Evidence for the presence of N^2-(1,3-dicarboxypropyl)-L-amino acids in crown-gall tumors induced by *Agrobacterium tumefaciens* strains 181 and EU6

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Using crown-gall tumors induced by *Agrobacterium tumefaciens* strains 181 and EU6 several unusual compounds have been isolated in a single fraction. Physicochemical analysis of the compounds in this fraction showed that they are N^2-(1,3-dicarboxypropyl)-L-amino acids derived from neutral and acidic amino acids. Asparagine and glutamine derivatives (asparaginopine and glutaminopine) are the main components. Synthesis of asparaginopine from asparagine, α-ketoglutarate and NADPH, and degradation of asparaginopine to asparagine have also been demonstrated using enzyme prepared from Pinto bean strain 181 tumor.

Asparaginopine *Glutaminopine* Agrobacterium tumefaciens *Crown-gall Tumor* *Ti-plasmid*

1. INTRODUCTION

A group of compounds sharing a common structure of HOOCRHC–NH–CHR′COOH have been isolated from crown-gall tumors. These compounds are synthesized by 3 different Ti plasmid-encoded enzymes in tumors induced by 3 different types of *Agrobacterium tumefaciens* strains [1,2]. Synthesis requires NADPH or NADH, α-keto acid and an amino acid. Plasmid-encoded enzymes include:

(1) Octopine synthase which synthesizes N^2-(1-carboxyethyl)-L-arginine, -L-lysine, -L-ornithine and -L-histidine (octopine, lysopine, octopinic acid and histopine, respectively);
(2) Nopaline synthase which catalyzes the syntheses of N^2-(1,3-dicarboxypropyl)-L-arginine and -L-ornithine (nopaline and nopalinic acid, respectively);
(3) Leucinopine synthase which synthesizes mainly N^2-(1,3-dicarboxypropyl)-L-leucine (leucinopine).

These amino acid derivatives are not found in tumors induced by strains 181 and EU6 [2–4], although an unknown Pauly reagent-positive compound has been reported [5]. We present evidence that tumors induced by strains 181 and EU6 also contain an enzyme that synthesizes mainly N^2-(1,3-dicarboxypropyl)-L-asparagine and -L-glutamine which we have designated as asparaginopine and glutaminopine.

2. MATERIALS AND METHODS

2.1. *Plant materials and crude extracts*

The growth of both normal and tumor tissues induced by various strains of *A. tumefaciens*, and the preparations of crude extracts from these tissues have been described in [2].

2.2. *Synthetic compounds*

N^2-(1-carboxyethyl)-L-aspartate and -L-glutamine were prepared as in [6]. Preparation of leucinopine was as in [2]. Other N^2-(1,3-di-

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carboxylpropyl)-L-amino acids were synthesized similar to [2]. The products were separated from α-ketoglutarate by adsorption on a Dowex 50, H^+ column and purified on an SP–Sephadex C-25 column (2.7 × 130 cm) eluted with ammonium formate buffer, pH 2.9. The derivatives were desalted on a Sephadex G-10 column (2.7 × 50 cm).

Radioactive N^2-(1,3-dicarboxypropyl)-L-amino acids were synthesized using the appropriate ^{14}C uniformly-labeled amino acids.

2.3. *Chromatographic methods*

SP–Sephadex C-25 columns (1.5 × 126 cm) were equilibrated in 0.2 M ammonium formate buffer (pH 2.9) and eluted with the same buffer. Eluted compounds were detected with a differential refractometer (Waters Associates, R-403). Pooled peak fractions (3.3 ml) were concentrated and desalted on a Sephadex G-10 column in H_2O.

DEAE–Sephadex A-25 column (2.7 × 80 cm) were prepared in 0.1 M Tris–HCl buffer (pH 8.3) containing 0.3 M NaCl; compounds were eluted by a linear gradient of 0.3–0.4 M NaCl and detected refractometrically.

Descending chromatograms were run on Whatman no.1 paper. The solvent systems were (by vol.): (A) *n*-BuOH–HOAc–H_2O (4:1:5); (B) *n*-BuOH–HOAc–Py–H_2O (6:1.2:4:4.8); (C) PhOH–H_2O (4:1); (D) PhOH–H_2O–HCOOH (90:30:1.2). Compounds were detected by xylose–aniline reagent [7].

2.4. *Oxidative degradation, acid hydrolysis and amino acid analysis*

$KMnO_4$ (10 mg/ml) was added to aqueous samples until no further precipitation was observed. After centrifugation amino acid content of the supernatant was analyzed by: (i) a Beckman model 120C amino acid analyzer; and (ii) two-dimensional paper chromatography using solvent systems A and C.

Additional identifications of glutamate and asparagine in samples were carried out with an SP–Sephadex column (1.6 × 130 cm) equilibrated and eluted with 0.2 M ammonium formate buffer, pH 3.6. Glutamate and asparagine eluted were detected refractometrically. Radioactive markers were detected by a scintillation counter.

An evacuated tube (Pierce Reacti-therm) was used for hydrolysis in constant boiling HCl at 105°C for 24 h. The hydrolysates were chromatographed on Whatman no.1 paper in solvent system A; compounds were detected with xylose–aniline and ninhydrin reagents.

3. RESULTS AND DISCUSSION

When crude extracts of strains 181 and EU6 tumor tissues were chromatographed on a cation exchange (SP–Sephadex) column, a unique refractometric peak appeared immediately after void volume (fig.1B). This peak was not detected in the extracts of either normal tissues or nopaline tumors (fig.1A). N^2-(1-carboxyethyl)-L-amino acids and N^2-(1,3-dicarboxypropyl)-L-amino acids derived from acidic and neutral amino acids including leucinopine also eluted at bed volumes similar to the unique peak compounds; aspartate (the first amino acid eluted) was identified in fractions (60–65) far behind the unique peak.

