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Short communication

Determination of abscisic acid by capillary electrophoresis with laser-induced fluorescence detection

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Abstract

A novel method based on capillary electrophoresis coupled to laser-induced fluorescence detection (CE–LIF) was developed for the determination of abscisic acid (ABA), which is an essential phytohormone during plant growth and development. ABA was labeled with 8-aminopyrene-1,3,6-trisulfonate via reductive amination in presence of acetic acid and sodium cyanoborohydride. The derivatization yield was maximized by optimizing several derivatization parameters including derivatization reagent concentration, reaction temperature and time. The conjugate was separated and quantitated by CE–LIF. The linearity of ABA was determined in the range from 0.1 to 10 μmol l^{-1} with a correlation of 0.9979. The derivatization limit of detection for ABA was found to be 56 fmol (corresponding to the concentration of 2.8×10^{-8} mol l^{-1}). The detection limit for ABA was 5.5 amol for an injection volume of 5 nl. As a preliminary application, the proposed method was successfully applied to determining trace amount of ABA in the crude extracts of tobacco without extra purification and enrichment procedure and showed a better selectivity and sensitivity than those conventional methods used in determination of ABA.

Keywords: Derivatization, electrophoresis; Tobacco; Plant materials; Abscisic acid; Plant hormones; Aminopyrenetrisulfonate

1. Introduction

The plant hormone abscisic acid (ABA) plays a key role in the process of plant growth and development, such as stomatal closure, seed germination, and the adaptation to environmental stress. In plant, the content of ABA will be increased if the plant was under stress conditions including drought, salinity or cold. Recently, it was shown that ABA could regulate the expression of many genes involved in seed development or in stress tolerance [1–4].

Since ABA has been proven to have vital physiological activity, analysis of trace amounts of ABA in plant is getting more and more important. To date, a number of analytical techniques have been developed for the determination of ABA, such as gas chromatography (GC) [5], GC–mass spectrometry (MS) with single ion monitoring (SIM) and GC–MS–MS [6–11], high-performance liquid chromatography (HPLC) [12–16], enzyme-linked immunosorbent assay (ELISA) [14,17], and radioimmunoassy (RIA) [14]. It has been reported that GC–MS, ELISA and RIA are well suited for the assay of large numbers of samples and show good selectivity for ABA. However, most of the above-mentioned techniques need complicate and intensive purification protocols for ABA in plant samples, which may consume many hours of tedious work. Recently, Gómez-Cadenas et al. described a rapid and sensitive method for direct analysis of ABA in crude plant extracts by liquid chromatography–electrospray tandem mass spectrometry [18]. The limitation of this proposed method is too expensive to apply for assay of large amounts of real samples. It is, therefore, important to exploit a simpler and more sensitive analytical method for determination of ABA.

Due to low sample consuming, short analysis time and high separation efficiency, capillary electrophoresis (CE) has become an important analytical technique, particularly in the analysis and separation of biological specimens [19]. The main challenge in developing CE method for phytochemical analyses is the presence of a complex mixture of various chemically different compounds in plant samples. There are a few papers reported for the determination of ABA by CE–UV detection [20,21]. The lack of a strong chromophore for ABA has been one of the limitations in its determination by CE. To overcome this shortcoming, Liu et al. reported an on-line sample concentration method for the determination

