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HPLC-based quantification of indole-3-acetic acid in the primary root tip of maize

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ABSTRACT

Auxin is a key regulator of plant growth and development. Because the growth regulation is mainly dependent of the change of free IAA levels in the target tissues, quantification of indole-3-acetic acid (IAA), the most abundant natural auxin, is indispensable in the study of auxin action. Currently, GC-MS is technically the best method to measure IAA, because of high sensitivity and specificity. However, its high cost for setting and maintenance makes it difficult for daily use in ordinary laboratory. Therefore, we established a standard method to quantify IAA based on HPLC, adopting fluorescence detector as the monitoring device to ensure the specificity and sensitivity. By applying this protocol, we quantified IAA from the tip of maize (*Zea mays*) primary root.

Key words : auxin quantification, indole-3-acetic acid (IAA), indole-3-propionic acid (IPA), HPLC, fluorescence detector (FLD), maize *(Zea mays*)

Introduction

Indole-3-acetic acid (IAA) is the most abundant natural auxin that plays diverse roles in plant growth and development (1). The activity of auxin can be regulated by changing the endogenous level of free IAA or the sensitivities of target tissues (2). Although the latter means that the differential responses to the given concentration of auxin, the exact mode of sensitivity change is achieved by modifying the dose-response curve to IAA in the target tissue (2). Therefore, detection and quantification of IAA in a living tissue is indispensable to study the plant growth regulation. Quantification is also a central part of auxin biosynthesis research (3).

The first trials to detect auxin activities were bioassays. Earlier, several bioassays including *Avena* curvature test (4) and split pea test (5) were widely used. However, the bioassay is not only dependent on the IAA amount, but also on the tissue sensitivities. Then, color reaction-based methods with indole were developed: e.g., Salkowski (6), van Urk (7) and Ehrlich (8) reagents. By combining the color reactions to TLC, it became possible to detect IAA and its metabolites at the same time (9) with the lower detection limit about 25 ng. However, the analyses based on the color reactions were essentially qualitative.

Immunoassay for IAA quantification was developed (10). Nowadays the immunoassay kit is commercially available from Phytodetek (USA). However, immunoassay is inconvenient, because IAA must be converted to methyl ester before quantification. In many cases error range is too wide to trace the endogenous level of IAA. High cost and short life span of the kit are also the weakness.

Currently, the most powerful methods to identify IAA and determine the quantity in a single preparation are gas chromatography-based (11). High resolution and sensitivities

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of GC permits the separation of IAA from similar substances in small amount of plant sample. By combining GC with diverse detection devices, especially with mass spectrometer, the IAA detection technologies have revolutionarily progressed. Quantification of IAA is possible only with several leaves of Arabidopsis by applying GC-MS-MS and comparing the signal intensity of IAA with that of stable isotope-labeled IAA added as internal standard (12, 13). Moreover, currently developing bench top GC-MS makes it easier than ever before to analyze IAA. Recently, LC-MS is also applied for the quantification of IAA (14). However, the most serious obstacle to routinely use GC-MS or LC-MS in each lab is the high cost for setting and maintenance.

HPLC is another choice to analyze IAA with high resolution and sensitivity, but requests relatively lower cost compared to GC-MS (11). The most frequently used column material for IAA quantification is the reverse phase C_{18}. On this column, IAA is well resolved with methanol or acetonitrile as the eluent. In HPLC-based methods, the most critical part is the monitoring system. Because indole metabolites absorb UV light, the UV monitor can be adopted. However, relatively low sensitivity of UV and diode array monitors is the weakness compared to electrochemical detector (ECD) and fluorescence detector (FLD). Currently, ECD and FLD are the most sensitive detectors that can be connected to HPLC. ECD can detect molecules without chromophore, if it can be oxidized (for review on HPLC see ref 15). HPLC-ECD based IAA quantification was recently reported (16). The application range of FLD is more limited, because it can be applied only for the detection of fluorescing molecules. However, fluorescence is a character of certain substances and can be used for specific monitoring. Therefore, we chose FLD as the detection system to increase both sensitivity and specificity of IAA quantification.

In HPLC based quantification without MS, radio-labeled internal standards are generally included (16), because the signal of stable isotope-labeled internal standard is indistinguishable with that of endogenous molecules. Radio-isotope is inconvenient to use for HPLC in normal laboratories. To overcome this problem, we selected indole-3-propionic acid (IPA), which was used for GC-MS by Mezzetti *et al.* (17), to use as non-isotopic internal standard that behaves similar with IAA during our analysis based on HPLC. In this paper, we evaluated the IPA as internal standard for IAA quantification, developed a standard protocol to measure IAA based on HPLC-FLD, and quantified IAA in the primary root tip of maize.

