OCTOPINE AS A MARKER FOR THE INDUCTION OF TUMOROUS GROWTH BY AGROBACTERIUM TUMEFACIENS STRAIN B_6

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SUMMARY:

Octopine [N^2-(D-1-carboxyethyl)-L-arginine] was detected in all tobacco and sunflower crown gall tumors incited by Agrobacterium tumefaciens (E. F. Sm. and Town.) Conn strain B_6 at levels between 1 and 2.5 μmoles/20 g fresh weight. Most tissue cultures derived from plant tumors contained octopine at levels between 0.3 and 1 μmole/20 g fresh wt. Normal plant tissues and tissue cultures derived from normal tissues contained no detectable octopine when assayed by a [^{3}H] arginine incorporation technique designed to detect low levels of octopine (less than 0.5 nmole/20 g fresh wt).

Agrobacterium tumefaciens (E. F. Sm. and Town.) Conn induces a tumorous growth (crown galls) on many dicotyledonous plants (1). Because axenic tissue cultures of crown galls maintain characteristic autonomous growth and apolar cell division, it is reasonable to suspect that bacterial DNA may be integrated into the plant genome (1). As a result, investigators have searched for compounds unique to crown gall tissues. Two such compounds, octopine [N^2-(D-1-carboxyethyl)-L-arginine] and nopaline [N^2-(1,3-dicarboxypropyl)-L-arginine] were found by Morel (2, 3). Further, he noted that the bacterial strain used to incite the tumors, rather than the plant species, determined which compound was present in the tumors (3). He therefore concluded that octopine and nopaline were products of bacterial gene expression. Recently, this conclusion has been disputed because octopine was detected in normal tobacco and sunflower tissues (0.33 μmoles/20 g fresh wt) (4), and in habituated

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tobacco callus and bean roots (0.20 μmoles/20 g fresh wt) (5). These observations suggest that genes coding for octopine biosynthesis reside in the normal plant genome; thus, octopine production may be merely increased in crown gall cells. Finally, further studies have not substantiated these results, suggesting that octopine is absent in normal tissues (6). Because of the significance of this controversy to the question of bacterial DNA integration, we examined the amounts of octopine present in normal and crown gall tissues using a different, highly sensitive method.

MATERIALS AND METHODS

Plant Material - Three-week-old crown galls were excised from plants wound-inoculated with Agrobacterium tumefaciens strain B_6 (7). Tissue culture lines of primary crown galls (tumors arising at the original site of inoculation), secondary crown galls (tumors spontaneously arising at sites distant from the site of inoculation), and normal tissues were then established from sunflower (Helianthus annuus, cv.'Mammoth Russian') (8) and tobacco (Nicotiana tabacum L. cv.'Wisconsin 38') (7).

Extraction and Separation of Amino Acids - Tissues (20 g fresh wt) were homogenized with a Polytron[1] tissue disrupter in an equal volume (w/v) of 10% trichloroacetic acid. The homogenate was centrifuged (10 min at 10,000 x g) and the clear supernatant adsorbed to a 0.9 x 10 cm column of Amberlite CG-120 ion exchange resin equilibrated with 2N HCl. The resin was washed with H_2O until the pH increased to 6. The amino acids were then eluted with 0.6 N ammonium hydroxide. The eluant was evaporated to dryness (in vacuo, 35°C) and the residue was dissolved in 1 ml H_2O. The amino acids in this fraction were separated using Beckman PA-28 ion exchange resin as described earlier (9). Elution was accomplished at 60°C by pumping pH 3.49 buffer through the column at a flow rate of 1 ml/min for 150 min, followed by pH 5.95 buffer for an additional 150 min. Fractions (2 ml) were collected. Octopine eluted from the resin well-separated from all other amino acids at 106 min (Figure 1). The amount of octopine eluting between 100-110 min was quantitated with the colorimetric assay for guanidine and normalized to 20 g fresh wt of tissue.

