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Solubilization and purification of the ATPase from the tonoplast of *Hevea*

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The tonoplast-bound ATPase of *Hevea brasiliensis* (caoutchouc tree) was solubilized with dichloromethan and purified 100-fold with two ammonium sulfate precipitation steps and a G-200 gel filtration step. The resulting ATPase activity eluted according to a molecular mass of approximately 200 kDa and chromatographed at an isoelectric pH of 5.3. Subunits of molecular mass 110 kDa, 68 kDa, 24 kDa and 12 kDa appeared after treatment with 1% sodium dodecyl sulfate or spontaneously during storage of the solubilized ATPase. Dodecyl sulfate/polyacrylamide gel electrophoresis yielded four polypeptides of molecular mass 54 kDa, 66 kDa, 23 kDa and 13 kDa. From protein determination by ultraviolet absorption and Coomassie stain it appears that the 54-kDa and the 66-kDa polypeptides exist in multiple copies. No close resemblance to the membrane-bound ATPase of mitochondria, plastids, plasmalemma, chromaffin granules and synaptic vesicles is seen. No antibody cross-reaction to F_1 of bacteria is observed. Therefore it is concluded that the vacuolar ATPase represents a novel type of ATPase.

Many properties of the tonoplast-bound ATPase such as pH-dependence, substrate specificity, ion-dependence and inhibitor sensitivity did not change when the enzyme had been solubilized and purified. The phosphatase activity was lost during the purification procedure. The stimulation of ATP-hydrolysis in tonoplast vesicles by uncouplers and ionophores was absent in the solubilized ATPase, and also the stimulation by chloride was significantly reduced. Anion channel blockers, such as triphenyltin and 4,4′-diisothiocyano-2,2′-disulfonic acid stilbene, which are strong inhibitors of membrane-bound ATPase, fully or partly lost their inhibiting effect after solubilization of the ATPase. These results are interpreted to indicate that ionophores do not directly affect the ATPase molecule, whereas chloride might have a small direct effect on the ATPase besides its effect as a permeating anion.

The latex of *Hevea brasiliensis* (rubber tree) contains a membrane fraction called lutoids, which according to enzymatic and cytological criteria can be regarded as equivalent to the tonoplast of higher plant cells [1]. An ATP-hydrolyzing enzyme, which is bound to the membrane, translocated protons into the intravesicular space [2]. The function of the ATPase is presumably the energization of transport of citrate, lysine and other solutes [3]. The ATPase of lutoids is in many respects, such as inhibitor sensitivity and stimulation by chloride ions and ionophores, similar to the ATPase of vacuoles from sugar beet, tulip petals, maize roots and sugarcane [4–7]. The lutoid ATPase can therefore be used as a model system for tonoplast ATPase of higher plants with the merits of a vacuolar material, which can be obtained in high yield by simple differential centrifugation of caoutchouc sap.

Despite lack of chemical critera it has been suggested that the tonoplast-bound ATPase is more closely related to mitochondrial and chloroplast ATPase than to plasmalemma ATPase [8]. An analogy might be drawn to the H^+-translocating ATPase of chromaffin granules, which is surely different from plasmalemma ATPase, but, perhaps, also from mitochondrial ATPase, thus constituting a novel type of ATPase [9].

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Abbreviation. SDS, sodium dodecyl sulfate.

The procedure used to solubilize the lutoid ATPase was that using organic solvents, introduced for preparation of ATPase from heart mitochondria [10]. This method is simple and rapid and thereby minimizes the proteolytic attack by intravacuolar enzymes. In mitochondria it tends to extract only that part of the enzyme with the catalytic site, whereas some firmly membrane-integrated polypeptides stay behind. Nevertheless it should be possible with this method to obtain valuable information about subunit composition of the tonoplast ATPase and about specific properties of this solubilized ATPase.

MATERIALS AND METHODS

All reagents were of analytical grade. When they were not mentioned below, they were obtained from Labosi (Paris, France), Fluka Feinchemikalien GmbH, (Ulm, FRG), Merck (Darmstadt, FRG) and Sigma Chemical Co. (Saint-Louis, USA). ATP as Na salt or Tris salt was from Boehringer (Mannheim, FRG). Nigericin and gramicidin were a gift of J. Berger (Hoffmann-La-Roche, Basel). Bacterial F_1 and polyclonal antiserum against bacterial F_1 was a generous gift of Dr Altendorf, Osnabrück.

Plant material

Latex was obtained from trees of *Hevea brasiliensis* Müll.-Arg. (clone Prang Besar 86) growing on the experimental

plantation of I.R.C.A. (Institut de Recherches sur le Caoutchouc en Afrique) Abidjan, Ivory Coast. The fluid cytoplasm was harvested in ice-cooled flasks as described previously [11].

The vacuoles (lutoids) were isolated, purified and lyophilized [11].

Preparation of tonoplast membranes

The lyophilized tonoplast membranes were dispersed in 25 mM Mes, 25 mM Hepes and 5 mM 2-mercaptoethanol, adjusted to pH 6.0 with Tris base. The preparation yielded tightly sealed vesicles [2].

