Bot. Bull. Academia Sincia **16**: 101-114 (1975)

OBSERVATIONS ON SOME SIMPLE PEPTIDES AND TWO UNKNOWN PAULY-POSITIVE COMPONENTS IN NORMAL PLANTS, CROWN-GALL TUMORS AND TOMATO NEMATODE GALL[1,2]

CHI-CHENG CHANG, BI-YU LIN and SHENE-PIN HU

Institute of Botany, Academia Sinica, Nankang, Taipei, Taiwan, Republic of China

(Received for publication December 10, 1974)

Abstract

A combination procedure of SP-Sephadex column chromatography and paper chromatography can reveal on paper chromatogram nine peptide fractions in plant extracts. Stems of five different plants, crown-gall tumors induced on stems of these plants by *Agrobacterium tumefaciens*, B_6 and nematode gall induced on tomato stem by *Miloidogyne incognita* were examined by this procedure as to their contents of these peptide fractions. Normal stems showed different patterns of peptide contents among themselves; the patterns of peptide contents in crown-gall tumors were also different from those of stems from which they derived. Crown-gall tumors and tomato nematode gall all contained a simple peptide fraction which was undetectable in normal stems. An unknown Pauly-positive compound which could not be detected in normal stems was also found in both crown-gall tumors and tomato nematode gall. Another unknown Pauly-positive compound not found in normal stems existed only in crown-gall tumors.

Introduction

There have been several reports on the comparative studies of soluble nitrogenous constituents in the normal plants and in the tumorous tissues by paper chromatographic method (Simonsen *et al.*, 1962; Roberts and Simonsen, 1962; Lioret, 1957). From one of these work a unique compound, lysopine, was discovered in crown-gall tumors which was not detected in normal plants (Lioret, 1957; Biemann *et al.*, 1960). Similar compounds, octopinic acid, octopine and nopaline, in crown-gall tumors were reported later (Ménagé and Morel, 1964, 1965; Goldmann *et al.*, 1969). Our study also revealed that two-dimentional paper chromatograms of extracts from crown-gall tumors all showed a

(1) This investigation was supported by the National Science Council, Republic of China.
(2) Paper No. 158 of the Scientific Journal Series, Institute of Botany, Academia Sinica.

ninhydrin-positive, peptide-like spot that was either absent or existing in much smaller amounts in the chromatograms from that of normal plants (Chang and Lin, 1973). In the course of study on this peptide fraction, we found that as many as nine simple peptide fractions could be shown in plant extracts by a combination of SP-Sephadex ion exchange column chromatography and paper chromatography. This paper reports the results of our survey of these compounds in five normal plants and in the crown-gall tumors derived from them. Nematode gall induced on tomato stem was also similarly analyzed. Results showed that both crown-gall tumors and tomato nematode gall contain a peptide fraction and an unknown Pauly-positive compound that can not be detected in the normal plants. Crown-gall tumors, but not tomato nematode gall, in addition contain another Pauly-positive compound that was undetectable in normal plants.

Materials and Method

Bacterium

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

Plants and inoculation procedure

Crown-galls were initiated on stems of young plants by injecting with syringe a culture of the bacterium into the stems of *Lycopersicon esculentum* Mill., *Phaseolus vulgaris* L. var. stripe-seeded, *Helianthus annus* L., *Nicotiana tabacum* L. and leaves of *Bryophyllum pinnata* Pers. grown in soil-filled pots in green house. Tumors were harvested about one month later. Stem parts above the tumor tissues and from healthy plants of the same age were taken for comparison. Nematode galls were induced on tomato stems according to the method described by Huang *et al.* (1970).

Extraction procedure and chromatographies

Plant tissues were cut into small pieces and immediately homogenized with 3 times their weight of methanol. The methanolic extract was squeezed through two layers of cheese cloth and further filtered through the washed Whatman No. 1 filter paper. The filtrate was then 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 extracted with ethyl acetate to remove chlorophyll. The water layer was evaporated to free the ethyl acetate and then passed through a column of Dowex 50 in hydrogen form. After the column was thoroughly washed with distilled water, the absorbed materials were replaced with 1 N NH_4OH. The effluent solution was evaporated to dryness in a rotary

evaporator. This cationic fraction constitutes the starting material to be applied to the SP-Sephadex, C-25, ion exchange column.

