Journal of Chromatography, 238 (1982) 151–156
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 14,485

IDENTIFICATION OF ENDOGENOUS N-(3-INDOLEACETYL)ASPARTIC ACID IN SCOTS PINE (*PINUS SYLVESTRIS* L.) BY COMBINED GAS CHROMATOGRAPHY–MASS SPECTROMETRY, USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY FOR QUANTIFICATION

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(Received October 13th, 1981)

SUMMARY

N-(3-indoleacetyl)aspartic acid (IAAsp), a conjugate of the phytohormone 3-indoleacetic acid (IAA), was conclusively identified by combined gas chromatography–mass spectrometry (GC–MS) in dormant shoots of Scots pine (*Pinus sylvestris* L.). A procedure based on reversed-phase liquid chromatography and fluorescense detection is described for quantitative analysis of IAAsp levels in pine tissue. The current year's shoots of dormant seven-year-old trees of Scots pine contained 60 ng/g IAAsp fresh weight. The sensitivity of this method for IAAsp, applied to this material, was 1 ng, and 200 pg for pure standard. The preceding clean-up of the extract included the use of Amberlite XAD-7, a polyacrylic ester, which transfers organic compounds from a water phase to small volumes of organic solvents, an excellent alternative to the often used buffer–organic solvent extractions.

INTRODUCTION

The substance 3-indoleacetic acid (IAA) has for many years been considered to be a natural growth-regulating compound in plants. During the past decade, this substance has been conclusively identified in a number of species[1], and recently in conifers, including Scots pine[2–5]. It is, however, obvious that the concentration of the free IAA in plant tissue depends on synthesis, degradation and conjugation of the acid. Accordingly, it is necessary to estimate these reactions before correlations between phytohormone contents and exogenous factors can be considered. This paper describes a quantification method developed for a specific part of conjugated IAA. It has been obvious for many years that the IAA molecule in many cases is conjugated rather than oxidized, and when exogenous IAA is supplied to plants it is usually converted into 1-(3-indoleacetyl)-β-D-glucose or N-(3-indoleacetyl)aspartic acid

0021-9673/82/0000–0000/$02.75

(IAAsp)[1]. Other conjugates of IAA have also been proposed, such as with inositol and with different amino acids other than aspartic acid (Asp)[6–8].

IAAsp was first reported in 1955 as a metabolite of exogenously applied IAA to pea tissue by Andreae and Good[9]. Following this paper, a number of reports dealing with the presence of IAAsp in IAA-fed[10] and unfed[11–14] tissue have been published. Paper or thin-layer chromatography with colour reagents specific to indole compounds were the usual methods and these were also used after the hydrolysis of IAAsp to free indoleacetic acid and free aspartic acid. Feung *et al.*[15] in 1976 used direct-inlet mass spectrometry (MS) for the identification of IAAsp in fed crown gall tissue but obtained mixed spectra. In 1980 IAAsp was identified in fed pine tissue by ^{1}H nuclear magnetic resonance (NMR) spectroscopy[16]. An endogenous conjugate between 4-Cl-IAA and Asp, the monomethyl ester of N-(4-Cl-3-indoleacetyl)aspartic acid, was identified in peas using mass spectrometry[17].

The hydrolysis method is often used in quantitative analysis where the amount of free IAA is correlated to that of IAAsp even though the origin of the IAA is unknown. Conjugates of IAA have different chemical properties and are also supposed to have different physiological functions in plants. It is therefore necessary to develop quantification methods for each conjugate separately. The purpose of the present study was to identify IAAsp as an endogenous substance in Scots pine, and to develop a reliable high-performance liquid chromatographic (HPLC) method for quantitative analysis of the substance.

EXPERIMENTAL

Synthesis of IAAsp

The synthesis of (±)-IAAsp was performed by the method of Mollan *et al.*[18]. The product was further purified on a 30 × 1.6 cm I.D. Sephadex G-10 column with 0.5 *M* ammonium hydrogen carbonate as eluent. The column was packed and pre-eluted with 200 ml of this eluent before use. A UV detector at 275 nm was used and the 25–75-ml fraction was collected. After evaporation, pink crystals, m.p. 190–191°C, were obtained in a yield of 37%. Final identification was made using IR, ^{1}H NMR and MS techniques, and all spectra were in agreement with those previously published[19].

