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Purification and Characterization of ATPases and Phosphatase of Light Membrane Vesicles Isolated from *Cucurbita pepo*

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*Cucurbita pepo*에서 분리한 Light Membrane Vesicle의 ATPase와 Phosphatase의 정제 및 특성

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ABSTRACT

Light membrane vesicles were isolated from the zucchini hypocotyl by floatation on ficoll density gradients and the proteins were solubilized with Triton X100. Three ATP-hydrolyzing enzymes were partially purified by ion-exchange and gel filtration chromatography and isoelectric focusing. There are plasma membrane-type ATPase whose activity was inhibited by vanadate but not by nitrate, tonoplast-type ATPase which was sensitive to nitrate but insensitive to vanadate and one having a phosphatase activity with a pI value different from that of an acid phosphatase. A fraction was obtained after DEAE-ion-exchange chromatography crossreacting with polyclonal antibodies against Ca^{2+}-ATPase from human erythrocytes.

INTRODUCTION

Compartmentation of the cytoplasm is the major characteristics of eukaryotic cells as distinguished from prokaryotic cells. The transport processes between the cell organelles are important elements for the metabolism of the cell.

Energy dependent primary proton transport across the membrane builds up an electrochemical potential called 'proton motive force' (PMF). This electrochemical potential drives the secondary transport of many molecules, e.g. ions (Gerson and Poole, 1972), glucose (Suzuki, 1982), IAA (Hertel *et al.*, 1983; Lomax *et al.*, 1985) and amino acids (Luettge et al., 1981). In the membrane of plant cells the primary transport is mainly ATP-dependent. Besides the H^+-ATPases there are the redox system of the plasma membrane (Lin, 1982) and the proton transport pyrophosphatase of the tonoplast (Rea and Poole, 1986).

The electrochemical gradient is also important for the regulation of growth and development of plant cells (Sze, 1985). Studies on the agents influencing the H^+-ATPase could provide explanations for the physiological controls in the plant cell.

The vacuole in plant cells is considered to be a storage for ions and metabolites and it is important for the homeostasis of the cytoplasmic ion concentrations (Teulieres *et al.*, 1985). The ADP/ATP ratio in the cytoplasm (Rausch *et al.*, 1985) and the change of redox conditions of the SH-groups of H^+-ATPase may serve as a control mechanism of tonoplast-bound H^+-ATPase (Hager and Biber, 1984).

Membrane vesicles from the plant cells are useful model systems for the characterization of H^+-ATPases. Ho-

wever, since only an enrichment of a specific membrane type is achieved, a contamination with membranes having similar properties cannot be excluded.

In order to circumvent this problem tonoplast-bound H^+-ATPase should be solubilized, purified and reconstituted in liposome membranes. Reconstituted proteoliposomes may be used as a model system for the study of precise control mechanism of tonoplast-bound H^+-ATPase.

A simple and rapid floatation method was developed to isolate light membrane vesicles which were enriched in intact small vacuoles (J. Gross, personal communication). This method also could not exculde a contamination with light vesicles from other organelles such as ER and the plasma membrane. From these light membrane vesicles ATP hydrolyzing enzymes were partially purified and characterized and results are presented in the present communication.

MATERIALS AND METHODS

Plant cultivation. Zucchini seeds, *Cucurbita pepo* L. cv. Cocozelle v. Tripolis, were purchased from Kuepper (Darmstadt). The seedlings were grown for 5 days at 26℃ on moist cellulose packing material. They were kept in dim green light and irradiated 2 h/day with red light.

Preparation of membrane vesicles. Zucchini hypocotyl segments (ca. 5 cm) were chopped with a razor blade in 1 mg/g fresh weight 25 mM MOPS-NaOH-buffer (pH 7.2) with 10% (w/w) sucrose. The homogenate was filtered through nylon cloth (mesh 100 μm). The resulting debris was centrifuged for 10 min at 2,000×g with JA rotor (Beckman). Oleosomes were sucked off and discarded. The supernatant was adjusted with 36% (w/w) ficoll and 60% (w/w) sucrose in MOPS-buffer to 6% (w/w) ficoll and 10% (w/w) sucrose. This membrane suspension was underlayed by 4% (w/w) ficoll, 10% (w/w) sucrose. The ficoll gradient was centrifuged for 30 min at 20,000×g with SRP 28 Al rotor. After centrifugation the floating light vesicles were sucked off and collected. All processes were carried out at 0-5℃.

Sedimentation and solubilization of isolated vesicles. The collected vesicles were treated with 0.008% (w/w) Triton X100 and centrifuged for 30 min at 96,000×g with 50 Ti rotor (Beckman). The resulting pellet was solubilized in 0.5% (w/w) Triton X100 on ice and centrifuged for 30 min at 96,000×g in a 50 Ti rotor. The supernatant was used for further experiments.

