Journal of Chromatography, 514 (1990) 343–347
Elsevier Science Publishers B.V., Amsterdam

CHROM. 22 595

Note

High-performance liquid chromatographic analysis of octopinic acid, lysopine and nopalinic acid as sensitive indicators of *Agrobacterium*-induced crown gall tumours

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(First received January 12th, 1990; revised manuscript received May 15th, 1990)

Octopinic acid, lysopine and nopalinic acid are members of a group of iminocarboxylic acids collectively termed opines (Fig. 1) which are characteristically found in crown gall tumours —plant tumours induced by the soil-living bacterium *Agrobacterium tumefaciens*[1]. Depending on the strain of *A. tumefaciens* which initiated the tumour, the tissue will contain either the octopine family (I–IV) or the nopaline family (V and VI). Octopine also occurs in some marine invertebrates[2].

The presence of opines in plant tissue is generally taken to indicate that the tissue has been genetically transformed by *A. tumefaciens*. Thus opines are useful markers of transformation. Opines occur in *Agrobacterium*-transformed plant tissue at levels from *ca.* 20 μg/g dry weight (d.w.) (octopine) to *ca.* 20 mg/g d.w. (nopaline)[3]. The validity of using opines as markers of transformation has been questioned by several workers[4–6]. Thus it has been reported that untransformed soybean callus can synthesise nopaline when cultured on a medium supplemented with arginine[6]. In addition lysopine has been reported to occur in untransformed tomato and tobacco tissue[4]. However, there are no reports of untransformed plant tissue containing, or able to synthesize, either octopinic acid or nopalinic acid.

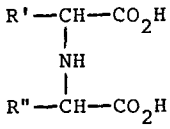
Detection of opines in transformed plant tissue has generally relied on detection of the guanidino group of octopine or nopaline using either the Sakaguchi reagent[7] or the more sensitive fluorescence reagent phenanthrenequinone[8], after paper chromatography or paper electrophoresis of the tissue extract.

The non-guanidino opines octopinic acid, lysopine and nopalinic acid can be detected on paper chromatograms using ninhydrin[9–11] and ninhydrin has also been used in conjunction with ion-exchange column chromatography to detect octopinic acid and lysopine[12]. The detection limits for these opines are not reported.

Gas chromatography of the heptafluorobutyric-*n*-propyl derivatives of the non-guanidino opines has been used[3]; however, this method requires a time-consuming derivatization step and does not have the sensitivity of the fluorescence method.

This paper reports the detection of octopinic acid, lysopine and nopalinic acid in

0021-9673/90/$03.50



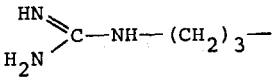
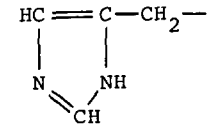
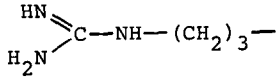
	OPINE	R'	R"
I	Octopine		CH_3—
II	Octopinic acid	H_2N—$(CH_2)_3$—	CH_3—
III	Lysopine	H_2N—$(CH_2)_4$—	CH_3—
IV	Histopine		CH_3—
V	Nopaline		HO_2C—$(CH_2)_2$—
VI	Nopalinic acid	H_2N—$(CH_2)_3$—	HO_2C—$(CH_2)_2$—

Fig. 1. Structures of the iminocarboxylic opines found in crown gall tumours. Depending on the strain of *A. tumefaciens* which initiated the tumour, the tissue will contain either the octopine family (I–IV) or the nopaline family (V and VI).

transformed plant tissue, using reversed-phase separation of the *o*-phthaldialdehyde (OPA) derivatives of these opines.

MATERIALS AND METHODS

Octopine and octopinic acid were obtained from Sigma. Lysopine was synthesized by hydrolysis of homooctopine with barium hydroxide[13]. Nopaline was synthesized as described by Cooper and Firmin[14]. Nopalinic acid was synthesized by hydrazinolysis of nopaline[10]. Paper chromatography and detection of the non-guanidino opines with ninhydrin was carried out as described in ref. 10.

Tissue extraction and derivatization

Samples [*ca.* 10 mg fresh weight (f.w.)] of plant tissue were extracted by grinding with acid-washed sand and aqueous ethanol (70%, v/v, 20 μl/mg f.w. of tissue). After

centrifugation (10 000 *g*, 5 min) the supernatant was stored at −20 °C until required. OPA derivatives were prepared immediately prior to injection onto the column by mixing 10 μl of the tissue extract with 90 μl of OPA reagent [OPA reagent is 10 ml, 0.5 *M*, pH 10.4 potassium borate + 100 μl OPA solution (80 mg/ml methanol) + 20 μl 2-mercaptoethanol]; 10–100 μl of the derivative was injected onto the column. For further details about preparation and stability of the OPA reagent see ref. 15.

High-performance liquid chromatography (HPLC)

Separation of the OPA derivatives was carried out on a reversed-phase C_8 column (Brownlee RP300, 7 μm), 25 cm × 4.6 mm I.D., fitted with a 3 cm × 4.6 mm I.D. guard column. The column was eluted with a pH 6.8 citrate (0.1 *M*, pH adjusted with KOH) methanol gradient (see Fig. 2 for details) at 1 ml/min. OPA derivatives in the eluate were detected by fluorescence (excitation 340 nm, emission 455 nm, bandwidth 10 nm).

