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PURIFICATION AND PROPERTIES OF ADENOSINETRIPHOSPHATASE FROM *ZEA MAYS* SEEDLING MICROSOMES

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Abstract—A simple method was developed for selective solubilization of membrane ATPase from etiolated corn seedlings using 0 01% Triton X100 and 0 01% deoxycholate containing 200 mM KI An 81-fold enriched enzyme preparation, with specific activity of 133 μmol P_i/mg protein/hr, was obtained The enzyme stored in 25 mM Tris-HCl buffer (pH 7 5) at 4° showed rapid loss of activity The enzyme was stabilized by 1 mM EDTA with addition of 1 2 mM Mg^{2+} Mg^{2+} and Ca^{2+} (1 2 mM) increased enzymatic activity by 12 and 10 8% respectively, whereas Na^+ and K^+ brought about a 20% increase in ATP-hydrolysis The effect of combined mono- and di-valent ions was neither synergistic nor additive Ouabain exerted no effect on enzyme activity The enzyme showed two pH optima (6 0 and 7 5) in the presence of Na^+ and K^+, and one optimum at pH 6 5 in the absence of these ions On polyacrylamide gel the enzyme was resolved into two protein bands, both exhibiting ATPase activity It is suggested that the soluble enzyme from the microsomal fraction of corn seedlings contains two ATP-hydrolyzing enzymes, one of them being stimulated by Na^+ and K^+ ions

INTRODUCTION

IT HAS been shown that ATPase (ATP phosphohydrolase, E C 3 6 1 3) associated with the microsomal fraction takes part in coupled transport of sodium and potassium ions in mammalian cells [1] This finding is mainly based on the observation that ATPase is synergistically stimulated by sodium and potassium ions and that the transport of these ions and enzyme activity is inhibited to the same degree by cardiac glycosides Although no such relationships have been found in higher plants, the activity of Na^+, K^+-stimulated ATPase (Na^+, K^+-ATPase) has been demonstrated in various subcellular fractions,[2–4] including purified cell walls [5] Recently Hodges *et al* [6] obtained an ion-stimulated ATPase associated with plasma membranes from oat roots

The aim of the present study was to isolate ATPase from corn seedlings microsomes Assuming that Na^+, K^+-ATPase is bound to lipid structure of the membranes and thus is water insoluble,[7,8] ionic and non-ionic detergents were used for its solubilization

RESULTS AND DISCUSSION

The effect of various concentrations of Triton X100 and deoxycholate on the degree of solubilization of Na^+, K^+-ATPase from corn seedlings microsomes is illustrated in Fig 1 The results indicate that the specific activity is highest on extraction with 0 01% deoxycholate

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(DOC) and 0 01% Triton X100 However the specific activity of the soluble enzyme obtained with 0 01% DOC was 80% greater than with 0 01% Triton X100 During extraction of microsomes with 0 01% DOC the recovery of ATPase activity in soluble and insoluble fractions was about 100% In contrast, total activity found in soluble and insoluble portions after treatment with 0 01% Triton X100 was only 72%

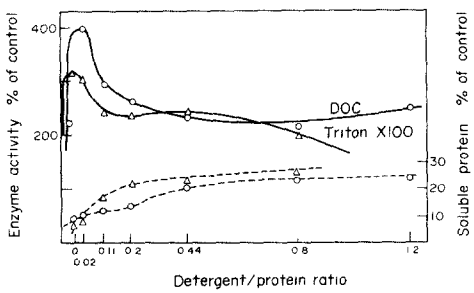

FIG 1 INFLUENCE OF TRITON X100 AND DEOXYCHOLATE-PROTEIN RATIO ON SOLUBILIZATION OF MICROSOMAL ATPase (- - -) AND SOLUBLE PROTEIN (——)

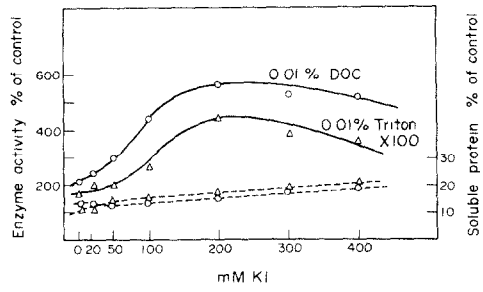

FIG 2 EFFECT OF KI ON SOLUBILIZATION OF TRITON X100 AND DOC TREATED ATPase (- - -) AND SOLUBLE PROTEIN (——)

