Adenosine Triphosphatase from Soybean Callus and Root Cells

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ABSTRACT

The ATPase activity of a membrane fraction from soybean (Glycine max L.) root and callus cells, presumed to be enriched in plasma membrane, has been characterized with respect to ion stimulation, pH requirement, and nucleotide specificity. The enzyme from both sources was activated by divalent cations (Mg²⁺ > Mn²⁺ > Zn²⁺ > Ca²⁺ > Sr²⁺) and further stimulated by monovalent salts. Preparations from root cells were stimulated by monovalent ions according to the sequence: K⁺ > Rb⁺ > Choline⁺ > Na⁺ > Li⁺ > NH₄⁺ > Cs⁺ > tris⁺. Membrane preparations from callus cells showed similar stimulatory patterns except for a slight preference for Na⁺ over K⁺. No synergism between K⁺ and Na⁺ was found with preparations from either cell source.

The pH optimum for ATP hydrolysis in the presence of 50 mM KCl and 3 mM MgSO₄ was 6.5 for both preparations and slightly higher in the presence of 3 mM MgSO₄ alone. The order of nucleotide preference was found to be: ATP > ADP > GTP > CTP > UTP. Maximal glucan synthetase activity at high (1 mM), but not at low (1 μM), substrate was found to be coincident with the position of this fraction on the sucrose gradient.

Procedures have recently been developed for the isolation of plasma membrane from higher plant cells utilizing differential and isopycnic centrifugation (6). These plasma membrane preparations contain a membrane-associated adenosine triphosphatase (ATPase) which is activated by divalent ions and further stimulated by monovalent ions. The majority of the research to date has been performed on the cereals such as oats (6, 9), barley (13), and corn (8, 10).

To our knowledge, the only report of plasma membrane isolation and its associated ATPase from a dicotyledonous plant is that of Robertson et al. (15). They reported a relatively small ATPase stimulation by K⁺ at pH 6.5 but did not investigate optimal ionic concentrations or the influence of other ions such as H⁺, Na⁺, or Ca²⁺.

We report the existence of a similar ATPase activity associated with a membrane fraction from soybean, presumed to be enriched in plasma membrane. Although the isolation of plasma membrane from soybean has been reported (5), its associated ATPase(s) has not been previously characterized.

We found considerable ATPase stimulation by K⁺ in soybean membrane preparations and have also established the optimal ion concentration, pH, and nucleotide specificity for ATPase activity.

Preliminary work had indicated an alteration in the ratio of K⁺ to Na⁺ content in callus cells versus similar cells in tissue. We, therefore, investigated possible differences in ATPase ion stimulation in preparations from soybean callus and root tissue.

MATERIALS AND METHODS

Plant Material. Soybean seeds (Glycine Max L.) were germinated between moist layers of absorbent paper at room temperature after surface sterilization with a diluted commercial Clorox solution. Roots used for membrane preparation were excised from 5- to 7-day-old seedlings and washed three times with cold distilled H₂O before use.

Callus, originally prepared from soybean hypocotyl tissue, was cultured on semisolid agar containing modified Murashige and Skoog medium (M and SS(V) (17). The cultures were grown in a chamber at 28 ± 1°C under continuous illumination (approximately 7000 lux) provided by a mixture of Growlux WS and cool white lamps. At least 1 month prior to use, the callus was transferred to a dark chamber at room temperature to lower cell Chl content.

Preparation of Membrane Fractions. Membrane fractions were obtained by the method of Hodges and Leonard (6). All manipulations during membrane isolation were carried out at 0 to 4°C. The excised roots were homogenized in a mortar and pestle in a medium containing 0.25 mM sucrose, 3 mM tris-EDTA, 25 mM tris-MES (pH 7.2), and 3 mM dithioerythritol. The resulting brei was strained through four layers of cheesecloth and centrifuged at 13,000g for 15 min. The resulting supernatant was centrifuged for 30 min at 29,000 rpm (80,000g) in a Spinco 30 rotor. The pellets were resuspended in the homogenizing medium, combined, and again centrifuged at 29,000 rpm for 30 min. This pellet (microsomes) was suspended in 18% (w/w) sucrose and layered above either a continuous or discontinuous sucrose gradient in 1 M tris-MES (pH 7.2). The continuous and discontinuous gradients were centrifuged 2 and 3 hr, respectively, at 26,000 rpm (95,000g) in a Spinco SW 27 rotor. Membrane fractions were removed from discontinuous gradients with a Pasteur pipette and from continuous gradients by dropwise elution using an ISCO model 184 gradient fractionator equipped with an ISCO UA-5 absorbance monitor.

