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Separation, detection, and quantification of imine-linked opines by high-performance liquid chromatography

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Abstract

Protocols for reversed-phase high-performance liquid chromatography (RP-HPLC) with fluorescence detection were developed for the separation, detection and quantification of imine-linked opines, following 4-fluoro-7-nitrobenzoxadiazole (NBD-F) derivatization. Both natural opines (mannopine, mannopinic acid, cucumopine, octopine, octopinic acid, and nopaline) and opine analogs (nor-mannopine, galactopine, glucopine) were included in the study. The on-column detection limits for NBD-opines varied from 0.1 pmol to 5 pmol. These methods were applied to quantify mannopine on the leaf surfaces of transgenic tobacco (*Nicotiana tabacum*) plants expressing mannityl opine synthesis (*mas*) genes from *Agrobacterium tumefaciens*.

Keywords: Derivatization, LC; Opines; Mannopine

1. Introduction

Opines are a unique class of natural substances which are synthesized in plant tissues genetically transformed by the soil bacterium *Agrobacterium tumefaciens*, the etiologic agent of crown gall disease [1]. It is thought that these opines provide an exclusive source of carbon and nitrogen for *A. tumefaciens* cells within the galls or in the soil surrounding the roots of the galled plant [2]. Transgenic opine-producing plants are currently under investigation because of their potential to selectively enhance colonization by engineered opine-catabolizing beneficial bacteria, such as those used for biological control of plant diseases or pests or for phytoremediation [3–8]. Such studies require methods to accurately quantify opines on the surfaces of transgenic plants which are available as sources of carbon and nitrogen for the engineered bacteria and prompted the development of the derivatization and HPLC-detection protocols reported here.

Two structural classes of opines have been documented [1,2,9], the sugar-phosphodiesters (agrocinopines) and the sugar- or organic acid-amino acid imines (primary amine derivatives). These opines have traditionally been detected by paper chromatography or high-voltage paper electrophoresis (HVPE) followed by chemical staining, using ninhydrin for opines with primary amines (lysopine, octopinic acid, and nopalinic acid) [2,10], phenan-

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threnequinone for opines with guanidino groups (octopine and nopaline) [2,11], or alkaline silver nitrate for opines with α-diols (agropine, agropinic acid, mannopine, and mannopinic acid) [2,12,13]. The main disadvantages of these detection methods are (i) the inability to accurately quantify the compounds; and (ii) the limited sensitivity (i.e. high detection limit). The detection limit of mannityl opines with HVPE and silver staining is approximately 1 μg/spot, while the detection limit of octopine and nopaline with HVPE and phenanthrenequinone staining is approximately 2 to 5 μg/spot [14]. Gas chromatography of non-guanidino opines following heptafluorobutyric-*n*-propyl derivatization is available [15].

Firmin [16] reported a precolumn derivatization of opines with *o*-phthalaldehyde (OPA) and separation by high-performance liquid chromatography (HPLC). However, imino opines without primary amines (such as octopine and nopaline) do not react with OPA. This paper describes a new detection method in which NBD-F, a sensitive reagent which reacts with both primary and secondary amines [17,18], can be used to derivatize opines prior to separation by HPLC and fluorescence detection. The method was subsequently used to quantify mannopine on the leaf surface of transgenic tobacco plants expressing *A. tumefaciens* mannityl opine synthesis (*mas*) genes [19].

2. Experimental

2.1. Chemicals

Eleven imine-linked opines were used in this study (Fig. 1). They represent four families: Agropine (agropine, mannopine, and mannopinic acid), octopine (octopine, allo-octopine and octopinic acid), nopaline (nopaline), and mikimopine (cucumopine). Three opine analogs (nor-mannopine, glucopine and galactopine), which have been used as alternatives to natural opines for utilization by mannityl opine catabolizing bacteria [20], were also included. Mannopine, nopaline, octopine, allo-octopine and octopinic acid were purchased from Sigma (St. Louis, MO, USA); galactopine, glucopine, and mannopinic acid were chemically synthesized according to

