N. J. Microbiology, 2(2), 1982, pp 195 – 206

PARTIAL CHARACTERIZATION OF A TRYPSIN STIMULATED CYTOPLASMIC SOLUBLE ADENOSINE TRIPHOSPHATASE OF *AGROBACTERIUM TUMEFACINES*

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ABSTRACT

A cold labile trypsin-stimulated adenosine triphosphatase, ATPase (EC. 3.6.1.3) activity has been found associated with the partially purified cytoplasmic soluble fraction of *Agrobacterium tumefaciens* strain C-58. Chromatographic, electrophoretic and catalytic studies are also reported.

Trypsin activated the enzyme 31% at the pH optimum of between 8.0 and 9.0. The optimum temperature of activity for the enzyme was between 28°C and 50°C, whereas the enzyme was better maintained for several days at 4°C than either at 28°C or 37°C.

INTRODUCTION

Adenosine triphosphatase(s) are widely distributed in both procaryotic and eucaryotic cells. In both types of cells, the enzyme can be found either bound to the cytoplasmic membrane or free in the soluble fraction of the cell. The ubiquitous nature of membrane-bound ATPase in several gram positive and gram negative bacteria suggests that the enzyme must serve a function in these organisms. There is good experimental evidence that ATPase acts as a coupling enzyme in the oxidative phosphorylation of ADP to ATP (Butlin, J.D. G.B. Cox, and F. Gibson 1971). Tsuchiya and Rosen (1976) have also shown that an ATPase dificient mutant of *E. coli* strain NR 70 was unable to synthesize ATP under an artificially imposed membrane potential.

While there is a lot of information in the literature on membrane-bound ATPase, there is scarcity of information on the characterization of the cytoplasmic soluble ATPases. ATPase from *Thiobacillus ferrooxidans* (Adapoe, C. and M. Silver, 1975) is one of the very few reported cytomplasmic soluble ATPases from bacterial sources.

The present is on the cytoplasmic soluble ATPase of *Agrobacterium tumefaciens*, a phytopathogen.

MATERIALS AND METHODS

Chemicals

Nucleotides were products of p-L Biochemicals, Inc. Milkwaukee, Wisconsin, or of Sigma chemical company, Saint Louis, Missouri. Dicyclo-hexyl-carbodiimide (DCCD) was purchased from Eastman Kodak Company, Rochester, New York. All other chemicals used were either ragent or A.C.S. grade.

Bacteria

Virulent *Agrobacterium tumefaciens* strain C-58 was obtained from Dr. Mary-Dell Chilton of the University of Washington in Seatle, Washington. The virulence of C-58 was confirmed throughout the period of this investigation by inoculation of stem tissue of life plant, *Kalanchoe daigremontiana* and Tobacco, *Nicotiana tabacum*. Tumor tissues developed at sites of needle puncture after 14 days of incubation at 28°C.

Growth Conditions

The bacteria were cultivated at 26°C on a shaking incubator in a liquid dextrose 0.5% basal medium enriched with 1% yeast extract, 0.5% sodium chloride and 1% Peptone as previously described by Hamilton and Fall (1971). The bacteria were grown in a 2-liter Erlenmeyer falsk containing 1.0 litres of the medium described above.

Exponential phase cells were harvested by centrifuging at 17,000 x g.

Extraction of Enzyme

Twenty g of cells were cracked by a mortar and pestle, using alumina as abrasive in 20ml of IMTRIS 2mM Mg^{++} at pH 7.5 and the mixture subjected to centrifugation at 30,000 x g for 1 hour. A buffer of this high ionic strength will prevent the release of the membrane bound ATPase, which is tightly bound to the membrane and could only be released by low ionic strength buffers. The supernatant fluid from the above centrifugation was the source of the cytoplasmic soluble ATPase.