Ion-exchange chromatography. A Sepharose CL-6B column of a size of ϕ 1.2 cm×1 8.9 cm was equilibrated with 0.5% (w/w) Triton X100. Elution was achieved at a flow rate of 10 ml/h.

Gel filtration chromatography. We used a ϕ=1.08 cm, l=23 cm Sephadex G-100 column, a ϕ=1.6 cm, l=18 cm Sephadex G-150 superfine column and ϕ=1.08 cm, l=23 cm Sephadex G-200. The elution rate was 12.5 ml/h for Sephadex G-100, 3 ml/h for Sephadex G-150 superfine and 7 ml/h for Sephadex G-200, respectively.

Concentration of protein solution. Fractions collected by chromatography were concentrated with dry Sephadex G-25. Fractions were added to Sephadex G-25 in the test tube on ice. After 5 min the squash was centrifuged through a plastic fritt for 10 min at 3,000×g with JA 20 rotor (Beckman) to remove Sephadex G-25.

Isoelectric focusing (IEF). 7% (w/v) polyacrylamide gel with 0.5% (w/w) Triton X100 was used for IEF. For the preparation of a gel covering the pH-range 4-6 the Ampholyte (Serva) of type pH 3-10 (Servalyt 3-10) and type pH 4-6 (Servalyt 4-6) were mixed 1 : 9. The gel was focused for 4 h with 1,000 V. After focusing the gels were cut into 1×1 cm^2 pieces. The pieces were equilibrated in MOPS-buffer with 0.5% (w/w) Triton X 100. After sedimentation of the gel particles the supernatants were concentrated with dry Sephadex G-25.

Enzyme-linked immunosorbent assay (ELISA). Proteins solubilized with 0.5% Triton X100 could not be immobilized on the test plate. The wells were pretreated with 0.25% glutaraldehyde for 30 min at 25℃. For the estimation of control value PBS (Phosphate buffered saline) was used and for blocking the free binding sites 3% BSA in PBS was used. Polyclonal antibodies from rabbit directed against Ca^{2+}-ATPase from human erythrocyte was obtained from Dr. Krebs, ETH, Zurich. The serum was used at a 1 : 10^3 dilution. Protein A-horseradisch peroxidase complex (Bio-Rad) was used for the detection of antibody-antigen reaction. The peroxidase activity was assayed spectrophotometrically with H_2O_2 and O-phenylenediamine as substrates. After 10 min incubation at 25℃ the reaction was stopped with 10% H_2SO_4. The entire solution of each testwell was diluted with H_2O and the absorption difference between 490 and 600 nm was determined.

Enzyme tests. The activity of phosphatase was assayed with p-nitrophenol. Acid phosphatase was measured with 3 mM p-nitrophenylphosphate as a substrate at pH 4.5. Alkaline phosphatase activity was assayed in 25 mM MOPS-Buffer at pH 7.2. The activity of ATP-hydrolysis was assayed by determining orthophosphate with

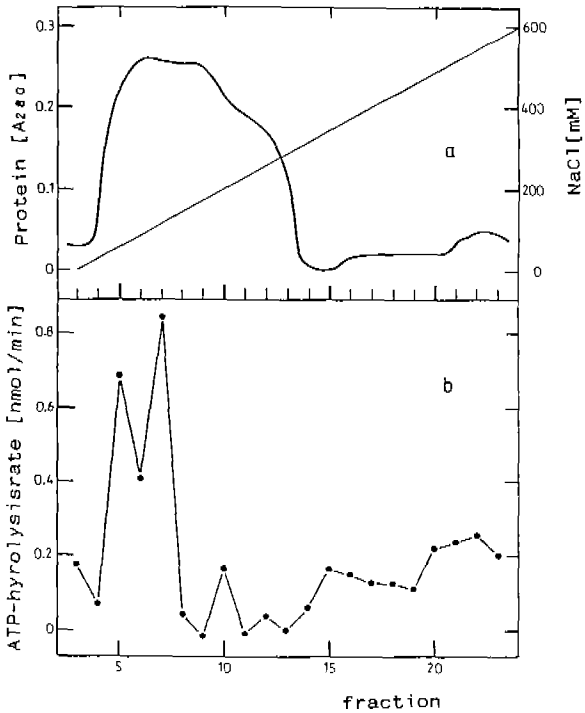

Fig. 1. DEAE-ion-exchange chromatography of proteins from vesicles in a linear salt gradient (0-600 mM NaCl). The vesicles were isolated from 170 g material and the proteins solubilized with Triton X100. a, protein profile. b, separation of ATP-hydrolysis-activity. Each fraction (ca. 1.2 ml) was concentrated 6 times with Sephadex G-25 and 50 μl was used for the enzyme test.