Extraction of tonoplast ATPase by organic solvents

Tonoplast membranes, usually prepared at 2–5 mg of protein/ml, were suspended in a solution (washing solution) containing 10 mM Tris, 1 mM EDTA and 1 mM ATP, adjusted at pH 7.5 by additon of H_2SO_4. Usually 1 mM phenylmethylsulfonyl fluoride was added to inhibit any intervention of vacuolar protease on the proteins. The suspension was vigorously vortex-mixed and 0.5 vol. of organic solvent was rapidly added in drops. After completion of the addition the shaking was continued for 10 min. The emulsion was centrifuged in a bench centrifuge for 2–5 min. The upper, aqueous layer was removed carefully and diluted 1:1 with fresh washing solution. The organic solvent layer and the insoluble material at the interface were discarded. The aqueous solution was centrifuged at $100000 \times g$ for 20 min to yield a clear supernatant and a tightly packed yellowish-brown pellet. The supernatant obtained contained a great part of the ATPase activity.

Ammonium sulfate purification

The ATPase released from tonoplast membranes and present in the aqueous extract was purified by two $(NH_4)_2SO_4$ fractionation steps. Firstly, solid $(NH_4)_2SO_4$ up to 0.176 g · ml^{-1} was added slowly to the well-stirred aqueous extract. When all the salt had been added, the sample was stirred for further 15 min in ice bath before centrifuged at $12000 \times g$ for 10 min to precipitate the finely dispersed protein. The supernatant was carefully removed, and solid $(NH_4)_2SO_4$ was further added to obtain 60% saturation (0.214 g · ml^{-1}). The addition of salt was made as described above. After 15 min incubation and following centrifugation the supernatant was removed and discarded and the yellowish sediment was dissolved in a small volume of washing solution. In some cases, the final solution was centrifuged for 4–5 min in an Eppendorf Microfuge (type 1514) to remove some insoluble material. When necessary, a second precipitation with 60% $(NH_4)_2SO_4$ was made at the same conditions as above.

The enzyme remained usually stable and fully active for several weeks when stored at 2 °C in 60% $(NH_4)_2SO_4$. When required for assay, the precipitate was dispersed by gentle shaking and an aliquot was taken out and centrifuged at $15000 \times g$ for 4–10 min. The pellet was dissolved in the medium for the measurement of ATPase activity.

ATPase assay

ATPase assay was performed at 30 °C in 1 ml of a reaction mixture composed of 100 ml Pipes and 0.1 mM ammonium molybdate, adjusted to pH 7.0 by Tris-base.

For assays performed at fixed substrate concentration the mixture also contained 10 mM $MgSO_4$ and 5 mM ATP. The reaction was started by the addition of either tonoplast membranes (containing about 500 µg of protein) or solubilized ATPase (corresponding to 100 µg of protein). Unless indicated otherwise, incubation proceeded for 30 min at 30 °C before the addition of 0.250 ml of 20% (w/v) trichloroacetic acid. Then, the mixtures were centrifuged at $7500-15000 \times g$ for 5 min at ambient temperature. The liberated phosphate present in the supernatant was determined according to the procedure of Taussky and Shorr [12] or the method of Heinonen and Lahti [13].

Corrections were made for the P_i content of reagent and enzyme, by using samples in which trichloroacetic acid was added before the enzyme. For the estimation of K_m and V_{max} values, the mixtures contained 5 mM $MgSO_4$ and 0.05–5 mM ATP.

One unit (I.U.) of the ATPase activity is defined as the amount of enzyme that catalyzes the hydrolysis of 1 µmol of ATP/h under the assay conditions. Specific activity is expressed as units per mg protein.

Protein determination

Protein was estimated by the method of Lowry with the modifications described elsewhere [14]. In these conditions, any interference with the compounds present in the incubation mixture was eliminated.

Estimation of molecular mass

The molecular mass was estimated by using gel filtration chromatography of standard proteins and purified ATPase at 8 °C. Sephacryl S-200 or S-300, equilibrated with 0.1 M Tris solution buffered at pH 7.5 with sulfuric acid, was packed in a 2.5-cm diameter column to a bed height of 96 cm. The effluent volume of each protein was measured individually or together. Each protein (0.5–3 mg), dissolved in 0.5–2 ml of buffer containing 10% (v/v) glycerol, was applied to the top of the column and the effluent was collected in fractions (3.9 ml) by using an automatic fraction collector with a drop-counting attachment. The flow rate was 10 ml · h^{-1}. An elution diagram was plotted by measuring the protein activity in the collected fractions. The effluent volume corresponding to the maximum concentration of each enzyme was estimated by extrapolating both sides of the elution peak to an apex.

In addition, each elution peak was analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) as performed and described elsewhere [15]. Proteins to be loaded onto the gel were incubated for 60 min at 25 °C in presence of 2% (w/v) sodium dodecyl sulfate. Gels were prepared (at 20 °C) as an exponential gradient from 10–18% or 7.5–15%. After 4–5 h electrophoresis at 25 mA and 8 °C, the gels were stained with Coomassie brilliant blue R 250 and destained in 25% methanol plus 7% acetic acid.

Isoelectric-focussing was performed with Servalyt-precotes (Serva, Heidelberg) gels, pH range 3–10, in aspartic acid/glutamic acid buffer and arginine/lysine/ethylendiamine buffer for 3 h at 10 °C with a final voltage of 1700 V.

Electron microscopy

Samples of the dissolved 60% $(NH_4)_2SO_4$ precipitate of the ATPase-containing peak from the molecular sieve gel-

filtration were subjected to negative stain according the procedure of Horne [16].

