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A gas chromatography–mass spectrometry method for the determination of delta-aminolevulinic acid in plant leaves

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ABSTRACT

Delta-aminolevulinic (δ-ALA) acid is an important intermediate for tetrapyrroles biosynthesis and it has recently received great attention in plant physiology and human toxicology. However, the colorimetric method which is the most common method for determination of δ-ALA is time consuming and is not specific. In this study, a method for determination of δ-ALA in plant tissues was developed based on the trimethylsilyl (TMS) derivative of the pyrrole formed from the reaction of δ-ALA with ethyl acetoacetate via Knorr condensation. The δ-ALA in the HCl extract was reacted with ethyl acetoacetate to form a pyrrole. Then, the pyrrole compound was extracted using ethyl acetate and the solvent was evaporated to dryness. The dried sample was derivatized to its TMS ester and analyzed using GC–MS. The concentration of δ-ALA in citrus leaves incubated with levulinic acid was also determined by the conventional colorimetric method. The linear range was 10–200 ppm in the full scan mode and 0.1–20 ppm in the selected ion monitoring (SIM). The limit of detection was 6 ppm in the full scan and 0.05 ppm in SIM mode, representing a four-fold increase in sensitivity compared to the colorimetric method. The GC–MS method developed in this study is simple, accurate, sensitive, and could also be used to measure δ-ALA in other biological samples.

1. Introduction

Delta-aminolevulinic acid (δ-ALA) is an intermediate for tetrapyrroles (chlorophyll, heme, siroheme, and phytochromobilin) biosynthesis in plants, animals, fungi, yeast and many photosynthetic bacteria [1]. The tetrapyrroles biosynthesis is a multistep and a multibranched pathway [1]. In the first step, glutamyl-tRNA synthetase attaches glutamate to t-RNA to form tRNAGlu complex. Next, the activated carboxyl group of glutamyl-tRNA is reduced by glutamyl-tRNA reductase to a formyl group to generate glutamate-1-semialdehyde (GSA). Then, GSA is converted into δ-ALA in intermolecular amino-exchange reactions catalyzed by GSA aminotransferase. In fungi, yeast, some bacteria and animals, δ-ALA is synthesized from succinyl-coenzyme A and glycine by ALA synthase. Subsequently, ALA-dehydratase (ALAD) combines two δ-ALA to form a pyrrole molecule, porphobilinogen (PBG). In the next step, PBG deaminase polymerizes four molecules of PBG to form the linear tetrapyrrole, 1-hydroxymethylbillane. The linear tetrapyrrole is converted to a closed macrocycle compound (uroporphyrinogen III) by uroporphyrinogen III synthase. For all tetrapyrroles synthesis, the steps from glutamate to uroporphyrinogen III are necessary.

Since δ-ALA is an important precursor in tetrapyrroles synthesis in plants, it has been targeted in many transgenic [2,3] and physiological studies [4]. In addition, δ-ALA has received great attention in clinical studies because its accumulation in human blood and urine at high levels is an indicator of lead toxicity [5]. In human, ALAD is necessary for heme biosynthesis and its activity can be inhibited by environmental toxins such as lead [5]. Lead deactivates ALAD by displacing the zinc from the enzyme's active site and consequently leads to the accumulation of δ-ALA, which can act as a γ-aminobutyric acid (GABA) receptor agonist in the nervous system and results in neuropathogenic effect [5].

The colorimetric method developed by Mauzerall and Granicks [6] is the most common method used for determination of δ-ALA in urine and plant samples. However, this method is time consuming because δ-ALA should be separated by ion-exchange chromatography before being reacted with Ehrlich's regent [6]. In addition, this method is not selective and many compounds including urea, pigments, and oxidizing and reducing agents may react with Ehrlich's regent [6]. In 1987, Tomokuni et al. [7] developed a new method based on Hantzch reaction for determining δ-ALA in urine using high performance liquid chromatography (HPLC) coupled with a fluorescence detector. Although this fluorometric

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method correlated well with the conventional colorimetric method at high concentrations, δ-ALA concentrations obtained with the fluorometric method were lower than those measured with the colorimetric method at concentrations below 5 mg/L [8]. Tomokuni et al. [8] concluded that the colorimetric method overestimates the levels of δ-ALA in the urine of lead workers when it is present at low concentration (less than 5 mg/L) due to matrix interference. The fluorometric assay was developed by Tomokuni et al. [7] was modified [9] in order to determine δ-ALA using smaller volume of the urine samples. In addition, it was recently adapted for determination of δ-ALA in swine waste [10].

Besides its usefulness as an index for lead toxicity, high δ-ALA level in blood and urine is also an indication of hepatic porphyria [11] and hereditary tyrosinemia type I (HT I) diseases [12]. Due to the high interest in δ-ALA in clinical studies, more methods have been recently developed for determination of δ-ALA in biological samples using different analytical instruments such as HPLC-mass spectrometry (HPLC–MS) [13], HPLC-electrochemical detection (HPLC-ECD) [14], capillary electrophoresis-photodiode array (CE-PDA) [15], and CE-mass spectrometry (CE-MS) [16].