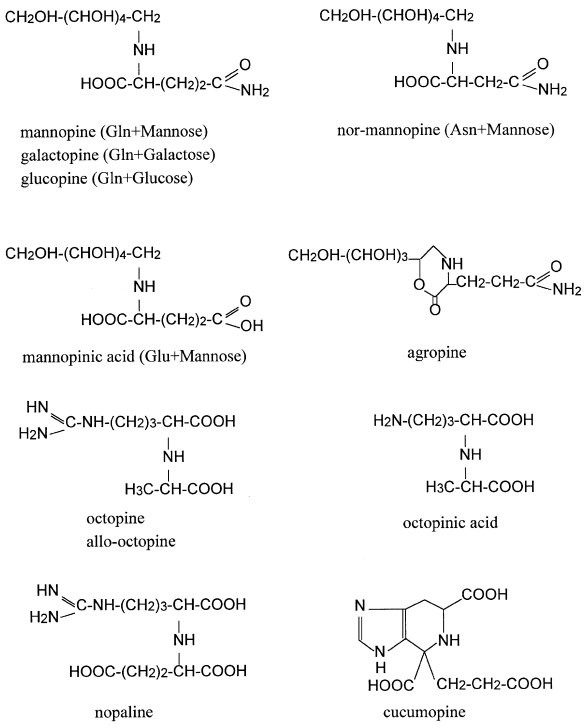

Fig. 1. Structural formulae of opines used in this study.

Tempé et al. [21] by Y. Dessaux and P. Guyon; cucumopine was chemically synthesized according to Davioud et al. [22] by E. Davioud; and agropine was enzymatically synthesized according to Dessaux et al. [23] by Y. Dessaux and P. Guyon. The purity was about 95–99% for galactopine, glucopine, mannopinic acid, and cucumopine, and 70% for agropinic acid as estimated by HVPE. Melting points for these opines are as follows: (i) agropine (natural) 175–176°C (Tate et al. [24]); (ii) agropine (chemically synthesized) 177–178°C (Tate et al. [24]); (iii) agropine (enzymatically synthesized) 181°C (Dessaux et al. [23]); (iv) agropinic acid 165–166°C (Tate et al. [24]); (v) cucumopine 205–210°C (Davioud et al. [22]); (vi) mannopine 173–175°C (Tate et al. [24]); (vii) mannopinic acid–data not available; (viii) L-allooctopine (in picrate) 222–230°C according to the authors (review by J. Tempé [25]); (ix) D-octopinic acid 256–271°C according to the authors (review by J. Tempé [25]); (x) D-octopine 262–306°C according to the authors (review by J. Tempé [25]); (xi) nopaline 195°C (Tempé [25]). No

data are available for the mannityl opine analogs (i.e. glucopine and galactopine). NBD-F was purchased from Sigma. Acetonitrile (CH_3CN) and methanol (MeOH) were HPLC grade; phosphoric acid (H_3PO_4), trifluoroacetic acid (TFA) and all other chemicals were of analytic reagent grade, and were purchased from Fisher Scientific (Pittsburgh, PA, USA).

2.2. Mobile phases

Two isocratic elutions were employed for separations of standard opines: a 10% aqueous CH_3CN with 0.1% TFA was used to separate amino acid–sugar opines; a 16% aqueous CH_3CN with 0.12% TFA was used to separate other opines. The flow-rate was 0.4 ml/min. Washates (leachates) from plant leaves were eluted by a modification of the multistep gradient elution of Kotaniguchi et al. [18], using a starting buffer A, 0.15 *M* H_3PO_4–CH_3CN (85:15, v/v), and a second buffer B, 0.1 *M* K_2HPO_4–CH_3CN–MeOH (40:21:39, v/v/v). The mobile phase was begun at 100% A for 11 min, followed by linear increase of B to 70% from 11 to 13 min, isocratic hold at 70% B from 13 to 35 min, and then linear increase of B to 100% from 35 to 45 min. The flow-rate was 0.7 ml/min. Before each run, the column was equilibrated with buffer A for 15 min. All solvents were filtered through a 0.45-μm microfilter (Sigma) and degassed prior to use.

