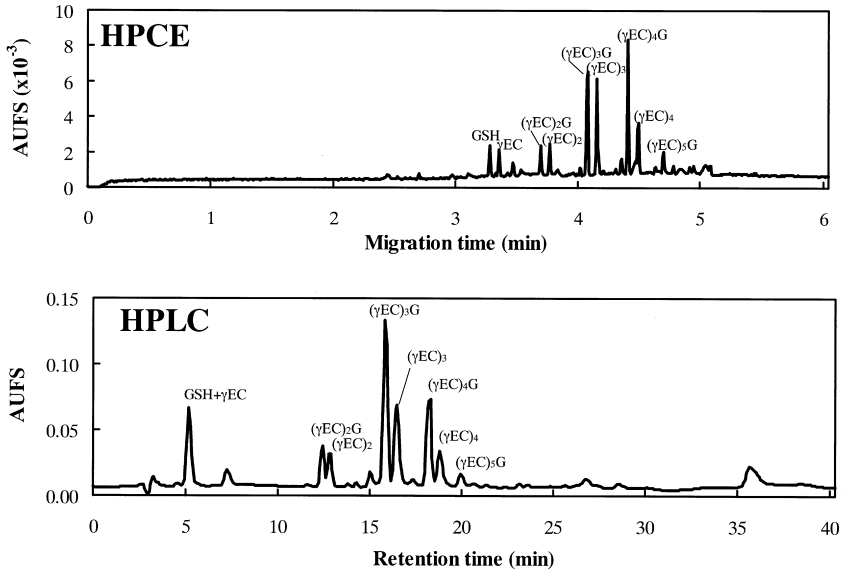

Fig. 4. Comparison of HPCE electropherogram and postcolumn derivatization HPLC chromatogram for the supernatant fraction of CIIIMT-containing root cultures.

pH 7.5 and 8.0, the peak areas were still small. The peak areas were largest at pH 8.5, and this pH was selected in other experiments.

3.4. Calibration curve

To compare the peak area for both GSH and γEC, the calibration curve linearity was followed in a 1:1 mixture of the two compounds. The concentration of added ABD-F was set at 20 m*M*. Both calibration curves were linear (R^2=0.999 and 0.991 for GSH and γEC, respectively) up to 1.5 m*M* each (3.0 m*M* total SH) and almost overlapped each other (data not shown), which suggested that the reactivity and peak area of both compounds were almost identical.

3.5. Comparison of HPCE with postcolumn derivatization HPLC

Fig. 4 shows the HPCE electropherogram and postcolumn derivatization HPLC chromatogram for the supernatant fraction of CIIIMT-containing root cultures. In HPCE, the peaks with migration times of 3.27 and 3.33 min were assigned to GSH and γEC, respectively, by co-analysis with authentic compounds. Thus, the GSH and γEC were separated well in HPCE. On the other hand, GSH and γEC were not separated in HPLC, at least under the conditions used for PC determination. In HPCE, moreover, respective CIIIMT species were also separated well. Furthermore, the analysis was performed within 6 min in HPCE, while 20 min were required in the postcolumn derivatization HPLC analysis. Consequently, the HPCE method accompanied by sample pretreatment with EDTA and ABD-F was found to be very useful for the analysis of GSH and γEC with CIIIMTs.

Acknowledgements

This study was supported in part by a grant from the Japan Health Sciences Foundation.

References

[1] W.E. Rauser, Plant Physiol. 109 (1995) 1141.
[2] J.E. Gawel, B.A. Ahner, A.J. Friedland, F.M.M. Morel, Nature 381 (1996) 64.
[3] S.B. McLaughlin, Y. Wand, Trends Plant Sci. 1 (1996) 249.
[4] H. Kubota, K. Sato, T. Yamada, T. Maitani, Plant Sci. 106 (1995) 157.

[5] J.H.R. Kägi, M. Vašák, in: C.D. Klaassen, K.T. Suzuki (Eds.), Metallothionein in Biology and Medicine, CRC Press, Boca Raton, FL, 1991, p. 49.
[6] Y. Hayashi, C. Nakagawa, N. Mutoh, M. Isobe, T. Gotoh, Biochem. Cell Biol. 69 (1991) 115.
[7] E. Grill, E.-L. Winnacker, M.H. Zenk, Proc. Natl. Acad. Sci. USA 84 (1987) 439.
[8] V.J. Hilser Jr., G.D. Worosila, S.E. Rudnick, J. Chromatogr. 630 (1993) 329.
[9] H.-J. Gaus, A.G. Beck-Sickinger, E. Bayer, Anal. Chem. 65 (1993) 1399.
[10] T. Toyo'oka, K. Imai, Anal. Chem. 56 (1984) 2461.
[11] T. Maitani, H. Kubota, K. Sato, T. Yamada, Plant Physiol. 110 (1996) 1145.
[12] H. Strasdeit, A.-K. Duhme, R. Kneer, M.H. Zenk, C. Hermes, H.-F. Nolting, J. Chem. Soc. Chem. Commun. (1991) 1129.
[13] K. Imai, T. Toyo'oka, Methods Enzymol. 143 (1987) 67.