A NEW AMINO ACID DERIVATIVE PRESENT IN CROWN GALL TUMOR TISSUE

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Received November 29,1976

SUMMARY:

A new amino acid derivative has been found in primary and secondary sunflower crown gall tissue cultures and in fresh crown gall tumors from sunflower plants wound-inoculated with *Agrobacterium tumefaciens* B_6. Normal plant tissue does not contain detectable levels of the compound. Radioactive labeling and cochromatography experiments strongly suggest that the natural derivative is identical to synthetic N^2-(1-carboxyethyl)-L-histidine (histopine). Crown gall tissue cultures contain 1 μmole of histopine/20 g fresh weight. *A. tumefaciens* strain B_6, but not strain C_{58}, can utilize natural histopine and incorporate the products into macromolecules.

Agrobacterium tumefaciens (E. F. Sm. and Town.) Conn induces tumorous growths (crown gall) on many dicotyledonous plants. A unique characteristic of crown gall tumors is the production of high levels of unusual amino acids [e.g., octopine N^2-(D-1-carboxyethyl)-L-arginine (1) or nopaline N^2-(1,3-dicarboxypropyl)-L-arginine (2)]. The bacterial strain rather than the species of plant determines whether octopine or nopaline is produced in the tumor (2). For example, strain B_6 produces crown gall tumors which always contain octopine, whereas strain C_{58} produces tumors containing nopaline. Further, a bacterial strain can usually utilize as a sole source of carbon and nitrogen only that unusual amino acid that it induces in the tumor (3).

Octopine is just one in a family of N^2-(1-carboxyethyl)-amino acids found in crown gall tumors induced by strain B_6. Other members include the L-lysine derivative (lysopine) (4), and the L-ornithine derivative

Research cooperative with the College of Agricultural and Life Sciences, University of Wisconsin, Madison, and the Agricultural Research Service, U. S. Department of Agriculture.

octopinic acid) (5). Notably the reported derivatives include all but one of the common basic amino acids, the exception being L-histidine. Thus far, nopaline is the only reported member of the N^2-(1,3-dicarboxypropyl)-amino acid family found in tumors induced by strain C_{58}.

The bacterial genes coding for virulence, unusual amino acid utilization by the bacteria, and unusual amino acid synthesis in the tumor are all carried on the large *A. tumefaciens* plasmid first described by Zaenen *et al.* (6). Merlo (7) reported that a small specific piece of the plasmid DNA would reanneal to crown gall tumor DNA but not to normal plant DNA. Thus, evidence which suggests that tumor induction involves the transfer to plant cells and expression of bacterial genes is beginning to accumulate. To date, however, only the expression of tumorigenesis and synthesis of the unusual amino acid derivatives have been described as functions of presumably transferred genes. The total number of tumor-specific amino acid derivatives as well as the number of gene products necessary to synthesize the amino acids is still in doubt. Because fully defining the unusual amino acid derivative content of tumor cells may provide information concerning the extent of gene transfer, I examined several types of plant tissue for new derivatives.

MATERIALS AND METHODS

Plant Material - Sunflower seeds (*Helianthus annuus*, variety Mammoth Russian) were germinated in vermiculite and when seven days old, inoculated with *A. tumefaciens* strain B_6 (8). Two weeks after inoculation, the primary sunflower crown galls were removed for further experimentation. The establishment of PSCG-11 tissue culture line (from a primary B_6 sunflower crown gall), SSCG (from a secondary B_6 gall), and HSSS (habituated sunflower tissue) has been described (8). All tissue cultures were maintained at 27° in the dark on Linsmaier and Skoog's medium (9) lacking phytohormones.

Utilization of Amino Acid Derivatives by *A. tumefaciens* - Five ml of minimal medium (8) containing 0.05% octopine and natural ^{14}C-histopine (25,000 CPM) as the sole sources of carbon and nitrogen were inoculated with 0.02 ml of an overnight culture of either *A. tumefaciens* strain B_6 or C_{58}. After 24 h the cells were removed by centrifugation and the culture supernatant retained for measurements of octopine and radioactivity. The cells were lysed (8) and macromolecules precipitated from 7% TCA. The precipitated material was dissolved in 0.5M NaOH for radioactivity measurements.

Labeling and Chromatographic Procedures - Surface-sterilized primary crown galls or axenically-grown tissue culture cells were labeled for 24 h by transfer to agar medium supplemented with 2.7 μM L-[ring-2-^{14}C]-histidine (55 m Ci/mmole, Amersham/Searle Corp.[1]) or 130 μM [1-^{14}C]-pyruvic acid (7.3 m Ci/mmole, New England Nuclear). Tissues were homogenized in 1.5 volumes (w/v) of 10% trichloroacetic acid. The homogenate was centrifuged for 20 min at 20,000 x g, and the clear supernatant liquid (2-3 ml) adsorbed to a 0.9 x 50 cm column of Aminex Q-150S ion exchange resin equilibrated with pH 3.49 buffer. Elution of the amino acids was accomplished by the two-buffer method described earlier (10).

