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ACCUMULATION OF A PEPTIDE IN CROWN-GALL TUMORS[1][2]

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Abstract

Paper chromatographic analyses of normal and crown-gall tissues of *Lycopersicon esculentum*, *Phaseolus vulgaris* var. stripe-seeded, *Helianthus annus*, *Nicotiana tabacum*, *Bryophyllum pinnata* and *Daucu carota* revealed the accumula- of a ninhydrin positive compound, other than lysopine or octopinic acid, in crown-gall tumors. Acid hydrolysis of this compound released nine ninhydrin positive components.

Introduction

Crown-gall tumors have been found to accumulate several unusual imino acids that might be absent or present in minute amounts in normal plant tissues (Lioret, 1957; Biemann *et al.*, 1960; Seitz *et al.*, 1964; Ménagé *et al.*, 1964; Ménagé *et al.*, 1965; Goldmann *et al.*, 1969; Petit *et al.*, 1970). We report here our finding that a peptide is also accumulated in the tumors.

Materials and Methods

Bacterium

Agrobacterium tumefaciens (Smith and Town.) Conn, strain B_6 was used to initiate the crown-galls. The culture medium and growth condition were that described by Heberlein and Lippincott (1965).

Plants and inoculation procedure

Crown-galls were initiated on stems of plant seedlings by injecting with syringe a culture of the bacterium into the stem tissues. The following plants grown in soil filled pots in the green house were used to obtain stem galls: *Lycopersicon esculentum* Mill., *Phaseolus vulgaris* L. var. stripe-seeded, *Helianthus annus* L., *Nicotiana tabacum* L. and *Bryophyllum pinnata* Pers. Galls were

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harvested about one month later. Normal stem sections just above and below the gall tissues and from comparable healthy plants were taken for comparison.

To obtain tumors on carrot (*Daucus carota* L.), sterile disks of carrot root bought from a local market were spread with the bacterial culture and incubated on 1.5% agar in petri dishes. Tumor masses grown on the surface of the disks were removed three weeks later. Disks without bacterium inoculation were taken as control.

Extraction procedure and paper chromatography

Plant tissues were cut into small pieces and immediately homogenized with cold 75% methanol (Ten times their fresh weight). The methanolic extract was filtered through the Whatman No. 1 paper and the filtrate evaporated to dryness in a rotary evaporator at 35 C. The residue was taken up in water (two tenth of the tissue fresh weight) and passed through a column of cation exchanger (Rexyn 101, H^+). The column was washed extensively with distilled water until the effluent became colorless. The adsorbed materials were then replaced with 0.5 N NH_4OH and the effluent solution was evaporated to dryness in a rotary evaporator. Aliquots of this fraction, dissolved in water and equivalent to 0.15 g fresh tissues, were applied to the Whatman No. 1 filter paper for two-dimensional paper chromatograhy. Development by descending solvent flow employs: 1. butanol-acetic acid-water (4:1:5) in the first dimension for 9 hours; 2. water-saturaed phenol (ammonia atmosphere) in the second dimension for 7 hours at room temperature. Papers were sprayed with 0.3% ninhyrin in alcohol.

Chemicals

Lysopine was obtained from Dr. Manasse of the Boyce Thompson Institute, Yonkers, New York, U. S. A. through Dr. J. A. Lippincott. D-Octopinic acid was obtained from Sigma Chemicals Co., St. Louis, Missouri, U. S. A.

Results

A comparison between chromatograms from normal tissues and that from crown-gall tumor tissues revealed that crown-gall tumors all contained a ninhydrin positive compound (P) which is either absent from the chromatograms from the normal tissues of *Lycopersicon esculentum*, *Phaseolus vulgaris* var. stripe-seeded and *Daucus carota* (Fig. 1–6) or present in much smaller amount in normal tissues of *Nicotiana tabacum* and *Helianthus annus*. Free amino acid patterns of crown-gall tissues were generally similar to that of corresponding normal tissues. Tumor on *Bryophyllum pinnata* contains much less this compound P that its extract had to be cleared by ethyl acetate extraction before passing through Rexyn 101 column. This allowed greater

