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IDENTIFICATION OF A CROWN-GALL TUMOR GROWTH FACTOR AS GABA

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Abstract—A crown-gall tumor growth factor (TGF-II) isolated from bean leaves inoculated with *Agrobacterium tumefaciens* strain 13333 is shown to be γ-aminobutyric acid (GABA). This identification is based on the comparative behavior of purified TGF-II and authentic GABA with respect to elution from preparative ion exchange and molecular sieve columns, ninhydrin reaction, TLC, co-chromatography on an automated amino acid analyzer, MS analysis and biological activity. GABA is detected by bioassay only in bean leaves infected with the bacterium and is in growth limiting supply when only a few tumors are present per leaf. GABA promotes tumor growth when as little as 1 ng is applied per leaf.

INTRODUCTION

BEAN LEAVES inoculated with *Agrobacterium tumefaciens* strain ATCC 13333 form tumors but fail to produce a tumor growth factor (TGF-I) characteristic of infection with many other strains of *Agrobacterium*.[1,2] Because the growth of strain 13333-induced tumors increases with increasing number of tumors formed per leaf, it was postulated that these tumors produce a growth factor which differs from TGF-I. We wish to report the isolation of this second tumor growth factor (TGF-II) and evidence which indicates it is γ-aminobutyric acid (GABA).

RESULTS

As shown in Table 1, extracts with tumor growth-promoting activity can be obtained from bean leaves with crown-gall tumors induced by *Agrobacterium tumefaciens* strain ATCC 13333, but not from uninoculated leaves. Only phosphate buffer extracts of these tumorous leaves significantly promoted growth of strain B6 tumors, while methanol extracts of tumorous leaves and both types of extract of control leaves were inactive. Since TGF-I is extracted with aqueous methanol,[3] these results indicate that a separate tumor growth factor, TGF-II, is present in strain 13333-inoculated leaves.

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[2] LIPPINCOTT, J. A., LIPPINCOTT, B. B. and CHANG, C.-C. (1972) *Plant Physiol.* **49**, 131.
[3] EL KHALIFA, M. D. and LIPPINCOTT, J. A. (1968) *Physiol. Plant.* **21**, 742.

Purification of TGF-II was effected by partitioning into ethyl acetate and successive chromatography on ion exchange resins, molecular sieve gel, and silica gel TLC (see Experimental). Activity was monitored at each stage of purification by bioassay. Purified TGF-II contained essentially a single detectable component on TLC and reacted only with ninhydrin among the many reagents used for detection. Preliminary analysis on an automated amino acid analyzer suggested that TGF-II might be an aminobutyric acid.

TABLE 1. TUMOR GROWTH PROMOTING ACTIVITY OF PHOSPHATE BUFFER AND AQUEOUS METHANOL EXTRACTS OF CONTROL AND 13333-INOCULATED PRIMARY BEAN LEAVES

Additions to strain B6 inoculated leaves	No. leaves with tumors	Mean tumor no. per leaf (range)	No. tumors measured	Mean tumor diameter (S.U.)	% Increase in tumor volume
H_2O Control	16	28·2 (12–49)	47	5·7 ± 0·1	—
Carnosine (1 mM)	16	24·2 (4–46)	45	6·6 ± 0·2	55
Extracts of non-inoculated leaves					
Phosphate buffer	14	25·4 (12–41)	42	5·8 ± 0·1	5
Aq. MeOH	16	28·9 (13–43)	47	5·6 ± 0·1	0
Extracts of 13333-inoculated leaves					
Phosphate buffer	14	19·6 (4–35)	39	6·7 ± 0·2	62
Aq. MeOH	16	22·3 (10–42)	47	5·9 ± 0·2	10

The data in Table 2 show that the mobility of purified TGF-II and authentic GABA on silica gel and silica gel-kieselguhr thin-layer adsorbents is identical in several solvents. Authentic GABA and purified TGF-II, chromatographed separately on the amino acid analyzer, had essentially identical elution times, and a mixture of the two eluted as a single symmetrical peak. GABA was also chromatographed on the same ion exchange and molecular sieve columns employed in the purification of TGF-II and was found to elute at the same positions as TGF-II.

TABLE 2. MOBILITIES OF AUTHENTIC GABA AND PURIFIED TGF-II ON TLC

Thin-layer adsorbents and solvents	$R_f \times 100$ GABA	TGF-II
Silica Gel		
BuOH–HOAc: H_2O (4:1:1)	48	49
PrOH–14·8 M NH_4OH: H_2O (6:3:1)	54	53
Silica Gel-Kieselguhr		
BuOH–2-butanone: 8·7 M NH_4OH (5:5:1)	3	3
EtOAc Formate: H_2O (12:1:7)	3	3
H_2O Saturated BuOH–Phenethyl Alcohol (1:1)	5	4
Methylethyl ketone–*n*-C_2H_5N–H_2O–HOAc (70:15:15:2)	15	15
PrOH–BuOH–14·8 M NH_4OH (2:2:1)	34	32
BuOH–HOAc–H_2O (4:1:1)	56	58
EtOH–14·8 M NH_4OH–H_2O (50:7:7)	63	64
PrOH–14·8 M NH_4OH–H_2O (6:3:1)	74	74

MS analyses of GABA and purified TGF-II were identical. Both showed prominent peaks in order of decreasing intensity at *m/e* 30, 85, 41, 42 and 56. The base peak at *m/e*

30 is the fragment (CH_2–NH_2) and is characteristic of most primary aliphatic amines.[4,5] The next most abundant peak at *m/e* 85 is probably due to ring formation by loss of water from the M^+ (MW = 103). The small parent ion (2–3% of the base peak) observed in the GABA sample was too small to be identified positively in the TGF-II sample because of background even though the sample was pure.