SP-Sephadex, C-25, ion exchange columns (115 cm×2.5 cm) were prepared in 0.2 M ammonium formate buffer, pH 3.4. Cationic materials obtained from 500 grams of fresh plant tissues were adjusted to about pH 2.4 and applied to each column. The compounds were eluted from the columns by ammonium formate buffer with the pH gradient from 3.4 to 3.6. The gradient was obtained by placing 1200 ml of 0.2 M ammonium formate, pH 3.4 in the mixing flask and 1200 ml of 0.2 M ammonium formate, pH 3.6 in the reservoir flask. The effluent was monitored by a Water Associates differential refractometer, model R403 with the sensitivity set at 128× attenuation. Ten ml fractions were collected. Eighty microliter effluent (or 160 μl for normal plant tissues) from every third tubes (tube 34 to tube 144) were spotted on a 46×57 cm Whatman No. 1 paper side by side along a line 6 cm from the bottom. The papers were developed by descending solvent flow in butanol-acetic acid-water (4:1:5, v/v/v) for about 16 hours until the solvent front reached the other end of the Paper. Papers were sprayed with 0.3% ninhydrin in alcohol or with Pauly reagent (see Smith, 1960).

Isolation of compounds from fractions of SP-Sephadex columns was also performed with one-dimentional paper chromatography using the same solvent system. Paper sections of the chromatogram containing the desired compounds were cut out and the compounds were washed out from the paper with 50% ethanol. The purity of the isolated compound was examined by the two-dimentional paper chromatography employing water-saturated phenol in ammonia atmosphere in the second dimension for 7 hours.

Chemicals

Lysopine was obtained from Dr. R. Manasse of the Boyce Thompson Institute, Yonkers, New York, U. S. A. through Dr. J. A. Lippincott.

SP-Sephadex, C-25 (40–120 μ) cation exchanger was obtained from Pharmacia, Uppsala, Sweden.

Results

Simple peptides in plant tissue

One dimentional paper chromatogram of every third effluent fractions, from tube 34 to tube 144, of each SP-Sephadex column were shown in figures 1–6. The amino acids coming out in this area were the acidic and neutral amino acids namely aspartic acid, glutamic acid, glycinine, alanine, valine, leucine, isoleucine, threonine and asparagine. Asparagine had the lowest R_f value among these major amino acid components. Lysopine, which showed up only

in chromatograms of crown-gall samples, but not in that of normal plant tissues and that of tomato nematode gall, appeared below asparagine. The other visible ninhydrin positive compounds with R_f values below that of lysopine were all peptide in nature. Beyond tube 144 no other ninhydrin-positive spot of that nature can be found. These peptides can be divided into two groups; one group occoupied a higher R_f position of 0.18, the other occupied a lower R_f position of 0.075.

There were six species of these peptides with the higher R_f value (No. 1 to No. 6) as revealed by the six sections of purple color spots along the same R_f value level (No. 1: tubes 45–48; No. 2: tubes 54–60; No. 3: tubes 69–78; No. 4: tubes 84–99; No. 5: tubes 105–111; No. 6: 132–135). Only tomato crown-gall tissue contained visible amounts of all these peptides (Fig. 1). Other tissues always lacked one or several of them (Fig. 1–6). In general gall tissues contain much higher amounts or more visible species, but sometimes a normal tissue may have high amount of one species which was invisible from the chromatograms of corresponding crown-gall tissues (Fig. 2). Although they have the same R_f value on chromatogram developed with butanol-acetic acid-water (4:1:5, v/v/v), development with the second solvent system (water saturated phenol in ammonia atmosphere) produced different R_f values. No. 1, No. 2 and No. 6 peptide fractions have R_f values of 0.07, 0.025 and 0.07 respectively, No. 3, No. 4 and No. 5 are peptides with R_f values of about 0.5. The three species of the second group of peptides (No. 7, No. 8 and No. 9) all have R_f values around 0.4 in the second solvent system. The exact composition of these peptides are still not certain because it is difficult to obtain a very pure compound. However, on hydrolysis each isolated peptide fraction gave rise to at least four strong ninhydrin-positive spots on two-dimentional paper chromatograms, two of them were always glutamic acid and glycine.

Comparison of the peptide pattern between normal tissue and the two kinds of galls

Neither normal plants nor tumors possess a common pattern of the peptide contents. However, there is a common feature of the crown-gall tumors and the nematode gall; they all contain the No. 5 peptide fraction which was absent from all of the chromatograms of the normal stem extract. The contents of other peptide fractions in a normal stem and their contents in the crown-gall tumor derived from it may be either the same (*Phaseolus vulgaris* and *Lycopersion esculentum*) or different (others). The chromatogram of normal tobacco extract showed two ninhydrin-positive spot sections whose R_f values were close to, but actually somewhat higher than, that of lysopine and No. 5 peptide. These fracions were examined two-dimentionally, and