Materials and Methods

Plant materials

Maize (*Zea mays* cv. Golden Cross Bantam 70) kernels were washed in tab water, soaked in distilled water at room temperature on a rotary shaker (30 cycles/min), and grown for 3 d in the dark on a paper roll system (18) that was prepared to fit in a 100-ml beaker. Tissue segments (1 cm) were successively isolated from the very tip of the primary root.

Extraction and concentration

Harvested segments were weighed and extracted with 100% methanol (2.5 ml/g.f.w) in a mortar and pestle. IPA was added as internal standard (10 nmol/g.f.w.). The extract was cleared by centrifugation (16,000 g × 10 min) at 4 °C. The resulting supernatant was transferred to a new tube and concentrated in a speed vacc (Centrivap Concengrator, Labconco) until the volume decreased to less than one-tenth of the initial.

Serial partitioning

To increase the polarity of the sample before partitioning against ethyl acetate, one volume of pure water was added. The pH of the plant extract was adjusted higher than 9 with 1 M KOH to keep IAA and IPA ionized and then partitioned against 100% ethyl acetate. The aqueous and organic phases were separated by centrifugation (16,000 g × 2 min), and the lower aqueous phase was transferred to a new tube. The pH of the solution was lowered below 3 with concentrated acetic acid to conserve IAA and IPA in protonated forms. The acidic sample was partitioned against 100% ethyl acetate and cleared by centrifugation. The upper organic

Table 1. HPLC solvent gradient program optimized for the separation of IAA and IPA. Solution A: 10% methanol, 0.3% acetic acid; Solution B: 90% methanol, 0.3% acetic acid; Solution C: 100% acetonitrile.

Time (min)	% Ratio of solvents			Gradient type
	Solution A	Solution B	Solution C	
0	40	60	0	
5	40	60	0	Linear
20	0	100	0	Linear
22	0	0	100	Linear
30	0	0	100	

phase was recovered and completely dried in a speed vacc (Centrivap Concengrator, Labconco), and then dissolved in a minimal volume (generally in 30 µl) of 100% methanol. During the serial partitioning described above, the recovery of standard IAA and IPA was evaluated after each step by spectrophotometry at 282 nm (DU-7500, Beckman, USA).

HPLC

Plant extracts and standard substances were resolved on a reversed phase C_{18} column (Apollo C_{18}, 5 µm, Alltech, USA) with a HPLC system (Waters 600E). A solvent gradient program was optimized for IAA and IPA separation in the presence of 0.3% acetic acid (**Table 1**). The elution profile was traced by a dual monitoring system with a UV- (Waters 486) and a fluorescence (Waters 470) detector. The chromatogram was analyzed with software, MultiChro™ (Yullin Technology, Korea).

Results and Discussion

Indole-3-propionic acid (IPA) was selected as a commercially available non-isotopic internal standard. IPA is very similar to IAA and absent in plants. Side chains of active auxins have even numbers of carbon as we can see in IAA and indole-3-butyric acid. IPA has three carbons in its side chain, i.e., it has one more carbon than IAA and one less than IBA. Therefore, we expected that IPA is a substance that can be ideally used as internal standard for IAA quantification. Very recently, IPA was applied as internal standard for IAA quantification based on GC-MS (17).

Before using IPA as internal standard, we examined the

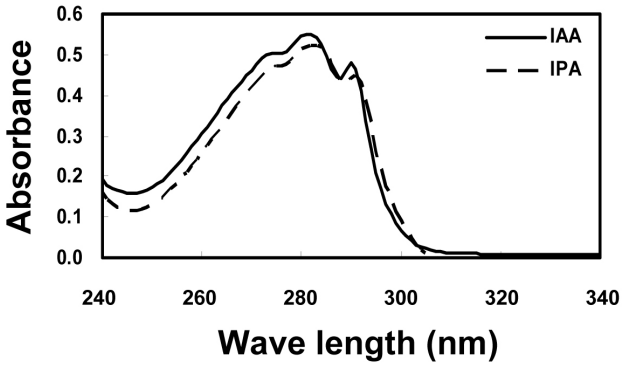

Fig 1. UV-absorption spectrum of indole-3-acetic acid (IAA) and indole-3-propionic acid (IPA). Substances were dissolved in 100% methanol.