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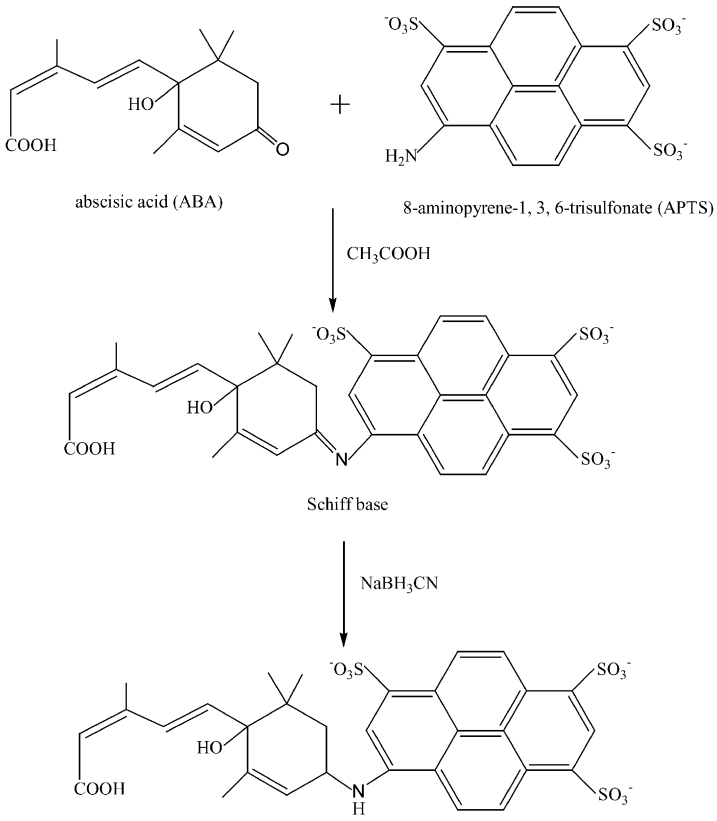

Fig. 1. Reaction procedure for the derivatization of ABA with APTS followed by reductive amination.

of plant hormones [21]. This method can largely improve detection sensitivity, but cannot eliminate the interference of other co-existing compounds. Thus, pretreatment procedures with C_{18} column or other purification techniques were required if it was applied to analysis real-world sample.

Laser-induced fluorescence (LIF) detection has been shown to be one of the most sensitive methods available for detection in CE. To our knowledge, there are no reports about ABA analysis by using CE–LIF. ABA lacks a suitable fluorophore for the LIF detection. An appropriate fluorescent derivatization reagent was required for the analysis of trace ABA. In this paper, a CE–LIF method was firstly developed for the rapid separation and sensitive detection of ABA by using a fluorescent labeling reagent, 8-aminopyrene-1,3,6-trisulfonate (APTS), which has been used for assay of carbohydrates and as a fluorescent derivatization agent for modifying aldehydes and ketones in biochemical field [22–25]. Most recently, Wang and Hsieh reported determination of chitin oligosaccharides from enzymatic digestions by CE–LIF after determination with APTS [26]. Up to now, the use of APTS in analytical field was only focused on the analysis of carbohydrates. Since ABA contains ketone group that can react with APTS. ABA may be derivatized with APTS by reductive amination.

In this work, the feasibility of APTS as the derivatization reagent for ABA has been investigated (Fig. 1). It was found that APTS reacted with ABA to form a highly fluorescent derivative in presence of acetic acid and sodium cyanoborohydride. The APTS-ABA derivative was separated by capillary zone electrophoresis (CZE) and detected by LIF. The method was successfully applied to determine the trace amount of ABA in crude extracts of tobacco leaves with good results.

2. Experimental

2.1. Chemicals

ABA and sodium cyanoborohydride were purchased from Sigma–Aldrich (St. Louis, MO, USA). 8-Aminopyrene-1,3, 6-trisulfonate (APTS) trisodium salt was obtained from Molecular Probe (CA, USA). Boric acid, sodium borate, glacial acetic acid, and methanol were purchased from Shanghai Chemical Co. (Shanghai, China). Unless otherwise specified, all reagents were of analytical reagent grade. All aqueous solutions were prepared from deionized water purified with a Milli-Q system (Millipore, Bedford, MA, USA). ABA was dissolved in methanol and stored at −20 °C prior to use.

2.2. Apparatus

CE separations were performed on a G1600A Agilent ^{3D}CE system (Agilent, CA, USA) equipped with a LIF de-

tection system consisting of a Ar-ion laser (Model: 163D02, Spectra-Physics, CA, USA), a LIF detector ZETALIF 2000 (Picometrics, Montlaur, France) and Agilent interface 35900E (Agilent). The excitation was performed by a Ar-ion laser with 20 mW at a wavelength of 488 nm. The emission intensity was measured at a wavelength of 520 nm filtered by a band pass filter and a notch filter was used to attenuate background radiations. Fluorescence was detected by a photomultiplier tuber. Data collection and process were performed on a G1601A Agilent ^{3D}CE Chemstation (Agilent). Fused-silica capillaries from Yongnian Optic Fiber (Hebei, China) of 65 cm (50 cm to detector) × 50 μm i.d. × 365 μm o.d. were used.