RESULTS

Extraction of ATPase by organic solvents

The organic solvents were added to the suspended membranes in a 1:2 ratio (organic solvent/membrane suspension, v/v), thoroughly agitated and then centrifuged at low speed. Part of the enzymatic activity was then recovered in soluble form from the upper aqueous phase. The interphase, consisting mainly of membraneous material and of rubber particles, was discarded. It was the separation of the rubber particles from the ATPase activity which made the method of organic solvent treatment especially superior over other solubilization methods. The highest yield of solubilized ATPase was obtained with dichloromethane: about half of the original enzymatic activity was found in the aqueous phase (Table 1). Extraction by chloroform was less successful, diethyl ether, *n*-butyl acetate, ethyl acetate and carbon tetrachloride were much less efficient solvents (Table 1). Benzene, butanol, pentane and some other organic solvents denatured the ATPase. The extraction by dichloromethane solubilized 10% of the membrane protein, so that the soluble ATPase was already enriched in the aqueous phase. The remaining membranous material contained about 30% of the original total ATPase activity. Attempts to solubilize that ATPase failed, because the yield became very low (not more than 10%) and a lot of other membrane proteins were thereby also extracted. The fact that only about 80% of the original ATPase activity could be recovered in the different fractions together after dichloromethane extraction indicates that some denaturation or inactivation had occurred.

Effect of extraction time, temperature and extraction medium on solubilization of the ATPase

Different conditions in the extraction procedure were varied to test their importance for high yield of solubilized ATPase. The extraction with dichloromethane was complete after about 10 min of agitation (Fig. 1), further extraction did not increase the yield. The presence of the protease inhibitor phenylmethylsulfonyl fluoride was important since in its absence a decrease of solubilized enzyme activity was observed after a few minutes (Fig. 1). Obviously an intravacuolar protease was set free during the extraction procedure and dichloromethane did not inactivate or inhibit it sufficiently. The use of other well known inhibitors of protease activity was not as effective. In addition, the degree of inactivation measured in the absence of any protease inhibitor varied and depended on the lutoid sample used. However, to avoid such variability, the phenylmethylsulfonyl fluoride was systematically used during the extraction phase.

The yield was dependent on the temperature of the extraction medium, with increasing yield of ATPase and protein at increasing temperature up to 35–40 °C (Fig. 2), but since the amount of extracted protein increased less, the specific activity was optimal between 20 °C and 30 °C. The low activity observed at 0 °C was not the consequence of irreversible inactivation in the cold because the ATPase could be preserved at low temperature for a long time.

The yield of solubilized ATPase was slightly increased in presence of nucleotides (ATP, ADP), EDTA and EGTA, and dithiothreitol. Mg^{2+} severely reduced the yield of extracted enzyme and of protein (Table 2). The effect of nucleotides and chelators can therefore mostly be explained by their Mg^{2+}-complexing ability. Highest yield of solubilized ATPase was obtained in presence of ATP, EDTA and phenylmethylsulfonyl fluoride together.

The optimal pH of the membrane suspension for extraction of tonoplast-bound ATPase was between 7 and 8, whereas at pH 6 or 9 the extraction of ATPase was five times less efficient. Thus, the most convenient procedure for solubilization of tonoplast ATPase was 10–15 min in 20 mM Tris-sulfate, pH 7.5, 1 mM EDTA, 0.5 mM dithiothreitol and 0.5 mM phenylmethylsulfonyl fluoride at ambient temperature. The protein concentration of the suspension was kept at 2 mg · ml^{-1}.

Table 1. *Release of ATPase activity from* Hevea *tonoplast membranes by treatment with various organic solvents*
The conditions were the same as those described for dichloromethane treatment in Materials and Methods

Organic solvent	ATPase activity	Specific activity
	I.U.	I.U. · mg protein^{-1}
None (crude membranes)	360	1.9
Dichloromethane	179	32
Chloroform	104	22
Diethyl ether	42	12
n-Butyl acetate	28	9.2
Ethyl acetate	27	5.1
Carbon tetrachloride	17	4.3
Residual membranes after dichloromethane extraction	112	1.1

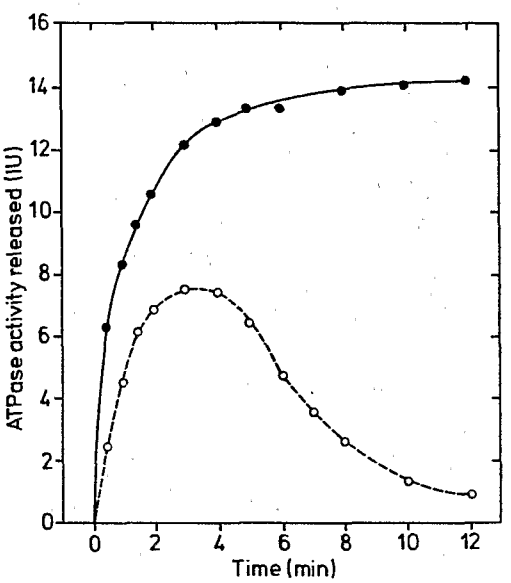
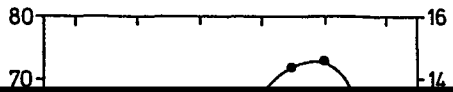


