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Short communication

Fast determination of glutathione by capillary electrophoresis with fluorescence detection using β-cyclodextrin as modifier

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ABSTRACT

A rapid analytical method was developed for the quantitative determination of glutathione (GSH) using capillary electrophoresis and fluorescence detection. A fluorescence derivatization reagent, naphthalene-2,3-dicarboxaldehyde (NDA) was successfully applied to label GSH. The optimal derivatization reaction was performed with 5.0 mM NDA, 20 mM borate buffer (pH 9.2) with the reaction time of 4 min at room temperature. The capillary electrophoresis analysis of GSH could be achieved in less than 120 s using 10 mM sodium tetraborate (pH 9.2) containing 2.5 mM β-cyclodextrin (β-CD) as the running buffer, and the detection limit of 2.5×10^{-9} M (S/N = 3) was obtained. This method was successfully applied to analyze the content of GSH in tobacco BY-2 cells.

1. Introduction

Glutathione (γ-glutamyl-cysteinyl-glycine, GSH), a low-molecular-weight thiol, plays an important role in the control of plant growth and development. Since it highly participates to the fight against reactive oxygen species (ROS), GSH is implied into numerous antioxidant mechanisms. Recently, the role of GSH in cellular regulation other than scavenging ROS has been found [1]: tracheary element differentiation, a programmed cell death process to transform the cells into a water-conducting pipe, the G1-S transition in the cell cycle, flowering, anthocyanin accumulation, enzymatic regulation, translational and transcriptional regulations, and detoxification of xenobiotics and heavy metals. Recent findings on the GSH-associated events in plants have been comprehensively reviewed by Ogawa [1].

Owing to its biological and clinical significance, numerous methods have been developed to determine its concentration in different samples. Capillary electrophoresis (CE) method coupled with UV detection has been used [2], but the sensitivity is comparably low. To improve the sensitivity, many fluorescence reagents have been explored for the derivatization of GSH, such as dansyl chloride [3], 4-chloro-7-nitrobenzo-2-oxa-1,3-diazol (NBD-Cl) [4], 5-bromomethylfluorescein (5-BrF) [5], fluorescein-5-maleimide (FM) [6], 5-iodoacetamidofluorescein (5-IAF) [7,8], monobromobimane (MBB) [9] and 4-(aminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (ABD-F) [10,11]. However, the derivatization selectivity of above reagents was not good enough.

On the other hand, *o*-phthalaldehyde (OPA) [12–14] and naphthalene-2,3-dicarboxaldehyde (NDA) [15–17] have been widely used as fluorescence reagents for the derivatization of amino acids and primary amino compounds in the presence of nucleophilic reagent, such as thiol and CN^-. Recently, Rammouz et al. [18] have given an in-depth review on the use of NDA for the analysis of primary amines in HPLC and CE. Also OPA [19–21] and NDA [22–25] have been demonstrated to be selective reagents for the detection of GSH, since GSH containing both primary amine and thiol moieties, could react with OPA and NDA without the presence of any other additional nucleophile. In comparison with OPA, the utilization of NDA as the derivatization reagent possessed some advantages [24], such as even higher fluorescent yields of the resultant adduct, better selectivity, and less interference.

In this work, we developed a rapid CE method coupled with fluorescence detection for the determination of GSH, which was labeled with NDA. The separation time was less than 120 s with 2.5 mM β-cyclodextrin (β-CD) as the additive of running buffer, and the detection limit of 2.5×10^{-9} M was achieved. Compared to other CE and fluorescence detection of GSH, the separation time of this method was lower than that obtained by FM, 5-IAF, MBB, NBD-Cl, and ABD-F, but similar to that of 5-BrF; the sensitivity was better than that achieved by 5-BrF, FM, 5-IAF, MBB and NBD-Cl, but was similar to that obtained by ABD-F. Also the method was successfully applied to the determination of GSH in the tobacco BY-2 cell lysates.