Membrane isolation from callus cells was identical to that of root cells except for the homogenizing procedure. After filtering the brei, the material retained on the cheesecloth was rehomogenized in a small aliquot of homogenizing medium, filtered, and combined with the first filtrate.

Enzyme Determinations. Enzyme assays utilizing phosphate substrates were carried out at 38°C in a total volume of 1 ml containing MgSO₄ (nominally 3 mM), KCl (nominally 50 mM), 3 mM phosphatase substrate, and 33 mM tris-MES buffer at the appropriate pH. Before use, ATP was converted to the tris form by Dowex 50-W ion exchange chromatography (6); other nucleotides were purchased in the tris form (Sigma). The Pi released was determined by the Fiske and Subbarow procedure (3). NADH-Cyt c reductase was assayed at room temperature by following the reduction of Cyt c at 550 nm (6). Succinate-INT reductase (succinate: 2(p-i odophenyl)-3-(p-nitrophenol)-5 phenyltetrazolium chloride oxidoreductase) assays were carried out at 38°C by the method of Pennington (14).

Latent IDPase activity in membrane fractions was determined by assaying for Pi released from tris-IDP in 3 mM MgSO₄, 50 mM KCl, and 33 mM tris-MES at pH 7.5 (11). Membrane fractions were incubated at 4°C for 4 days prior to assay.

Glucan synthetase was assayed by a modified method of Van Der Woude et al. (16). The modification enabled incorporated
**RESULTS**

**Separation of Membranes from Soybean Root and Callus Cells.** Callus and root preparations gave very similar enzyme distributions and protein profiles on linear sucrose gradients. Both preparations gave a broad peak of ATPase activity at pH 9 and two peaks of ATPase activity at pH 6.5 (Fig. 1A). The pH 6.5 ATPase activity peaks at 25% (w/w) sucrose and in the 31 to 35% sucrose region. This latter peak of activity at pH 6.5 appears to consist of two overlapping peaks (Fig. 1A). Increasing centrifugation times to 16 hr produced virtually the same ATPase distribution.

NADH-Cyt c reductase exhibited a peak of activity at approximately 25% sucrose (Fig. 1B). A high ratio of absorbance at 254/280 nm (280 nm data not shown) was also found at this position on the gradient. Succinate-INT reductase and malate dehydrogenase activities were highest at sucrose densities >39% (Fig. 1B). A 25% increase in IDPase activity of the 25% sucrose region was found after 4 days at 4 C, over activity at the time of isolation.

**ATPase Activity in the 30 to 38% Sucrose Region.** Because maximal K+ stimulation of ATPase activity at pH 6.5 was found at 30 to 38% sucrose (Table I), this portion of the gradient was further investigated as a possible location of plasma membrane. Closer examination of this region showed a 6-fold increase in specific activity of ATP hydrolysis, over that of the crude root homogenate, and a decrease in specific activity of other nucleotide hydrolysis (Table II). A decrease was also found in the specific activity of hydrolysis of β-glycerol phosphate (4) and p-nitrophenylphosphate at pH 5.5 and 9.5 (6). The over-all pattern of nucleotide specificity was found to be similar for callus and root preparations (Table II).

**Optimal pH for ATPase Activity.** The pH optima for ATPase activity in the presence of 3 mM Mg++ was 7.5 for root and 7 for callus isolates. Adding 50 mM KCl shifted the optimal pH to 6.5 for isolates from both sources (Fig. 2).

