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Short communication

Comparison of methanol and acetonitrile eluents for the quantitation of chelators specific to soft-metal ions by HPLC

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ABSTRACT

HPLC eluent systems employing acetonitrile and methanol were evaluated for the quantitation of glutathione (GSH) and phytochelatin (PC_n), a family of peptides implicated in heavy-metal detoxification in higher plants. The detection system is based on the dequenching of copper(I)–bathocuproine disulfonate and is specific for soft-metal chelators. Although both elution systems yielded comparable analytical performance for each PC_n, the acetonitrile system had a lower sensitivity for GSH and a steadily increasing baseline. The inferior properties of the acetonitrile system may be due to complex formation between acetonitrile and Cu(I) ions. Both methods were applied to measure peptide levels in the primitive red alga *Cyanidioschyzon merolae*. Coefficients of variation (CVs) were less than 5%, except for GSH and PC_4 determinations in the acetonitrile system, in cases when CV values were found to be 8.8% and 6.3%, respectively. Recoveries were greater than 96%, except for GSH determination in the acetonitrile system, with a recovery of 84.4%; however, the concentration measured in the acetonitrile system did not differ from that measured in the methanol system at a significance level of 0.05.

1. Introduction

The toxicity of Cd(II) in both the plant and animal kingdoms is widely known. To alleviate metal toxicity, peptides named phytochelatins (PCs) are synthesized in high plants [1], algae [2], and some fungal species [3]. A PC has a general structure of (γ-Glu-Cys)$_n$-Gly (PC_n), with $n \geq 2$, and it can bind Cd(II) through the thiolate groups of the peptide. PC_n peptides are synthesized using glutathione (γ-Glu-Cys-Gly; GSH) and/or previously synthesized PC_{n-1} as substrates in a reaction catalyzed by phytochelatin synthase (PCS), where Cd(II) is required to activate the enzyme [4].

Quantitation of these peptides has relied solely on their thiol groups. Separation of the peptides on an HPLC column and a post-column reaction with 5,5′-dithiobis(2-nitrobenzoic acid) yields the 2-nitro-5-thiobenzoate anion. Monitoring the absorption of this anion affords moderate levels of sensitivity [5] and is frequently employed in PCS enzyme assays [6]. Derivatization of the thiol groups with the fluorescent reagent monobromobimane (mBBr) prior to HPLC analysis and the use of a fluorescence detection system also result in high sensitivity [5]. However, this method is rarely applied in enzyme assays because excessive levels of GSH may remain in the PCS assay solution and interfere with the fluorescent labeling of PC_n. Recently, an HPLC detection system for chelators that favorably bind to soft-metal ions was established in which competition for Cu(I) between the fluorescent reagent bathocuproine sulfonate (BCS) and the chelators of interest was exploited [7,8]. After HPLC separation and post-column mixing with a solution containing Cu(I)–BCS complexes, the chelators capable of binding Cu(I) prevented binding of Cu(I) ions to BCS, thereby allowing free BCS molecules to fluoresce. The present method employs post-labeling strategies that afford a sensitive PCS activity assay. Additionally, its sensitivity to PC_n was comparable to that of the mBBr pre-label method [9]. Differences were observed in baseline levels and signal intensities of GSH when eluting with acetonitrile and methanol.

2. Experimental

2.1. Chemicals and reagents

Phytochelatins PC_2, PC_3, and PC_4 were purchased from Hayashi-Kasei (Osaka, Japan); BCS and GSH were purchased from Sigma–Aldrich (Tokyo, Japan). $CuSO_4 \cdot 5H_2O$ and ascorbic acid were purchased from Wako Pure Chemicals (Tokyo, Japan), HPLC grade acetonitrile and methanol were from Kokusan Chemical (Tokyo, Japan), and *N*-cyclohexyl-2-aminoethanesulfonic acid was obtained from Nacalai Tesque (Kyoto, Japan).