2. Experimental

2.1. Apparatus and chemicals

The setup used in this work is the same as we reported previously [17]. Briefly, a laboratory-built system based on an upright

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fluorescence microscope (Olympus, Japan), a photo-multiplier tube (PMT), a ±30 kV high-voltage dc power supply was used, and a uncoated fused-silica capillary of 42 cm (30.5–31 cm length to the detector window) × 50 μm I.D. × 365 μm O.D.(Yongnian Optical Conductive Fiber Plant, China) was used for separation. A 100-W high-pressure mercury lamp was used as the excitation radiation. The optical sub-system in the microscope consisted of a 40× objective, a NIB excitation cube including an excitation filter (EX 400–490 nm), a dichroic mirror (DM 510 nm) and a barrier filter (BA 515 nm). The signal from the PMT was monitored using photon-counting device (Beijing Bingsong Photon Technological Corporation, China) and collected by a computer with photon-counting software, and processed with Origin software packages.

GSH standards and NDA were purchased from Sigma (St. Louis, MO). β-CD was purchased from Merck. Other chemical reagents were of analytical grade and used without further purification. The stock solutions of GSH were prepared at a concentration of 1.0×10^{-2} M in 0.01 M hydrochloric acid, and stored in a refrigerator (4 °C) for a maximum period of a week. Further dilutions were made with de-ionized water, which was purified with Milli-Q system (Millipore, Bedford, MA, USA). The stock solution of NDA was prepared in methanol at a concentration of 10.0 mM, stored at 4 °C, and diluted to desired concentration with methanol prior to use.

2.2. Pre-capillary derivatization procedures

One hundred microliters of GSH standards solution, 10 μl of 2.0×10^{-2} M sodium tetraborate buffer (pH 9.2), and 10 μl of 5.0×10^{-3} M NDA solution were added sequentially. The resultant solution was thoroughly mixed, then allowed to stand for 4 min at room temperature in dark. Then the derivatized solution was immediately injected for CE without dilution.

2.3. Capillary electrophoresis

Ten millimolar sodium tetraborate buffer (pH 9.2) containing 2.5 mM β-CD was used as the CE running buffer and prepared daily. New capillary was pre-treated with 1.0 M NaOH, and water for 30 min sequentially. Prior to use, the capillary was rinsed with 0.1 M NaOH, and water for 5 min, respectively, and followed by preconditioning with running buffer for 10 min. Separations were carried out at constant voltage of 21 kV and the operating current was 13.5–14 μA. Sample injection was performed by hydrodynamic mode with sampling height at 14 cm for 42 s.

2.4. Sample preparation

The tobacco (*Nicotiana tabacum*) BY-2 cells were cultured in modified MS medium supplemented with 1.0 mg/l of 2,4-dichlorophenoxyacetic acid (2,4-D) and 3.0% sucrose at 28 °C in darkness. 0.052 g (fresh weight) of BY-2 cells was grounded in liquid nitrogen, and immediately grinded in the mortar for 5 min in the presence of 1 ml ethanol, which was used to precipitate the protein. Then, de-ionized water was added to dilute the extracts. The extracts were transferred to a centrifugal tube and the final volume of extract was 12.3 ml. The extracts were centrifuged at 344 × *g* for 3 min, and the supernatant was further diluted 1:9 with water. The diluted lysate was immediately derivatized with NDA following the procedure mentioned above.