During preparation of the ethyl esters, the TGF-II sample became contaminated with pump oil. However, the peaks of greatest significance at *m/e* 30, 86 and 69 were still apparent. Significantly, the ester yields a peak at *m/e* 86 rather than at 85 as for the free acid. The *m/e* 86 ion is formed by loss of (OC_2H_5) from the M^+ (MW = 131). The *m/e* 69 fragment is then formed by the additional loss of 17 (OH). Although the M^+ for the ester is somewhat larger than for the free acid, contamination prevented positive identification of the M^+ in the TGF-II sample.

TABLE 3. TUMOR GROWTH PROMOTING ACTIVITY OF AUTHENTIC GABA

Additions to strain B6 inoculated leaves	No. leaves with tumors	Mean tumor no. per leaf (range)	No. tumors measured	Mean tumor diameter (S.U.)	% Increase in tumor volume
Experiment 1					
H_2O	16	22·8 (4–38)	47	5·9 ± 0·2	—
1 mM GABA	15	20·1 (9–39)	41	6·5 ± 0·2	34
Experiment 2					
H_2O	16	20·9 (3–69)	45	5·5 ± 0·1	—
1 mM GABA	16	18·9 (3–34)	44	6·7 ± 0·2	81
Experiment 3					
H_2O	13	7·0 (2–19)	30	5·5 ± 0·2	—
0·1 μM GABA	12	7·3 (2–16)	25	5·9 ± 0·2	23
3·0 μM GABA	15	4·7 (1–19)	24	6·3 ± 0·2	50
30·0 μM GABA	11	4·2 (1–8)	25	6·3 ± 0·2	50

Results in Table 3 demonstrate that authentic GABA is highly active in promoting tumor growth in the same bioassay used to detect TGF-II and to follow its purification. Although the extent of this tumor growth promotion may vary from one experiment to the next due to uncontrolled changes in leaf sensitivity, we consistently obtain activity when 0·1 ml of 1 mm GABA is applied to each leaf and have obtained tumor growth promotion with concentrations of GABA as low as 0·1 μM. This high specific activity is consistent with the tumor growth promoting activity of purified TGF-II preparations relative to quantitative estimates (based on ninhydrin reaction) of the amount of amino acid present.

DISCUSSION

The specific activity of GABA, or TGF-II, in the tumor growth bioassay is at least 100× greater than any of the compounds previously identified which promoted tumor growth. It appears, therefore, that GABA and GABA metabolism are important in the changes which occur during the tumor conversion process and which ultimately support the abnormal growth of crown-gall tumors.

Since application of as little as 1 ng of GABA per leaf can produce a detectable tumor growth response, GABA is most probably not acting simply as a carbon–nitrogen source.

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[5] BIEMANN, K., SEIBL, J. and GAPP, F. (1959) *J. Am. Chem. Soc.* **83**, 3795.

This is particularly apparent as many other amino acids are inactive in promoting tumor growth in this bioassay.[6] A possible role at the membrane level such as that shown for GABA in neurotransmission[7] appears more plausible. Further studies on TGF-II production by tumors induced by other strains of *Agrobacterium*, its relation to other crown-gall tumor growth factors, and its possible mode of action will be presented elsewhere.

EXPERIMENTAL

TGF-II extraction and purification. TGF-II was extracted following the procedures of El Khalifa and Lippincott[3] for the extraction of TGF-I, but using 10 mM Na phosphate buffer (pH 7) as the extraction medium. These preparations were extracted 3× with EtOAc and evaporated to dryness on a rotary evaporator at 40°. The residue was dissolved in H_2O and chromatographed on Bio Rex 70 cation exchanger using H_2O as the eluant. The active fractions were concentrated and this material eluted from G-10 Sephadex (Pharmacia) with 0·1 M NH_4HCO_3. Active fractions were again concentrated by flash evaporation and finally applied to a SP-Sephadex C-25 cation exchange column and eluted with 0·2 M ammonium formate buffer (pH 4·1).

Tumor growth bioassay. Column fractions and authentic GABA were assayed for tumor growth-promoting ability as previously described[8] by applying 0·1 ml portions to each of 16 primary bean leaves 3 days after they were inoculated with *A. tumefaciens* strain B6. Tumors were counted and measured at day 6 from inoculation at 30× magnification. Tumor dia. are given in terms of the ocular scale used in their measurement. One scope unit (S.U.) equals 0·033 mm. Tumor vol. is calculated based on a spherical model.[8]

TLC. Chromatograms were developed by the ascending method and employed either silica gel or silica gel-kieselguhr. Layers were sprayed with ninhydrin reagent and developed at 105°.

Automated amino acid analysis. Obtained with a Durram 500 analyzer equipped with a PDP8E computer.

MS analysis was performed using a modified Bendix Time-of-Flight Mass Spectrometer[9] and the free acids as well as the ethyl esters of authentic GABA and purified TGF-II prepared following the method of Biemann and Vetter.[10]

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