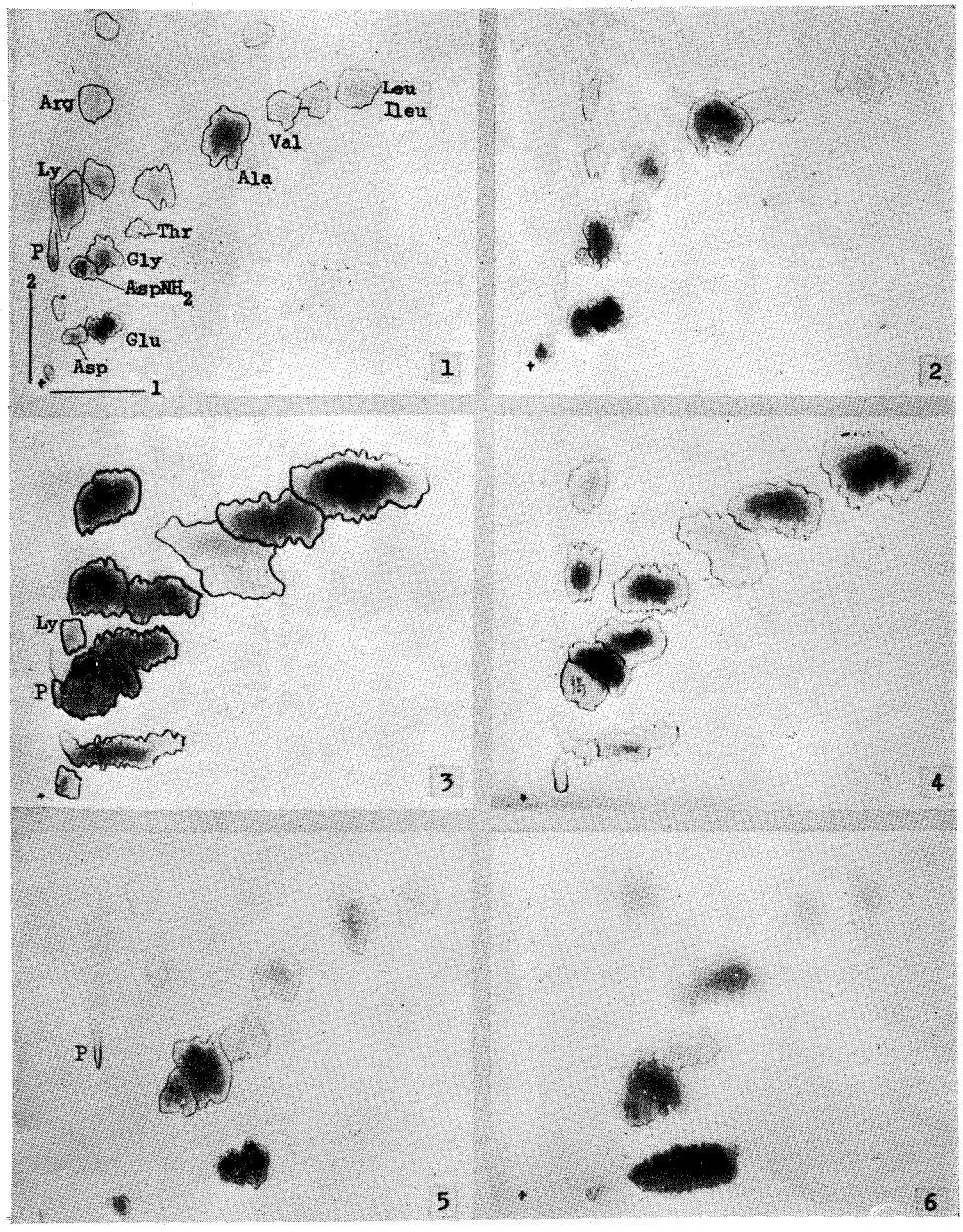

Fig. 1-6. Two-dimensional chromatograms of methanolic extracts of normal and crown-gall tissues obtained as described in the text. Figs. 1, 3, 5 are chromatograms from crown-gall tumors of *Lycopersicon esculentum*, *Phaseolus vulgaris* var. stripe-seeded and *Daucus carota*, respectively; Figs. 2, 4, 6 are chromatograms from their corresponding normal stem tissues. P: the peptide which accumulates in tumors; Ly: lysopine. Amino acids labeled are aspartic acid (Asp), asparagine (Asp NH_2), arginine (Arg), glutamic acid (Glu), glycine (Gly), threonine (Thr), alanine (Ala), valine (Val), leucine (Leu) and isoleucine (Ileu).

amount to be applied to the paper to show the presence of this compound. By tripling the amount of the sample applied to the paper, we subsequently also noticed the presence of this compound in the normal stem tissues of *Lycopersicon esculentum*. In the second solvent system, some "tailing" of this compound always occurred.