Aqueous $KMnO_4$ oxidation of the compounds in the unique peak yielded mainly glutamate, asparagine and glutamine and small amounts of

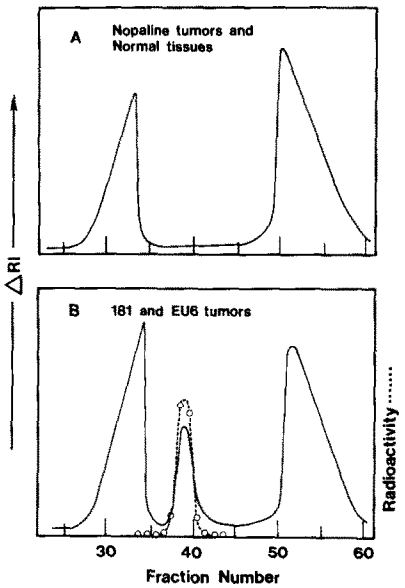

Fig.1. Early refractometric profiles of SP–Sephadex column chromatography of crude extracts from normal and tumor tissues; (○···○) Radioactive N^2-(1,3-dicarboxypropyl)-L-asparagine added to the tumor crude extracts.

other amino acids (table 1 and other chromatographic analyses mentioned in the methods). Under the same conditions N^2-(1,3-dicarboxypropyl)-L-asparagine, leucinopine, nopaline and nopalinic acid yielded glutamate and other amino acids (asparagine, leucine, arginine and ornithine, respectively) (unpublished, [8,9]). These data suggest that the unique peak is composed of several species of N^2-(1,3-dicarboxypropyl)-L-amino acids with asparagine and glutamine derivatives being the main components.

The primary components in the unique peak were eluted at the same bed volumes as N^2-(1,3-dicarboxypropyl)-L-asparagine and -L-glutamine on the DEAE–Sephadex anion exchange column (fig.2). A minor peak was also eluted at the bed volume of glutamate or aspartate derivatives.

Chromatography on a molecular sieve column, Sephadex G-10, also showed that compounds in the unique peak have molecular sizes in the vicinity of N^2-(1,3-dicarboxypropyl)-L-asparagine, -L-glutamine or leucinopine (0.45 bed volume).

Acid hydrolysis of the main components in the unique peak yielded products identical to that of N^2-(1,3-dicarboxypropyl)-L-asparagine or -L-glutamine.

Paper electrophoresis [2] of the peak compounds showed that they migrated as cations at pH 2.0 and as anions at pH-values above 3.0. N^2-(1,3-dicarboxypropyl)-L-asparagine, -L-glutamine and leucinopine display similar electrophoretic mobilities. Paper chromatographic R_f-values of asparagine and glutamine derivatives cannot be distinguished from that of the main peak components: 0.13 in solvent A; 0.2 in solvent B, and 0.52 in solvent D. Like leucinopine, the natural and synthetic compounds gave positive reactions on chromatograms to xylose–aniline [7] and chlorination–starch–iodide tests [10].

Table 1

Oxidation products of the unique peak compounds detected by amino acid analyzer

Amino acids	Relative amounts (nmol)
Glutamic acid	170
Asparagine + glutamine + serine	93 (using serine k)
Aspartic acid	10
Glycine	6
Alanine	3
Phenylalanine	1
Leucine	1
Threonine	1

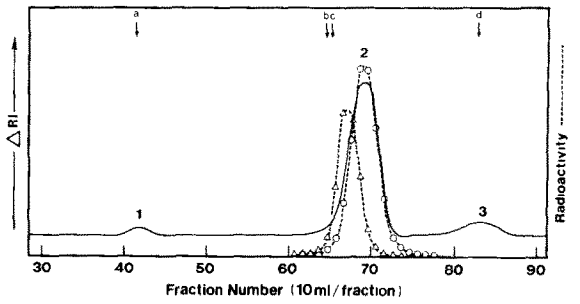

Fig.2. DEAE-column chromatography of the compounds in the unique peak. Corrections have been made for changes in the refractive index (RI) due to the increasing NaCl concentration. Radioactive markers are N^2-(1,3-dicarboxypropyl)-L-asparagine (○···○) and -L-glutamine (△···△). Arrows indicate the elution volumes of other compounds. (a) N^2-(1-carboxyethyl)-L-glutamine; (b,c,d) N^2-(1,3-dicarboxypropyl)-L-leucine, -L-serine, and -L-glutamate, respectively.

Tissue culture of strain 181 tumors also convert L-[U-^{14}C]asparagine in the growth medium to a product eluted at the same bed volume as the unique peak. Glutamine appears to be the only other amino acid that can effectively act as a substrate for the synthesis of the unique compounds. Moreover, this conversion appears to be tumor-type-specific. Leucinopine-type tumors converted much less asparagine to the product; while virtually none of the products were obtained by either octopine or nopaline tumors.

Crude enzymes obtained from Pinto bean strain 181 tumors by precipitation with 50–70% $(NH_4)_2SO_4$ saturation in the presence of α-ketoglutarate and NADPH converts radioactive asparagine into a product corresponding to the unique peak. The requirement for α-ketoglutarate cannot be replaced by either pyruvate or oxaloacetate. Conversely, radioactive N^2-(1,3-dicarboxypropyl)-L-asparagine in the presence of

NADP was also degraded to radioactive asparagine by the crude enzyme.

In conclusion, an enzyme that synthesizes N^2-(1,3-dicarboxypropyl)-L-amino acids is present in strains 181 and EU6 tumors. This enzyme prefers the amino acids asparagine and glutamine as substrates. We called the two main products asparaginopine and glutaminopine.

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