RESULTS AND DISCUSSION

The fluorescence traces obtained from extracts of normal and *Agrobacterium*-transformed plant tissue after derivatization with OPA are shown in Fig. 2. Gradient elution was required to separate the opines from other amino acids present in the extract. The OPA derivatives of the opines show a similar instability to that found with the OPA derivatives of ornithine and lysine; thus the derivatized sample should be injected onto the column immediately after it is prepared.

The short half life of the OPA derivatives of octopinic acid, lysopine and nopalinic acid can be useful in the event of ambiguity in peak identification since delaying injection of the derivatized sample by 15 min results in a three-fold decrease in the size of the opine peak relative to peaks given by most other amino compounds *e.g.* glutamate and aspartate. Replacing 2-mercaptoethanol with ethanedithiol, an alternative reducing agent which is reported to increase the stability of several OPA-amino acids, including OPA-ornithine and lysine[16], did increase the stability of OPA-octopinic acid, -lysopine and -nopalinic acid, presumably for the same reasons that OPA-ornithine and -lysine are stabilized. However, the resulting increased retention times together with some loss of resolution would require modification of the elution conditions. N-Acetyl-L-cysteine, another alternative reducing agent[17], also increased the stability of the OPA-opine derivatives but resulted in loss of resolution of the OPA-opines from other amino acids present in the extract.

The guanidino opines octopine and nopaline do not react with OPA under the conditions described here to yield UV-absorbing or fluorescent derivatives.

Reducing the pH of the citrate buffer below 6.8 resulted in loss of resolution of the opines from other amino acids. Octopinic acid, lysopine and nopalinic acid had a response factor (normalized to glutamic acid) of *ca.* 0.5. The limit of detection for these opines was *ca.* 0.5 pmol. By contrast, the limit of detection for these opines with ninhydrin on paper was *ca.* 20 nmol.

The method described in this paper provides a rapid and sensitive assay for the non-guanidino opines octopinic acid, lysopine and nopalinic acid, and has been used successfully with several other plant species *e.g.* sunflower, pea, flax and tobacco.

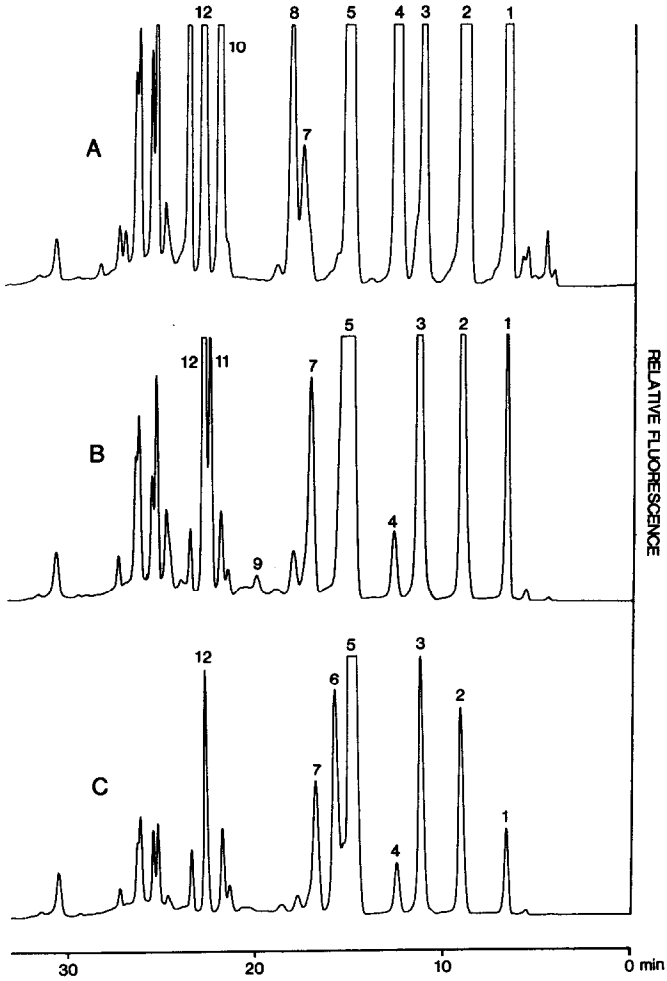


Fig. 2. HPLC of OPA derivatives from (A) untransformed mint (*Mentha citrata*), (B) mint transformed with an octopine strain of *A. tumefaciens* (strain A384) and (C) mint transformed with a nopaline strain of *A. tumefaciens* (C58). The traces shown are equivalent to 50 μg f.w. of tissue with the exception of sample B, which was injected at a higher level (100 μg f.w.) because of the lower relative concentration of octopinic acid. The column was eluted with a pH 6.8 (0.1 *M*) citrate–methanol (B) gradient: 0 min: 20% B, 13.2 min: 35% B, 19.8 min: 75% B, 26.4 min: 75% B, 33 min: 20% B. A further 10 min were allowed for re-equilibration. All increments are linear. Peaks: 1 = aspartate; 2 = glutamate; 3 = asparagine, 4 = serine; 5 = glutamine; 6 = nopalinic acid; 7 = arginine; 8 = threonine; 9 = octopinic acid; 10 = alanine; 11 = lysopine; 12 = γ-aminobutyric acid.

ACKNOWLEDGEMENTS

I am grateful to Andrew Spenser, AFRC Institute of Food Research, Norwich, U.K., for supplying crown gall tumours and untransformed plant tissue, and to Dr. J. Tempé, Institut des Sciences Végétales, Centre National de la Recherche Scientifique, France, for supplying a sample of homooctopine.

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