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THE PREPARATION AND PROPERTIES OF AN ISOLATED PLANT MEMBRANE FRACTION ENRICHED IN (Na^+-K^+)-STIMULATED ATPase

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SUMMARY

Membrane fragments were isolated from a homogenate of *Phaseolus vulgaris* cotyledon tissue by a centrifugation scheme designed to yield purified plasma membrane. The membrane fraction was enriched in basal ATPase (EC 3.6.1.3) activity by 3–6-fold relative to homogenate on a specific activity basis and in (Na^+-K^+)-stimulated ATPase activity by 6–9-fold. Glucose-6-phosphatase (EC 3.1.3.9) and 5'-nucleotidase (EC 3.1.3.5), markers for microsomal membranes in this tissue, as well as the mitochondrial enzyme succinate dehydrogenase (EC 1.3.99.1) were relatively absent. By electron microscopy it was apparent that the fraction was a clean preparation of membranes with primarily vesicular conformations. The increased specific activity of the basal enzyme and in particular that of the (Na^+-K^+)-stimulated ATPase, together with the relative absence of microsomal and mitochondrial membranes, suggest that the preparation is partially purified plasma membrane.

INTRODUCTION

ATPase (EC 3.6.1.3) activity has been found in a variety of subcellular fractions from plant tissue including purified cell wall, mitochondrial and soluble fractions[1,2]. Its presence has not, however, been demonstrated in isolated preparations of plant plasma membrane. HALL[3] quite recently illustrated by enzyme histochemistry that ATP hydrolysis occurs on the plasma membrane of maize root cells, but there was evidence of ATPase activity in the cytoplasm as well indicating that the enzyme is not exclusively associated with the cell membrane. Given that ATPase may be a feature of at least some plant plasma membranes, it seems reasonable that such an enzyme could be characterized by a specific cation response. Cation sensitivities for particulate plant ATPases have been reported[1,4,5]. For example, FISHER AND HODGES[1] found that the ATP hydrolyzing activities of particulate fractions from *Avena sativa* roots were activated by added Mg^{2+} and further stimulated by K^+ in the presence of Mg^{2+}. Moreover, the largest proportion of the total K^+-stimulated activity was present in the microsomal fraction. It is known that plasma membrane forms vesicles of predominantly microsomal size during relatively severe tissue homogenization[6]. Thus, such a response to K^+ may be due to the presence of plasma membrane fragments and their associated ion-sensitive ATPase activity in the microsomal fraction.

In this study, membrane fragments possessing cation-sensitive ATPase activity were isolated from a homogenate of *Phaseolus vulgaris* cotyledon tissue. Isolation was accomplished by using a modification of the method originally developed by NEVILLE[7] for obtaining purified plasma membrane from rat liver tissue. The present communication describes the preparation and properties of this membrane fraction.

MATERIALS AND METHODS

Glucose 6-phosphate, ATP (Tris salt), iodonitrotetrazolium violet and AMP were obtained from Sigma Chemical Co. OsO_4 was from British Drug Houses Ltd. and all other chemicals and solvents were Fisher reagent grade.

Growth conditions

Seeds of *Phaseolus vulgaris* were germinated and grown in moist vermiculite in the dark at 29° and the cotyledons harvested after 4 days. The seeds were not soaked prior to planting.

Isolation of the membrane fraction

Membrane fragments were isolated from the cotyledon tissue essentially by the method of NEVILLE[7], which was originally developed for preparing purified plasma membrane from rat liver. Some modifications were introduced to accommodate the plant tissue. All preparative procedures were carried out at 5°. For each preparation 70–90 g of cotyledons were cut up finely with scissors and homogenized with a mortar and pestle in 0.05 M $NaHCO_3$ (pH 7.5) (3 ml/g of tissue). The homogenate was strained through four layers of fine-mesh cheese cloth, diluted to 500 ml with $NaHCO_3$ solution, stirred thoroughly and filtered again through four layers of fine-mesh cheese cloth. The filtrate was centrifuged at 1500 × *g* for 10 min and a two-layered pellet consisting of a light brown fluffy layer overlying a tightly packed layer of starch was obtained. The supernatant was syringed off and the fluffy layer only resuspended in 30 ml of $NaHCO_3$ solution. This suspension was centrifuged at 1220 × *g* for 10 min giving rise to a pellet of predominantly fluffy material. The procedures of resuspension and centrifugation were repeated until a homogeneous pellet of fluffy material with no visible trace of starch layer was obtained. The pellet was then resuspended in 30 ml of $NaHCO_3$ solution and incubated with stirring for a further 4 h in order to lyse the membranous vesicles and release any trapped protein. At the end of this period a final pellet was obtained by centrifugation at 1220 × *g* for 10 min.

