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Characterization of the Membrane-bound Adenosine Triphosphatase from Corn Roots

Moon, Hye Yeon, Kwang Soo Roh* and Woong Seop Sim
(Department of Biology, Korea University, Seoul and
*Department of Biology, Keimyung University, Daegu)

옥수수 뿌리로부터 分離한 Membrane-bound ATPase의 特性에 關한 硏究

文惠延 · 盧光洙* · 沈雄燮
(高麗大學校 理科大學 生物學科 ·
*啓明大學校 理工大學 生物學科)

ABSTRACT

The membrane-bound ATPases were separated on sucrose gradient from corn roots and characterized by pH optima, sensitivity to monovalent salt, Km and Vmax. The pH optima for the activity of all the ATPases associated with 13,000*g* pellet and 13,000~80,000*g* pellet were 5 and 9, respectively. The ATPases in Fractions B and C of the 13,000*g* pellet were more active at pH 5 than pH 9. While, in the case of Fractions D, E and F, they were reverse. The activities of the ATPase in Fractions A and C of the 13,000~80,000*g* pellet were greater at pH 5 than pH 9. On the other hand, the ATPases in Fractions B, D, E, and F were more active at pH 9 than pH 5. The optimum concentraction of ATP for the assay was about 3 to 5 mM. The Km's for the membrane-bound ATPases in 13,000*g* pellet and in 13,000~80,000*g* pellet were 0.25 mM. While Vmax values for 13,000*g* pellet were from 8.0 to 12.5 μM Pi/mg protein/hr. according to pH values, those for 13,000~80,000*g* pellet were from 35.7 to 55.6 μM Pi/mg protein/hr. Activities of the membrane-bound ATPases in both 13,000*g* pellet and 13,000~80,000*g* pellet were stimulated with increasing the concentration of K^+.

INTRODUCTION

It is well known that ion transport in plants requires respiratory energy (Laties, 1959; Lundegardh, 1955; Robertson, 1960). The actual energy source for transport is ATP. It has also been shown that an ATPase is intimately involved in this ATP-driven reaction (Skou, 1965). The existence of ion sensitive ATPases in plant tissues has been

reported for several plant species (Brown *et al.*, 1965; Dodds and Ellis, 1966; Fisher and Hodges, 1969; Gruener and Neumann, 1966; Hall and Butt, 1969; Kylin and Gee, 1970).

ATPase is believed to mediate energy transfer from ATP to the ion transport system in plant roots (Hodges, 1973). This hypothesis is supported by the reports on high correlation between ATPase activities and ion absorption rates (Fisher *et al.*, 1970), and association of the ATPase with plasma membrane of oat roots (Hodges *et al.*, 1972). In many cases, plant ATPase have been found to possess distinctive cation sensitivities, particularly for Na^+, K^+ and Mg^{++} and in this sense are similar to the cation-stimulated ATPase found on the surface membrane of many mammalian cells (Berman *et al.*, 1969).

However, membrane systems of such structures as plasma membrane, tonoplast, mitochondria, nuclei, endoplasmic reticulum and Golgi also possess ATPase activity (Hall, 1971; Macklon and Higinbotham, 1970; Pierce and Higinbotham, 1970). Hence, *in vitro* assays of ATPase activity employing a crude preparation of membranes (*i.e.*, differentially centrifuged fractions) may measure several different ATPases. It would be desirable to know how many membrane-bound ATPases can be distinguished in cell extracts and to identify the various membrane systems containing these enzymes. It is the purpose of this investigation reported here.

MATERIALS AND METHODS

Plant material. Maize seeds (*Zea mays* L. cv. Golden growth bandam) were allowed to germinate at 30°C for 5 days in the sterilized dark condition.

Preparation of 13,000 *g* pellet and 13,000~80,000 *g* pellet. Prior to the experiment, the five-day-old roots (6~8 cm in length) were excised, washed 3 times in distilled water and chilled prior to homogenizing in an ice-jacketed mortar and pestle. All manipulations were at 0 to 4°C. The roots were ground in a medium consisting of 0.25 M sucrose, 3 mM EDTA and 25 mM tris-HCl buffer, pH 7.2. Four milliliters of grinding medium were used per gram fresh weight of root tissue. The homogenate was filtered through four layers of cheese-cloth and successively centrifuged at 13,000 *g* for 15 min. and 80,000 *g* for 30 min. Before discontinuous gradient centrifugation, the 13,000 *g* pellet and 13,000~80,000 *g* pellet were suspended in a sucrose solution (2%, w/w less than the top of the gradient) containing 1 mM $MgSO_4$ and 1 mM tris-HCl, pH 7.8.