It has been proved that solubilization of the enzyme by detergents depends on the ratio of detergents to protein [9] Therefore, the optimum ratio of DOC and Triton X100 to protein was determined and found to be 0 01 and 0 02 respectively (Fig 1) In all subsequent experiments protein concentration of the microsomal fraction was set at 4 5 mg/ml, and optima concentrations of DOC and Triton were used

TABLE 1 PURIFICATION OF Na K -ATPase FROM THE MICROSOMES OF *Zea mays* SEEDLINGS

Fraction	Total protein (mg)	Total units*	Sp act (units mg protein)	Yield (%)	Purification factor
Microsomes	748 0	1219	1 63	100	1
Supernatant after treatment with 0 01% Triton X100	71 7	289	4 03	23 7	2 5
Supernatant after treatment with 0 01% DOC + 200 mM KI†	4 8	638	133 0	52 4	81

* 1 unit = 1 μmol Pi/hr

† Precipitate after extraction with Triton X100 was extracted with 0 01% DOC + 200 mM KI

Uesugi *et al* [10] have found that addition of NaI to 4% Lubrol enhanced the solubility of microsomal Na^+, K^+-ATPase from bovine brain In the present experiments, best results were obtained with addition of 200 mM KI to the detergent (Fig 2) Comparison of the results of enzyme extraction with 0 01% Triton X100 and 0 01% DOC containing 200 mM KI, showed only slight differences in the amount of protein solubilized (Figs 1 and 2) On the other hand, the specific activity of ATPase liberated with DOC was 65% higher, as compared with Triton X100 According to preliminary studies Triton X100 mainly extracts Mg^{2+}-dependent ATPase, and deoxycholate—the Na^+ K^+ stimulated enzyme

[9] SWANSON P D BRADFORD H F and MCILWAIN H (1964) *Biochem J* **92**, 235

[10] UESUGI S KAHLENBERG A MEDZIHRADSKY F and HOKIN L E (1969) *Arch Biochem Biophys* **130** 253

Combined use of both detergents achieved a selective solubilization of ATPase from corn seedlings As shown by the results presented in Table 1, initial extraction of microsomes with 0 01% Triton X100 and re-extraction of the residue with 0 01% DOC containing 200 mM KI liberates the enzyme exhibiting specific activity of 133 μmol Pi/mg protein/hr, this represents 81-fold purification and 60% yield Precipitation with ammonium sulphate inactivated the enzyme

Since the enzyme lost 50% activity during 2 week's storage at 4° in Tris-HCl buffer (pH 7 6), the effect of various stabilizers used for animal ATPase[11,12] was studied According to the results reported in Fig 3, an addition of 20% glycerin or of 200 mM NaCl plus 100 mM KCl (salt concentrations at which ATPase showed optimum activity) failed to stabilize the enzyme, as opposed to 1 mM EDTA plus 1 2 mM Mg^{2+} which exerted a pronounced stabilizing effect The enzyme stored in this medium for 312 hr at 4° lost only 15% of its initial activity

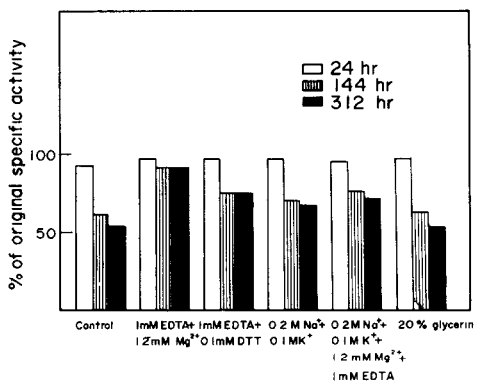

FIG 3 EFFECT OF DIFFERENT LIGANDS ON THE STABILITY OF THE SOLUBLE ATPase

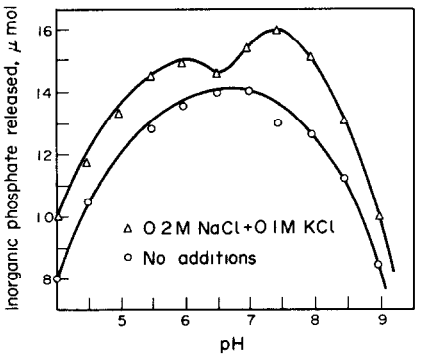

FIG 4 EFFECT OF pH ON SOLUBLE ATPase ACTIVITY

Preliminary investigations on the effect of some monovalent and divalent ions and pH on the kinetics of ATPase were performed Mg^{2+} and Ca^{2+} (1 2 mM) activated the enzyme by 12 and 10 8% respectively and activity fell to 75% at 10 mM Mg^{2+} and to 68% at 10 mM Ca^{2+} Na^+ (200 mM NaCl) and K^+ (100 mM KCl) brought about a 20% increase in activity If the sum of these ions was maintained at 100 mM and the ratio of Na^+ to K^+ was varied, there was no significant change in ATPase activity indicating the absence of synergistic effects of Na^+ and K^+