Selected fractions eluting from the Q-150S resin were concentrated and desalted by adsorbing them to a 0.9 x 10 cm column of Amberlite CG-120 resin equilibrated with 2N HCl. Elution with NH_4OH and evaporation was accomplished as described earlier (10).

Colorimetric Assay - Imidazoles were detected by mixing equal volumes of sample, 1% (w/v) sulphanilic acid in 1N HCl, 5% (w/v) $NaNO_2$ in H_2O, and 15% (w/v) Na_2CO_3. Histidine and other imidazoles gave an immediate red color (11). The assay was quantitative if the absorbance (A_{500}) of the samples was measured immediately. Histidine was used as a standard.

Thin-Layer Chromatography - Cellulose plates (Cellulose Mn 300, Brinkmann Instruments) were used to confirm the identity of the radioactive material and synthetic derivative. Samples were mixed, spotted, and chromatographed with methanol:H_2O:pyridine (20:5:1) solvent system I), isopropanol:formic acid:H_2O (40:2:10) (system II), or t-butanol:methyl ethyl ketone:sat. NH_4OH:H_2O (25:15:5:5) system III). After air drying, imidazoles were detected by spraying the plate with the reagents for the colorimetric assay in the order listed. The red-colored spot was scraped from the plate and radioactivity measured in a liquid scintillation spectrometer (12).

Chemical Synthesis - N^2-(D, L-1-carboxyethyl)-L-histidine (histopine) was synthesized by a modification of the method Biemann used to synthesize lysopine (4). It consisted of condensing 100 mg L-histidine with 1 ml D, L-α-bromopropionic acid in the presence of 0.2 g $Ba(OH)_2$ and 5 ml of 50% ethanol. The reaction proceeded at 55° for 36 h. The final condensation product was isolated by making the mixture 0.2 M in H_2SO_4 to precipitate the barium salt and adsorbing the acidified product to a 0.9 x 10 cm column of Amberlite CG-120 ion exchange resin equilibrated with 2N HCl. After washing the resin with H_2O, the product was eluted with 0.6 N NH_4OH. The eluant was evaporated to dryness (*in vacuo*, 35°) and the residue was dissolved in 1 ml H_2O.

The ethyl ester (MW 283) of the product (P) was examined by mass spectrometry. A molecular ion was detected at *m/e* 283 and the base peak was at *m/e* 81. Other prominent ions were at *m/e* 210 (P-$COOC_2H_5$) and 202 (P-imidazol plus CH_2). Other peaks were also consistent with Structure I.

RESULTS AND DISCUSSION

Since ^{3}H-octopine could be detected in crown gall tissues (PSCG-11 and SSCG) grown on medium supplemented with ^{3}H-arginine (10), I attempted

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

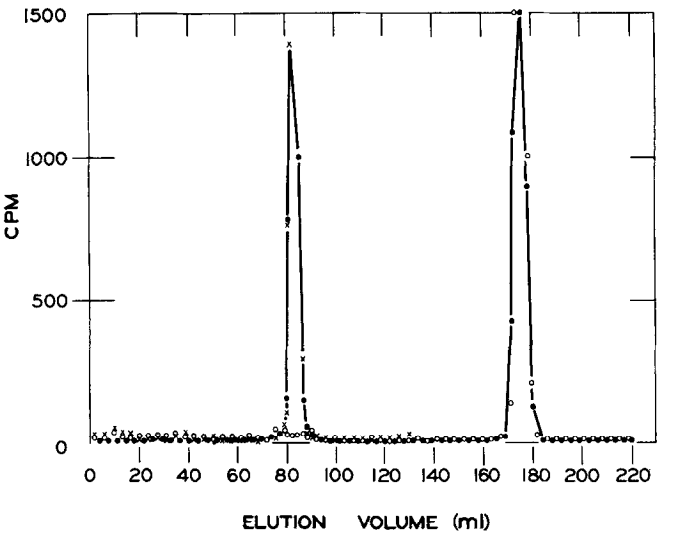


Figure 1. Chromatogram of amino acids present in the acid-soluble fraction of PSCG-11 (•-•), SSCG (x-x) or HSSS (o-o) tissue labeled 24 h on medium containing ^{14}C-histidine. Radioactivity was measured in an aliquot of each fraction collected from the Q-150S resin. Histidine elutes from 170-180 ml.

to detect N^2-(1-carboxyethyl)-L-histidine (histopine) by growing tissues for 24 h on medium supplemented with ^{14}C-histidine. Because ^{14}C-histidine was the only acid-soluble compound found in labeled HSSS tissue (Figure 1), I concluded that normal sunflower tissues do not synthesize detectable amounts of any imidazole-containing compounds other than histidine. The results were quite different, however, when crown gall tissue (PSCG-11 or SSCG) was grown for 24 h in the presence of ^{14}C-histidine (Figure 1). Up to one third of the total radioactive material eluted from the resin between 80-84 ml of effluent, a position 25 ml in advance of octopine (10). In the case of PSCG-11 tissue, the remaining two thirds of the radioactivity eluted with histidine (170-180 ml). The SSCG results presented in Figure 1 did not include histidine. Subsequent experiments have shown that the results for SSCG are identical to PSCG-11. Results similar to those presented for PSCG-11 (Figure 1) were also noted when fresh crown galls were excised from plants and labeled in culture with ^{14}C-histidine. Results similar