Enzymatic Assay

ATPase activity was assayed by colorimetric measurement of inorganic phosphate (Pi) released from ATP, by the method of Fiske and Subbarow (1925) as modified by Dulley (1975). The reaction mixture (1ml) contained 0.1ml of 2.5 x 10^{-2}M nucleotide, 0.3ml (70 – 100 ug protein) enzyme source and 0.6ml of 0.1M Tris-ECI (pH 7.5).

When the effects of cations and known inhibitors were tested, 0.1ml of the agent was pre-incubated with the reaction mixture. Final volume of the reaction mixture was always adjusted to 1 ml with the buffer. The mixture of enzyme and buffer was pre-incubated for 10 min at 37° before the addition of substrate, and the incubation was continued for 30 min at 37°C in a water bath shaking incubator.

A control mixture contained all the components of the test medium except the enzyme source so as to correct for the endogenous breakdown of the substrate.

Protein concentration was determined by the method of Lowry et al. (1951), or by U.V. absorption.

Enzyme Purification

All operations were carried out at 2-5°C as follows: The cytoplasmic soluble fraction (obtained as described above) was applied on a DEAE-Sephadex A-50 column and fractions were collected by elution with a stepwise gradient using Tris buffers in the following order: 0.1M Tris-HCl + 5mM Mg^{++} pH 7.5, 0.0M Tris-ECI + 5mM Mg^{++} PH 7.5 and 0.3M Tris-HCl

+ 5mM Mg^{++} pH 7.5 respectively. Active fractions wrer not eluted until after the application of buffer of 0.3M Tris-HCl + 5mM Mg^{++} pH 7.5. Pooled active fractions were used in the study of the enzyme without further treatment. For electrophoretic studies, the extract was concentrated 10-fold with dry sephadex G-25 without any loss of activity. Ammonium sulphate concentration, on the other hand, inhibited the activity of the enzyme. As a result of the noted inhibitory action of ammonium sulphate, the salt was not used to concentrate the extract before application onto the DEAE – Sephadex column.

A unit of enzyme is the amount of enzyme that releases 1 u mole of inorganic phosphate in 30 min.

Sodium Dodecyl sulfate (SDS) Gel Analysis

SDS gel analysis was performed by the method of Laemmli and King (1971).

Effect of Physico-Chemical Conditions on enzyme activity:

Effect of various compounds

Enzyme activity was assayed under standard conditions except that the following substances at the indicated concentrations were added. 2,4 Dinitrophenol ($1x10^{-3}$ M), Sodium azide ($1x10^{-3}$ M), Dicyclo-hexylcarbodiimide ($1x10^{-2}$ M), Ethylene diaminetetra-acetic acid ($1x10^{-3}$ M), Potassium fluoride ($1x10^{-3}$ M), and Trypsin (500 microgram). The activity of the standard reaction mixture was taken as 100%.

Stability of enzyme at selected temperatures

Samples of cytoplasmic ATPase preparation obtained by DEAE – Sephadex chromatography were stored at 4°C, 28°C and 37°C respectively. At intervals aliquots were withdrawn and the activity was assayed under the standard conditions.

Effect of cations on enzyme activity

Enzyme activity was assayed under standard conditions except that the reaction mixture was made 10 micromole with respect to the added cations. The cations tested were Mg^{++}, Mn^{++}, Ca^{++}, Zn^{++} and Ni^{++}.

Effect of mercuric chloride (Hgc12) on enzyme activity

Enzyme activity was assayed under standard conditions except that to the reaction mixture was added 1×10^{-2}M Hgc12.

Effect of pH on enzymatic activity

Activity was measured under standard conditions except that 50mM buffers (Acetate – NaOH for pH below 6, Tris-HCl, pH 6.0 – 8.0, and glycine – NaoH pH 9.0 – 12.5) were used. Percentage activity was computed using highest activity as 100%.

Effect of incubation temperature on enzyme activity

Ezyme activity was assayed under standard conditions except that four different temperatures (4°C, 28°C, 37°C, and 50°C) of incubation were checked.