Ouabain, in concentrations of 0 1–1 mM, failed to exert any effect on the rate of ATP hydrolysis both in the presence and absence of ions These results differentiate plant ATPase from the enzyme of animal origin

In the presence of ions the enzyme preparation showed two pH optima (6 0 and 7 5) and in the absence of ions one optimum (pH 6 5) (Fig 4) On polyacrylamide gel, it was resolved into two protein bands with ATPase activity These findings suggest that the preparation comprised two ATP-hydrolyzing enzymes, one of them being Na^+ K^+-ATPase, namely, upon incubation of gel in ion-free medium, one of the bands exhibited very slight activity, which increased, however, in the presence of monovalent ions

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EXPERIMENTAL

Plant material As plant material shoots of 4-day-old etiolated seedlings of corn *Zea mays* cv Vigor were used All reagents were of *pro analysis* grade

Extraction and solubilization of membrane ATPase Plant material was homogenized in ratio of 1 5 (w v) in 50 mM Tris-imidazole buffer containing 3 mM EDTA 1 mM $MgCl_2$ and 0 25 M sucrose pH 7 5 The homogenate was pressed through two layers of cloth and the filtrate was kept for further use The residue was homogenized another 2 × The three filtrates were combined and centrifuged at 1600 *g* for 10 min The ppt was discarded and the supernatant was centrifuged at 10 000 *g* for 15 min After discarding the ppt the supernatant was centrifuged at 44 000 *g* for 50 min The ppt containing the microsomal fraction was suspended in 25 mM Tris-HCl buffer with addition of 2 mM EDTA pH 7 5 Specific activity of microsomal ATPase usually varied within the range of 1 3–1 65 μmol Pi/mg protein, hr To the microsomal suspension (4 5 mg protein ml) in 25 mM Tris-HCl buffer (pH 7 5) containing 2 mM EDTA Triton X100 (final concn 0 01%) was added The sample was shaken at 0 4 for 10 min and centrifuged at 165 000 *g* for 30 min The supernatant was discarded The ppt was washed (2 ×) with 25 mM Tris-HCl buffer (pH 7 5) and after centrifugation at 165 000 *g* for 30 min suspended in this buffer with addition of DOC (final concentration 0 01%) and 200 mM KI The resulting microsomal suspension was incubated at 40 for 10 min and then centrifuged at 165 000 *g* for 30 min The supernatant containing soluble enzyme, was dialysed for 24 hr against 25 mM Tris-HCl buffer pH 7 5

Protein determination Protein was determined by the modified biuret method according to Gornall *et al*[13] and by the procedure of Lowry *et al*[14]

Determination of ATPase activity For enzyme activity estimations the enzyme preparation was diluted and adjusted with detergent or K^+ in this way that detergent concentration was always 0 1% and of K^+ was 50 mM in the final incubation mixture Amount of the enzyme in each incubation mixture was always the same To 1 ml microsomal extract 1 ml incubation mixture made up of 25 mM Tris-HCl buffer 2 mM ATP (disodium salt) 2 mM EDTA, 100 mM NaCl pH 7 5 and if necessary 50 mM KCl was added In some cases ion concentration was modified or other ions were used Samples were incubated at 30 for 30 min The enzyme was inactivated with 0 5 ml of cold 1 2 M $HClO_4$ and phosphorus was determined by the method of Fiske-Subbarow[4] When Triton X100 interfered with the determination of Pi by the Fiske method (in concentrations over 0 01%) the modified method of Post and Sen[15] was applied

Disc gel electrophoretic separation and location of ATPase Disc electrophoresis was performed according to Davis[16] with development in 7 0% gel at pH 8 5 Samples (50–100 μl) of the microsomal extracts (previously concentrated 10- to 20-fold in dialysing bags) were applied to the gel After electrophoresis the gels were stained either for protein with Amido black or for ATPase activity by submerging the gels into a solution containing 100 mM Tris-HCl buffer, 5 mM ATP 50 mM $CaCl_2$ 100 mM KCl and 200 mM NaCl at pH 8

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