Fig. 1. *Time-dependent release of ATPase from the tonoplast.* Tonoplast vesicles (containing 25 mg of protein) were suspended in 10 ml of 10 mM Tris-sulfate, 1 mM EDTA, pH 7.5, with (●——●) or without (○——○) 1 mM phenylmethylsulfonyl fluoride. At the times indicated aliquots of membrane suspension were withdrawn and assayed for ATPase extraction by dichloromethane, as described under Materials and Methods. The solubilized ATPase was measured at 30 °C with 5 mM ATP and 10 mM $MgSO_4$



activity, together with a protein peak, shortly after the void volume (Fig. 3). A lot of non-ATPase proteins were eluted into later fractions. The specific activity of the ATPase peak

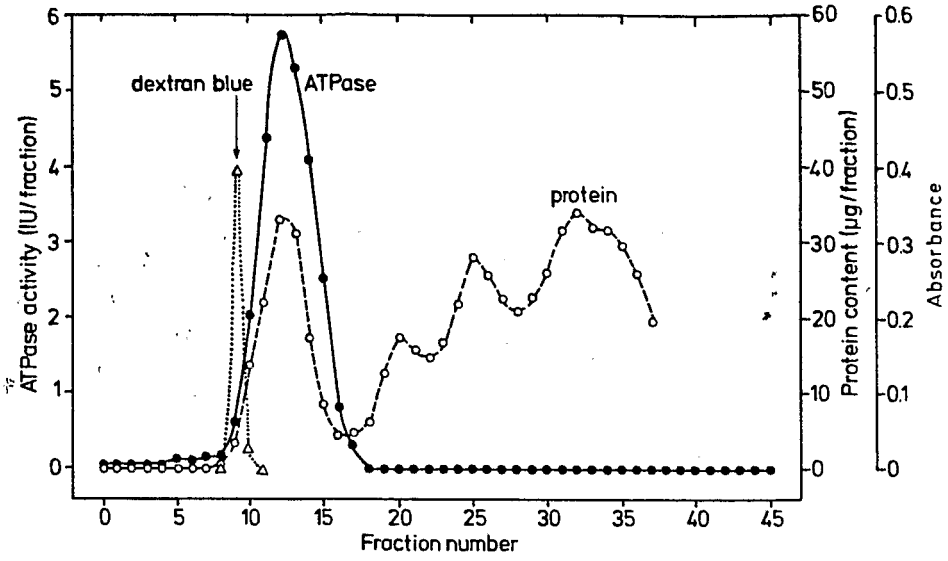


Fig. 3. *Sephadex G-200 column chromatography of dichloromethane-solubilized ATPase from tonoplast membranes.* The 60% $(NH_4)_2SO_4$ precipitate was chromatographed in descending fashion on a column (0.9 cm × 45 cm) of Sephadex G-200, previously equilibrated with washing Tris-SO_4 buffer. The column was loaded with 1 ml of 60% $(NH_4)_2SO_4$ precipitate (0.25 mg of protein, containing 30.1 units of ATPase). the flow rate was maintained at 7.1 ml · h^{-1} by a peristaltic pump. Fractions (about 1.2 ml) were collected and analyzed for ATPase activity (●——●) and protein content (○——○). The elution of blue dextran was followed as absorbance at 278 nm (△——△)

Table 3. *Purification of dichloromethane-released* Hevea *tonoplast ATPase*
ATPase activity was measured at 37 °C in 1 ml of 50 mM Pipes buffer pH 7.0, containing 5 mM ATP and 10 mM $MgSO_4$

Purification step	Protein concentration	Volume	Total protein	Total activity	Specific activity	Yield	Purification
	mg · ml^{-1}	ml	mg	units	units · mg^{-1}	%	fold
Tonoplast membranes	5.0	100	500	910	1.82	100	1
Dichloromethane extract	0.31	180	55.8	529	9.48	58.1	5
Supernatant from 30% $(NH_4)_2SO_4$	0.089	270	24.0	481	20.04	52.9	10.9
First precipitate from 60% $(NH_4)_2SO_4$	1.51	8	12.1	452	37.4	49.6	20
Second precipitate from 60% $(NH_4)_2SO_4$	0.85	6	5.1	402	78.8	44.2	43
Eluate of Sephadex G-200	0.10	15	1.5	350	233.3	38.5	128

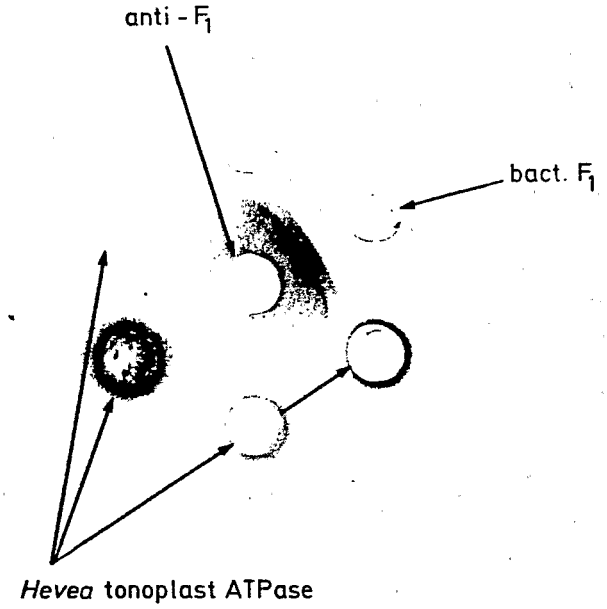


Fig. 4. *Antibody diffusion test of tonoplast proteins with anti-F_1.* Solubilized proteins from tonoplast membranes were allowed to diffuse against antiserum to bacterial F_1. The reaction with bacterial F_1 is also shown for control