Lysopine (Ly), a nindydrin positive imino acid found in tissue cultures of crown-galls (Lioret, 1957; Biemann *et al.*, 1960) was not visible on chromatograms from crown-gall tumors of *Helianthus annus, Daucus carota and Bryophyllum pinnata*. Octopinic acid, another ninhydrin positive iminto acid found in crown-gall tissues (Ménagé *et al.*, 1965), was not visible on all the chromatogram; its position should be right below lysopine.

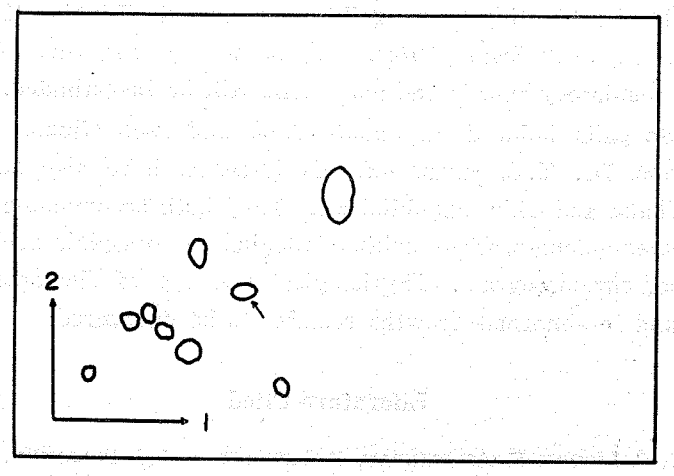

Fig. 7. Two-dimensional paper chromatography of the ninhydrin positive compounds released by acid hydrolysis of the compound P. Nine spots were shown on the chromatogram. Solvent systems were the same as that for the chromatograms in Fig. 1-6, but the first dimension was run for 12 hours instead of 9 hours. The arrow indicates the position of glycine.

Compound P area was eluted from chromatograms from tomato and carrot crown-gall tumors with 50% alcohol. It was further purified by running on Whatman No. 1 paper in the first solvent system for 14 hours and then precipitation with alcohol. On hydrolysis in 6 N HCl at 110 C for 24 hours under reduced pressure, nine ninhydrin positive components were released from this compound, as shown by the same two-dimensional paper chromatography (Fig. 7). Efforts have been made to identify these ninhydrin positive products, using the protein amino acids as standards, by chromatographies on filter paper, silica gel TLC sheet and polyamide layer after dansylation (Woods and Wang, 1967). One of them matched with glycine in all three chromatographic systems only; the others seem to be non-protein amino acids.

Discussion

The comparative study thus revealed the existence of an unknown compound in plants the exact nature of it remains to be elucidated. At the moment we can only assumed that the ninhydrin positive components, or some of them, are amino acids linked by peptide bonds in this compound. The detection of this peptide in normal tissues of a variety of plants suggests that it is a common plant constituent which will accumulate in crown-gall tissues. The same situation has been implied for lysopine (Seitz *et al.*, 1964). In this study we also observed that lysopine does not exist, at least, in great quantity in certain crown-galls.

Most of the imino acids accumulated in crown-gall tumors have been found to be capable of promoting crown-gall tumor growth (Lippincott *et al.*, 1970; Lippincott *et al.*, 1972; Swain, 1972). The possibility that this peptide might also play a regulatory role in the plant cells will be investigated.

Nematode galls induced on tomato stem and root (Huang *et al.*, 1970), obtained from Dr. T.C. Huang of this institute, have also been similarly analyzed (Chang and Chiu, unpublished). They both accumulated this peptide in even greater amounts, while neither lysopine nor octopinic acid was detectable on paper chromatogram. Physiological meaning of the accumulation of this compound in abnormal growths remains to be uncovered.

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植物癌腫對於一種未知胜的堆積現象

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蕃茄，四季豆，胡蘿蔔，向日葵，菸草和落地生根植株上的癌腫組織和正常組織的甲醇抽出物，經過離子交換去掉中性和酸性物質後，用二向濾紙色層分析法比較這二種組織所含能和 ninhydrin 呈色的化合物。我們發現游離氨基酸的變化不大，被認爲是癌腫所特有的 lysopine 在各種癌腫中的量差異很大。但是癌腫組織都含有一種未知化合物，而正常組織不是含此化合物很少就是無法看出。這種化合物會被 6N HCl 水解產生九種和 ninhydrin 呈色的化合物，很像是一種胜。