3. Results and discussion

3.1. Choice of derivatization conditions

NDA was first developed by Orwar et al. [24] to be a selective reagent for the derivatization of GSH to produce high fluorogenic derivatives, because NDA could react with both amino and sulfhydryl groups of the GSH molecule, and no additional CN^- was required. In this work, the derivatization conditions of NDA with GSH were further investigated. Fig. 1 shows the effect of pH value of derivatization buffer on the relative fluorescence intensity (RFI). It was seen that when the pH value was varied from 5.0 to 9.2, the RFI rapidly increased due to the increasing concentration of fluorescent molecule, since pH value decreases the concentration of the non-fluorescent molecule increases [26]; and when the pH value was above 9.2, the RFI decreased slowly. Thus, pH 9.2 is selected for derivatization. To ensure the complete reaction, the effect of concentration of NDA on RFI was investigated. The RFI increased with the increase in concentration at 0.31–5.0 mM, and then leveled off with further increasing the concentration. Therefore, 5.0 mM NDA was chosen for further study. The effect of the reaction time varying from 1 to 50 min on RFI was also studied. The reaction almost completed in 1 min, and when the reaction was longer than 5 min, the RFI began to decrease slowly due to the degradation of the derivative. Therefore, 4 min was used as the reaction time. In summary, the derivatization reaction was carried out with 5.0 mM NDA, and 20 mM borate buffer (pH 9.2) with the reaction time of 4 min at room temperature in dark.

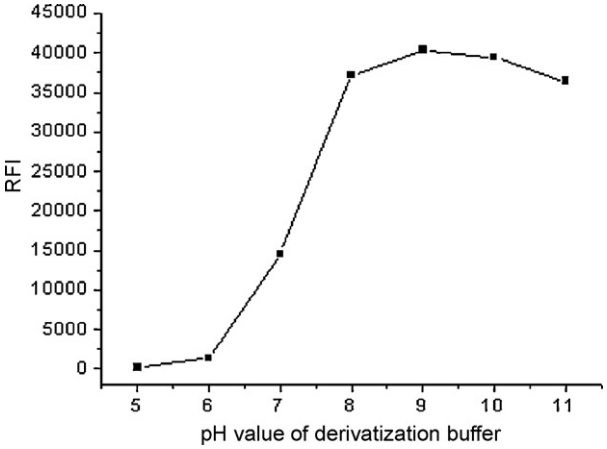

Fig. 1. Effect of pH value of derivatization buffer on the RFI. *Conditions*: CE buffer, 20 mM sodium tetraborate (pH 9.2); separation voltage, 21 kV; hydrodynamic injection, 14 cm (height) for 40 s; the concentration of GSH, 1.0×10^{-6} M.

3.2. Optimization of CE separation procedure

It is well known that the acidity of the running buffer plays an important role in CE because of its effect on the electroosmotic flow as well as the over-all charges of the analytes. The influence of pH value of running buffer (6.0, 7.0, 8.0, 9.2, 10.0) was studied. It was found that the migration time decreased while the pH value increased from 6.0 to 8.0 due to the increased electroosmotic flow, then increased with further increasing the pH value probably because of the increased dissociation of NDA-labeled GSH, and when pH value was 9.2, the RFI was the highest. Considering both RFI and migration time, pH 9.2 is most suitable for the detection of NDA-labeled GSH. The influence of the concentration of borate running buffer at pH 9.2 was examined at 10, 20, 30 and 40 mM. The migration time increased with the increase in the running buffer concentration; and the RFI decreased with increasing the concentration of running buffer. So the optimal concentration of borate buffer was 10 mM.

Owing to the formation of inclusion complexes between β-CD and a variety of guest molecules, β-CD was widely used as an additive to improve selectivity in CE [27–32]. In some cases, the detection sensitivity could also be improved [17,33]. In this work,

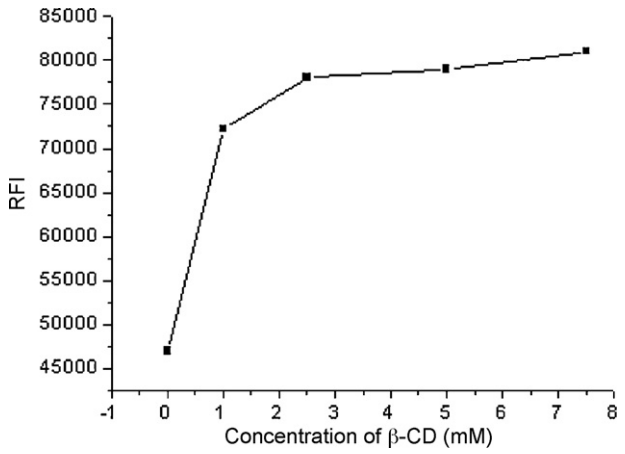

Fig. 2. Effect of concentration of β-CD on the RFI. *Conditions*: CE buffer, 10 mM sodium tetraborate (pH 9.2) containing different concentrations of β-CD; other conditions as in Fig. 1.