2.3. Derivatization procedure

A 2 μl volume of 0.1 mmol l^{-1} ABA in a 500-μl micocentrifuge tube (Eppendorf, Germany) was mixed with 2 μl of 60 mmol l^{-1} APTS in 15% acetic acid and 6 μl of 1 mol l^{-1} sodium cyanoborohydride in tetrahydrofuran. The tube was capped. Then, the reaction solution was vortex-mixed, centrifuged briefly at 8000 × *g* to ensure all the reactants in the tips of the tubes, and heated in water bath at 60 °C for 3 h. After the derivatization, the vials were kept at −20 °C till the analyses was conducted. Before CE analysis, the resulting mixture was diluted to 50 μl with water prior to be injected in to capillaries.

2.4. Capillary electrophoresis procedures

New capillaries were pre-treated with 1 mol l^{-1} NaOH followed by deionized water for 60 min at room temperature. Prior to use, the capillary was rinsed with 0.1 mol l^{-1} NaOH for 5 min, with deionized water for 5 min, and then with running buffer for 10 min. The pressure was chosen as 940 mbar in rinsing procedure. This above flushing cycle was repeated to ensure the separation reproducibility for each injection.

25 mmol l^{-1} borate buffer (pH 9.35) was chosen as running buffer. Buffer solutions were filtered through 0.22 μm membrane filter prior to electrophoresis. Samples were injected by pressure at 50 mbar for 5 s. Separations were performed at 25 °C using a running voltage 22 kV.

2.5. Plant material and extraction procedure

A 1 g amount of fresh tobacco (*Nicotiana tabacum* cv. SRI) leaf was snipped and grinded to fine powder in the presence of liquid nitrogen. 1 ml of 70% (v/v) aqueous methanol was mixed with this powder in 1.5 ml of microcentrifuge tube. After vigorous vortexing, the mixture was kept in an ice-bath overnight, then centrifuged at 4 °C (5000 × *g* for 10 min). The supernatant was collected without further purification, and directly reacted with APTS using the same procedure for the derivatization of standard ABA solution. The recoveries of ABA analysis were obtained by spiking the ABA standard in crude extracts of tobacco leaf.

3. Result and discussion

3.1. Optimization of derivatization conditions

The APTS derivatization reaction mechanism involves the reaction of the primary amino group of APTS with the ketone group of ABA to form Schiff base. The intermediate is subsequently reduced to a more stable secondary amino compound by $NaBH_3CN$. In this derivatization reaction procedure, Schiff base formation is a relatively labile, reversible interaction that is readily cleaved in aqueous solution by hydrolysis, and the ketone group of ABA shows lower reactivity toward APTS than aldehyde group. It is, therefore, important to control the formation of Schiff base for the high sensitive detection of ABA. In order to achieve the best possible compromise between high yield of ABA derivative and low by-products, several parameters affecting the derivatization reaction were studied, including the amount of APTS, reaction temperature and time.

As described above, the hydrolysis of the Schiff base in aqueous solution can influence the yield of ABA derivatives. The Schiff base formation and hydrolysis of ABA by APTS is considered to be a pair of competitive reactions. The excess of APTS can facilitate the generation of derivative. In order to obtain quantitative reaction, it is necessary to use excess reagent in derivatization reaction. The effect of APTS concentration on the derivatization reaction was studied within the range of 5–80 mmol l^{-1}. When the concentration was beyond 40 mmol l^{-1}, the peak height of the ABA-APTS derivative can reach maximum and almost unchangeable. However, when the more derivatization reagent was used, the unreacted dye peak increased, which would lead to possible inference with sufficiently separation of ABA. 60 mmol l^{-1} was chosen as optimum concentration for ABA derivatization.