Table 2. Recovery of IAA and IPA after a serial partitioningat at pH > 9 and pH <3. The amount of the substances was measured by a wavelength scan with a spectrophotometer (Beckman DU 7500). The recovery of substances was calculated from the absorbance values before and after the serial partitioning at the three representative wave lengths. Data are presented as M ± SD (n = 3).

Wave length	IAA	IPA
273 nm	97.4 ± 5.8	97.2 ± 0.1
282 nm	91.6 ± 2.6	90.2 ± 0.4
290 nm	89.9 ± 3.8	89.4 ± 0.6

UV absorption spectrums of IAA and IPA. Spectrums of both IAA and IPA appeared as of a typical indole with a shoulder at 273 nm, 282 nm and 290 nm (**Fig 1**). Although the absolute values were slightly higher with IAA than with IPA, the differences were only minute.

The specific absorption spectrums of IAA and IPA were utilized to determine the recovery of the two substances after serial partitioning against ethyl acetate at pH > 9 and at pH < 3 (**Table 2**). Before and after the serial partitioning, the absorption spectrums of IAA and IPA between 240 nm and 340 nm were scanned and compared at the three wavelengths where the characteristic shoulders of indole-specific absorption appeared. At the three wavelengths, the recovery of both IAA and IPA was over 89% and was more importantly same with that of each other. Therefore, we got the first evidence indicating that IPA could be used as internal standard for IAA in our pre-purification steps.

We analyzed IAA and IPA on a reversed phase HPLC column under a methanol gradient program in the presence of 0.3% acetic acid as described in materials and methods. To trace the elution of the substances, we used both UV-

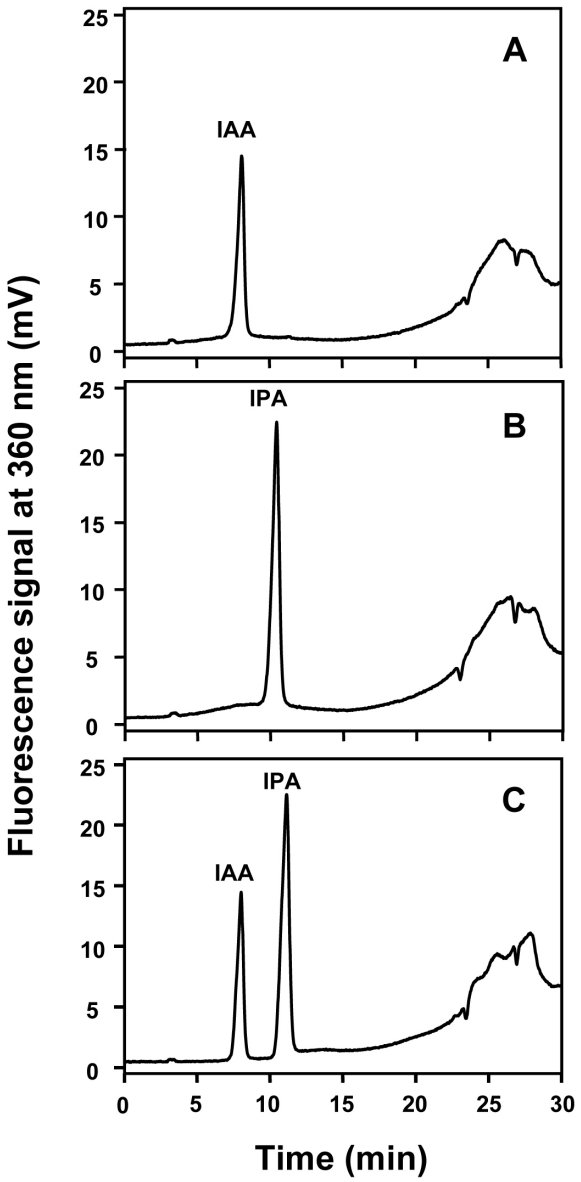

Fig 2. Chromatogram of IAA (A), IPA (B). and their mixture (C). Materials were prepared in 100% methanol, manually injected (20 pmol in 10 μl for both substances), and resolved on a reverse phase HPLC column (Apollo C_{18}, Alltech) under a methanol gradient conditions as described in Table 1. For the monitoring of the elutes with fluorescence at 360 nm, excitation was set to 280 nm.