Plant material

The current year's shoots of seven-year-old Scots pine (*Pinus sylvestris* L.) were collected in October 1980 at a natural stand 25 km north-east of Umeå, Sweden. The plant material was stored at −80°C until analysed.

Reagents

The following reagents were used: methanol (redistilled; analytical-reagent grade for HPLC analysis), buffer chemicals (Merck, Darmstadt, G.F.R.; analytical-reagent grade), ethanol (p.a.), diethyl ether (p.a.), butanol (p.a.), poly-N-vinylpyrrolidone (PVP, purchased as Polyclar At powder, GAF Corp., New York, NY, U.S.A.), Celite (30–80 mesh for GC, BDH), Sephadex G-10 and LH-20 (Pharmacia, Sweden), Amberlite XAD-7 (purchased as Servachrom®-XAD-7, pract, 300–1000 μm, Serva, G.F.R.). Purification and sieving of XAD-7 were performed as follows.

The synthetic resin was washed seven times with water in a beaker and the fines were discarded by decanting. Further washing was performed with methanol (seven times), the resin was dried by filtration, sieved (30–45 mesh) and finally washed with diethyl ether in a Soxhlet apparatus (2 × 12 h), and then dried in air.

Columns for clean-up

All columns were wet-packed in the respective solvents. For the combined Celite–PVP–Sephadex LH-20 column (45 × 1 cm I.D.) water was used when packed. The bottom layer of Sephadex LH-20 (19.5 cm) was eluted with water (70 ml) before the next layer of PVP (24.5 cm) was added. This was then eluted with water (50 ml) and the Celite layer (1 cm) added. The column was further eluted with 150 ml of 0.1 *M* phosphate–citrate buffer, pH 4.5, before use. The first XAD-7 column (15 × 0.5 cm I.D.) was packed in water and eluted with 20 ml of 0.1 *M* phosphate–citrate buffer, pH 4.5, before use. The second XAD-7 column, of the same size, was packed and eluted in the same way as the first, but the pH of the buffer was 2.7.

Extraction procedure (Fig. 1)

Pine shoots (each 10 g) were homogenized (Ultra Turrax) in cold methanol (200 ml) and extracted at −18°C for 18 h. After filtration, 10 ml of 0.1 *M* phosphate buffer (pH 8.0) were added and the solution was evaporated to 5–10 ml in a rotary evaporator (<40°C). The same buffer was added to a final volume of 15 ml, the pH was corrected to 4.5 and the sample centrifuged. The extract was applied to the combined