it was found that the detection sensitivity and migration speed of NDA-labeled GSH can be obviously increased with the addition of β-CD in the running buffer. As shown in Fig. 2, the RFI increased rapidly with the increase in concentration of β-CD up to 2.5 mM, and then nearly leveled off. The effect of β-CD concentration on the RFI of NDA-labeled GSH was also investigated by the fluorescence spectroscopy, similar results were obtained. So 2.5 mM β-CD was used as the additive in CE.

Under the above-optimized conditions, the effect of separation voltage (18–25 kV) was studied. It was found that the RFI remained stable when the voltage was not higher than 22 kV. Considering the analysis time and the sensitivity, 21 kV was chosen for separation. With sampling height at 14 cm, the injection time (20–60 s) was optimized. The RFI rapidly increased with injection time ranging from 20 to 40 s, and then increased slightly with further increasing injection time. Thus, 42 s was used for optimum injection.

3.3. Calibration, reproducibility and detection limit

Fig. 3 shows the electropherograms of GSH standards prepared with water at different concentrations under the optimum conditions. It was noted that the concentrations of GSH shown in text and

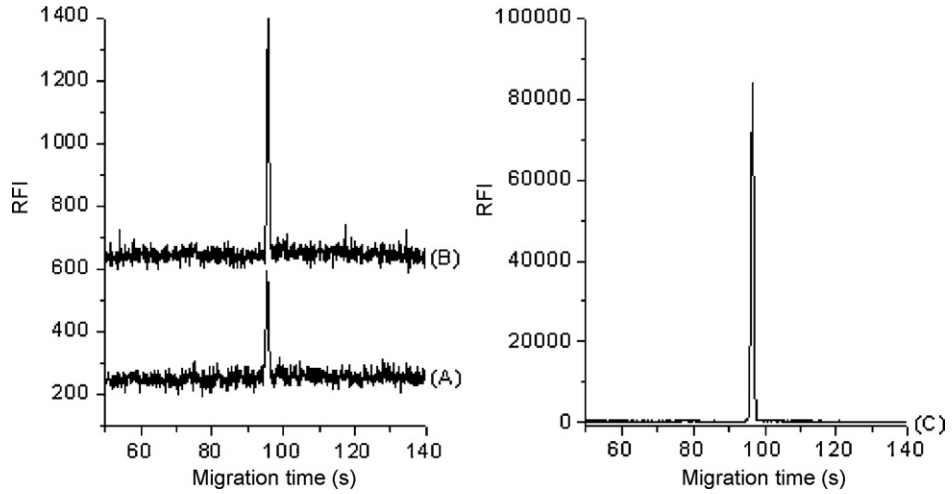

Fig. 3. Electropherograms of GSH with different concentrations under the optimum conditions. *Conditions*: CE buffer, 10 mM sodium tetraborate (pH 9.2) containing 2.5 mM β-CD; separation voltage, 21 kV; hydrodynamic injection, 14 cm (height) for 42 s. The concentrations of GSH in (A)–(C) were 5.0×10^{-9}, 1.0×10^{-8} and 1.0×10^{-6} M respectively.