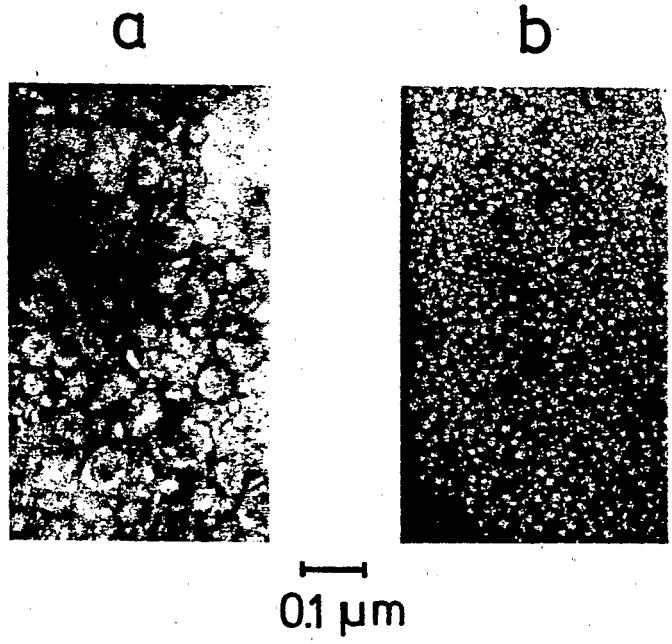


Fig. 5. *Electron micrograph of the lyophilized lutoids and the purified ATPase.* The lyophilized and resuspended lutoid fraction (a) and the solubilized and purified ATPase (b) were depicted in negative stain according Horne [16]. Bar scale for both micrographs is 0.1 µM

SDS-polyacrylamide gel electrophoresis of the ATPase

The dissociation of the protein into polypeptides by SDS and subsequent electrophoresis revealed four protein bands (Fig. 8) with apparent molecular mass of 66 kDa, 54 kDa, 23 kDa and 13 kDa (and sometimes faint bands at 50 kDa and 43 kDa). The SDS-PAGE electrophoresis of those four protein peaks from column chromatography, which were obtained after mild SDS treatment (Fig. 7), gave polypeptides of 54 kDa (peak II), 66 kDa (peak III), 23 kDa (peak IV) and 13 kDa (peak V). (Sometimes high molecular mass components 260 kDa, 210 kDa and 116 kDa in SDS-PAGE of peak II showed up; they seemed to be aggregates, since they appeared mostly after prolonged storage of samples in solution).

The conclusion from the results of SDS electrophoresis is that the solubilized ATPase consists of two polypeptides of 54 kDa (which seem to be closely associated and do not dissociate by mild SDS treatment), and three polypeptides of 66 kDa, 23 kDa and 13 kDa. The different polypeptides were sensitive to protein-specific stains in very different ways; whereas the Lowry procedure and ultraviolet absorption gave relatively similar results, peaks IV and V (23 kDa and 13 kDa) were only faintly stained by Coomassie blue. The subunit stoichiometry when calculated according the protein tests of Lowry or ultraviolet absorption was therefore 1:2:1:1, whereas the Coomassie blue stain suggested a much higher copy number for the two large polypeptides (66 kDa and 54 kDa).

pH dependence, divalent cation requirement and ATP dependence

The purified enzyme exhibited a pH dependence with a broad maximum of activity at pH 6.5–7.5, which differed

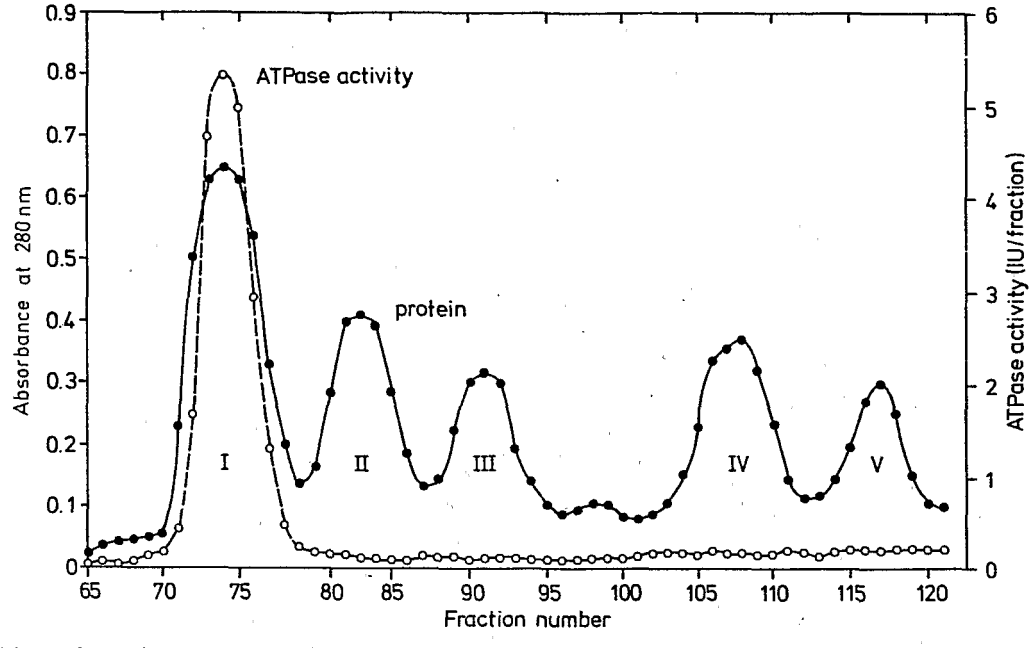


Fig. 6. *Sephacryl S-200 column chromatography of the solubilized ATPase.* The purified ATPase fraction was chromatographed as described in Materials and Methods. The column was loaded with 2.0 ml of purified ATPase fraction from Sephacryl S-200 chromatography