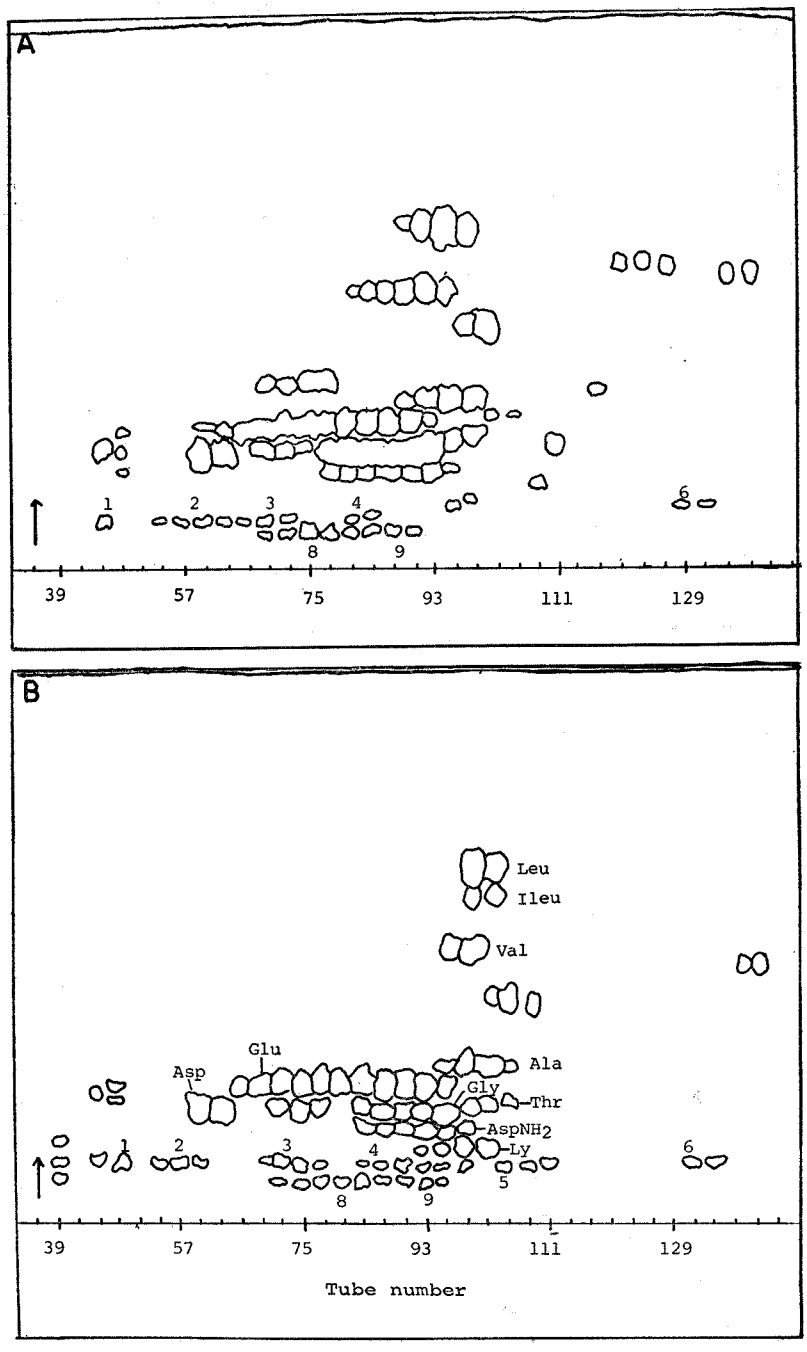

Fig. 1. One-dimentional paper chromatograms of fractions eluted from 115×2.5 cm columns of SP-Sephadex. A: tomato stem; B: crown-gall on tomato stem. Cationic fractions from Dowex 50 (H+) column of methanolic extracts of 500 grams fresh plant tissues were applied to the column. Only every third tubes, from tube 34 to tube 144, were applied to the paper for one-dimentional chromatography. Some amino acids eluted in this region are labeled. Numbers label different sections of ninhydrin-positive peptide spots. Solvent: n-butanol: acetic acid: water (4:1:5, v/v/v).