RESULTS

Enzyme Fractionation

The chromatographic profile of the cytoplasmic soluble ATPase on DEAE-Sephadex A-50 showed two major peaks of activity (Fig. 1), which were eluted with 0.3M Tris buffer as indica-

ted. Peak I was used for work reported here.

The enzyme was about seven fold purified by DEAE-Sephadex A-50 fractionation (Table I). The pooled active fractions from DEAE-Sephadex step lost all activity when subjected to cellex-D fractionation.

Effect of physico-chemical conditions on the activity of the enzyme.

Known inhibitors of members ATPase including potassium fluoride, Dinitrophenol, Dicyclohexyl-carbodiimide (DCCD), sodium azide and ethylene diamine tetracetic acid (EDTA) had no effect on the activity of the enzyme (Table 2). However, trypsin activated the enzyme 31% above the control level.

With respect to the stability of the enzyme at different storage temperatures, less susceptibility to inactivation at $4^{o}C$ than at other temperatures was observed (Fig. 2). The reason for the instability of the cytoplasmic soluble ATPase at $28^{o}C$ and $37^{o}C$ is not known at present but could be due to a natural inhibitor of soluble ATPase and/or contaminating proteases that are active at these temperatures.

The enzyme was slightly stimulated by all the divalent cations tested (Table 3).

Mercuric chloride ($HgCI^{2}$) at a concentration of $1 \times 10^{/2}$ M inhibited the cytoplasmic soluble ATPase by 34%.

The pH optimum of activity for the cytoplasmic soluble ATPase was found to be between 8.0 and 9.0 (Fig. 3). When different incubation temperatures were checked, the optimum temperature of activity was found between 28 and $50^{o}C$ (Fig. 4). When aliquots of the sephadex G-25 concentrated enzyme were subjected to SDS-polyacrylamide gel electrophoresis, polypeptide chains of molecular weights of 30,000, 60,000 and 200,000. Daltons were found by comparison with standard marker proteins (Fig. 5).

Table 1:

Summary of Purification of Cytoplasmic Soluble ATPase form c-58 Strain of Agrobacterium tumefacines

Steps	*Total Protein, mg*	*Total Activity Micromoles Pi (released/30 min)*	*Specific Activity Micromoles Pi (released/30 min/mg Protein)*
Crude – extract	196	149.9	0.765
DEAE – Sephadex- A-50	40	233	5.84

TABLE 2:

Effect of Various Compounds on Cytoplasmic Soluble ATPase Activity
Values represent average of triplicate assays.

Compound Added	*Micromoles PO_4 Released*	*Relative Activity, %*
None	0.875	100
$1X10^{3}$M 2, 4 DNP	0.850	96
$1X10^{-3}$ NaN_3	0.850	96
$1X10^{-2}$M DCCD	0.875	100
$1X10^{-3}$ EDTA	0.850	96.9
$1X10^{-3}$M KF	0.825	94
500 ug Trypsin	1.15	131

* 2,4 DNP – 2, 4 Dinitrophenol

NaN_3 – Sodium azide

DCCD – Dicyclo-hexyl-carbodiimide

EDTA – Ethylene diamine tetra-acetic acid

KF – Potassium Fluoride

TABLE 3:

Effect of Divalent Cations on Cytoplasmic Soluble ATPase Activity.
Values represent average of triplicate assays.