2.3. Derivatization

For standard opine analysis, 15 μl of a mixed opine solution (0.75–37.5 nmol each), 15 μl of sodium borate buffer (0.2 *M*, pH 8.0), and 10 μl of NBD-F (15 m*M* in CH_3CN, prepared daily from a 60 m*M* stock in −20°C) were added to a 1.5-ml Eppendorf tube. The tube was capped, covered with aluminium foil, and heated on a Dry Heat Incubator (Fisher Scientific) at 60°C for 2.5 min. The tube was cooled on ice and 710 μl of 5 m*M* HCl were added to stop and dilute the reaction. A 10-μl volume of the NBD-opine derivative was injected onto the column.

For analysis of opines produced by transgenic tobacco plants (*Nicotiana tabacum* cv. Xanthi, line 2-26), leaves were gently washed with deionized water in a sterile plastic bag (approximately 20 μl water/cm^2 leaf). Leaf washates were centrifuged at 16 000 *g* for 5 min. A 15-μl volume of the leaf washate supernatant was allowed to react with NBD-F as described above. After cooling on ice, the reaction mixture was acidified by adding 110 μl of 50 m*M* HCl and 10 μl of the NBD-F derivative was injected onto the column.

2.4. HVPE analysis of derivatized agropine

Solutions of mannopine and agropine pre and postderivatization were analyzed by HVPE to determine whether agropine was degraded to agropinic acid during derivatization. Agropinic acid was not derivatized by NBD-F and the complete degradation of agropine to agropinic acid would account for the inability to derivatize agropine. Electrophoresis was carried out using a formic acidacetic acid buffer at pH 1.8 as described previously [4,18]. Samples of mannopine (10 μmol/ml), derivatized mannopine solution (10 μmol/ml), agropine (10 μmoles/ml), and derivatized agropine solution (10 μmoles/ml) were compared with a standard consisting of a mixture of 10 μmol/ml each of agropinic acid, agropine, mannopinic acid and mannopine.

2.5. HPLC

A 600E multisolvent delivery system equipped with a U6K universal injector and a model 600 controller (Waters, Milford, MA, USA) was used. Separation of the NBD-derivatives was carried out on a reversed-phased C_{18} column (Nova-Pak, 150× 3.9 mm, pore size 60 Å, Waters) at ambient temperature. A Waters 474 scanning fluorescence detector equipped with a 16-μl flow cell was used with an excitation wavelength of 470 nm and an emission of 530 nm. A Waters 996 photodiode array detector was simultaneously applied to peak identification at 470 nm absorbance. The fluorescence intensity and UV absorbance were recorded by a Millennium chromatographic data system (Waters).

3. Results and discussion

Of the imino opines used in this study, all but

agropine reacted with NBD-F. The NBD-F derivatives had both a fluorescence response (at excitation 470 nm and emission 530 nm) and UV absorbance (at 470 nm). Although the UV absorbance was approximately one order less than the fluorescence response with regard to signal-to-noise ratio, it was nevertheless useful for peak identification. Subsequently, the fluorescence detector was used throughout the study for sample analyses while the UV detector was used only as a control. The NBD-F derivatives of amino acid–sugar opines (i.e. nor-mannopine, mannopine, galactopine, glucopine, and mannopinic acid) were eluted out and separated within 15 min under isocratic elution of 10% aqueous acetonitrile (Fig. 2A). Residual glutamine, used to synthesize mannopine, galactopine, and glucopine, was detected at 17.1 min. Two background peaks of the derivatizing agent, NBD–NH_2 and NBD–OH, eluted at 22 and 24.6 min, respectively. Amino acid–organic acid opines and cucumopine were isocratically eluted out with 16% aqueous acetonitrile within 25 min (Fig. 2B). Residual arginine, one component of nopaline and octopine, also was detected at 5.8 min and NBD–NH_2 and NBD–OH eluted at 10.7 and 13.5 min, respectively. Octopine, however, eluted as two peaks under these conditions, suggesting that there may have been two stereoisomers present in the sample. Alternatively, it is possible that the NBD-F may derivatize another N atom of the compound, forming a di-NBD-octopine derivative; this was not observed for either nopaline or octopinic acid, suggesting that it is unlikely.