It has been documented that both Schiff base formation and reduction amination are affected by the variation of reaction temperature [24]. The influence of reaction temperature on derivatization efficiency was investigated under three different temperatures, 40, 60, and 80 °C. When the reaction temperature was 40 °C, the derivatization yield was low because the low temperature can reduce the reactivity of $NaCNBH_3$ toward Schiff base. However, when the reaction temperature was up to 80 °C, the yield of derivative was also decreased because high reaction temperature may result in the cleavage of Schiff base. Therefore, 60 °C was selected for reaction temperature.

The effect of the reaction time from 0 to 4 h at 60 °C was also tested. It was found that the derivative yield reached maximum plateau in 2 h. Prolonging reaction time could not improve the yield of derivatization reaction. The rate of derivatization reaction was slow due to the low reactivity of ketone group toward APTS as previously reported that

the derivatization reaction of ketohexose with APTS needed longer derivatization time [22]. Thus, 3 h was chosen as derivatization time in our study. The ABA derivative was stable at least 2 days at −20 °C in the dark.

3.2. Analytical conditions

As shown in Fig. 1, the obtained derivative was negatively charged, so the CZE mode was employed to analyze ABA. It has been reported that APTS derivatives can be well separated by CZE in basic or acidic medium [23,26]. In addition, borate buffer was usually employed as the running buffer for CZE separations of APTS derivatives because it can provide a high and reproducibility to ensure validity of analysis [27].

In this study, 25 mM borate buffer at pH 9.35 was chosen as background electrolytes. The samples with volume of approximately 5 nl were hydrodynamically injected at anode (50 mbar for 5 s). The applied field strength for the separation was 340 V cm^{-1} with a current of 35 μA.

3.3. Reproducibility, linear calibration, and limit of detection

In order to evaluate the characteristics of this method, the reproducibility, linear calibration, and limit of detection for ABA was determined. The reproducibility of this method was tested by examining six sequential runs of ABA standard solution (in concentration of 10 μmol l^{-1}) for migration time and peak area. The relative standard deviations (RSDs) were 1.2% for migration time and 4.5% for peak area. The high reproducibility of the method indicated that CE–LIF with APTS was reliable for analyzing ABA.

The quantitative analysis of ABA was performed by derivatizing different amount of ABA standard from 0.1 to 10 μmol l^{-1} under the optimum derivatization and separation conditions. The linearity between the peak area (y, mAU) and the concentration (x, μmol l^{-1}) was investigated. Our results indicated that the linear regression equation was $y = 265.4x + 54$ and the correlation coefficient for ABA was 0.9979, indicating good linearity.

The derivatization limit of detection for ABA was determined using the derivatization of the diluted ABA standard solution and found to be 56 fmol (2.8×10^{-8} mol l^{-1}), at a signal-to-noise of 3. The mass on-column detection limit of ABA was 5.5 amol or 1.1 nmol l^{-1} in terms of concentration (S/N = 3). These results were presented that the method could provide sufficient detection sensitivity for detecting the trace amount of ABA in plant tissue.