Cation	*Micromoles PO_4*	*Relative Activity %*
None	1.35	100
10mM Mg^{++}	1.40	103
10mM Mn^{++}	1.40	103
10mM Ca^{++}	1.50	111
10mM Zn^{++}	1.50	111
10mM Ni^{++}	1.45	107

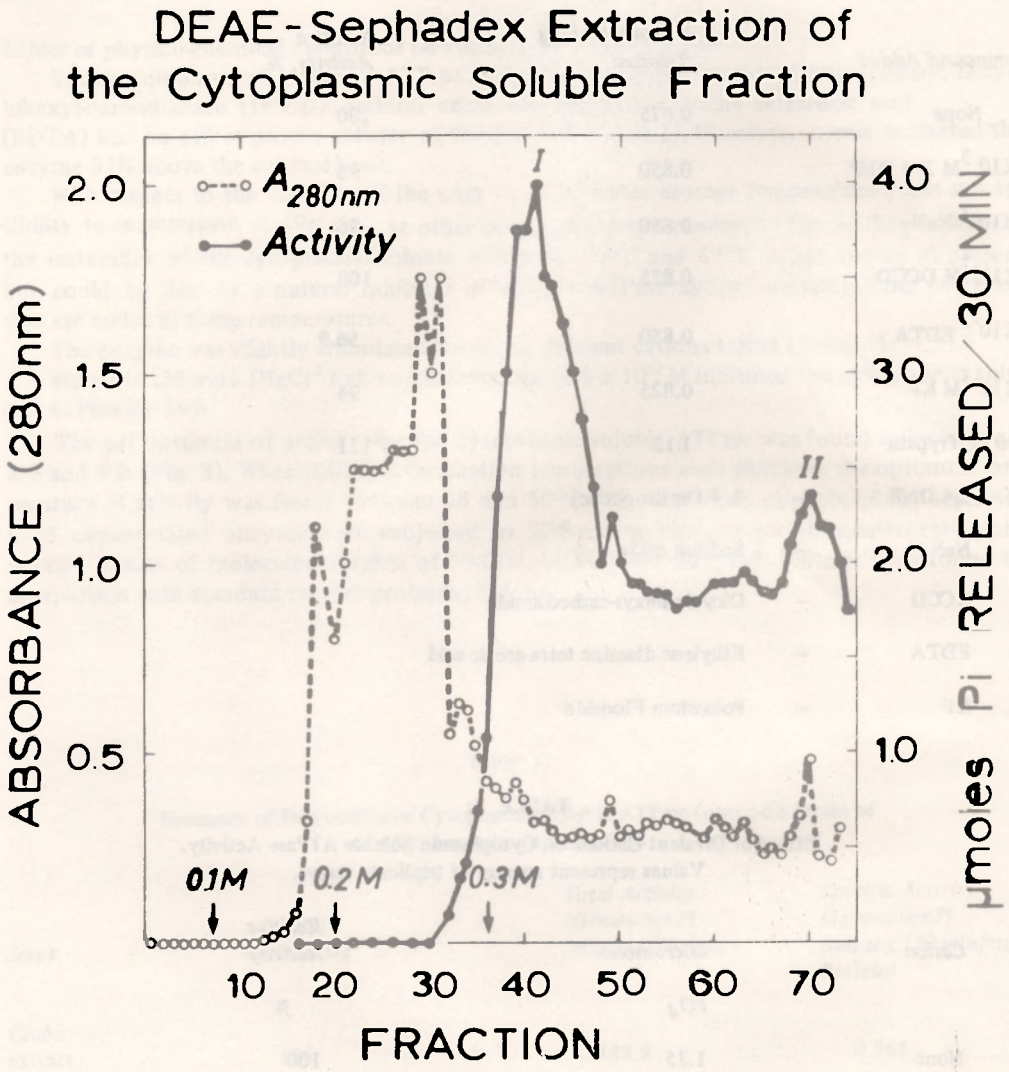

Fig. 1. Chromatographic profile of the cytoplasmic soluble ATPase on DEAE-Sephadex A-50 column (46 x 3cm). ATPase activity was assayed under standard conditions as described in "methods" ——, ATPase activity; 0 — 0, protein concentration measured by the absorbance at 280nm. Arrows indicate where tris-HCl butters, pH 7.5 of indicated concentrations were applied. Fraction volume was 4ml. Peak I was used for this work.