(Waters 486) and fluorescence monitor (Waters 470). The wavelength of 282 nm was used both for UV-monitoring and for the excitation to record the fluorescence at 360 nm. As expected, the fluorescence signal was much stronger than UV absorption with the same amount of materials (data not shown). Therefore, the elution of IAA and IPA was mainly traced with the fluorescence detector. Under the separating conditions, IAA and IPA were eluted at 8.1 min and at 10.3 min, respectively (**Fig 2A and B**). When the mixture of these two substances was injected, two peaks appeared at the exact elution time (**Fig 2C**) as observed with pure substances, indicating that IAA and IPA did not disturbed the mobility of the other on the HPLC column. The elution time of IAA and IPA remained stable even when the injected amount of the substances was changed (**Table 3**). The peak area of IAA was slightly smaller than that of IPA. However, the peak areas were linearly proportional to the amount of injected substances both for IAA and IPA (**Fig 3**), R^2 = 0.997 for IAA

Table 3. Retention time of IAA and IPA from a reverse phase RP-C_{18} HPLC column. The sample was injected in 100% methanol and resolved with a gradient program as appeared in Table 1 in a HPLC system. The elution of IAA and IPA was monitored with a fluorescence detector (Em at 360 nm, Ex at 282 nm; Waters 470, USA). Data are presented as M ± SD (n = 3).

Substanc	Injected amount (pmol)	Retention time (min)
IAA	20	8.0 ± 0.1
	40	8.1 ± 0.0
	60	8.1 ± 0.3
IPA	20	10.2 ± 0.1
	40	10.4 ± 0.3
	60	10.3 ± 0.2

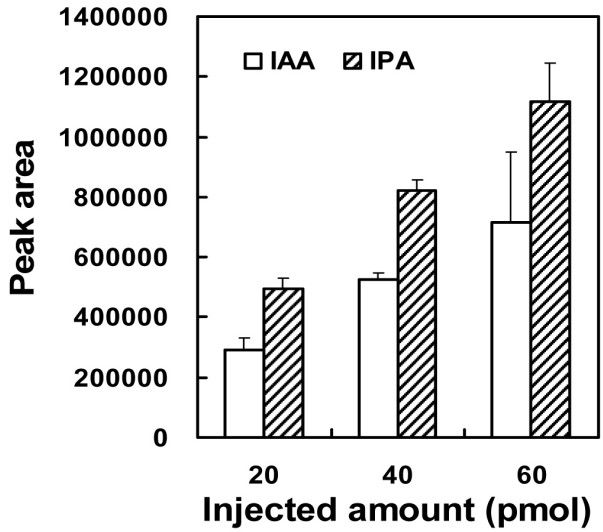

Fig 3. Peak area of eluted IAA and IPA obtained by HPLC-FLD when various amount of substances were injected.

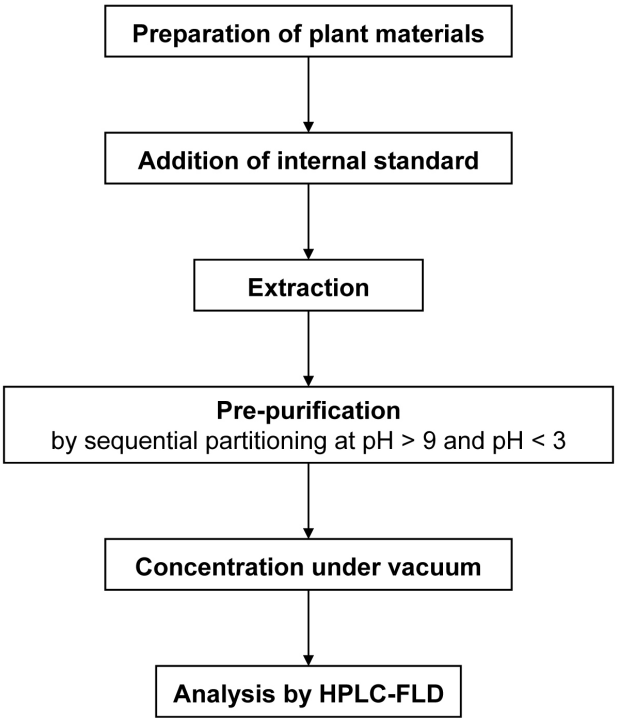

Fig 4. Schematic illustration of the standard protocol to quantify IAA by HPLC-FL

and R^2 = 0.999 for IPA, making it possible to use the fluorescence-based HPLC for the quantification of IAA. Based on the results, we established a standard protocol to extract and quantify IAA form plants (**Fig 4**)