2.2. Analytical procedure

After injection of a 20 μL sample, the peptides were separated on an octadecylsilane column (4.6 mm $\phi \times$ 150 mm, TSKgel ODS-80^{TM};

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Tosoh, Tokyo, Japan), and the eluate was merged with a post-column solution containing 0.2 μM $CuSO_4$, 0.5 μM BCS, and 5 μM ascorbic acid in a 50 mM *N*-cyclohexyl-2-aminoethanesulfonic acid–NaOH buffer (pH 10.0). Cu(II) ions were reduced to Cu(I) ions by a reaction with the ascorbic acid. A stream of He gas was bubbled through the post-column solution to prevent reoxidation of the Cu(I) ions. In the acetonitrile elution system, the mobile phase consisted of 5% acetonitrile for the first 5 min. This was followed by a linear gradient elution from 5 to 20% acetonitrile over 25 min in a background of 0.1% trifluoroacetic acid (TFA). The methanol system began with 8% methanol for 5 min prior to an 8–25% linear gradient of methanol over 25 min in a background of 0.1% TFA. The mobile phase was eluted using a PU-1580 pump (Jasco, Tokyo, Japan) at a flow rate of 1.0 mL/min. The post-column solution was eluted using a PU-980 pump (Jasco, Tokyo, Japan) at a flow rate of 1.0 mL/min. The fluorescence intensity was monitored using an FP-920 fluorescence detector (Jasco) at 395 nm with an excitation wavelength of 280 nm.

2.3. Culturing of *Cyanidioschyzon merolae* and the determination of peptide concentrations

The alga *Cyanidioschyzon merolae* (10D strain) was distributed from the Microbial Culture Collection of the National Institute for Environmental Studies (Tsukuba, Japan). The algal cells were grown as described previously [10] using modified Allen's medium consisting of 40 mM $(NH_4)_2SO_4$, 4 mM $MgSO_4{\cdot}7H_2O$, 4 mM KH_2PO_4, 1 mM $CaCl_2$, 100 μM Fe-ethylenediaminetetraacetic acid, 100 μM H_3BO_3, 30 μM $MnCl_2{\cdot}4H_2O$, 1.5 μM $ZnCl_2$, 4.5 μM Na_2MoO_4, 0.6 μM $CoCl_2{\cdot}6H_2O$, and 0.6 μM $CuCl_2$. The pH of the solution was adjusted to 2.8 with H_2SO_4. The cells were cultured phototrophically at 37 °C under continuous illumination at 32 μmol photon m^{-2} s^{-1} with continuous shaking at 100 rpm.

Upon reaching an optical density of 1.0 at 750 nm, the culture was supplemented with 1.0 mM $CdSO_4$ and incubated for another 2 h. The cells were collected by centrifugation at 1500 × *g* for 15 min at 4 °C. After centrifugation, 380 μL of 0.1% TFA was added, and the cells were sonicated on ice. The homogenate was centrifuged at 22,000 × *g* for 5 min at 4 °C. The resulting supernatant was adjusted to 50 mM $HClO_4$ prior to filtration through a membrane filter with a pore size of 0.22 μm (Millipore, Bedford, MA, USA). While measuring PC_n concentrations, the solution was diluted with 0.1% TFA by a factor of 2 in both elution systems. While measuring GSH concentrations, the solution was diluted with 0.1% TFA by factors of 10 and 100 in the acetonitrile and methanol elution systems, respectively. Sample volumes of 20 μL were subjected to analysis.

3. Results and discussion

3.1. Method validation

Fig. 1a shows an HPLC chromatogram of a solution containing GSH (5 μM) and PC_2–PC_4 (500 nM each) obtained with the acetonitrile elution system. The peak corresponding to GSH elution exhibited a retention time of 3.23 min, followed by peaks corresponding to PC_2, PC_3, and PC_4 at retention times of 7.16, 15.05, and 19.91 min, respectively. Each of the peptides was sufficiently resolved using this elution system. Although the baseline level remained nearly unchanged at retention times up to 10 min, it continuously increased between 10 and 35 min.

The methanol elution system also yielded a well-resolved chromatogram for the peptides, with peaks corresponding to GSH, PC_2, PC_3, and PC_4 appearing at retention times of 3.33, 8.24, 17.93, and 25.38 min, respectively (Fig. 2a). In contrast to the acetonitrile system, however, no baseline elevation was observed when eluting with methanol. In fact, baseline levels in the methanol system decreased slightly in a linear fashion during the course of the analysis (Fig. 2a).