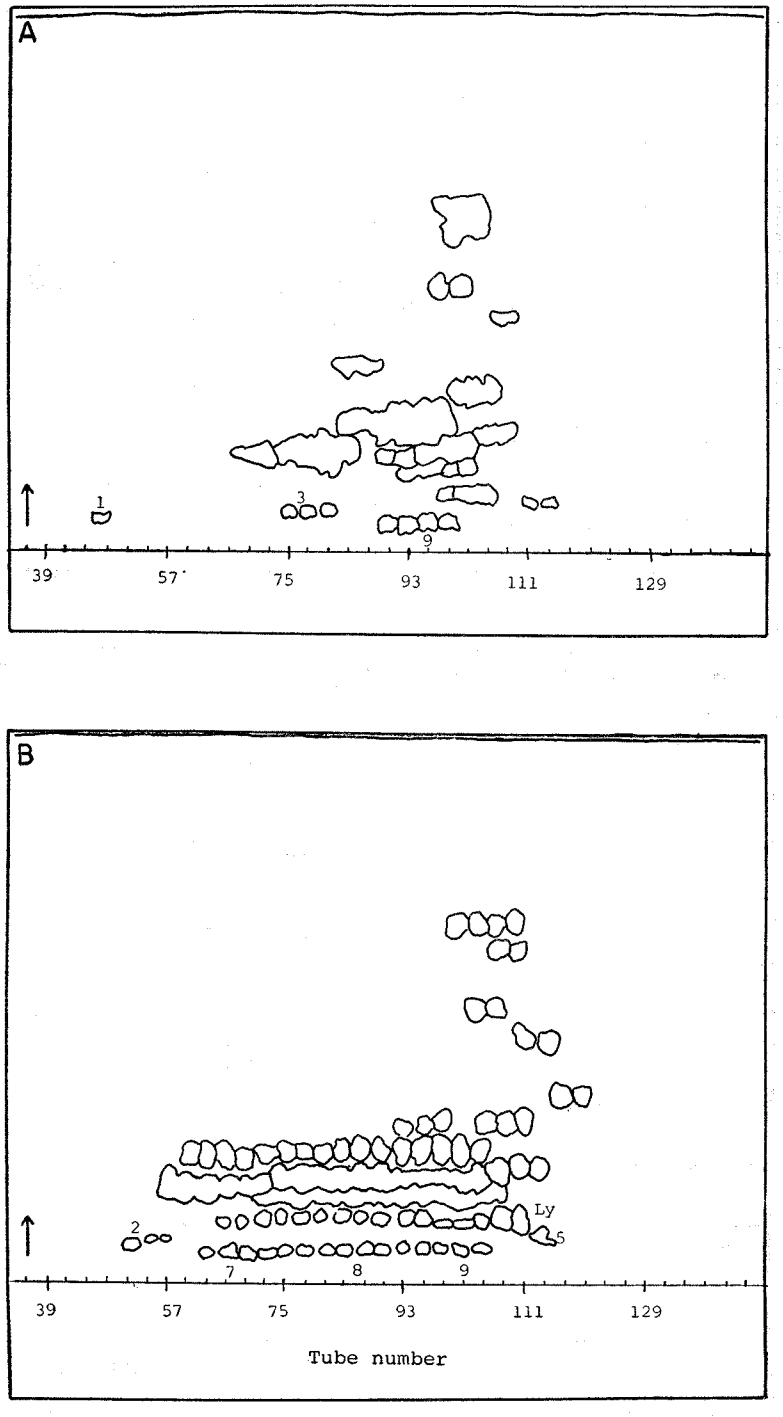

Fig. 2. One-dimentional paper chromatograms of fractions eluted from columns of SP-Sephadex. A: tobacco stem; B: crown-gall on tobacco stem. Experimental conditions were the same as described in Fig. 1.