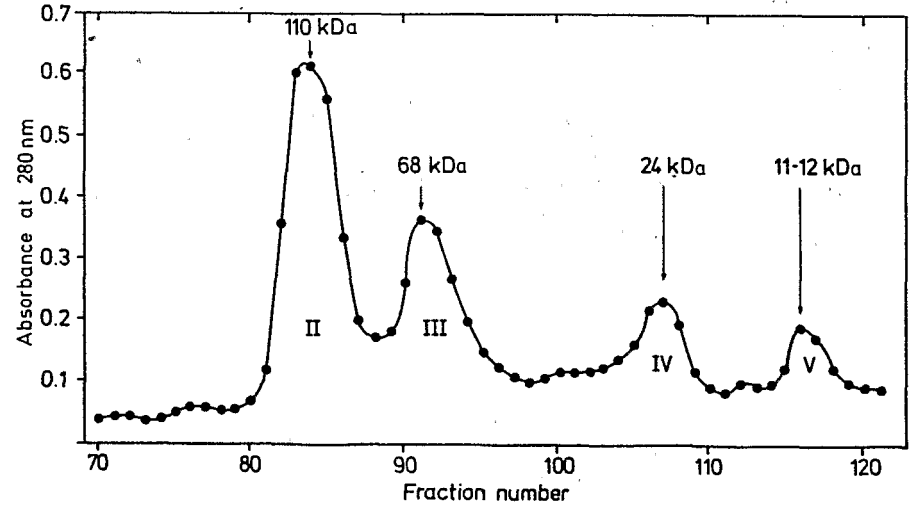


Fig. 7. *Sephacryl S-200 column chromatography of the purified ATPase after 1% SDS treatment.* The protein peak from S-200 chromatography, which contained the ATPase activity, was dissolved in 1% SDS and then chromatographed on Sephacryl S-200

slightly from those reported for the membrane-bound ATPase (pH 6.5–8.2 [17]). At pH 4.5–5.0 the hydrolytic activity was completely abolished, but the enzyme was not permanently inactivated. Full activity could generally be recovered when the pH was brought back to neutrality.

The purified enzyme had an absolute requirement for Mg^{2+}. Virtually no activity has been detected in its absence or with addition of EDTA (1–2 mM). In certain conditions, it could be completely replaced by Mn^{2+} (Fig. 9). But, in contrast to Mg^{2+}, Mn^{2+} at relatively high concentration became ineffective. A relatively low rate was obtained with Ca^{2+}, e.g. 30% of the observed maximal rate with Mg^{2+}. No activity or less than 15% of that obtained by Mg^{2+} was found with Co^{2+}, Zn^{2+}, or Cu^{2+}.

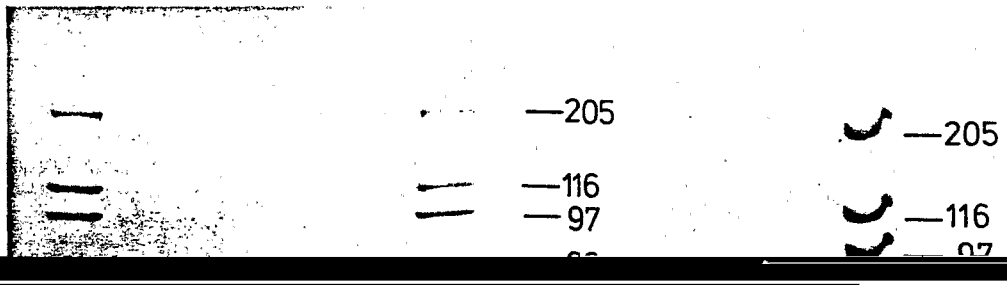
Incubation of the purified ATPase with increasing concentrations of ATP (ranging from 0.05–5.0 mM) in the presence of 5 mM $MgSO_4$ produced a dose-dependent hyperbolic increase in the ATPase activity. The K_m value for ATP (in the presence of Mg^{2+}) changed slightly from 0.75 mM for the membrane-bound ATPase to 0.45 mM for the solubilized and purified enzyme.