**Ion Requirement of the pH 6.5 ATPase.** Magnesium was the preferred divalent cation and had an optimum concentration of 3 mM (Fig. 3 and Table III). For monovalent ions, a wide range of stimulation was found with K+ salts (Table IV). Surprisingly, a number of K+ salts which had been found to stimulate Avena plasma membrane ATPase (9) such as potassium acid phthalate, gave only a slight stimulation with our preparations. In the absence of Mg++, we found very little stimulation of ATPase activity by KCl (not shown).

For both root and callus preparations, there appears to be no synergism of ATPase stimulation by Na+ and K+ (Fig. 4). Callus preparations consistently exhibited a slightly greater stimulation with Na+ than with K+, while the reverse was true for root isolates (Fig. 4).

**Glucon Synthetase Activity.** Maximal glucon synthetase with 1.5 µM UDP-glucose as the substrate was found at the 25 to 30% sucrose interface; when the substrate was raised to 1 mM, maximal activity was found in the 30 to 34% region of the gradient (Table I). At low substrate, this enzyme is supposedly a marker for Golgi apparatus and at higher substrate levels a marker for plasma membrane (16). Glucan synthetase at high substrate and the pH 6.5 ATPase are the only two enzymes we have found, thus far, to be enriched by this isolation procedure in the 30 to 34% sucrose region, over their activity in the crude homogenate.

**DISCUSSION**

Due to the location of maximal K+ stimulation of ATPase activity and high substrate glucon synthetase activity as well as the similar coenrichment of these enzymes, we speculate that the 30 to 35% sucrose region is the location of the plasma membrane in these isolates. This is somewhat surprising since in both
 contained tris-MES mM. The 30 found to other dicots by activated and cereal resembling in increase Mg²⁺ were 3 266. FIG. 3. Influence of pH and K⁺ on ATPase activity in callus and root preparations taken from the 30 to 38% sucrose interface. Data labeled Mg²⁺ were obtained in the presence of 3 mM Mg²⁺, 33 mM tris-MES, and 3 mM tris-ATP at the particular pH. Data labeled KCl represent the increase in activity caused by the addition of 50 mM KCl to the reaction mixture.

FIG. 2. Effect of Mg²⁺ and Ca²⁺ on ATPase activity in membranes at the 30 to 45% interface from soybean callus tissue. The reaction mixture contained tris-MES buffer at 33 mM (pH 6.5) and tris-ATP (pH 6.5) at 3 mM.

animal and cereal preparations, the plasma membrane is found closer to 38% sucrose (6, 7, 16). However, Hardin et al. (5) found similar results for plasma membrane from soybean hypocotyl.

The ATPase activity associated with this membrane fraction is activated by Mg²⁺ and further stimulated by K⁺ in a manner resembling the ATPase activity in cereal preparations (1, 8) and other dicots (15); however, with our preparations, Ca²⁺ acts as

FIG. 3. Effect of Mg²⁺ and Ca²⁺ on ATPase activity in membranes at the 30 to 45% interface from soybean callus tissue. The reaction mixture contained tris-MES buffer at 33 mM (pH 6.5) and tris-ATP (pH 6.5) at 3 mM.

FIG. 4. Effect of various mixtures of Na⁺ and K⁺ on ATPase activity in preparations from callus and root tissue. In addition to NaCl and/or KCl, the reaction mixture contained 3 mM tris-ATP (pH 6.5), 3 mM MgSO₄, and 33 mM tris-MES (pH 6.5).