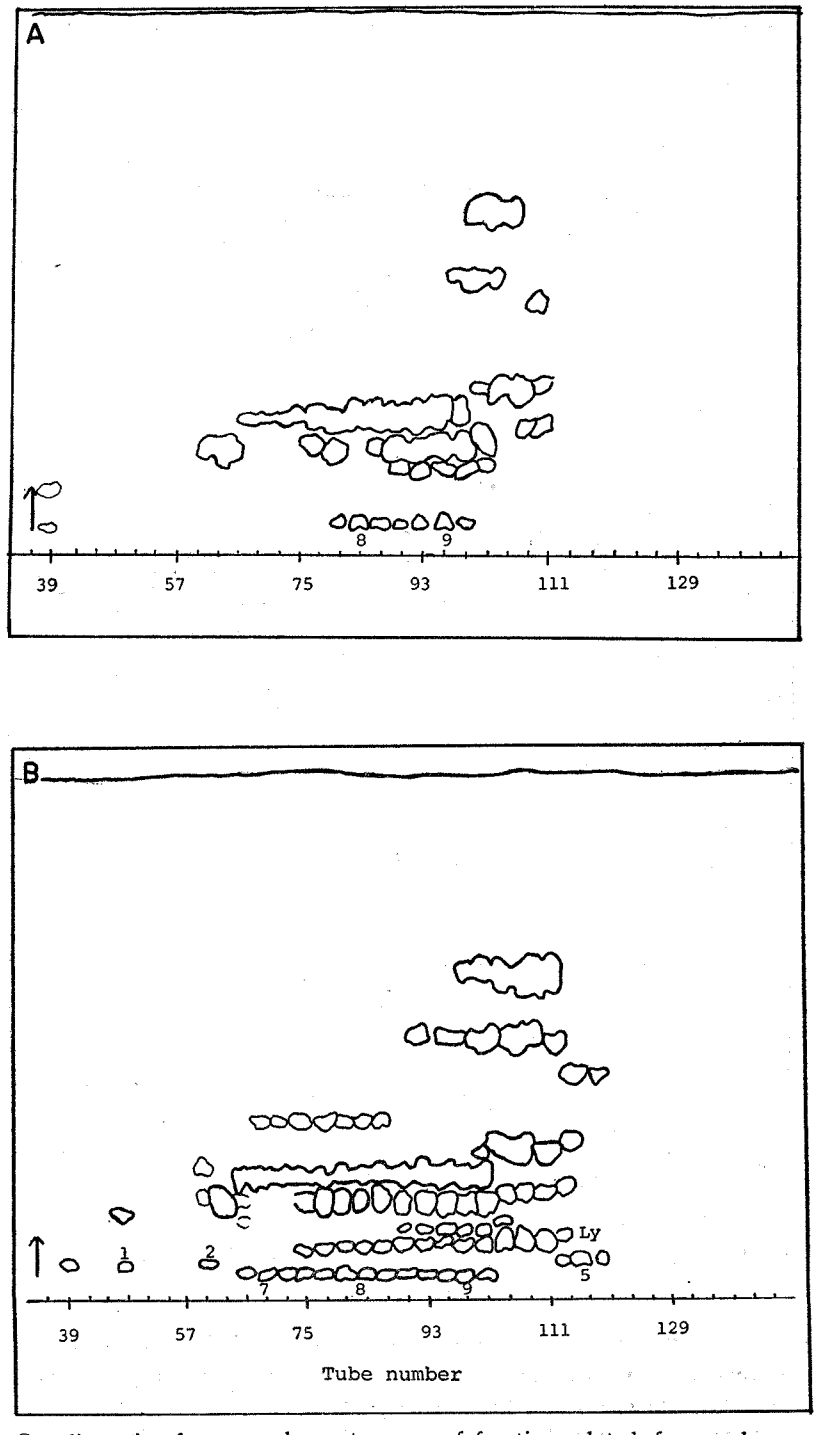

Fig. 3. One-dimentional paper chromatograms of fractions eluted from columns of SP-Sephadex. A: sunflower stem; B. crown-gall on sunflower stem. Experimental conditions were the same as described in Fig. 1.