Colorimetric Assay for Guanidine - The procedure for determining octopine was that of Ménagé et al. (10) as modified by Johnson et al. (4). Standard curves for octopine and arginine were linear between 4 and 300 nmoles (A_{530} 0.02 to 2.0). The limit of detection for the results reported in Tables 1 and 2 was 1 nmole of octopine/g fresh wt of tissue.

Thin-Layer Chromatography - Cellulose plates (Cellulose MN 300, Brinkmann Instruments) were used to confirm the identity of isolated octopine. Samples were chromatographed in a solvent system of methanol:H_2O:pyridine (20:5:1) (M. D. Chilton, personal communication). After air drying, the amino acids were detected by spraying the plate with 0.2% ninhydrin in acetone. Subsequently, octopine was visualized under ultraviolet light after a second spraying with 0.01% phenanthrene quinone in 75% ethanol containing 5% sodium hydroxide (4). Rf's were compared to those of standards.

[1]Mention of companies or commercial products does not imply recommendation or endorsement by the U. S. Department of Agriculture over others not mentioned.

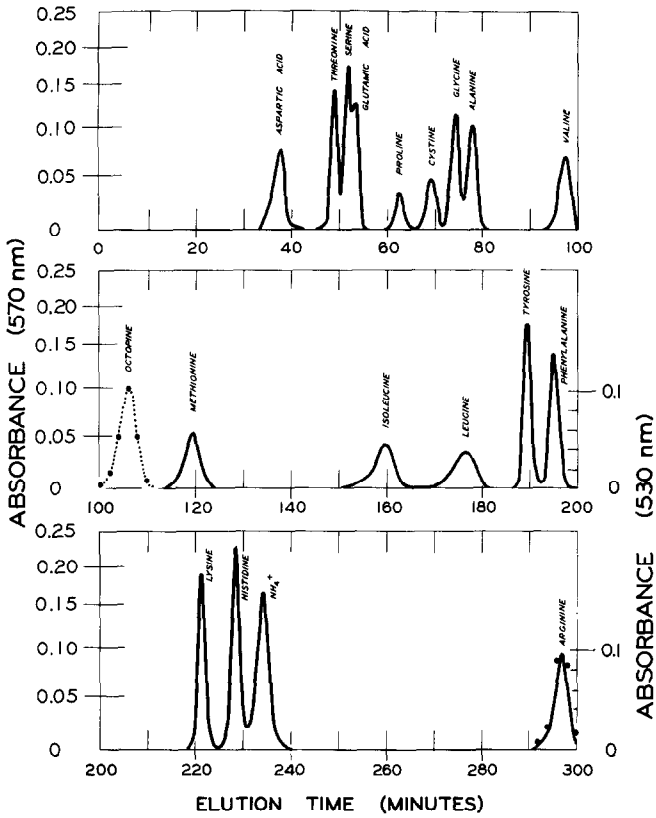

Figure 1. Chromatogram of authentic amino acids. Ninhydrin-reacting materials (--) were measured at 570 nm. Guanidine-containing material (•--•) was measured at 530 nm.

Radioactive Assay for Guanidine - A radioactive labeling method of analysis was developed to detect low concentrations of octopine. Tissues were labeled by transferring them for five hours to fresh medium supplemented with 0.06 μM 3-[^{3}H]-L-arginine (25 Ci/mmole, New England Nuclear). After separating the amino acids by the procedures outlined above, the specific radioactivities (μCi/μmole) of octopine and arginine were recorded (9). Since twice background levels of radioactivity could be measured if present in 0.3 ml aliquots from the column fractions, a lower limit of detection was set at 10_pCi of radioactivity in octopine or arginine/g fresh wt.