Table 4. *Stoichiometry of subunits after 1% SDS-treatment*
The purified ATPase was treated with 1% SDS and chromatographed on Sephacryl S-200. The four protein peaks (Fig. 7) were collected and the protein content was estimated by ultraviolet absorption at 280 nm

Subunit	Protein	Protein/ molecular weight
	mg	ng/kDa
Peak at 110 kDa	3.28	29.8
Peak at 68 kDa	1.84	27.0
Peak at 24 kDa	0.68	28.3
Peak at 12 kDa	0.35	29.0

Substrate specificity

Table 5 shows the rate of hydrolysis of a number of nucleotides and phosphate esters by the purified ATPase. In all cases the best substrate was ATP; CTP, ITP and UTP were hydrolyzed at relatively low rates whereas other nucleotides could not serve as substrate at all (data not shown). The different analogs of ATP decreased the rate of ATP hydrolysis: adenosine 5′-[β,γ-imido]triphosphate; adenosine 5′-[β,γ-methylene]triphosphate and trinitrophenyl-ATP showed 50% inhibition at 8 µM, 10 µM and 12 µM, respectively. The hydrolysis of trinucleotides other than ATP was slightly less by the solubilized enzyme than by the membrane-bound enzyme. The acid phosphatase of *Hevea* vacuoles, typically adsorbed on lyophilized tonoplast membranes was not present in the solubilized ATPase preparation, therefore the same rate of ATP hydrolysis was obtained in presence and absence of molybdate (Table 5).



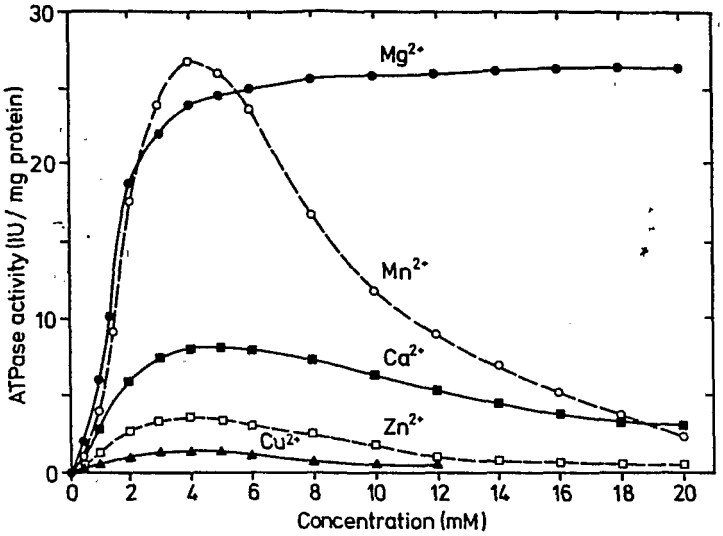


Fig. 9. *Effect of divalent cations on the solubilized tonoplast ATPase.* Purified enzyme (100 μg) was incubated in the medium containing different concentrations at 37°C during 30 min. The reactions were started by 1 mM ATP addition: (●), Mg^{2+}; (○), Mn^{2+}; (■), Ca^{2+}; (□), Zn^{2+} and (▲), Cu^{2+}

Table 5. *Substrate specificities of the ATPase from* Hevea *tonoplast*
The liberation of P_i from various substrates was determined after incubation of 30°C for 30 min. The final incubation volume was 1 ml. The incubation medium contained 50 mM Pipes, 5 mM $MgSO_4$, 5 mM substrate and 0.1 mM ammonium molybdate, adjusted at pH 7.0 with Tris base. Rates of hydrolysis of each substrate are presented as a percentage of the rate with ATP. It is assumed that each molecule

Table 6. *Effect of anions and monovalent cations on ATPase activity from* Hevea *tonoplast*
Each salt was tested at a concentration of 50 mM (with respect to the cation). Other assay conditions were as described in Materials and Methods. The ATPase activity, measured in presence of 5 mM $MgSO_4$, was taken as reference and each activity was expressed as a percentage of this value, which was 2.85 units · mg protein^{-1} and

Table 7. *Effect of inhibitors and ionophores on the activity of* Hevea *tonoplast ATPase*
Membrane-bound and solubilized ATPase activities were assayed in the presence of the indicated compounds as described in Materials and Methods. The rate of activity without any addition was taken as 100%. Control activity in the absence of any compound was 1.85 and 25.1 units · mg protein^{-1} for the membrane-bound and solubilized ATPase, respectively

Inhibitor or ionophore	Membrane-bound ATPase	Solubilized ATPase
	%	%
Na_3VO_4 (50 μM)	100	95–102
Oligomycin (5 μg · ml^{-1})	100	100
N,N'-Dicyclohexylcarbodiimide (25 μM)	20–25	15–20
p-Chloromercuribenzene sulfonate (0.2 mM)	25–30	20–30
4,4'-Diisothiocyano-2,2'-disulfonic acid stilbene (50 μM)	16–20	40–50
Triphenyltin chloride (0.1 mM)	5–10	95–100
Carbonylcyanide *p*-trifluoromethoxyphenyl hydrazone (10 μM)	195–210	85–100
Nigericin (5 μM) + K_2SO_4 (10 mM)	190–200	90–105
Gramicidin (5 μM)	150–165	100

anion channel blockers, inhibited (in absence of any Cl^-) the membrane-bound ATPase more than the solubilized form. Therefore it is questionable whether the tonoplast ATPase possesses an essential chloride channel.

Uncouplers, such as carbonylcyanide *p*-trifluoromethoxyphenylhydrazone or S-13, and ionophores such as nigericin ad gramicidin, only stimulated the membrane-bound ATPase activity significantly (Table 7) and not the solubilized ATPase. It is clear from these results that the stimulatory effect of the ionophores on the membrane-bound ATPase was most probably due to the relief of the protonmotive potential in the vesicular system.

DISCUSSION

The solubilization and purification of *Hevea* tonoplast ATPase was performed as a first step to gain information about the polypeptide composition of the enzyme. This information makes it possible to compare the tonoplast ATPase with the well-known ATPases from mitochondria, plasmalemma and chromaffin granules [9, 18] and to draw conclusions about ontogeny and phylogeny of this ATPase.