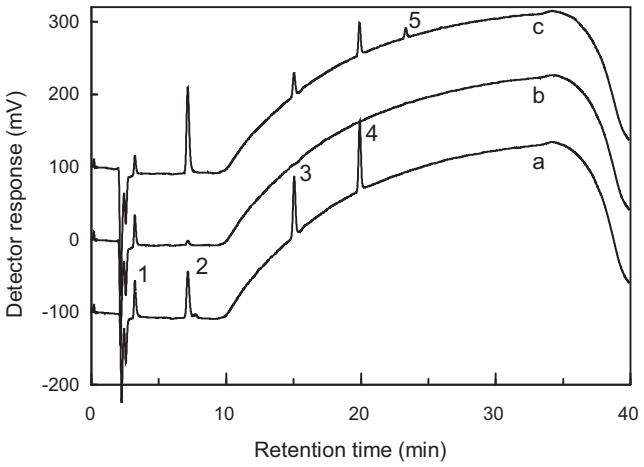

Fig. 1. HPLC chromatograms of GSH and PC_n using the acetonitrile elution system. Peaks corresponding to GSH, PC_2, PC_3, and PC_4 are marked 1, 2, 3, and 4, respectively. (a) A standard solution containing 5 μM GSH and 500 nM PC_n. (b) A minute PC_2 signal is apparent in the chromatogram of the extract obtained from *C. merolae* cells incubated in the absence of Cd. In (c), all of the above peptides and an additional unknown peak (5) are evident in the chromatogram of the extract obtained from *C. merolae* cells incubated in 1.0 mM Cd.

The increased baseline levels observed during sample elution with acetonitrile may be due to the formation of complexes between acetonitrile and Cu(I) [11]. The binding of Cu(I) by acetonitrile would result in an increase in the relative concentration of unbound, fluorescent BCS molecules. This is consistent with an increase in baseline levels as more acetonitrile is added to the post-column solution during sample elution.

Furthermore, peak intensities for GSH in the acetonitrile system were lower than those in the methanol elution system (Fig. 1a *versus* Fig. 2a). This trend was evident despite 10-fold concentration differences. The lower sensitivity of GSH is evidenced by a comparison of calibration slopes obtained using the two elution systems. The slope of the line obtained using the acetonitrile system was 0.076, whereas that of the line obtained using the methanol system

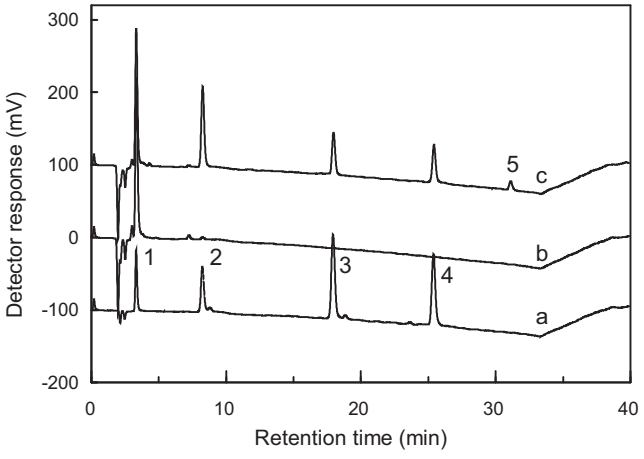

Fig. 2. HPLC chromatograms of GSH and PC_n using the methanol elution system. Peaks corresponding to GSH, PC_2, PC_3, and PC_4 are marked 1, 2, 3, and 4, respectively. (a) A standard solution containing 500 nM GSH and 500 nM PC_n. (b) A minute PC_2 signal is apparent in the chromatogram of the extract obtained from *C. merolae* cells incubated in the absence of Cd. In (c), all of the above peptides and an additional unknown peak (5) are evident in the chromatogram of the extract obtained from *C. merolae* cells incubated in 1.0 mM Cd.

Table 1
Calibration line equations and limits of detection for GSH and PC_n using the acetonitrile elution system.

Peptides	Linear regression equation	Linearity range (nM)	R^2	Detection limit (nM)[a]
GSH	$y = 0.076x + 1.758$	0–1000	0.9919	72.6
PC_2	$y = 1.605x + 1.887$	0–1000	0.9955	4.15
PC_3	$y = 2.457x - 41.304$	0–1000	0.9982	1.74
PC_4	$y = 2.671x + 37.312$	0–1000	0.9932	1.27

[a] Detection limit is defined as the concentration of peptide yielding a peak area corresponding to three times the standard deviation of the peak area obtained from three replicate measurements of GSH (500 nM), PC_2 (50 nM), PC_3 (20 nM), and PC_4 (20 nM) concentrations.

Table 2
Calibration line equations and limits of detection for GSH and PC_n using the methanol elution system.