RESULTS

Octopine was found in fresh crown galls from infected tobacco plants (2.4 μmoles/g fresh wt) and sunflower plants (1.04 μmoles/g fresh wt) (Table 1). Slightly lower amounts were measured in all of the primary and secondary sunflower tissue cultures derived from fresh crown galls (0.28 to 0.47 μmoles/g fresh wt). However, only one of the two tobacco tissue culture lines of crown

Table 1. Octopine Content of Various Normal and Crown Gall Tissues.

PLANT TISSUE	OCTOPINE (μmoles/20 g fresh wt)
CROWN GALL	
Fresh Stem Tissues	
Tobacco	2.40
Sunflower	1.04
Sunflower Tissue Cultures	
Primary Crown Gall (PSCG-4)	0.45
Primary Crown Gall (PSCG-7)	0.28
Primary Crown Gall (PSCG-11)	0.41
Secondary Crown Gall (SSCG)	0.47
Tobacco Tissue Cultures [a]	
Primary Crown Gall (E-1)	0.41
Primary Crown Gall (TCG)	ND [d]
NORMAL	
Fresh Stem Tissues	
Tobacco	ND
Sunflower	ND
Sunflower Tissue Cultures [b]	
Habituated (HSSS)	ND
Normal (SSS)	ND
SSS + 0.4 μmoles octopine	0.31
Tobacco Tissue Cultures [c]	
Habituated (DV_{12})	ND
Normal (OLT)	ND

a/ E-1 tissue, a gift of M. D. Chilton, was induced by A. tumefaciens strain B_6-806 on N. tabacum L. cv. Xanthi (4). TCG was isolated by us (7).

b/ HSSS is habituated tissue which arose spontaneously from normal (SSS) sunflower tissue (8).

c/ DV_{12}, obtained from J. P. Helgeson, is habituated tissue which arose spontaneously from normal (OLT) tobacco tissue.

d/ Not detectable by the colorimetric assay.

gall origin contained enough octopine to be detected by the colorimetric assay (Table 1). Finally, octopine could not be detected in plant parts or tissue culture lines derived from normal plant parts. It was possible that those

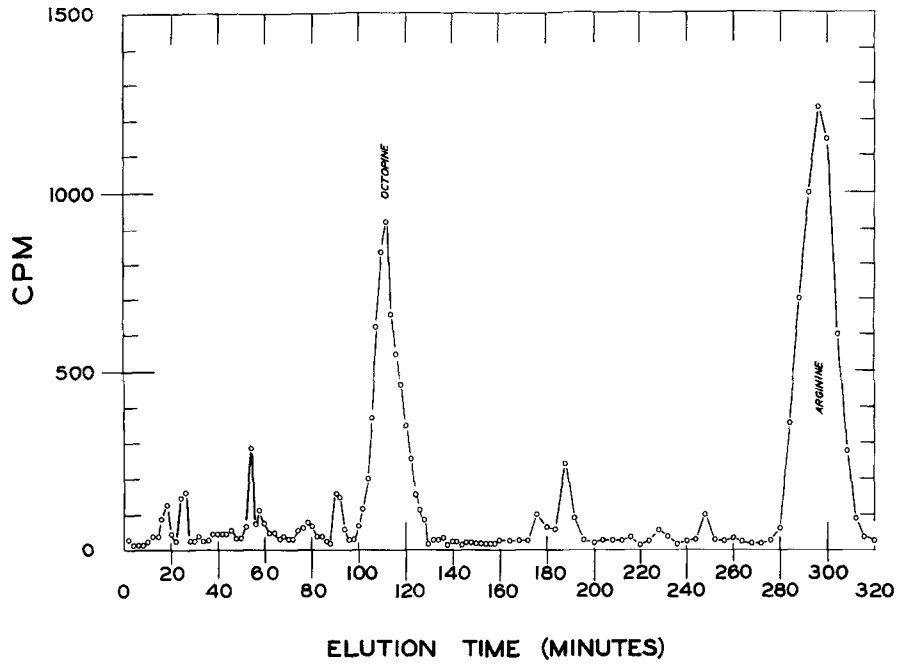

Figure 2. Chromatogram of amino acids present in the acid-soluble fraction of SSCG tissue labeled 5 hr on medium containing [^{3}H]-arginine. Radioactivity was measured in an aliquot of each fraction collected from the PA 28 resin.

tissues appearing to lack octopine may, in fact, rapidly degrade octopine when disrupted. However, this possibility was eliminated when 75% of the authentic octopine was recovered following addition to 20 g of normal tissue (SSS) before homogenization (Table 1).