Isolation of membranes on discontinuous sucrose gradient. Two millilters of membrane preparation (10~20 mg protein) were laid onto a 36 ml discontinuous gradient consisting of 4 ml of 45% (w/w) and 6.4 ml each of 38, 34, 30, 25 and 20% sucrose (w/w) in 1 mM $MgSO_4$ and 1 mM tris-HCl buffer, pH 7.2. The membrane-loaded gradient were centrifuged for 2 hrs. at 95,000 *g* in a Beckman SW 27 rotor.

Each membrane protein band was removed by puncturing tube bottom with a needle and identified as shown in Fig. 1.

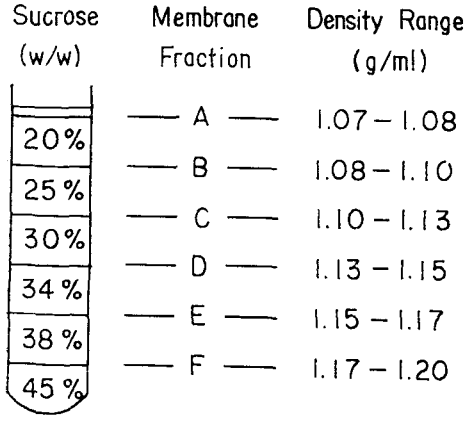
ATPase assay. The reaction mixture for ATPase assay contained appropriate concentrations of ATP, 1.5 mM $MgSO_4$, 50 mM KCl, 33 mM tris-HCl buffer at desired pH and 300 to 500 μg of protein in a total volume of 1 ml. The reaction was carried out at 38°C for times up to 1 hr. The reaction was terminated by the addition of 10 ml of 1.25% (w/v) cold ammonium molybdate in 2.5 N sulfuric acid. ATPase activities were measured by determining the amount of inorganic phosphate released. The amount of inorganic phosphate was determined by the Fiske and Subbarow procedure (Fiske and Subbarow, 1925).

Protein assay. Protein was determined by the biuret methods (Gornall *et al.*, 1945).

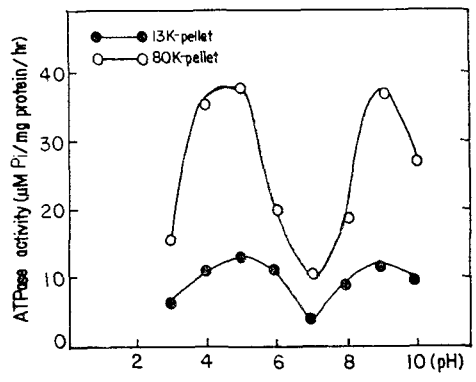
RESULTS

Optimal pH for ATPase activity. The pH optimal for the activity of the membrane-bound ATPases in 13,000*g* pellet were 5 and 9. The membrane-bound ATPases in 13,000~80,000*g* pellet also show the same pH optimal of 5 and 9 (Fig. 2).

Distribution of the ATPase activity in various membrane fractions. In order to separate and characterize the membrane-bound ATPases, the 13,000*g* pellet and 13,000~80,000*g* pellet from the same homogenate were fractionated by discontinuous sucrose gradient centrifugation. The activities of the membrane-bound ATPases in each membrane fraction were measured at pH 5 or 9.



(Fig. 1)



(Fig. 2)

Fig. 1. Discontinuous sucrose gradient used for separation of membrane fractions of corn roots. 13,000*g* pellet and 13,000~80,000*g* pellets were the overlay.

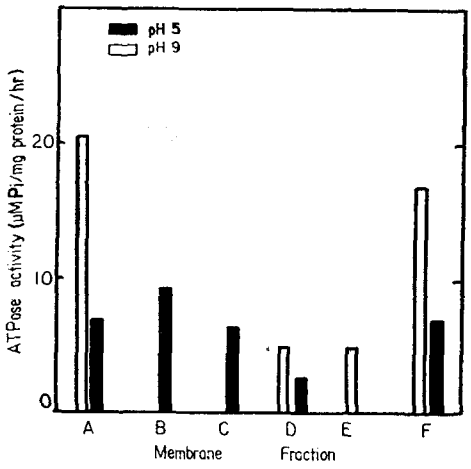
Fig. 2. Effect of pH on the activities of membrane-bound ATPase in 13,000*g* pellet and in 13,000~80,000*g* pellet from corn roots.