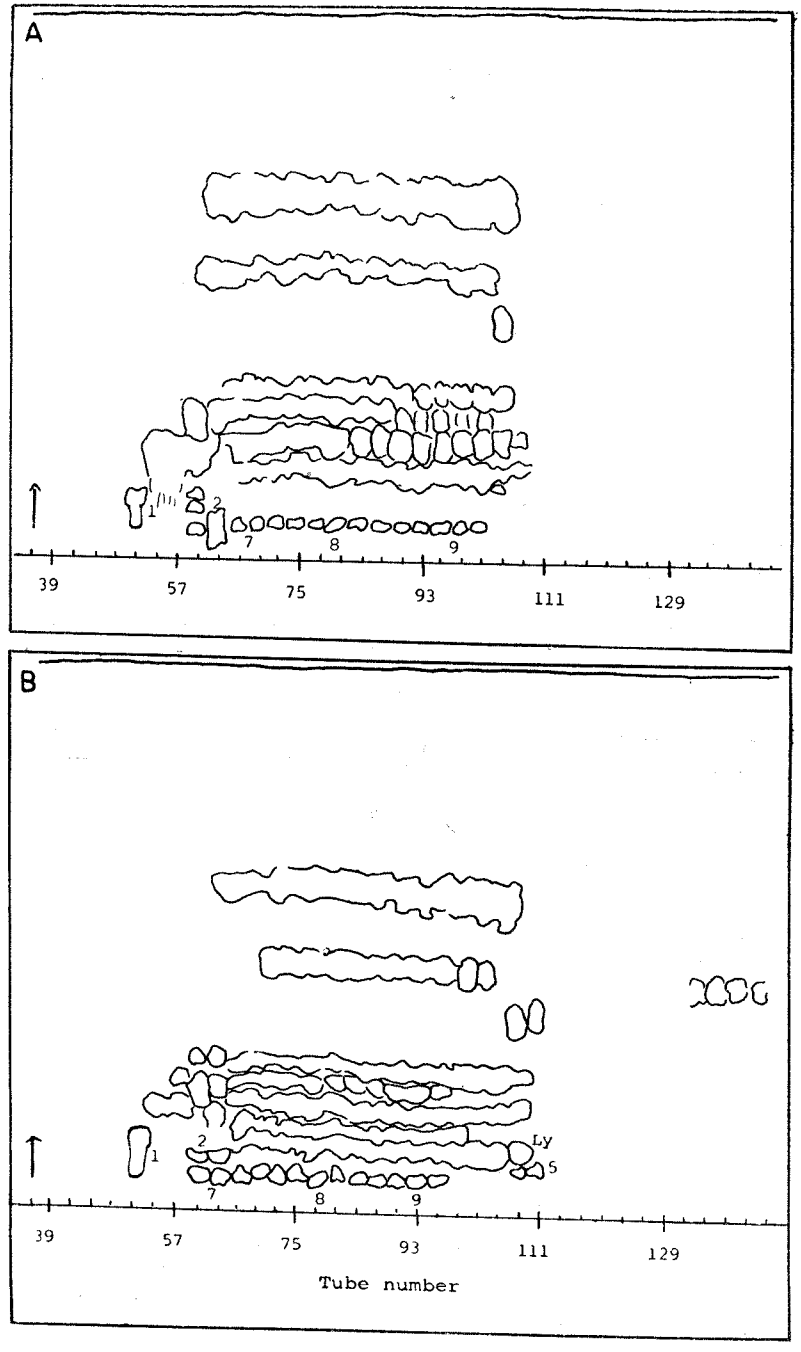

Fig. 4. One-dimentional paper chromatograms of fractions eluted from columns of Sp-Sephadex. A: stems of ***Phaseolus vulgaris*** L. var. stripe-seeded; B: crown-gall on stem. Experimental conditions were the same as described in Fig. 1.

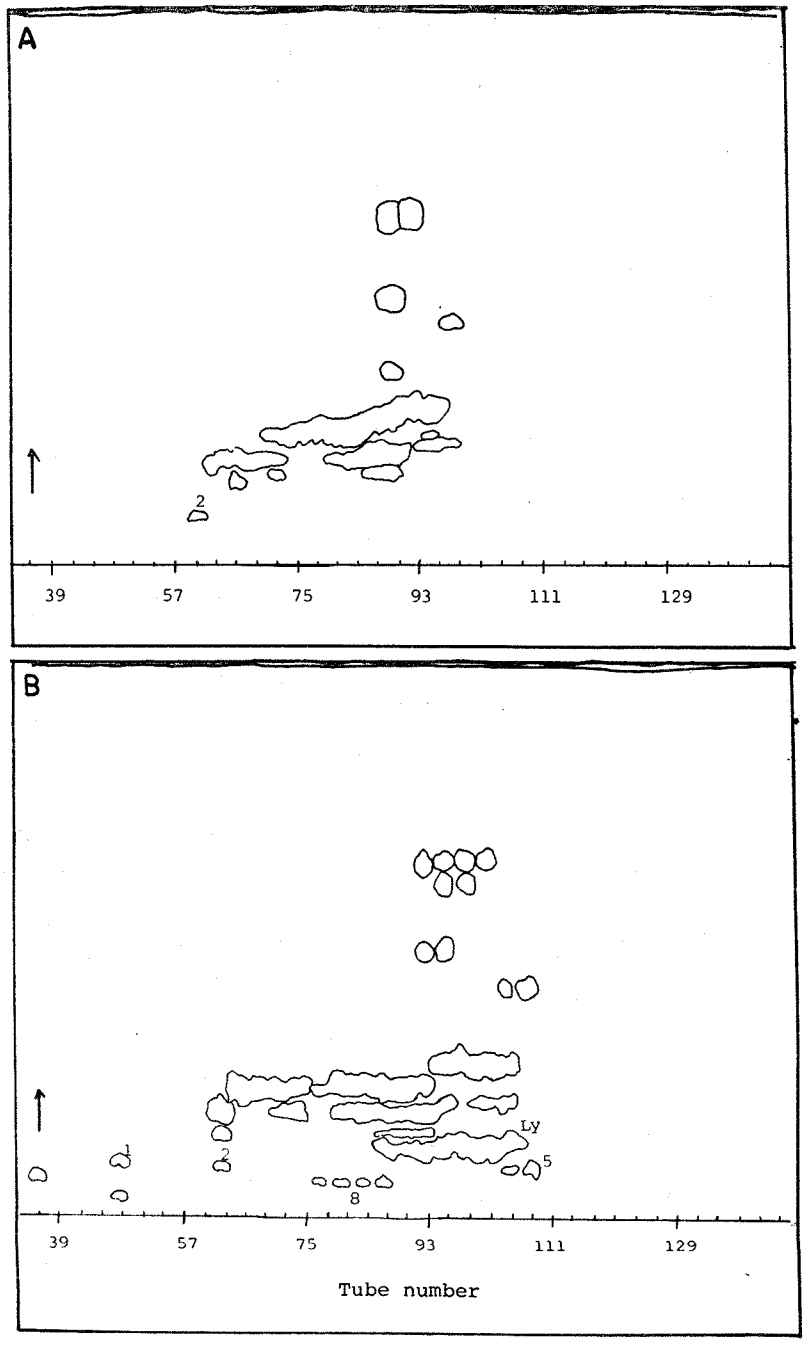

Fig. 5. One-dimentional paper chromatograms of fractions eluted from columns of SP-Sephadex. A: stems of *Bryophyllum pinnata* Pers.; B: crown-gall on leaves. Experimental conditions were the same as described in Fig. 1.

