# CYCLIC-AMP PHOSPHODIESTERASE ACTIVITY IN CROWN-GALL TUMOR FORMATION

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## SUMMARY

Cyclic-AMP phosphodiesterase activity was found in crown-gall tumor tissue obtained from the inoculation of the primary leaves of Pinto beans with Agrobacterium tumefaciens strain B6. Considerably less enzyme activity was found in normal leaf tissue, or wounded leaf tissue inoculated with either sterile broth or a non infectious strain of A. tumefaciens. Some of the implications of these results are discussed.

## INTRODUCTION

Within the past several years an intensive study has been undertaken on the role of cyclic-AMP in animal cancer. These studies have revealed that (1) cyclic-AMP can inhibit the growth and formation of certain animal tumors (1, 2); (2) lower concentrations of cyclic-AMP are found in transformed cells than in the corresponding normal tissue (3, 4); (3) altered levels of adenyl cyclase and cyclic-AMP phosphodiesterase activity are found in tumor tissue when compared to the normal tissue (5, 6).

We have recently shown in our lab that cyclic-AMP can inhibit the formation of crown-gall tumors induced by infectious strains of Agrobacterium tumefaciens on the primary leaves of Pinto beans by 50% (7).

Based on the above findings and similarities between crown-gall tumors and animal cancer, it is important to determine the level of cyclic-AMP phosphodiesterase activity in normal and plant tumor tissue.

## MATERIALS AND METHODS

Stock cultures of Agrobacterium tumefaciens strain B6 were obtained from Dr. James Lippincott of Northwestern University. The bacteria were grown in a medium containing 0.8% nutrient broth, 0.1% yeast extract, and 0.5% sucrose for 48 hours in a New Brunswick Scientific Shaker at 27° C.

Tumors were induced in the primary leaves of Phaseolus vulgaris var. Pinto using the method described by Lippincott and Heberlein (8). This basically involves spreading 0.1 ml of a properly diluted bacterial suspension (containing $5 \times 10^8$ cells/ml) on the seven day old primary leaves of Pinto beans which had been previously dusted with #400 grit carborundum. One week after inoculation 3 grams of normal and tumor tissue were cut out using a 3.1 mm cork bore. The tissue was weighed and homogenized in a Waring blendor with 15 ml of a $10^{-2}$ M Tris buffer pH 7.4 containing 0.01 mg/ml $MgCl_2$. The homogenate was then centrifuged for 10 minutes at 12,000 x g. The supernatant was decanted and used as the source of the crude phosphodiesterase.

Assay of phosphodiesterase: 0.5 ml of curde enzyme was added to 0.5 ml of 50 mM MES* buffer pH 6.0 which contained 0.4 g of $MgCl_2$, 4mM B-mercaptoethanol and 1 uc (3H) Adenosine 3', 5' cyclic monophosphate. The reaction mixture was then placed in a water bath at 37° C and allowed to react for 3 hours. At the end of this time period a 60 ul aliquot was spotted on Whatman 3 MM chromatography paper using a solvent system which contained 1 M ammonium acetate and 95% ethanol (3:7). Standards of cyclic-AMP, 5'-AMP, 3'-AMP, and adenosine were also spotted. The chromatogram was allowed to develop for 6 hours after which time the standards spots were identified using a UV hand lamp. The corresponding spots from the reaction mixture eluate were then cut out and placed in scintillation vials containing toluene and 2,5-diphenyloxazole (5 g/liter toluene). The vials were counted in a Beckman LS-100 liquid scintillation counter. Most of the radioactivity was present in the adenosine spot indicating the presence of some nucleotidase activity in the crude enzyme extract. The presence of nucleotidase activity has been found in almost all phosphodiesterase extracts form higher plants (9). Enzyme activity is expressed in units/mg protein. A unit of phosphodiesterase activity is defined as the amount of enzyme necessary to hydrolyze 1 p mole of (3 H) cyclic-AMP/hr at 37° C. Protein concentrations were determined according to the method of Lowry et al (10).