When tissues were labeled on medium containing [^{3}H]-arginine, 90% of the radioactive material eluted from the resin with octopine and arginine (Figure 2). The remaining 10% of the radioactivity was associated with several other small amino acid peaks. All of the tissues tested contained substantial amounts of radioactive arginine (0.9 to 1.4 μCi/μmole) (Table 2). However, only tissue culture lines of crown gall origin (PSCG-4, -7, -11, SSCG, and E-1) contained measurable amounts of radioactivity in octopine.

A lower level of detection for the radio-labeling assay was estimated by assuming that the specific radioactivity of any octopine that may be present in normal tissues would be similar to the specific radioactivity of

Table 2. Specific Radioactivities of Octopine and Arginine in Normal and Crown Gall Tissue Cultures Labeled 5 hr on [^{3}H]-Arginine.

TISSUE [a]	OCTOPINE (μmoles/20g fr wt)	ARGININE (μmoles/20g fr wt)	OCTOPINE (μcuries/20g fr wt)	ARGININE (μcuries/20g fr wt)	OCTOPINE (μCi/μmole)	ARGININE (μCi/μmole)
PSCG-4	0.40	0.23	0.177	0.230	0.44	1.00
PSCG-7	0.20	1.53	0.069	1.420	0.35	0.93
PSCG-11	0.44	0.29	0.152	0.25	0.35	0.86
SSCG	0.20	0.40	0.180	0.56	0.90	1.40
E-1	0.33	1.22	0.130	1.60	0.40	1.31
TCG	ND [b]	0.59	ND [d]	0.54		0.90
HSSS	ND	0.34	ND	0.35		1.02
SSS	ND	0.60	ND	0.71		1.20
OLT	ND	-- [c]	ND	--		--
DV_{12}	ND	0.64	ND	0.50		0.78

a/ See Table 1 for description of tissues and Figure 2 for labeling procedures.
b/ Not detectable by the colorimetric assay.
c/ Not measured.
d/ No detectable radioactivity in a 0.3 ml aliquot of each fraction.

octopine recorded for crown gall tissues. Such an assumption is probably valid because the specific radioactivity of arginine, the presumed biosynthetic precursor of octopine, was similar for all tissues. Thus, normal tissue cultures can contain no more than 0.5 nmoles of octopine/20 g fresh wt.

DISCUSSION

Octopine was not detected in any of the normal tissues examined by either the colorimetric or radioactive assay. The apparent detection of considerable amounts of octopine in normal tissues (4, 5) cannot be attributed to differences in the assay procedure since the same colorimetric assay was used by each investigator. More likely, the difference lies in the fact that the PA-28 resin does a superior job of separating octopine from interfering compounds such as the guanidines reported by Bell and coworkers (11).

It is interesting to note that although TCG tissue is of crown gall

origin, it does not contain detectable levels of octopine. A possible explanation is that habituation, the ability to grow in culture on medium lacking hormones, readily occurs when Wisconsin 38, but not Xanthi, tobacco cells are placed in culture and that the habituated cells outgrow the tumor cells. A study of early events during culturing is needed.

In conclusion, we have confirmed Morel's observation that the presence of octopine is correlated with tumorigenesis. It is quite possible that genes for octopine biosynthesis are part of the normal plant genome and that A. tumefaciens activates these genes during tumorigenesis. However, any final conclusion as to the genetic origin of the ability to produce octopine is premature.

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