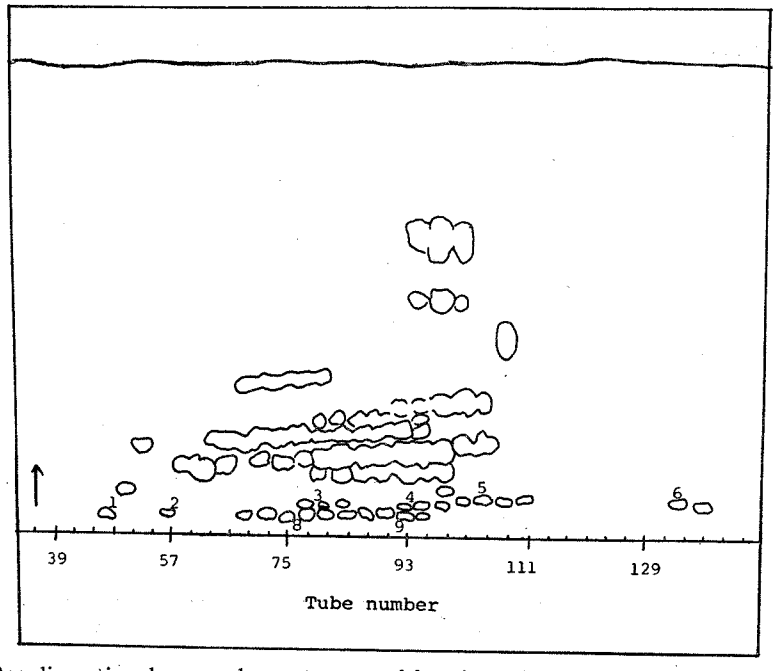

Fig. 6. One-dimentional paper chromatogram of fractions eluted from SP-Sephadex column. Sample applied was tomato nematode gall extract. Experimental conditions were the same as described in Fig. 1.

were proven to contain no lysopine and the No. 5 peptide.

Detection of a Pauly-positive compound in crown-gall tumors

On spraying the chromatograms with Pauly reagent, the chromatograms from crown-gall sample always showed a Pauly-positive (pink) spot section which overlapped with No. 5 peptide but ranged a few fractions (108–111) later at the same R_f level (0.18). This made isolated No. 5 peptide fraction always contaiminated with this Pauly-positive compound. Normal plant tissues and the tomato nematode-gall tissue do not contain this compound. This was checked with concentrates of the corresponding fractions from both the columns of normal plant and gall samples and proven to be true (Fig. 7). The R_f value of this Pauly-positive compound in the second solvent (phenol saturated with water, in ammonia atomsphere) was lower than that of the No. 5 peptide (0.4 in comparison to 0.5 of the peptide). It is, ninhydrin-negative and persisted on two-dimensional paper chromatogram (Pauly reagent spray after acetone washing of the paper) of the hydrolyzate of isolated No. 5 peptide section. It came off the column at about the same volume as octopine.

Comparison of the refractometric tracings of the effluents of SP-Sephadex columns

Fig. 7. One-dimentional paper chromatogram of part of the effluents (tubes 105-111) from SP-Sephadex columns. Right two: crown-gall on stem of *Phaseolus vulgaris* L. var. stripe-seeded; left two: normal stem tissue. Fractions were combined and concentrated in rotary evaporator. Portions were then applied to the paper. The developed paper was sprayed with Pauly reagent. The spot with the lowest R_f value (No. 5 peptide fraction) is missing from normal stem tissue.

When refractometric tracings were examined, a peak around tube 41 was always found in tracings obtained from crown-gall (Fig. 8. B) and nematode gall extracts. Extracts of normal stems did not give rise to this peak (Fig. 8. A). Chromatography on filter paper and Silica gel thin layer showed that this peak contain a component which reacted with Pauly reagent to give a yellow color; corresponding tubes from normal plant extracts did not contain this component.

Discussion

A variety of small peptides has been found in plant tissues, most of them are dipeptides and tripeptides. r-Glutamyl peptides in plants have been reviewed by Thompson *et al.* (1962) and other peptides by Carnegie (1963) and Waley (1966). Leaf *et al.* (1958) also reported some peptide peaks in ion exchange column analysis of nitrogen-fixing root nodules of Alnus. The exact nature of them was unknown, but on acid hydrolysis they all yielded aspartic acid, glutamic acid, alanine, serine and glycine. Some also yielded threonine and