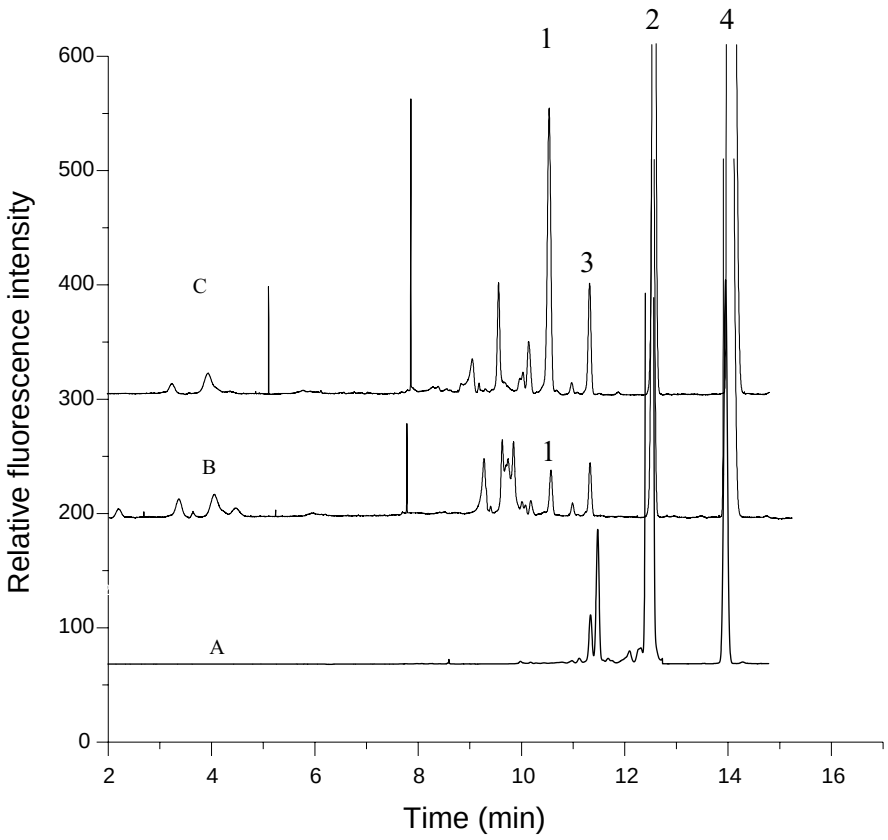

Fig. 2. Electropherogram obtained from (A) blank reaction, no addition of ABA, other conditions as described in Section 2.3, (B) extract from tobacco leaves, (C) the same sample spiked with 1 nmol of ABA standard. Peaks: (1) ABA, (2) APTS, (3, 4) unknown. Conditions: running buffer; 25 mmol l^{-1} borate buffer (pH 9.35); capillary: 65 cm (50 cm effective length) × 50 μm i.d.; injection: 5 s at 50 mbar; separation voltage: 22 kV; temperature: 25 °C.

Table 1
Analytical results of ABA in tobacco leaves with APTS

Tobacco leaves[a]	Added (pmol)	Found (pmol)	RSD (%, $n = 5$)	Recovery (%)
ABA	0	213	7.3	
	500	697	5.7	97
	1000	1254	5.5	104

[a] Fresh mass, 1 g.

3.4. Application to extract of tobacco leaf

Various extraction procedures have been published for ABA and other plant growth regulator. For the extraction of ABA from plant material, methanol was found to be the most effective solvent. The common extraction procedure involves the use of aqueous methanol or other organic solvent and extra purification protocol that remove lipids, chlorophyll, and other pigments. If real samples were not clean up, ABA could not be detected or hardly quantified by previous analytical methods. In this paper, 70% aqueous methanol was employed for the extraction of ABA in tobacco leaf and the crude extracts were directly used for ABA analysis.

The electropherograms of real sample and spiked standards are presented in Fig. 2. The ABA peak was well separated form other interference peaks and identified by spiking ABA standard. This method showed good ability of anti-interference. Because APTS only label the compound containing aldehyde or ketone group, other co-existing plant hormones, such as indole-3-acetic (IAA), gibberellic acid (GA), kinetin-6-furfurylaminopurine (KT) and 2-naphthaleneacetic acid (NAA), etc., cannot be reacted with APTS to form fluorescent labeling product. Moreover, they do not interfere with the determination of ABA.

The amount and recoveries of ABA in crude extracts of tobacco leaf were listed in Table 1. These results indicate that the analysis of trace amount of ABA in plant tissue can be well done by this CE–LIF method.

4. Conclusion

In summary, a CE–LIF method was developed for the analysis of ABA by using APTS as pre-column fluorescent derivatization reagent. Under the optimum derivatization conditions, the detection limit of ABA can reach nmol l^{-1} for an injection volume of 5 nl and thus are equivalent to or better than the detection limit obtained by other methods which were used in the determination of ABA. Our preliminary work showed that the proposed method had fairly good selectivity and sensitivity. Only small amounts of plant material are needed to complete the analysis. The method allowed the existence of pigments and other organic substances and was successfully applied for the direct assay of ABA in crude extract of tobacco leaves. Due to highly sensitivity and selectivity, this approach can omit laborious purification and enrichment steps and enable the simultaneous determination of many samples.

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