Table II. Nucleotide Specificity of Soybean Root and Callus Fractions.
The ability of cellular fractions to liberate Pi from the tri- salt of various substrates 3 mM was determined at 38 C and pH 6.5. Each reaction mixture contained 3 mM MgSO₄ and 33 mM tris-MES. Specific activity is presented in moles Pi released/my protein-hr⁻¹. Numbers in parenthesis are relative activities.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Homogenate</th>
<th>Microsomes</th>
<th>30 to 38% Zone</th>
<th>Microsomes</th>
<th>30 to 45% Zone</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>4.6 (100)</td>
<td>11.0 (100)</td>
<td>26.9 (100)</td>
<td>10.5 (100)</td>
<td>28.6 (100)</td>
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<tr>
<td>UTP</td>
<td>4.3 (93)</td>
<td>3.2 (29)</td>
<td>0.9 (3.5)</td>
<td>1.8 (17)</td>
<td>1.0 (4)</td>
</tr>
<tr>
<td>GTP</td>
<td>2.8 (60)</td>
<td>4.9 (45)</td>
<td>1.4 (5.3)</td>
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<td>---</td>
</tr>
<tr>
<td>CTP</td>
<td>0.6 (13)</td>
<td>2.5 (23)</td>
<td>1.3 (5)</td>
<td>2.9 (28)</td>
<td>0.7 (2)</td>
</tr>
<tr>
<td>ADP</td>
<td>4.9 (106)</td>
<td>2.9 (26)</td>
<td>2.4 (8.8)</td>
<td>---</td>
<td>0.6 (2.4)</td>
</tr>
</tbody>
</table>

Table III. Relative ATPase Activity in the Presence of Various Divalent Ions. Membranes taken from the 30 to 38% interface of a discontinuous sucrose gradient. Each divalent cation (3 mM) was added to the assay mixture as the chloride. Potassium, when added, was present as 50 mM KCl.

<table>
<thead>
<tr>
<th>Divalent Ion</th>
<th>ROOTS</th>
<th>CALLUS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mg</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Mn</td>
<td>85</td>
<td>89</td>
</tr>
<tr>
<td>Zn</td>
<td>41</td>
<td>42</td>
</tr>
<tr>
<td>Ca</td>
<td>37</td>
<td>34</td>
</tr>
<tr>
<td>Sr</td>
<td>34</td>
<td>31</td>
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</tbody>
</table>

Table IV. Monovalent Ion Stimulation of ATPase Activity from Root Preparations. Relative ATPase activity of the 30 to 38% interface was measured in the presence of 3 mM MgSO₄, 3 mM tris ATP and 33 mM tris-MES (both pH 6.5) plus 50 mM of the respective K⁺ or Cl⁻ salt. Kβ50 = potassium benenate sulfonate; KHP = potassium hydrogen phthalate; KRO = potassium acetate.

<table>
<thead>
<tr>
<th>Monovalent Ion</th>
<th>ROOTS</th>
<th>CALLUS</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCl</td>
<td>100%</td>
<td>73%</td>
</tr>
<tr>
<td>KNO₃</td>
<td>100%</td>
<td>71%</td>
</tr>
<tr>
<td>KNO₂</td>
<td>100%</td>
<td>68%</td>
</tr>
<tr>
<td>KC₆H₄O₄</td>
<td>100%</td>
<td>79%</td>
</tr>
<tr>
<td>KCI</td>
<td>100%</td>
<td>72%</td>
</tr>
<tr>
<td>KCl</td>
<td>100%</td>
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<tr>
<td>KCl</td>
<td>100%</td>
<td>67%</td>
</tr>
</tbody>
</table>
an enzyme activator, (Fig. 3) whereas in *Avena* and *Zea* plasma membrane preparations it acts as an enzyme inhibitor (ref. 1 and R. T. Leonard, unpublished).

Numerous ATPases have been shown to occur in higher plant cells (11). Further work will therefore be needed to determine the function of this ATPase; we speculate that the enzyme from soybean tissues may be involved in transmembrane fluxes of monovalent ions as has been suggested for the plasma membranes ATPase preparations from the cereals (2). A problem in evaluating such a hypothesis with our preparations is that the exact monovalent ion stimulation pattern seems as yet unclear.

Finally, we find the ATPase activities from soybean root and callus cells to be similar. It seems doubtful that the slight difference in ATPase stimulation by K+ and Na+ could, in itself, explain any differences in cellular K+ and Na+ content.

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**LITERATURE CITED**