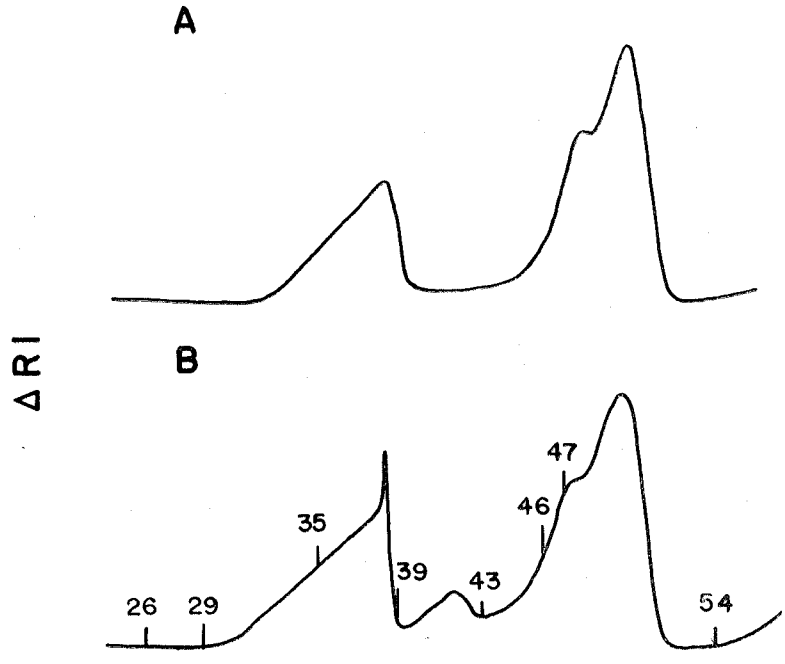

Fig. 8. Refractometric tracings of the effluents of SP-Sephadex columns from tube 24 to 54. A: Tomato stem; B: crown-gall tumors on tomato stem.

a basic amino acid. Another peptide gave glutamic acid, ornithine, citrulline, hydroxy-proline and an unknown ninhydrin-positive spot.

In most of the above cases only a single tissue or plant has been used for isolation or analysis of peptides. This report presents a comparative analysis of five different plants and of five crown-gall tissues and a nematode-gall tissue. Although the exact characterization of these peptides has not been made, it showed that peptide pools (Waley, 1966) in normal plants are more different from one another than the amino acid pools. Transformation of cells into the tumorous state also results in change in the peptide pools.

The No. 5 peptide fraction and the two Pauly-positive compounds in crown-gall tumors are of interest. Recent reports have indicated that lysopine and octopine may be minute constituents of normal plant tissues (Johnson *et al.*, 1974; Ackerman *et al.*, 1973; Wendt-Gallitelli *et al.*, 1973). Crown-gall tumors may be either of the octopine type or of the nopaline type according to the bacterium strains which induced them (Goldmann *et al.*, 1969). Two bacterium strains even induced tumor that accumulates neither octopine (or lysopine) nor nopline (Petit *et al.*, 1970; Lippincott *et al.*, 1973). It may be possible that this peptide and the two Pauly-positive compounds are also minute constituents of normal plant cells which are synthesized in enormous amounts in crown-gall tumors. Lysopine, octopine and nopaline, however, are

able to promote crown-gall tumor growth *in vivo* (Lippincott *et al.*, 1972; Swain, 1972). Lysopine can also promote the bud formation and gametophore development of the moss *Pylaisiella selwynii* (Spiess *et al.*, 1972). It would be, therefore, of interest to know if the presence of this peptide and the two Pauly-positive compounds in crown-gall tumors, or the presence of the peptide and one of the Pauly-positive compounds in nematode gall is of any physiological or morphogenetical significance.

Acknowledgment

The authors wish to express their appreciation to Dr. C.S. Huang of the Academia Sinica for providing the root knot nematode.

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正常植物和植物腫瘤中幾種簡單胜和二種未知 Pauly 氏試劑呈色物的分析

張啓正 林碧玉 胡雪萍

中央研究院植物研究所

離子交換試劑管柱和濾紙色層分析合併使用，可以將植物組織中的一些簡單胜分開而得到九個部份。利用這種方法，我們分析了這些胜在正常組織和腫瘤組織中的含量。這些組織材料是蕃茄、菸草、向日葵、四季豆和落地生根的莖、由 *Agrobacterium tumefaciens*, B_6 在植物莖上（或落地生根葉上）引起的腫瘤和由線蟲在蕃茄莖上引起的腫瘤。結果明顯的表示出各種正常莖含有這些胜的情形都不一樣；一種胜可能在菸草莖裏很多，但在其他植物莖却測不出來。在腫瘤組織中也有這種情形。並且由細菌所引起的腫瘤和由線蟲所引起的腫瘤組織中都含有一種正常植物莖或葉所沒有的胜。

我們同時也發現由 *Agrobacterium tumefaciens*, B_6 所引起的腫瘤比正常莖和線蟲腫瘤多了一種能和 Panly 氏試劑呈色的化合物。另一種能和 Panly 氏試劑呈色的化合物在由 *Agrobacterium tumefaciens*, B_6 所引起的腫瘤和線蟲腫瘤裏面都存在着，只有在正常莖裏測不出來。上述未知胜及二種能和 Pauly 氏試劑呈色的化合物的化學構造需要等待以後的研究才能够知道。