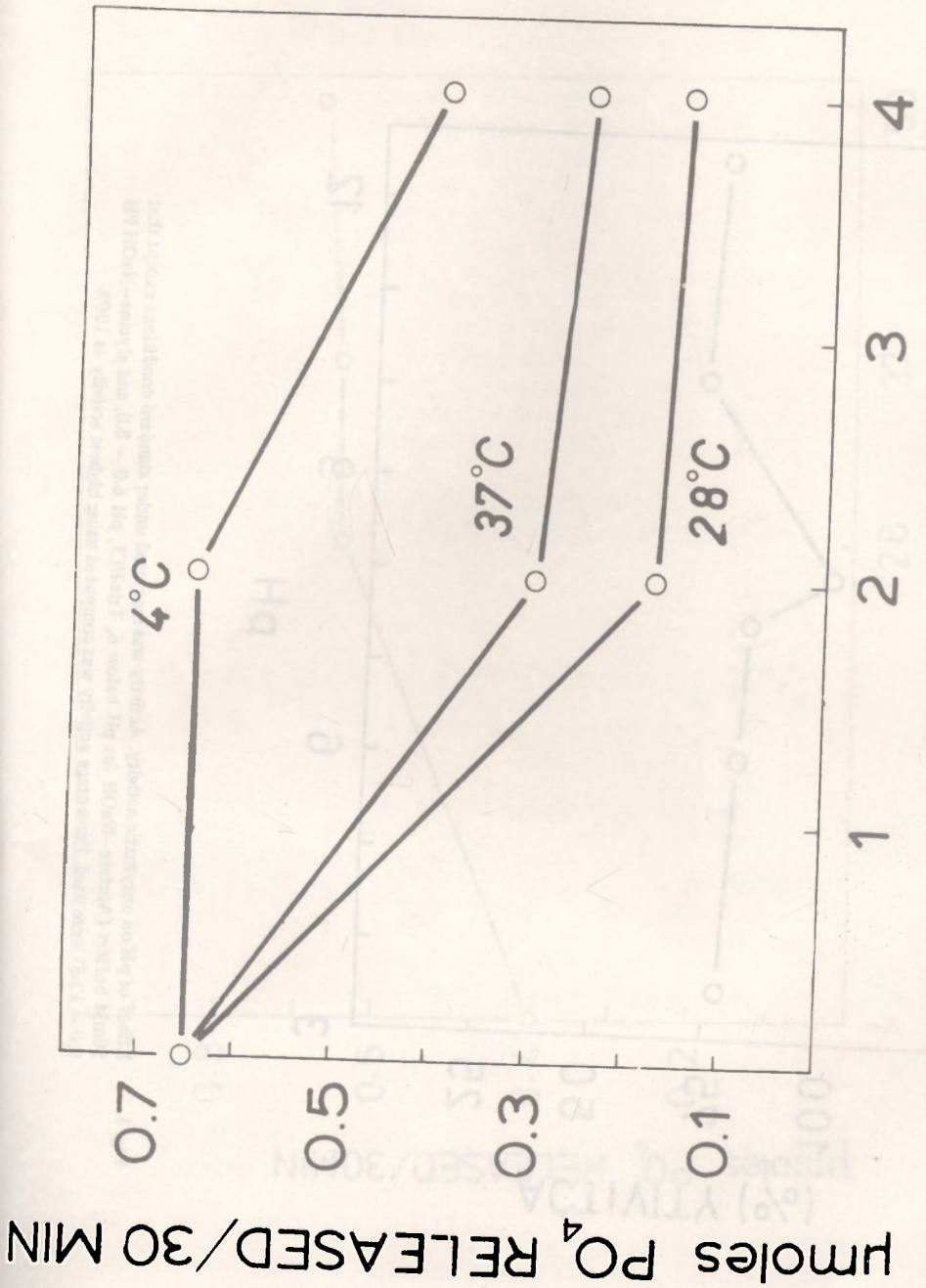

Fig. 2. Stability of enzyme at selected temperatures.