Fig. 3 shows the membrane-bound ATPase activities in various membrane fractions of 13,000g pellet. Fractions B and C had greater activity at pH 5 than pH 9, while Fractions D, E and F had greater activity at pH 9 than at pH 5. Among the membrane fractions, the greatest ATPase activity at pH 9 was that of the Fraction F. Based on the results for oat roots (Leonard *et al.*, 1973), this fraction should be rich in mitochondria when the 13,000g pellet is layered on the gradient. At pH 5, the greatest ATPase activitiy was that of the Fraction B. Fractions B and C had no ATPase activity at pH 9, on the other hand the ATPase activity at pH 5 was not showed in Fraction E.

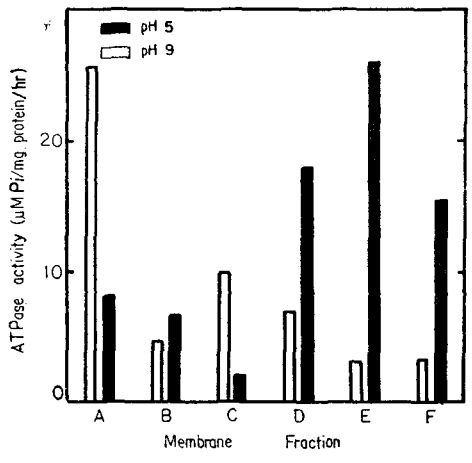
The membrane-bound ATPase activities in various membrane fractions of 13,000~80,000g pellet are shown in Fig. 4. The highest ATPase activity at pH 9 was found in Fraction C and that of the pH 5 was Fraction E. This fraction consists of plasma membrane (Leonard and VanDerWoude, 1976; Hodges and Leonard, 1974).

Optimum concentration of ATP and kinetics. Fig. 5. shows the effect of ATP concentration on the ATPase activities in 13,000g pellet. As shown in Fig. 5, the optimum concentration of ATP for the assay about 3 to 5 mM. The apparent Michaelis constant and maximum velocity for the ATPase were estimated from the data in Fig. 5 and are shown in Fig. 6.

The Km's and Vmax value at pH 5 for the membrane-bound ATPases in 13,000g pellet were 2.5 mM and 8.0 μM Pi/membrane protein, respectively. At pH 9, the Km's for the enzyme was also 2.5 mM, but the Vmax value was 12.5 μM Pi/mg membrane protein.



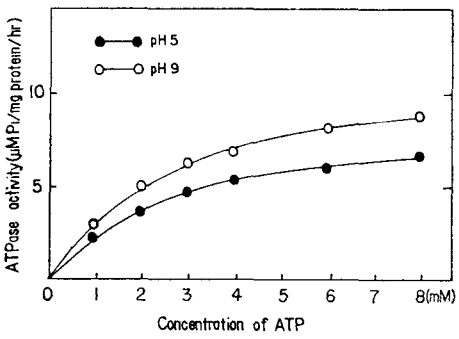
(Fig. 3)



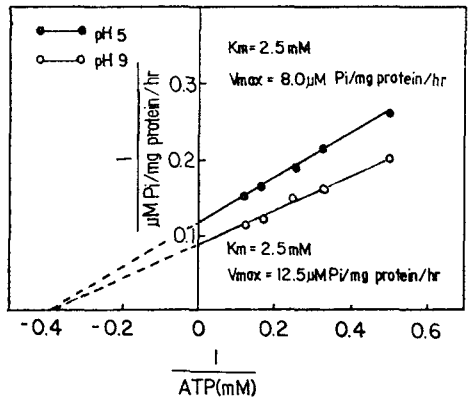
(Fig. 4)

Fig. 3. Distribution of ATPase activity on discontinuous sucrose gradient of Fig. 1. 13,000g fraction from corn root homogenate was the overlay.

Fig. 4. Distribution of ATPase activity on discontinuous sucrose gradient of Fig. 1. 13,000~80,000g fraction from corn root homogenate was the overlay.