The solubilization procedure with organic solvents could mean that in analogy to the solubilization of F_1 in mitochondria [10] only that part of the ATPase, which bears the catalytic site, was extracted. Reconstitution studies in the future should clarify whether the purified ATPase is a functional H^+-translocating ATPase.

The yield of ca. 30% assured that the purified enzyme was the main ATPase existing in tonoplast membranes, and did not derive from a small contaminant fraction. The lack of cross-reaction with antibody to bacterial F_1 further indicated that it was not the ATPase of mitochondrial or plastid origin.

The solubilized ATPase had a molecular mass of approximately 200 kDa, and it was probably assembled of five subunits with molecular masses of 66 kDa, 54 kDa, 23 kDa and 13 kDa, respectively. Such composition already excludes that the tonoplast ATPase was related to the plasmalemma ATPase, since this consists (perhaps exclusively) of a polypeptide of 100 kDa [19]. The subunit pattern is not strictly comparable to those reported for F_1 and CF_1 which is 3α (53–62 kDa) 3β (50–57 kDa) γ (25–36 kDa) δ (12.5–35 kDa) and ε (7.5–16 kDa), though at a first glance the polypeptide pattern (Fig. 8) looks strikingly similar to the 3α,3β,γ,δ,ε pattern of F_1, if the faint band at 43 kDa is included and if the strong Coomassie stain of the 66-kDa and 54-kDa band is taken as evidence for multiple copies. Also the ATPase of chromaffin granules with reported subunits of 125 kDa, 80 kDa, 40 kDa and 20 kDa [9, 18] appears significantly different.

Recently the ATPase of the cholinergic synaptic vesicle membrane was solubilized with dichloromethane and an ATP-hydrolyzing molecule of 250 kDa was obtained consisting of only one polypeptide of 50 kDa [20]. Regrettably, agreement or disagreement with published molecular masses cannot be taken as strong evidence in either direction. Conclusions on phylogenetic relations have to wait for comparison of antibody specificity and amino acid sequences.

The substrate specificity, the pH dependence and the inhibitor sensitivity were nearly the same for the native and the purified ATPase. The more interesting part was the stimulation by anions and ionophores. Whereas chloride stimulated and nitrate inhibited the solubilized ATPase and the membrane-bound ATPase, the uncouplers, ionophores and ammonium were only effective on the membrane-bound enzyme. The most plausible explanation is (as has been suggested before [21, 22]) that uncouplers and ionophores relieved the inhibition of the ATPase, by collapsing the electrochemical H^+ gradient across the tonoplast vesicles. A similar effect was expected, and partly also seen, with chloride, but since there was still some stimulation by chloride in the solubilized ATPase, some direct interaction of Cl^- with the ATPase is possible. Similarly, a dual effect of Cl^-, a direct one and one on the membrane conductivity had been postulated by Bennet and Spanswick [22, 23]. The physiological relevance of the Cl^- effect is still obscure, since *Hevea* lutoids and *Hevea* latex contain by far less Cl^- than needed to exert the stimulating effect. Perhaps it is another anion in the latex which plays a regulatory role and whose effect was mimicked by chloride.

In conclusion, the analysis of the purified vacuolar ATPase revealed a novel type of ATPase which is strikingly different from the plasmalemma-type ATPase, and vaguely more related to the F_0F_1-type ATPases, but again sufficiently different from these to be insensitive against F_1 antibody. So far F_0F_1-type ATPases were considered as enzymes which work physiologically for ATP-generation whereas plasmalemma-ATPases work for H^+ translocation. Thus the vacuolar ATPase (and perhaps some other ATPases) might belong to a novel family of ATPases.

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REFERENCES

1. D'Auzac, J., Cretin, H., Marin, B. & Lioret, C. (1982) *Physiol. Veg. 20*, 311–331.
2. Marin, B., Marin-Lanza, M. & Komor, E. (1981) *Biochem. J. 198*, 365–372.
3. Marin, B., Cretin, H. & D'Auzac, J. (1982) *Physiol. Veg. 20*, 333–345.
4. Walker, R. R. & Leigh, R. A. (1981) *Planta (Berl.) 153*, 140–149.
5. Lin, W., Wagner, G. J., Siegelmann, H. W. & Hind, G. (1977) *Biochim. Biophys. Acta 465*, 110–117.
11. D'Auzac, J. (1975) *Phytochemistry 14*, 671–675.
12. Taussky, H. H. & Shorr, E. (1953) *J. Biol. Chem. 202*, 675–685.
13. Heinonen, J. K. & Lahti, R. J. (1981) *Anal. Biochem. 113*, 313–317.
14. Marin, B. (1983) *Planta (Berl.) 157*, 324–330.
15. Laemmli, U. K. (1970) *Nature (Lond.) 227*, 680–685.
16. Horne, R. W. (1965) in *Techniques for electron microscopy* (D. Kay, ed.) pp. 328–348, Blackwell Scientific, Oxford.
17. D'Auzac, J. (1977) *Phytochemistry 16*, 1818–1885.
18. Amzel, L. M. & Pedersen, P. L. (1983) *Annu. Rev. Biochem. 52*, 801–824.
19. Gaffney, A. & Clarke, C. W. (1981) *Biochim. Biophys. Acta*