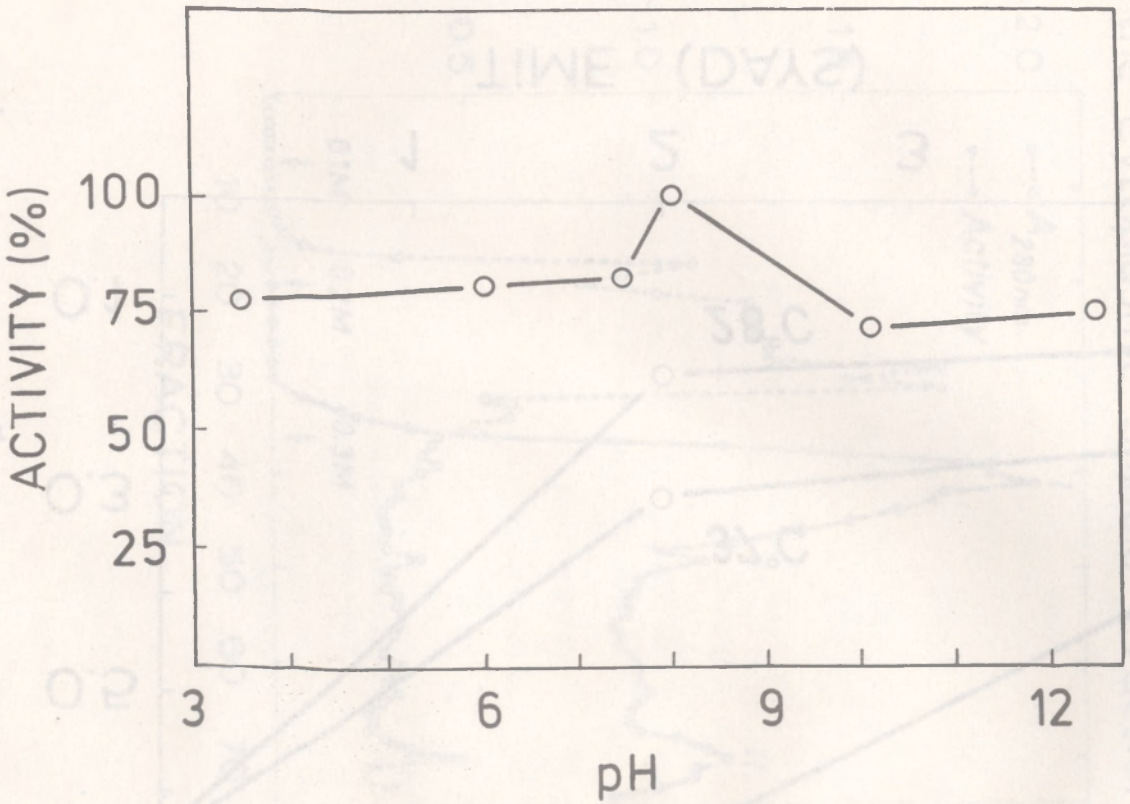

Fig. 3. Effect of pH on enzymatic activity. Activity was measured under standard conditions except that 50mM buffers (Acetate–NaOH for pH below 6, Tris-HCl, pH 6.0 – 8.0, and glycine–NaOH PH 9.0 – 12.5) were used. Percentage activity was computed using highest activity as 100%