An acidic mobile phase (approximately pH 1.8) was useful to quench the excess of the reagent, reducing the intensity of the peak of NBD-OH, the hydrolysate of NBD-F. While phosphoric acid as an acidic modifier gave higher fluorescence intensity than TFA, TFA provided better peak resolution.

The sensitivity of detection of the NBD-opine derivatives varied, ranging from 0.1 pmol for octopinic acid to 5 pmol for cucumopine. In the case of octopinic acid, the NBD-F may react with its pri-

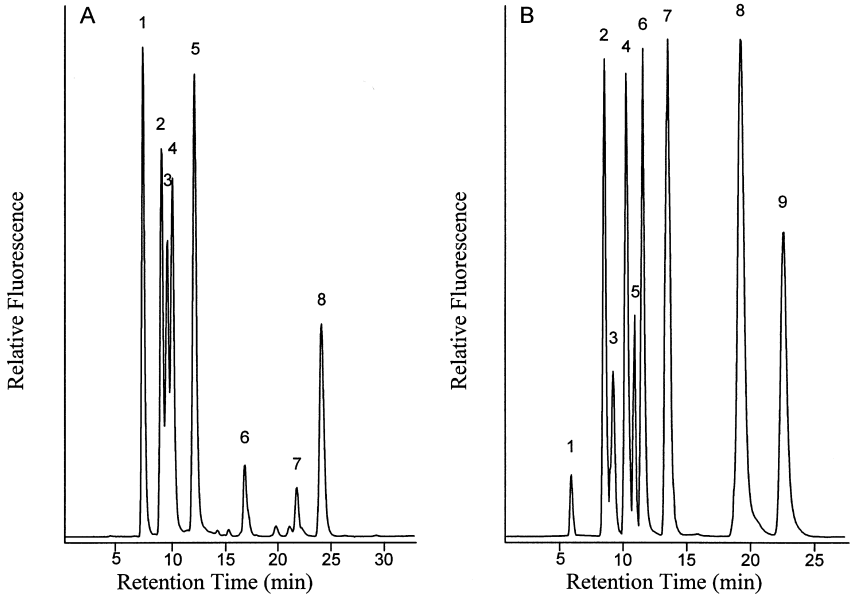

Fig. 2. Chromatograms of NBD-F-derivatized opines. Separation was carried out on a Nova-Pak C_{18} column (150×3.9 mm) at ambient temperature. The fluorescence detection was at e_x 470 nm and e_m 530 nm. (A) Isocratic elution was 10% aqueous acetonitrile with 0.1% TFA at a flow-rate of 0.4 ml/min. Peaks (each representing 100 pmol): 1=nor-mannopine; 2=mannopine; 3=galactopine; 4=glucopine; 5=mannopinic acid; 6=glutamine (residual contaminant in mannopine etc.); 7=NBD-NH_2; and 8=NBD-OH. (B) Isocratic elution was 16% aqueous acetonitrile with 0.12% TFA at a flow-rate of 0.4 ml/min. Peaks: 1=Arg (residual contaminant in octopine etc.); 2=octopinic acid (10 pmol); 3=cucumopine (500 pmol); 4=nopaline (500 pmol); 5=NBD-NH_4; 6=allo-octopine (100 pmol); 7=NBD-OH; 8 and 9=octopine (100 pmol).

mary amine. However, when proline, which has only a secondary amine, and glutamine were used to compare relative derivatization efficiency, the reactivities and fluorescence yields of NBD-proline and NBD-glutamine were similar to that of NBD-octopinic acid at the concentration range from 1 to 100 pmol (although the fluorescence response of the amino acid derivatives was about one order of magnitude higher than that of the opine derivative), suggesting that NBD-F reacts effectively with both primary and secondary amines under the experimental conditions. The structures of different opine molecules may determine the efficiency of derivatization. Nevertheless, the HPLC detection limits were much lower than with the traditional methods, for example, a detection limit of 3 nmol for mannityl opines using HVPE and silver stain, and 8–10 nmol for octopine and nopaline using HVPE and phenanthrenequinone stain.