The bacterial enzyme was obtained by centrifuging 48 hour cultures of A. tumefaciens at 12000 x g for 30 minutes. The pellet was then resuspended in a $10^{-2}$ m Tris buffer pH 7.4 containing 0.01 mg/ml $MgCl_2$. The suspension was then passed through (twice) a Aminco French Pressure Cell and the resulting slurry centrifuged at 12,000 x g for 30 minutes. The pellet was then taken up in 1.5 ml of the Tris buffer and this was used as the source of our crude bacterial enzyme. Phosphodiesterase activity was assayed as previously described.

## RESULTS AND DISCUSSION

Table I (Exps. A and B) show that a considerable amount of phosphodiesterase activity is present in tumor tissue while little if any activity is found in normal tissue or wounded tissue inoculated with sterile bacterial growth medium.

When the primary leaves of Pinto beans are inoculated with a non-infectious strain of A. tumefaciens (IIBNV6) which also attaches to the tumor binding site

*(2 N-Morpholino Ethane Sulfonic Acid)

Table I - CYCLIC-AMP PHOSPHODIESTERASE ACTIVITY IN PRIMARY LEAVES OF PINTO BEANS INOCULATED WITH A. TUMEFACIENS STRAIN B6 AND BACTERIA-FREE GROWTH MEDIUM

| Sample | Units of phosphodiesterase activity/mg protein | |
|---|---|---|
| | Exp. A | Exp. B |
| Tumor tissue obtained from A. tumefaciens strain B6 | 440.0 | 382.8 |
| Normal tissue obtained from non-inoculated plants | 18.2 | 16.5 |
| Wound tissue obtained from bacteria-free growth medium | 7.1 | 14.3 |

Table II - PHOSPHODIESTERASE ACTIVITY OBTAINED FROM PRIMARY PINTO BEAN LEAF TISSUE INOCULATED WITH INFECTIOUS AND NON-INFECTIOUS STRAINS OF A. TUMEFACIENS

| Sample | Units of phosphodiesterase activity/mg protein | |
|---|---|---|
| | Exp. A | Exp. B |
| Tumor tissue obtained from A. tumefaciens B6 | 424.8 | 361.0 |
| Tissue obtained from leaves inoculated with A. tumefaciens IIBNV6 | 22.0 | 50.2 |

Table III - CYCLIC-AMP PHOSPHODIESTERASE ACTIVITY FROM INFECTIOUS AND NON-INFECTIOUS STRAINS OF A. TUMEFACIENS

| Strain used | Units of phosphodiesterase activity/ mg proteins | Infectivity on Pinto bean leaf[a] |
|---|---|---|
| B6 | 136.8 | 100 |
| IIBNV6 | 121.9 | 0 |

[a]Data of Lippincott and Lippincott (11)

(11) the resulting leaf tissue obtained possessed very little phosphodiesterase activity when compared to the B6 tumor tissue (Table II Expts. A and B).

The enzyme activity found in the tumor tissue may be present as a result of the transformation process of a normal cell into a crown-gall tumor cell or could be coming from the inoculating bacteria. Table III shows that phosphodiesterase activity is present in both infectious and non-infectious strains of A. tumefaciens. The facts that although enzyme activity is present in the IIBNV6 strain, and there is little enzyme activity associated with leaf tissue inoculated with these bacteria, strongly suggest the possibility that the source of phosphodiesterase activity is the tumor itself and its presence represents a necessary event in crown-gall tumor formation. If this is the case, this would help explain the previously referred to findings (7) that the exogenous addition of cyclic-AMP can inhibit the formation of crown-gall tumors and would also suggest that like many animal tumors (3, 4) crown-gall tumors may also possess a lower concentration of cyclic-AMP than corresponding normal tissue.

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