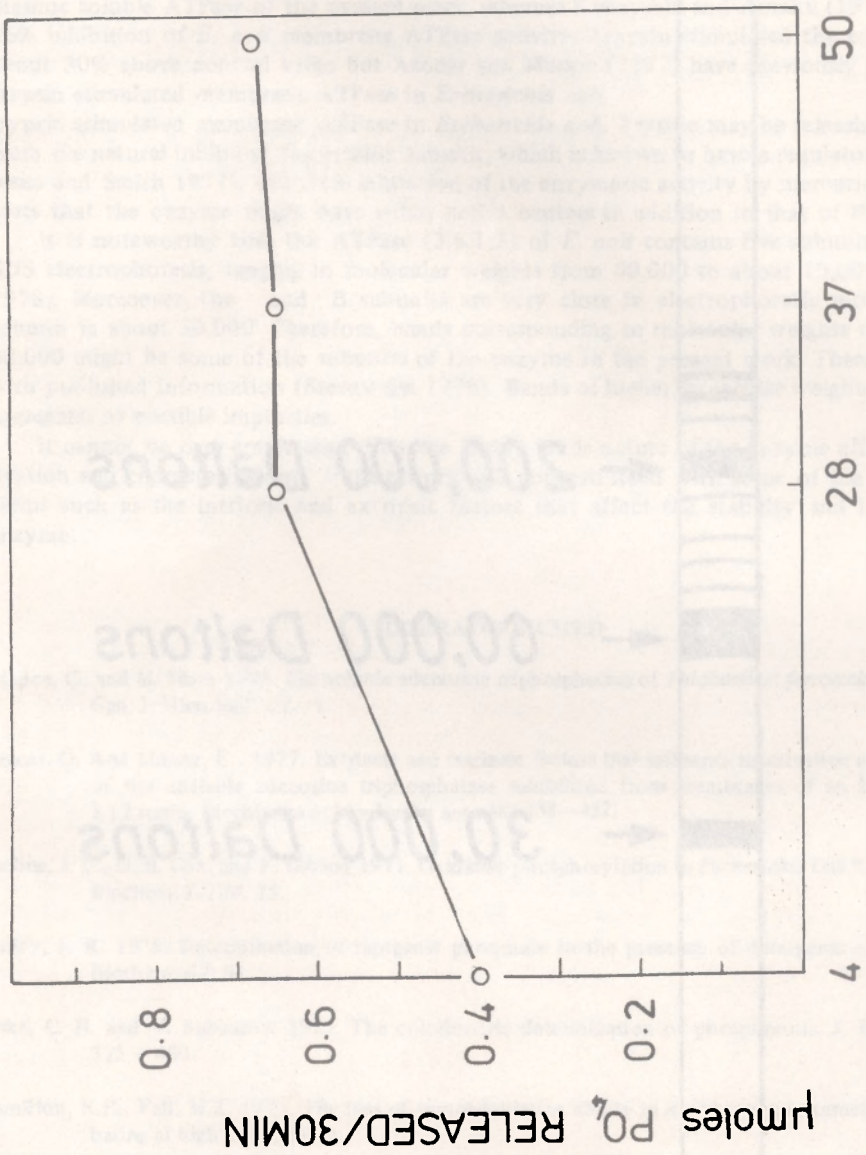

Fig. 4. Effect of incubation temperature on enzyme activity.