Further purification was achieved by floatation centrifugation through a discontinuous sucrose gradient in a Spinco SW-25.1 rotor. The final pellet from the previous procedure was resuspended in 7 ml of 5 mM $NaHCO_3$ (pH 7.5) and designated payload (input of partially purified membranes to the sucrose gradient). The bottom layer of the gradient was prepared by mixing 5 ml of payload with 9.5 ml of sucrose, density 1.34, and the gradient was completed by layering 15 ml of sucrose, density 1.16, over the bottom portion. Both solutions of sucrose were previously adjusted to pH 7.5 with $NaHCO_3$. The remaining payload was retained for chemical and biochemical determinations. After centrifugation for 2 h at 58750 × *g* a distinct layer was present at the interface and there was a pellet at the bottom of the tube. The layer was the purified membrane fraction and was collected with a syringe, centrifuged, resuspended in 5 mM $NaHCO_3$ (pH 7.5) and retained for subsequent assays.

All supernatants and residual pellets arising from the preparation were retained for enzyme recovery determinations.

Enzyme assays and protein determinations

Homogenates and fractions were assayed for ATPase (EC 3.6.1.3)[1], 5′-nucleotidase (EC 3.1.3.5)[8], glucose-6-phosphatase (EC 3.1.3.9)[9] and succinate dehydrogenase (EC 1.3.99.1)[10].

For the ATPase assays, particulate fractions were washed in 1 mM EDTA (pH 7.5, with Tris) by resuspension and centrifugation. The homogenates and supernatant fractions were simply diluted with EDTA solution to make a final concentration of 1 mM. The basic reaction mixture consisted of 20 mM Tris (pH 8.0), 3 mM ATP (Tris salt) and enzyme. When required, Mg^{2+}, K^+ and Na^+ were added as their chloride salts at final concentrations of 2, 40 and 40 mM, respectively. Washing the particulate fractions in EDTA solution ensured removal of essentially all residual Na^+ that might have been carried over from the $NaHCO_3$ homogenizing medium. For homogenates and supernatant fractions the dilutions inherent in the assay technique rendered the $NaHCO_3$ concentrations in the final reaction mixtures insignificant in terms of any contribution to the reaction. This was born out experimentally by comparing the basal ATPase levels of a homogenate prepared in 3 mM EDTA (pH 7.5) and of one prepared in the routinely used $NaHCO_3$ buffer. Both homogenates were prepared from the same batch of tissue and their specific activities were not significantly different. For determinations of ATPase pH profiles, the reaction mixtures were adjusted to the required pH values.

Protein determinations were routinely carried out by the method of LOWRY *et al.*[11].

Microscopy

Samples of freshly prepared membrane fractions were fixed in glutaraldehyde (3 % in 0.05 M phosphate buffer (pH 7.4)), post-fixed in OsO_4 (1 % in 0.05 M phosphate buffer (pH 7.4)) dehydrated in acetone and embedded in vestopal[12]. Thin sections were post-stained with uranyl acetate (1 % aqueous solution) and lead citrate (0.1 % in 0.1 M NaOH) prior to examination by electron microscopy.

RESULTS

Enzymatic properties of the purified membrane fraction

Quite substantial levels of ATPase activity were found in the purified membrane fraction and initially experiments were carried out to determine the effects of added cations on this activity. The results of two representative experiments for membrane fragments isolated from 4-day-old tissue are set out in Table I. Mg^{2+} augmented the basal activity by 50–70 % whereas each of Na^+ and K^+ caused about a 2-fold increase in total ATP hydrolysis. The effects of the monovalent and divalent ions in combination were neither additive nor synergistic. Consistently, the addition of Na^+ and K^+ together gave the highest stimulation and hence this combination was used routinely in establishing the enrichment of the capacity for cation-sensitive ATP hydrolysis in the purified membrane fraction. The pH optimum for total activity measured in the presence of Na^+ and K^+ was 8.0 for the purified fraction. Accordingly, all ATPase assays were routinely carried out at pH 8.0.

TABLE I

THE EFFECTS OF VARIOUS CATIONS ON THE ATPASE ACTIVITY OF A PURIFIED MEMBRANE FRACTION FROM 4-DAY-OLD COTYLEDONS

Activities are expressed as mg phosphorus per mg protein per h. A and B are separate experiments.