The NBD-opines were found to be quite stable when acidified with HCl. There was no decrease in either peak height or area of NBD-opines after 8 h at room temperature in the dark, suggesting that degradation of NBD-opines did not occur. The average R.S.D. of peak areas was 3.15% at 100 pmol of each opine.

Enzymatically synthesized agropine [23] was not derivatized by NBD-F or by any of the other reagents that are used to label secondary amines, such as dansyl-Cl [26], phenylisothiocyanate (PITC) [27] or thiamine-Cl [28]. Agropine (Fig. 1) is N^2-(1-deoxy-D-mannital-1′-yl)-L-glutamine, a lactone of the acyclic mannopine [24]. According to the proposed structure, the imino group remains unchanged after lactonization and should have been available to react with NBD-F, as in the case of mannopine. The possibility that agropine was not stable under the derivatization conditions and was converted to agropinic acid, a tertiary amine (lactam) opine [24], which would not be able to react with NBD-F, was eliminated by HVPE analysis of the agropine solution pre and postderivatization. HVPE analysis indicated that agropine was present in both solutions, at approximately the same concentration, and that there had been no degradation to agropinic acid (Fig. 3); hence, we must conclude that for some reason enzymatically-synthesized agropine cannot be derivatized with NBD-F or the other reagents mentioned above. Unfortunately, no chemically-synthesized agropine was available to determine whether it could be derivatized using the protocol described.

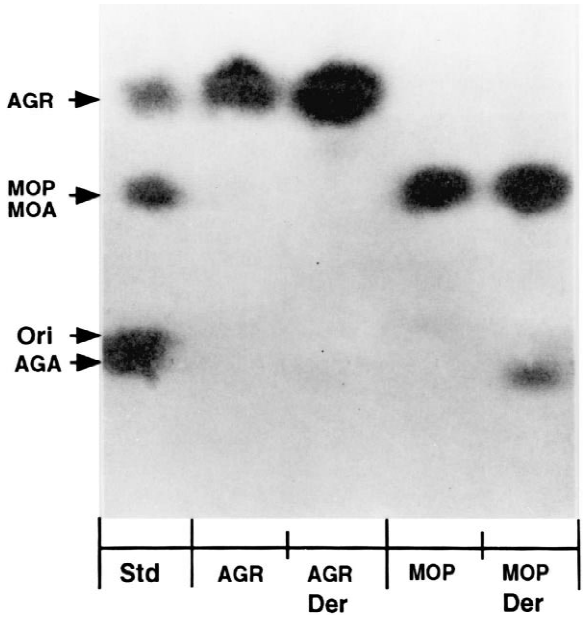

Fig. 3. High-voltage paper electrophoretogram of mannopine and agropine solutions pre and postderivatization with NBD-F. Lanes: 1=2 μl of a standard consisting of a mixture of 10 μmoles/ml each of agropinic acid (AGA), agropine (AGR), mannopinic acid (MOA) and mannopine (MOP); 2=2 μl of 10 μmol/ml of agropine (AGR); 3=2 μl of 10 μmol/ml of NBD-F-derivatized-agropine (AGR Der); Lane 4=2 μl of 10 μmol/ml of mannopine (MOP); 5=2 μl of 10 μmol/ml of NBD-F-derivatized-mannopine (MOP Der). Other abbreviations: origin (ori).