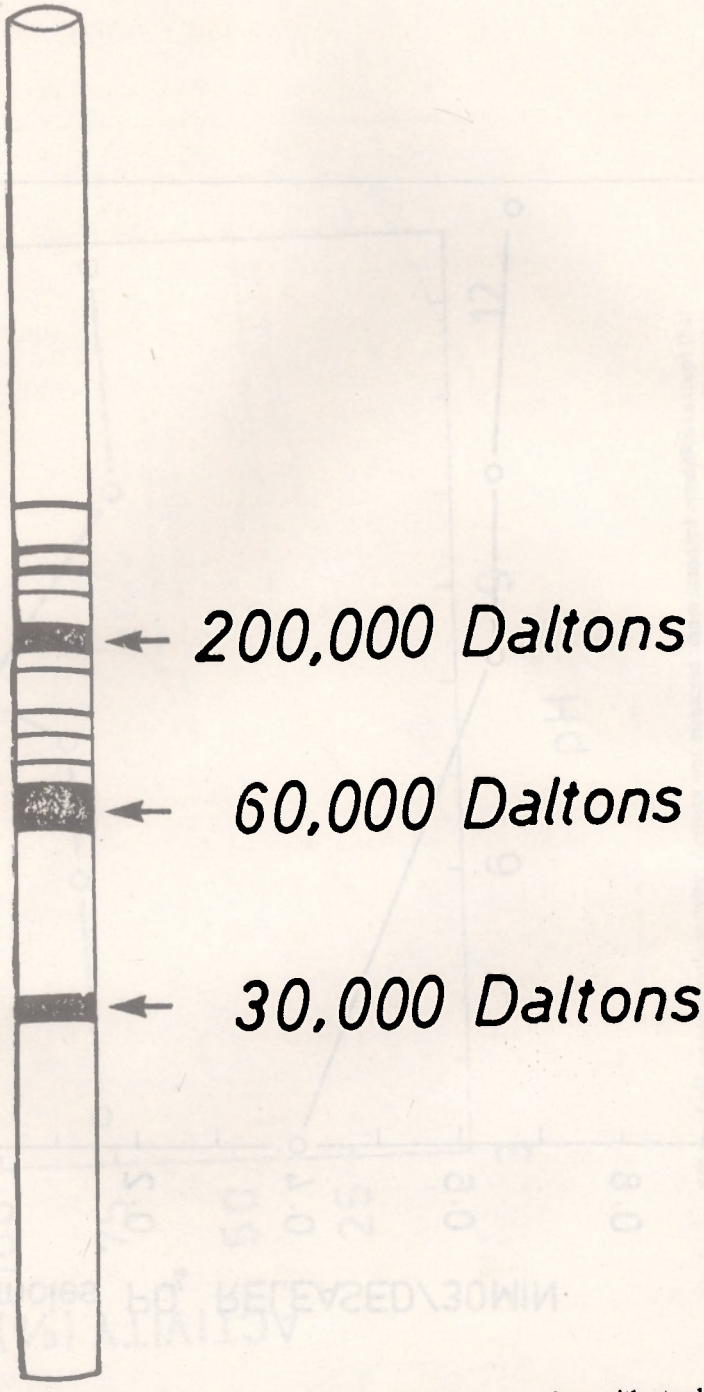

Fig. 5. Molecular weights of the polypeptide chains estimated by comparison with standard marker proteins.

DISCUSSION

The results reported in this study show that *Agrobacterium tumefaciens* produce a cytoplasmic soluble ATPase that has characteristics that appear different from those of reported membrane ATPases. DEED, a known inhibitor of membrane ATPase had no effect on the cytoplasmic soluble ATPase of the present work, whereas Kobayashi and Anraku (1972) reported a 26% inhibition of *E. coli* membrane ATPase activity. Trypsin stimulated the present enzyme about 30% above control value but Azocar and Munoz (1977) have previously reported on a trypsin stimulated membrane ATPase in *Escherichia coli.*
trypsin stimulated membrane ATPase in *Escherichia coli.* Trypsin may be releasing the enzyme from the natural inbibitor, the epsilon subunit, which is known to have a regulatory role (Sternweiss and Smith 1977). The 34% inhibition of the enzymatic activity by mercuric chloride suggests that the enzyme might have other active centers in addition to that of the sulphydryl.

It is noteworthy that the ATPase (3.6.1.3) of *E. coli* contains five subunits (& – E) by SDS electrophoresis, ranging in molecular weights from 60,000 to about 10,000 (Sternweiss, 1978). Moreoever, the and B subunits are very close in electrophoretic mobility and the. subunit is about 30,000 Therefore, bands corresponding to molecular weights of 60,000 and 30,000 might be some of the subunits of the enzyme in the present work. These units concur with published information (Sternweiss 1978). Bands of higher molecular weights are probably aggregates or possible impurities.

It cannot be over-emphasized that the highly labile nature of the enzyme affected its purification and characterization. Future work will concern itself with some of the unsolved problems such as the intrinsic and extrinsic factors that affect the stability and lability of the enzyme.

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