Additives to the basic reaction mixture	*A*	*B*
None	12.4	7.4
Mg^{2+}	18.3	12.3
K^+	22.0	18.7
Na^+	28.4	16.5
$Mg^{2+} + K^+$	19.9	20.2
$Mg^{2+} + Na^+$	17.4	15.8
$Na^+ + K^+$	29.1	20.5
$Mg^{2+} + Na^+ + K^+$	23.6	16.1

TABLE II

RELATIVE ENRICHMENTS OF ATPASE ACTIVITY IN A PURIFIED MEMBRANE FRACTION FROM 4-DAY-OLD COTYLEDONS

Enrichments are expressed as ratios of the specific activity in the purified membrane fraction to that of the corresponding homogenate. Basal activities were subtracted before calculating the ratios for (Na^+-K^+)stimulation.

Expt.	*Basal ATPase*	*(Na^+-K^+)stimulation of basal ATPase*
A	2.9	6
B	5.8	8.8
C	4.2	7.6

The purified membrane fraction was enriched in basal ATPase activity by 3–6-fold relative to homogenate on a specific activity basis (Table II). On the other hand enrichments of net (Na^+-K^+)-stimulated activity ranged from 6- to 9-fold on the same basis (Table II). Values for (Na^+-K^+)stimulation were determined by subtracting basal activities from the total activity measured in the presence of the ions and for individual experiments the enrichment of the cation-sensitive ATPase in the purified preparation was consistently greater than that of the basal activity (Table II).

Levels of contamination in the isolated fraction were determined by measuring succinate dehydrogenase and glucose-6-phosphatase, markers for mitochondrial and endoplasmic reticulum membranes, respectively. In addition, since 5′-nucleotidase has been shown to be a marker for plasma membrane in several mammalian tissues[6, 13, 14], it too was monitored in these studies. All three enzymes were essentially not detectable in the purified membrane fraction (Table III).

Recoveries of enzyme activities in the fractions arising from the preparative procedure ranged from 70 to 94 % of homogenate activity for ATPase, from 70 to 97 % for 5′-nucleotidase and from 79 to 85 % for glucose-6-phosphatase. Recoveries for succinate dehydrogenase were low (50–60 %). However, when homogenates prepared in the same manner were fractionated as described previously[15] by a more conventional centrifugation scheme yielding nuclear, mitochondrial, microsomal and soluble frac-

TABLE III

5′-NUCLEOTIDASE, GLUCOSE-6-PHOSPHATASE AND SUCCINATE DEHYDROGENASE ACTIVITIES OF HOMOGENATES AND PURIFIED MEMBRANE FRACTIONS FROM 4-DAY-OLD COTYLEDONS

Phosphatase activities are expressed as μg phosphorus per mg protein per h and succinate dehydrogenase as nmoles reduced iodonitrotetrazolium chloride per mg protein per h. Payload = input of partially purified membranes to the sucrose gradient. T = trace activity and corresponds to a net absorbance of 0.005 or less. n.d. = not detectable.

Enzyme	*Expt.*	*Homogenate*	*Payload*	*Purified membrane fraction*
5′-Nucleotidase	A	10.6	1.4	n.d.
	B	16.2	16.6	T
Glucose-6-phosphatase	A	23.2	n.d.	n.d.
	B	13.0	n.d.	n.d.
Succinate dehydrogenase	A	17.6	n.d.	n.d.
	B	18.8	T	n.d.

tions, recoveries of 62–92 % for succinate dehydrogenase were obtained. It would seem likely, therefore, that in the fractionation used to isolate the membrane fragments, low recoveries are attributable to the large dilution of the supernatant fractions, which contain the major proportions of this enzyme.

Electron microscopy

A representative electron micrograph of the purified membrane preparation is shown in Fig. 1. It is evident that the fraction is a clean preparation of membranous vesicles. There is no evidence of recognizable contamination by cytoplasmic organelles.