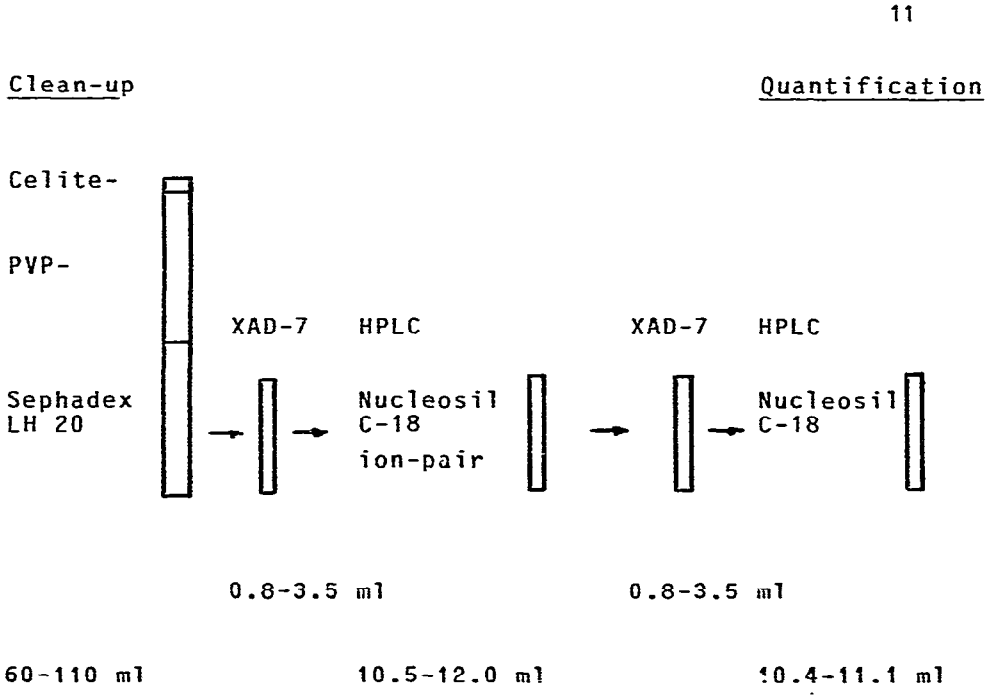

Fig. 1. Overview of the extraction procedure.

Celite–PVP–Sephadex LH-20 column. The 60–110-ml fraction was collected and applied to the XAD-7 concentrator column. The eluent was changed to ethanol, after which the 0.8–3.5-ml fraction was collected, evaporated to dryness in a stream of nitrogen (<40°C) and redissolved in 200 μl of 0.1 *M* phosphate buffer, pH 6.5, for HPLC analysis.

Equipment for HPLC and quantification of IAAsp

The HPLC system consisted of a Milton Roy Minipump connected via a Valco 50-μl loop injector to a 15 × 0.46 cm I.D. analytical column of 5-μm Nucleosil C-18 (Skandinaviska GeneTec AB, Sweden) and a Spectra-Physics SD-970 spectrofluorimetric detector with a 5-μl cuvette. The detector was adjusted to an excitation wavelength of 285 ± 5 nm. The emitted light was passed through an interference filter with a wavelength of 360 ± 10 nm. Two Nucleosil C-18 columns were used: the first in the preparative clean-up, and the second in the quantification step. The preparative column was eluted with methanol in 0.01 *M* phosphate buffer and 0.01 *M* tetrabutyl ammonium hydrogen sulphate. The methanol concentration was 25%, pH 6.5, and the flow-rate 1.5 ml/min. Fractions from four consecutive injections (10.5–12.0 ml) were collected and the methanol was evaporated with nitrogen. The 10-ml residue was adjusted to pH 2.7 and then applied to the second XAD-7 column as above. The 0.8–3.5-ml fraction was collected. After evaporation of ethanol, the extract was redissolved in 200 μl of 0.01 *M* phosphate buffer, pH 6.5, and applied to the second Nucleosil C-18 column for quantification. The eluent now consisted of methanol (25%), water (73%), and acetic acid (2%), and the flow-rate was 1.5 ml/min.

Equipment for GC–MS and identification of IAAsp

The gas chromatograph was equipped with a 25 m × 0.25 mm I.D. capillary quartz column OV-101 (Hewlett-Packard). The Grob-type injector was kept at 280°C. The column temperature was initially kept at 90°C for 1 min; it was then increased by 25°C/min to a final value of 280°C, which was held for 15 min. The gas chromatograph was connected to a Finnigan Model 4021 mass spectrometer equipped with a INCOS computer system. The temperature of the interface and the ion source was 250°C. The electron multiplier voltage was 1600 V and the spectra were recorded at 70 eV.

For the identification, fractions (10.4–11.1 ml) from four consecutive injections were combined after the second HPLC column. A few ml of butanol (azeotrope) were added and the extract was evaporated in a stream of nitrogen. The extract was then methylated with diazomethane in diethyl ether and a few drops of methanol and then purified on the second HPLC column as the IAAsp dimethyl ester. The eluent was methanol–water–acetic acid as before and the fraction (9.0–10.0 ml) was collected. After addition of butanol and evaporation, the extract was redissolved in a minimum amount (50 μl) of dichloromethane for GC–MS analysis. The retention time on GC was 9 min. Blank samples were treated in an identical way.

RESULTS AND DISCUSSION

The purification of pine extracts is very comprehensive and we started with the combined Celite–PVP–Sephadex LH-20 column used in our previous work[5,19,20].