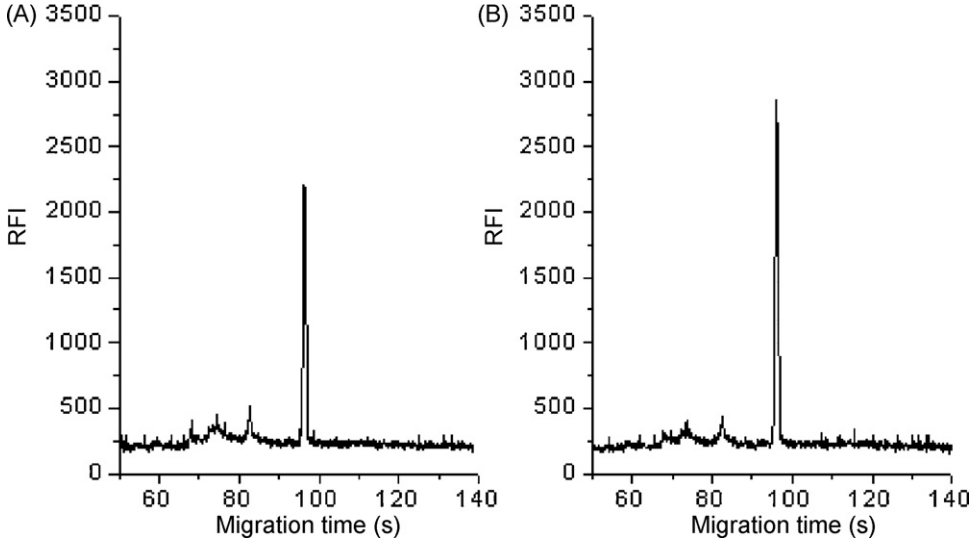

Fig. 4. Electropherograms of GSH analysis in the lysate of BY-2 cells. (A) Sample without the addition of standard; (B) sample with the addition of GSH standards with final added concentration at 1.0×10^{-8} M. Other conditions as in Fig. 3.

Table 1
Analytical results of GSH in the lysate of tobacco BY-2 cells with NDA.

Cell lysate	Added (mol/l)	Found (mol/l)	RSD (%, $n=4$)	Recovery (%)
GSH	0	2.60×10^{-8}	4.7	
	1.0×10^{-8}	3.725×10^{-8}	6.0	103.5

figure captions were those of the corresponding standard solution before derivatization. It was seen from Fig. 3 that the sensitivity of the method was sufficient for sample analysis, and the detection limit of 2.5×10^{-9} M (S/N = 3) was achieved. The reproducibility was performed by repeating injection ($n = 6$) of the GSH standard solution at the concentration of 1.0×10^{-6} M. The relative standard deviations (RSDs) of the migration time and peak height were 1.2 and 4.1%, respectively. Using the fluorescence intensity versus sample concentration, the linear calibration curve was obtained in the range of 5.0×10^{-9} to 2.0×10^{-6} M with regression coefficient (R) of 0.9999 ($n = 8$).

3.4. GSH analysis in BY-cells

Tobacco BY-2 cells are non-green, fast growing plant cells widely used as model systems for higher plants because of their exceptionally high homogeneity, high growth rate, and featuring still general behavior of plant cell. So it is considered as the "HeLa" cell in the cell biology of higher plants for research of cell division, cytoskeletons, plant hormone signaling, intracellular trafficking, and organelle differentiation [34]. On the other hand, GSH has been found to be involved in multiple processes in plants [1]. So, it is important to analyze the content of GSH in tobacco BY-2 cells. However, so far, there are no reports concerned on the analysis of GSH in the tobacco BY-2 cells. Fig. 4A shows the analysis of GSH in the tobacco BY-2 cell lysate. It can be seen that some amount of GSH was detected in the cell lysate. To identify the peak of GSH, GSH standard was spiked in the above cell lysate before derivatization (Fig. 4B). The analytical results of tobacco BY-2 cell lysate were summarized in Table 1. Using the linear calibration curve, the concentration of GSH in the diluted lysate was calculated to be 2.60×10^{-8} M; based on the data, the content of GSH in tobacco BY-2 cells was calculated to be 6.15×10^{-8} mol/g.

4. Conclusion

A rapid CE method coupled with fluorescence detection was developed for the determination of GSH with β-CD as the additive of running buffer. NDA was used to label GSH. Under the optimum conditions, the separation time was less than 120 s, and the detection limit of 2.5×10^{-9} M (S/N = 3) was obtained. The method was successfully applied to the analysis of GSH in the tobacco BY-2 cells.

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