We extracted IAA from the primary root tip of maize, pre-purified following the established procedures, and resolved on a C_{18} HPLC column (**Fig 5**). The amount of IAA was determined to 20.4 ± 6.1 nmol/g.f.w. in the first one cm of the primary root tip in maize (**Table 4**) based on the peak area obtained by fluorescence monitoring. In the immediate upper part of the primary root tip (between 1 and 2 cm), the IAA amount was 16.2 ± 11.8 nmol/g.f.w. All the values were corrected against the signal of IPA added as internal standard. We optimized the amount of IPA to 10 nmol/g.f.w. at which concentration the signal of endogenous IAA was similar to that of applied IPA. We referred to published level of IAA in maize to evaluate our measurement. Reported free IAA content looked lower (19, 20) than that we determined, e.g., 30 ng/g.f.w. (about 0.2 nmol/g.f.w) in the primary root tip between 2.5 and 5 mm from the tip from 3-d-old plants (19). However, simple comparison may be impossible, because the growth conditions that Pilet's group used (19, 20) were quite different with that we set; they grew plants at 21 °C in contrast that we incubated plants at 28 °C. Because maize growth at the young seedling stage is quite sensitive to the temperature and better at 28 °C than at 21 °C, the development of our plants seems to be far more progressed after 3 d from the germination than the plants of Saugy and Pilet. The increase of free IAA following plant

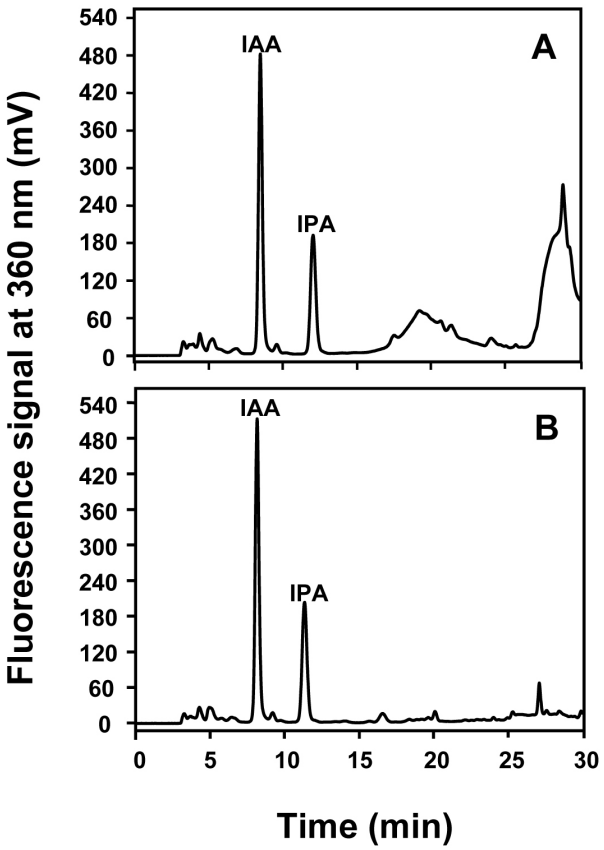

Fig 5. Elution profiles of plant extracts resolved by HPLC-FLD under the optimized conditions to separate endogenous IAA, and IPA added as internal standard. A: from 0-1 cm; B: from 1-2 cm from the tip of the maize primary root.

Table 4. Level of IAA in the tip of maize primary root after 3 d from germination. The amount of IAA was determined by the peak area recorded with a fluorescence detector (Ex = 282 nm; Em =360 nm) after the correction against the signal of IPA that was added as internal standard (10 nmol/g.f.w). Data originatefrom three independent measurements and are presented as M ± SD.

Root segments from	Quantity of IAA (nmol/g.f.w)
0 to 1 cm	20.4 ± 6.1
1 to 2 cm	16.2 ± 11.

growth (19) supports the idea that the higher level of IAA in our sample might be due to rapidly progressed development under optimal growth conditions. Use of different cultivars could also be the cause of differences.

Another source of IAA increase could be the alkaline hydrolysis of ester-conjugated IAA during the serial partitioning. In this case we might measure the total IAA including both free and conjugated IAA. When considering the procedures to measure conjugated IAA include alkaline treatment step for several hours (14, 19), however, it is difficult to imagine the short, generally less than 5 min, exposure to alkaline conditions so profoundly affected the IAA quantification.

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