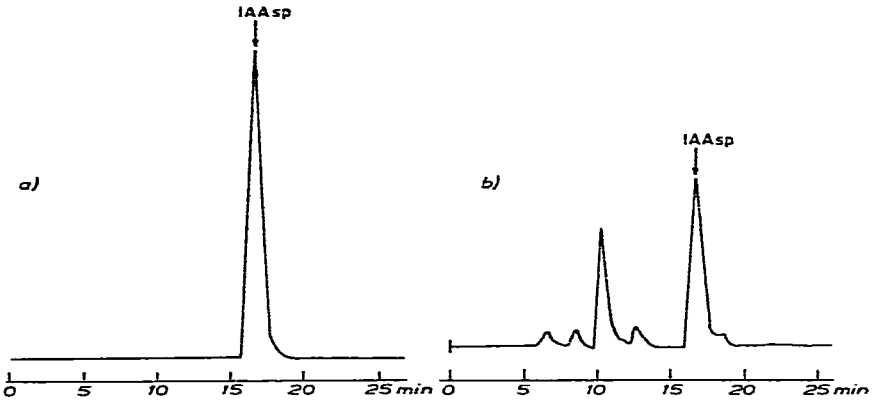

Fig. 2. HPLC chromatogram of (a) 100 ng standard IAAsp, (b) extract from 10-g pine shoots on Nucleosil C-18. Eluent: methanol–water–acetic acid (25:73:2).

where IAAsp is eluted with buffer. Extraction of this phase with organic solvents is difficult, however, since IAAsp is rather water-soluble even at low pH. For that reason we used column extraction on Amberlite XAD-7[21,22], which adsorbs organic compounds from water and releases them in a few ml of organic solvent. Ethanol was found to give high desorption efficiency and, moreover, to produce dry extracts after evaporation, since it forms an azeotrope with water. The XAD-7 column also had a favourable purification effect on the extract and, for that reason, we used two XAD-7 columns at two different pH values. The recoveries of IAAsp were quantitative at both pH values.

The identification of natural IAAsp in pine shoots was confirmed by retention time in two different HPLC systems, and by retention time as its dimethyl ester in both HPLC and GC. Final identification was afforded by combined GC–MS. All data agreed with those of the standard, and the mass spectrum also agreed with that published earlier[23].

Further HPLC clean-up was necessary before GC–MS identification since the residue of tetrabutylammonium ion from the first HPLC column resulted in a tremendous background noise in the GC analysis.

Owing to the huge amount of extractable compounds present in pine tissues, a multi-clean-up process had to be used for correct quantification of IAAsp. As can be seen in Fig. 2, a very clean extract is finally obtained. The tedious clean-up is thus worth the effort.

Today there is no doubt of the presence of IAAsp in plant tissue as a result of investigations that provide direct or indirect proof. Investigations of this type are often based on the ability of the tissue to convert applied IAA into a compound which, after hydrolysis, splits into compounds with the same chromatographic properties as indoleacetic acid and aspartic acid. However, there has been a lack of direct identification of IAAsp in unfed tissue. The present work thus provides the final proof for the presence of natural IAAsp in plant tissue.

The need for quantitative analysis of IAAsp has been still greater. Earlier indirect quantification of liberated IAA is based on the assumption that IAAsp is very

soluble in the organic phase in buffer/organic phase partitioning at low pH. We have found that this is correct for *n*-butanol. However, for diethyl ether or ethyl acetate (the solvents usually used in extraction of IAAsp) this is by no means true. The IAAsp losses can thus be very high if an unsuitable organic solvent is used. Neglecting this causes incorrect amounts of IAA after hydrolysis and thus a wrong picture is obtained of the actual amount of IAAsp present in the tissue.

The total yield of the extraction procedure is 35% and was determined in parallel where one sample was spiked with 1000 ng of standard IAAsp. It is intended to use [^{14}C]IAAsp, when available, for calculations of losses, which would afford better control of the yields in routine analyses.

ACKNOWLEDGEMENTS

We thank the staff at the National Board of Occupational Safety and Health, Umeå, for the GC–MS facilities put at our disposal. We offer our special thanks to Drs. Kurt Andersson and Arne Dunberg for the inspiring discussions we had with them and for their advice. The work was financially supported by the Swedish Council for Forestry and Agricultural Research (grants P316, P331 and P425), and the J. C. Kempe and Seth M. Kempe Memorial Foundations.

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