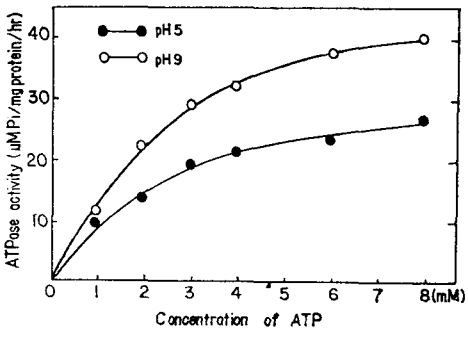
(Fig. 5)



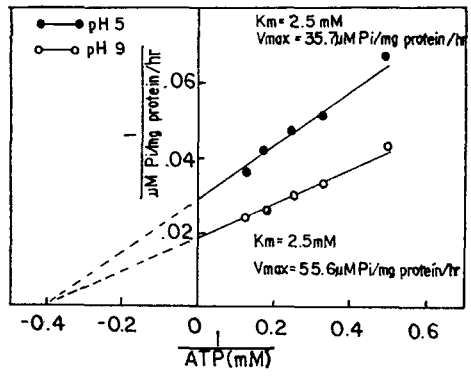
(Fig. 6)

Fig. 5. Effect of ATP concentration on the activities of membrane-bound ATPase in 13,000 g pellet. Reactions were run at 38°C for 1 hr. with 1 ml reaction mixture containing various concentration of ATP, 1.5 mM $MgSO_4$, 50 mM KCl, 30 mM tris-HCl buffer (pH 5 or 9) and membrane protein.

Fig. 6. Lineweaver-Burk plot of data in Fig. 5. Velocity is expressed as μM Pi/mg protein/hr, and concentration of substrate as molarity (mM). Kinetic constants were calculated by linear regression analysis.



(Fig. 7)



(Fig. 8)

Fig. 7. Effect of ATP concentration on the activities of membrane-bound ATPase in 13,000 ~80,000 g pellet.

Fig. 8. Lineweaver-Burk plot of data in Fig. 7. Velocity is expressed as μM Pi/mg membrane protein/hr and concentration of substrate as molarity (mM). Kinetics constants were calculated by linear regression analysis.

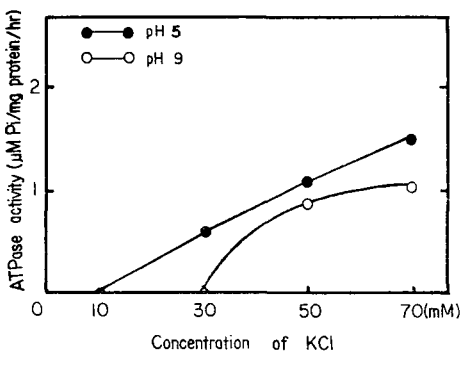
Fig. 7 shows the effect of ATP concentration on the membrane-bound ATPase activities in 13,000~80,000*g* pellet and their Lineweaver-Burk plots for ATP are shown in Fig. 8. At pH 5, the Km's for the enzyme in 13,000~80,000*g* pellet was 2.5 mM and Vmax value was 35.7 μM Pi/mg membrane protein. While, at pH 9, the Km's and Vmax values for the enzyme were 2.5 mM and 55.6 μm Pi/mg membrane protein, respectively.

Effect of monovalent cation. The effects of K^+ concentration on the ATPase activities of the 13,000*g* pellet and 13,000~80,000*g* pellet are shown in Fig. 9 and 10, respectively. As shown in Fig. 9 and 10, the ATPase activity at pH 9 increased sharply with increasing concentration of K^+ up to about 50 mM and then increased more gradually up through 70 mM K^+. On the other hand, the ATPase activity at pH 5 was stimulated in proportion to the increasing of K^+-concentration up to 70 mM (Figs. 9 and 10).

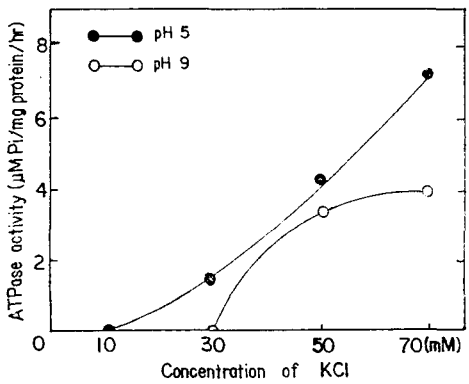
DISCUSSION

The pH profiles for membrane-bound ATPase activity in the 13,000*g* pellet and the 13,000~80,000*g* pellet had two peaks at pH 5 and 9, respectively (Fig. 2). Similar results had also obtained from *Salicornia pacifica* (Kim, 1979) and corn (Leonard and VanDerWoude, 1976). However, it is not known how many kinds of ATPase are in the 13,000*g* pellet and 13,000~80,000*g* pellet.