Although gas chromatography-mass spectrometry (GC–MS) is a robust and a sensitive method and is widely used in plant metabolomics studies [17], δ-ALA in plants is commonly determined using the conventional colorimetric method [2,4]. The non-volatile nature of the δ-ALA and its presence in a complex matrix in plants could be the main reasons behind the difficulty of its determination by GC. The non-volatile nature of δ-ALA can be easily overcome using many derivatization techniques. However, δ-ALA in a complex matrix cannot be detected by GC without sample purification. In fact, δ-ALA has rarely been determined by GC. To measure δ-ALA in normal plasmas, Gorchein et al. [18] derivatized the Knorr pyrrole to its pentafluorobenzyl ester and analyzed it by GC–MS.

The δ-ALA does not accumulate in greening (developing) plant tissues and is directly converted to PBG by ALAD. However, the accumulation of δ-ALA can be induced in greening tissues by the addition of levulinic acid, a specific competitive inhibitor of ALAD [2]. In the current study, we developed a GC–MS based-method for the determination of δ-ALA in leaves of higher plants. In this method, δ-ALA was first reacted with ethyl acetoacetate to produce a pyrrole compound by a Knorr condensation, and then the pyrrole compound was analyzed by GC–MS after trimethylsilyl (TMS) derivatization.

2. Experimental

2.1. Material and reagents

δ-Aminolevulinic acid, levulinic acid, porphobilinogen, ethyl acetate, ethyl acetoacetate, Ehrlich's reagent, mono basic sodium phosphate, dibasic sodium phosphate, perchloric acid, methoxyamine hydrochloride solution (MOX) in pyridine (2%), and *N*-methyl-(*N*-trimethylsilyl) trifluoracetamide (MSTFA) were purchased from Sigma (St. Louis, MO).

2.2. Plant materials

Citrus macrophylla Wester (Alemow) trees used in this study were about one year old and 60 cm height with multiple new shoots. All plants were kept in a greenhouse under controlled conditions; 25 °C temperature, 75% humidity and 16L: 8D h photoperiod until use.

2.3. Leaves incubation in levulinic acid

Three young leaves (one to two weeks-old) from each plant were collected from one plant and each leaf was immersed separately by its petiole in 1.5 ml of 100 mM levulinic acid in 10% dimethylsulfoxide solution for 0 or 6 h at ambient temperature. Five different plants were used for each treatment. Leaves were stored at −20 °C until the day of analysis.

2.4. Extraction of δ-aminolevulinic acid (δ-ALA) from citrus leaves

Leaves were ground with liquid nitrogen and about 0.20 g of the ground tissues was transferred to 2 ml centrifuge tube. One ml of 0.1 N HCl was added and the samples were vortexed for 2 min. The samples were left for 10 min at room temperature. The vortex procedure was repeated three times and the samples were centrifuged at 14,000 rpm for 6 min at room temperature to remove plant debris.

2.5. Colorimetric determination of (δ-ALA)

The concentration of δ-ALA in the 0.1 N HCl extract was determined using the conventional colorimetric assay as developed by Mauzerall and Granick in 1956. Briefly, 0.4 ml of the supernatant was transferred to a new centrifuge tube and was mixed with 0.4 ml of phosphate buffer (equal volume of 0.5 M NaH_2PO_4 and 0.5 Na_2HPO_4; pH: 6.8) and 80 μl ethyl acetoacetate. The mixture was placed in a hot water bath (85 °C) for 10 min. At the end of the incubation time, the samples were cooled to room temperature and centrifuged at 14,000 rpm for 6 min to separate insoluble products. After centrifugation, 0.4 ml of the supernatant was mixed with an equal volume of modified Ehrlich's reagent containing 2 N perchloric acid. The intensity of the pink color produced from the reaction of δ-ALA-pyrrole with Ehrlich's reagent was read at 553 nm using a UV-1700 spectrophotometer (Shimadzu, Torrance, CA, USA). A set of δ-ALA standards (10.0, 5.0, 2.5, 1.2, 0.6, 0.3 and 0.0 ppm in water) were reacted with ethyl acetoacetate and Ehrlich's reagent as mentioned above and were used to construct the standard curve.

2.6. Direct derivatization of δ-ALA and PBG

In our preliminary trials, δ-ALA and PBG were directly derivatized with MSTFA alone or with MOX and then with MSTFA. Briefly, about 10 μl of 1000 ppm of each standard was evaporated to dryness under a nitrogen stream. A 35 μl of MOX reagent was added to the dried sample and the sample was incubated at 85 °C for 35 min. At the end of the incubation time, 80 μl of MSTFA reagent was added and the mixture was incubated for another 35 min at 85 °C. For direct derivatization with MSTFA only, the dried standard was mixed with 100 μl of reagent and the mixture was incubated for 35 min at 85 °C. At the end of the reaction time, 1 μl of the derivatized standard was injected into the GC–MS running in the full scan mode. For the leaf samples, 0.5 ml of the HCl extract was dried under nitrogen stream and derivatized in the same way before being analyzed by GC–MS.

2.7. Derivatization of δ-ALA-pyrrole

A 0.4 ml of HCl extract was mixed with 0.4 ml of phosphate buffer and 20–80 μl ethyl acetoacetate and the mixture was incubated in a hot water bath (85 °C) for 10 min. At the end of the incubation time, the sample was cooled to room temperature and centrifuged at 14,000 rpm for 6 min to remove the solid products. The supernatant was transferred to a new centrifuge tube and δ-ALA-pyrrole derivative was extracted with 3 × 600 μl of ethyl