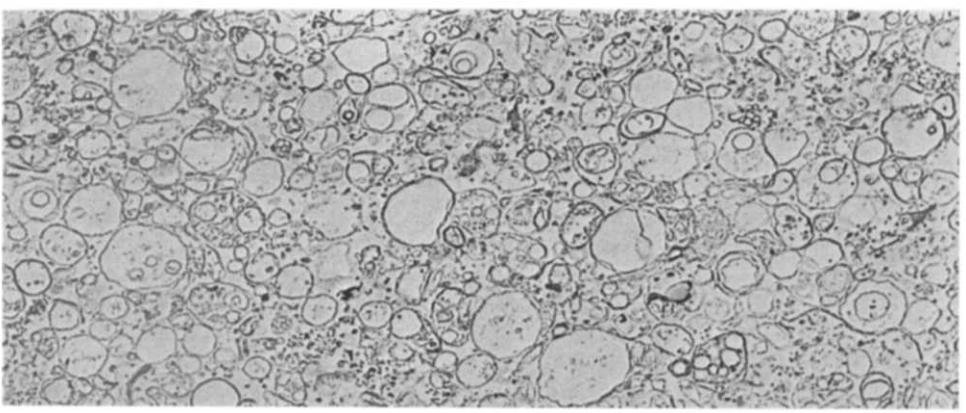

Fig. 1. Electron micrograph of a purified membrane fraction from 4-day-old cotyledons of *Phaseolus vulgaris*. X 9372.

DISCUSSION

It has been quite clearly established for a variety of mammalian cells that ATPase is associated with the plasma membrane[16,17]. Moreover a (Na^+-K^+)stimulation of

this activity has been shown to be integrally involved in the active transport of these ions across the plasma membrane[17]. For plant tissues there are indications from enzyme histochemical studies that ATP hydrolysis occurs at the surface membrane[3], but its significance with respect to active ion transport across the plasmalemma is not yet clearly resolved. FISHER AND HODGES[1] have reported that a membranous ATPase from maize root shows stimulation in the presence of Mg^{2+} and K^+ of sufficient magnitude to account for active transport of K^+, but the native subcellular localization of the enzyme was not determined. In this study, membrane fragments isolated by a procedure designed to yield purified plasma membrane were found to possess (Na^+-K^+)-stimulated ATPase activity.

Since the plasma membrane of cells in this tissue does not possess a distinguishing morphological feature by which it can be identified in the isolated state, it was not possible to assess the relative abundance of surface membrane in the isolated fraction by electron microscopy. However, the enrichment of ATPase in the fraction and in particular the increased enrichment of the net (Na^+-K^+)-stimulated activity (Table II), a property one would expect to be associated with the cell surface, indicate that it is a preparation of purified plasma membrane. It has, in fact, been previously shown for rat liver that purified surface membrane is 2.5-fold more enriched in (Na^+-K^+)-stimulated ATPase than in the less specific Mg^{2+}-stimulated ATPase[16]. Moreover, the enrichment of 6–9-fold obtained for the cation-stimulated ATPase in this study is comparable in magnitude to that reported for 5′-nucleotidase, an enzyme marker for mammalian plasma membrane, in fractions isolated from guinea pig tissue by the same method[14].

The procedure of NEVILLE[7] for isolating plasma membrane from mammalian tissue was later modified by EMMELOT *et al.*[18] in order to decrease mitochondrial contamination. The modification entailed adding three more sucrose layers to the density gradient. This modified gradient was not routinely used in this investigation because it was found that the added layers of sucrose decreased the yield of membrane and did not enhance the enrichment of ATPase in the final fraction. Any contribution to the fraction by endoplasmic reticulum or fragments of mitochondrial membrane is minimal judging by the relative absence from the preparation of marker enzymes for these potential contaminants. Succinate dehydrogenase is a well established marker for mitochondrial membrane and both glucose-6-phosphatase and 5′-nucleotidase have been previously shown to be present in microsomal fractions isolated from this tissue[15,19]. These latter two enzymes should, therefore, have served quite adequately as markers for microsomes if not for endoplasmic reticulum. Thus, with the possible exception of some contribution by protein body membranes, for which there is no enzyme marker, the preparation appeared to be relatively free of contamination by cytoplasmic membranes.

There are notable similarities as well as differences between this plasma membrane fraction purified from a plant tissue and that isolated from mammalian tissue by the same method[14,18]. They have similar densities as judged by their parallel centrifugation behaviours during the preparative procedure. ATPase activity is a feature common to both. The ATPase of the plant plasma membrane shows cation sensitivity, but unlike its mammalian counterpart, the (Na^+-K^+)stimulation is not Mg^{2+}-dependent nor are the effects of Na^+ and K^+ synergistic. Moreover 5′-nucleotidase, a marker for plasma membrane for several mammalian tissues[6,13], is essentially absent from the plant membrane fraction.

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