When the 13,000*g* pellet was centrifuged on the gradient, mitochondria membrane was rich in the interface between 38 and 45% sucrose (Leonard and Hodges, 1973). Furthermore, intact corn mitochondria showed high ATPase activity at pH 9 (Jung and Hanson, 1973). According to the above mentioned results, the ATPase activity at pH 9 in Fraction F of the 13,000*g* pellet (Fig. 3) seemed to be highly correlated with



(Fig. 9)



(Fig. 10)

Fig. 9. Effect of KCl on the activity of membrane-bound ATPase in 13,000*g* pellet.

Fig. 10. Effect of KCl on the activities of membrane-bound ATPase in 13,000~80,000*g* pellet.

mitochondria.

Based on the various reports (Leonard and VanDerWoude, 1976; Ray *et al.*, 1969), the Fraction A of the 13,000~80,000*g* pellet appeared to be associated with solubilized protein of unknown origin. Fraction B of 13,000~80,000*g* pellet was probably associated with endoplasmic reticulum (Leonard *et al.*, 1973; Leonard and VanDerWoude, 1976). Judging from the report by Leonard and VanDerWoude (1976), the ATPase in Fraction C of the 13,000~80,000*g* pellet (Fig. 4) seemed to be caused by Golgi apparatus. The ATPase in Fraction C had greater activity at pH 9 than pH 5 in contrast to the other fractions. This result agreed with that observed for the oat root system (Leonard *et al.*, 1973). The third ATPase of the 13,000~80,000*g* pellet was more active at pH 5 than pH 9, and the membrane had a peak density of 1.17 g/ml (Fig. 4). According to Hodges *et al.*, (1972) and Leonard and VanDerWoude (1976), the majority of the membrane vesicles in the 1.15 to 1.20 g/ml density region was plasma membrane, and these fractions had the greater ATPase activity at pH 6 to 6.5 than pH 9.

Vmax values for membrane-bound ATPases in the 13,000*g* pellet and the 13,000~80,000*g* pellet were, respectively, 8.0 and 35.7 μM Pi/mg membrane protein/hr at pH 5. At pH 9, Vmax values of the above mentioned ATPases were 12.5 and 55.6 μM Pi/mg membrane proteine/hr, respectively. These results were simimilar to those of various plant ATPases (Balke and Hodges, 1975; Erdei *et al.*, 1979; Kuiper and Kuiper, 1979 a, b; Wheeler *et al.*, 1979). The Km's values reported here (Figs. 6 and 8) were within the range of those reported for the various ATPases (Sen and Post, 1964; Opit and Charnock, 1965).

The above results showed that the ATPase in the 13,000*g* pellet and the 13,000~80,000*g* pellet had an equal affinity for substrate, but the activity of the ATPases in the 13,000~80,000*g* pellet was much greater than that in the 13,000*g* pellet.

The membrane-bound ATPase activity was further stimulated by K^+ when Mg^{++} was present in reaction mixture. But the K^+ alone had little effect on the ATPase activity (These data are not present). These results consisted with those of oat roots (Fisher and Hodges, 1969), soybean callus and root cells (Donald and Kennedy, 1977). The effect of K^+ concentration on the ATPase activity was similar to the reports by Kim (1979), and Fisher and Hodges (1969).

摘 要

옥수수 뿌리로부터 분리한 13,000*g* pellet과 13,000~80,000*g* pellet 내에 있는 membrane-bound ATPases의 특성을 究明하였다. 13,000*g* pellet과 13,000~80,000*g* pellet의 membrane-bound ATPases의 최적 pH는 5와 9였다. Discontinuous sucrose gradient centrifugation에 의한 13,000*g* pellet의 분획중 Fraction C는 pH 5에서, Fraction D, E 및 F는 pH 5에서보다 pH 9에서 더높은

활성을 나타냈다. 13,000~80,000*g* pellet의 분획에서 보면, Fraction A, C는 pH 9보다 pH 5에서, Fraction B, D, E 및 F는 pH 5보다 pH 9에서 더 높은 活性을 가졌다. pH 5와 pH 9에서 membrane-bound ATPases의 기질포화 농도는 3~5 mM이며 ATP에 대한 Km 값은 모두 0.25 mM이었다. Vmax 값은 8.0~55.6 μM Pi/mg membrane protein/hr의 범위에 있었다. Membrane-bound ATPase의 활성은 K^+ 이온에 의해 증가되었다.

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