Leachates from leaves of greenhouse-grown wild-type and transgenic tobacco plants that express mannityl opine synthesis (*mas*) genes were analyzed by HPLC (Fig. 4). Under the conditions described, the opines, mannopine and mannopinic acid, and 17 identified amino acids from leaves of transgenic plants were detected and separated (Fig. 4B). The amounts of mannopine and mannopinic acid ranged from 0.5 to 2.3 $\mu g/cm^2$ leaf area and 0.1 to 0.5 $\mu g/cm^2$ leaf area, respectively. These concentrations of mannopine are close to the levels of agropine previously estimated in washings of leaves from the same line of greenhouse-grown transgenic plants using HVPE, which ranged from 1.3 $\mu g/cm^2$ leaf area on mid-level leaves to 1.9 $\mu g/cm^2$ leaf area on lower leaves [4]. Mannopine and mannopinic acid

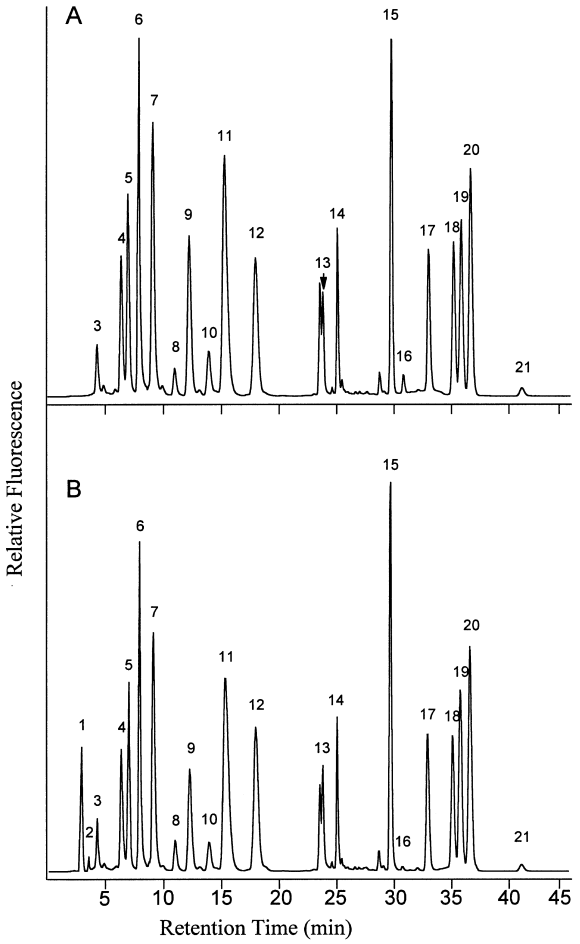

Fig. 4. Chromatograms of NBD-F derivatized tobacco leaf washates from (A) the wild type and (B) the transgenic plants. Separation was carried out on a Nova-Pak C_{18} column (150×3.9 mm) at ambient temperature. Elution conditions: 0% B from 0 to 11 min, 0 to 70% B from 11 to 13 min, 70% B from 13 to 35 min, and 70 to 100% B from 35 to 45 min. The flow-rate was 0.7 ml/min. Peaks: 1=mannopine, 2=mannopinic acid, 3=His, 4=Arg, 5=Asn, 6=Gln, 7=Ser, 8=NBD-NH_2, 9=Asp, 10=Glu, 11=NBD-OH, 12=Thr, 13=Ala, 14=Pro, 15=Val, 16=Cys, 17=Lys, 18=Phe, 19=Ile, 20=Leu, and 21=Tyr.

were not detected in leaves of the wild-type plants (Fig. 4A).

In conclusion, the NBD-F derivatization and HPLC analysis described provides a rapid and sensitive assay for opines with amino or imino groups. The protocol is currently being used to quantify the spatial distribution of mannopine in the gall, stems, roots, and leaves of plants with *A. tumefaciens* tumors [29] and to quantify mannopine on the surface of leaves of tomato (*Lycopersicon esculentum*) plants transformed with the *mas* genes [30].

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