Peptides	Linear regression equation	Linearity range (nM)	R^2	Detection limit (nM)[a]
GSH	$y = 2.751x + 5.353$	0–100	0.9910	1.94
PC_2	$y = 1.950x + 7.796$	0–600	0.9961	1.87
PC_3	$y = 3.641x + 67.226$	0–600	0.9934	1.04
PC_4	$y = 2.906x + 26.493$	0–600	0.9959	1.37

[a] Detection limit is defined as the concentration of peptide yielding a peak area corresponding to three times the standard deviation of the peak area obtained from three replicate measurements of GSH (10 nM) and PC_n (10 nM) concentrations.

was 2.751 (Tables 1 and 2). In contrast, comparable slope values were obtained in both systems for PC_n (Tables 1 and 2). These sensitivities are consistent with the similar detection limits obtained for both systems.

Complex formation between acetonitrile and Cu(I) may cause a buffering action with respect to the level of free Cu(I). Weak bonding between GSH and Cu(I) would modestly reduce Cu(I) levels and give rise to small enhancements in the fluorescence of the post-column solution. The strong binding of PC_n to Cu(I) may outweigh the buffering capacity of the Cu(I)—CH_3CN system, resulting in high signals in the acetonitrile elution systems. The absence of such a buffering action in the methanol elution system may account for the higher slope of the calibration line and the lower detection limit for GSH. The solvent may affect signal intensity as revealed by the calibration-line slopes for PC_n in the methanol elution system. The slope of the calibration line for PC_2 is lower than that for GSH, and the slope of the calibration line for PC_4 is lower than that for PC_3 regardless of the binding constant [12] (Table 2). Taken together, these findings indicate that both solvent systems exhibited comparable sensitivities for PC_n, whereas markedly lower sensitivity was observed for GSH in the acetonitrile elution system.

3.2. Determination of GSH and PC_n concentrations in *Cyanidioschyzon merolae* cultured with or without Cd

HPLC chromatograms obtained using the acetonitrile elution system of the extract of *C. merolae* in the absence and presence of Cd(II) show the Cd(II)-induced synthesis of PC_n (b and c in Fig. 1). A similar result was obtained using the methanol elution system (b and c in Fig. 2). Peptide concentrations in the algal cells treated with Cd(II) were determined using both eluent systems. The results for acetonitrile and methanol elution are shown in Tables 3 and 4, respectively. With the exception of GSH in the acetonitrile system, peptide recovery was greater than 96.0%. The poor recovery and high variability of GSH measurements in the acetonitrile system (Table 3) may be due to the lower sensitivity described above. Although the acetonitrile elution system yielded nominally lower GSH concentrations than the methanol elution system, a statistical analysis of peptide concentrations revealed no difference between the two eluents at a significance level of 0.05.

Table 3
Concentrations of GSH and PC_n in the extract of *C. merolae* cells incubated in a Cd-containing medium and their percent recovery using the acetonitrile elution system.

Peptide	Concentration (pmol/unit of *C. merolae* cells)[a]			Recovery (%)
	Initial	Added	Found	
GSH	683 ± 60.3	250	894 ± 38.0	84.4 ± 28.5
PC_2	342 ± 3.06	25	367 ± 1.24	100.0 ± 1.3
PC_3	49.9 ± 2.48	25	73.9 ± 1.62	96.0 ± 1.2
PC_4	45.1 ± 2.90	25	71.6 ± 2.09	106.0 ± 1.4

[a] One unit is defined here as the amount of *C. merolae* cells in 1 mL of culture with an OD_{750} of 1.0.

Table 4
Concentrations of GSH and PC_n in the extract of *C. merolae* cells incubated in a Cd-containing medium and their percent recovery using the methanol elution system.

Peptide	Concentration (pmol/unit of *C. merolae* cells)[a]			Recovery (%)
	Initial	Added	Found	
GSH	789 ± 29.6	250	1034 ± 17.2	98.0 ± 13.7
PC_2	345 ± 4.17	25	371 ± 2.35	104.0 ± 1.9
PC_3	44.1 ± 1.99	25	70.3 ± 2.53	104.8 ± 1.9
PC_4	41.9 ± 1.50	25	67.0 ± 1.55	100.4 ± 0.9

[a] One unit is defined here as the amount of *C. merolae* cells in 1 mL of culture with an OD_{750} of 1.0.

4. Conclusions

Two HPLC eluents, acetonitrile and methanol, were compared in the quantitation of GSH and PC_2–PC_4 using fluorescence dequenching of the Cu(I)–BCS complex. The elution systems exhibited comparable analytical performance for PC_n. However, the acetonitrile elution system was much less effective in determining levels of GSH.

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