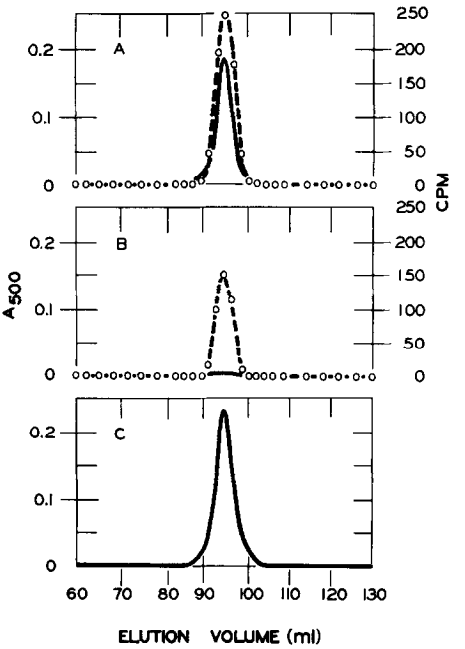


Figure 2. Chromatogram of synthetic and natural histopine on Q-150S resin at pH 3.12. A. Chromatogram of 500 µg of nonradioactive synthetic histopine (---) and 20 µg of natural ^{14}C-histopine (o--o) (pooled 80-84 ml from Figure 1). B. 15 µg of natural ^{14}C-histopine alone (o--o). C. 400 µg of synthetic histopine alone (---).

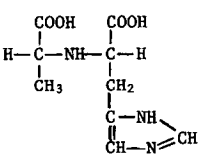
to those for HSSS (Figure 1) were noted when uninoculated sunflower stems were labeled. In one experiment, the material in PSCG-11 tissue eluting from the column between 80-84 ml was pooled, desalted, and concentrated as described in Materials and Methods. The colorimetric assay indicated there was 1 µmole of imidazole-amino acid/20 g fresh wt tissue.

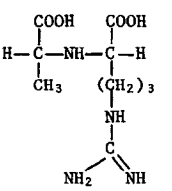
The presumed structure of histopine predicts that it should be labeled when PSCG-11 tissue is grown on medium containing ^{14}C-pyruvate. As expected, many acid-soluble compounds were labeled when PSCG-11 tissue was grown 24 h on ^{14}C-pyruvate medium. However, a peak of radioactivity (1-2% of the total) was detected at 82 ml of effluent.

The structure of natural histopine was further confirmed when authentic N^2-(1-carboxyethyl)-L-histidine was synthesized from α-bromopropionic acid and L-histidine and its properties compared to those of the naturally

occurring isolate. A 25-fold excess of nonradioactive synthetic histopine was mixed with naturally occurring ^{14}C-histopine. When the mixture was chromatographed on Q-150S resin at pH 3.12, both materials cochromatographed at 94 ml (Figure 2A). Natural histopine was detected by measuring radioactivity (Figure 2B) and synthetic histopine by the colorimetric assay (Figure 2C). Finally, the two materials were compared by thin-layer chromatography. Cochromatography occurred in all three solvent systems with an Rf of 0.73 in solvent I, Rf of 0.18 in solvent II, and an Rf of 0.02 in solvent III. In no case was radioactivity or imidazole-positive material observed at any other position on the chromatogram. In all three solvents histidine was well separated from histopine.

The radioactive labeling and cochromatography experiments strongly suggest that PSCG-11 and SSCG tissues synthesize a new amino acid derivative from histidine and pyruvate, histopine (Structure I). The stereochemistry of the asymetric carbon atom of the histidine chain is L, but the configuration of the asymetric carbon of the alanine chain is unknown. If the configuration is analogous to octopine (Structure II), the asymetric alanine carbon should be D as shown in Structure I.





As with octopine, histopine was utilized by *A. tumefaciens* strain B_6 but not by strain C_{58} (Table I). When medium containing octopine and histopine was inoculated with strain B_6, all of the octopine was utilized along with 66% of the ^{14}C-histopine. In addition, the histopine was used to synthesize acid precipitable material (macromolecules). In contrast,

Table I. Utilization of Octopine and Histopine by *A. tumefaciens* strain B_6.

A. TUMEFACIENS	OCTOPINE (mg)		^{14}C-HISTOPINE (cpm)		
	Input	Recovered	Input	Recovered Medium	Cells
B_6	2.5	0.01	25,000	11,100	9,400
C_{58}	2.5	1.9	25,000	19,300	110

See Materials and Methods for details. The source of ^{14}C-histopine was pooled 80-84 ml from Figure 1.

strain C_{58} could not utilize either octopine or histopine (Table I). Thus, histopine appears to be a strain-specific derivative like the other known amino acid derivatives. The question of whether histopine utilization is coded for by the same plasmid gene as is octopine is under investigation.

The author thanks Ms. M. Albert and M. Dietrich for technical assistance and Mr. S. Vicen for preparation of art work.

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