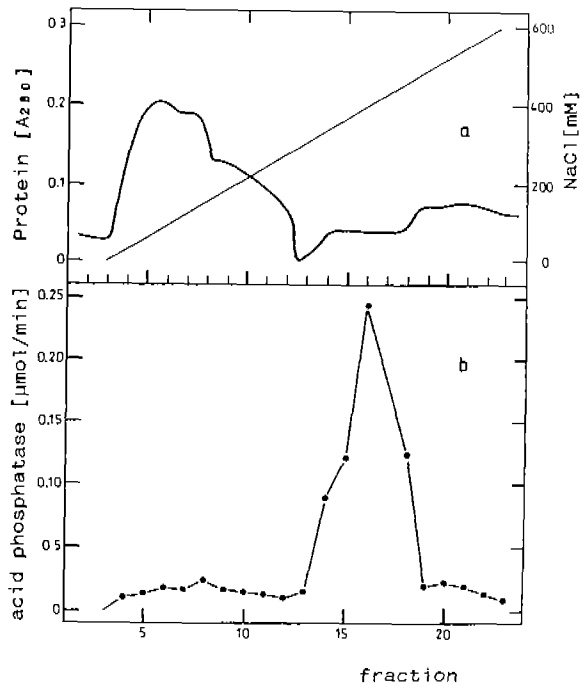

Fig. 2. DEAE-ion-exchange chromatography of proteins from vesicles in a linear salt gradient (0-600 mM NaCl). The vesicles were isolated from 160 g material and the proteins solubilized with Triton X100. a, protein profile. b, activity profile of acid phosphatase. Each fraction (ca. 1.2 ml) was concentrated 6 times with Sephadex G-25 and 50 μl was used for the enzyme test.

a spectrophotometer in the reaction mixture containing 3 mM ATP and 3 mM $MgCl_2$ at pH 7.2 (Lin and Morales, 1977). [γ-^{34}P]ATP was also used for the enzyme assay where 300 μM ATP, 3 mM $MgCl_2$ and [γ-^{32}P]ATP (3.75 KBq) were used as substrates at pH 7.2. After incubation for 30 min at 25℃ the reaction was stopped with 1% SDS and 1 mM ATP, 1 mM ADP and 1 mM AMP were added. The mixture was developed on the TLC (300 PEI/UV_{254}, Macherey Nagel). The position of orthophosphate was determined with UV light. Radioactivity was counted with a liquid scintillation counter.

RESULTS AND DISCUSSION

Partial purification of ATP-hydrolysis activities from vesicles. From 125-170 g zucchini hypocotyls, 3.1-4.3 mg light membrane vesicles were acquired. After fractionation of vesicle proteins with ion-exchange chromatography the ATP-hydrolysis activities of the different concentrated fractions were assayed (Fig. 1). Two main peaks were found in concentrated fractions 5-7 eluted at 60-130 mM NaCl. In concentrated fractions 14-19 (320-470 mM NaCl) and concentrated fractions 20-23 (500-570 mM NaCl) the activities were also detected. No activity was measured in concentrated fraction 9-13 (170-300 mM NaCl). Because of the probable assay error the activity could be detected in concentrated fraction 10.

Seperation of phosphatases with IEF. Since phosphatases can hydrolyze ATP in an unspecific manner the phosphatase content of the vesicle protein fractions were tested. Acid phosphatase is a soluble enzyme and a marker enzyme for intact vacuoles (Gross, 1982). Almost all acid phosphatase was collected in concentrated fractions 14-18 (350-480 mM NaCl) after ion-exchange chromatography on DEAE-Sepharose (Fig. 2). Therefore the two main ATP-hydrolysis-activity peaks of membrane vesicles do not correspond to the unspecific ATP-hydrolysis-acti-

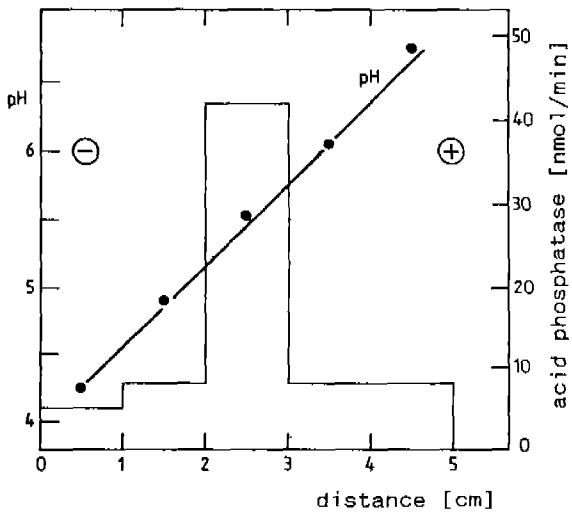

Fig. 3. Distribution of the acid phosphatase activity of proteins from fractions (elutriated at 300-500 mM NaCl) in DEAE-ion-exchange chromatography after the IEF (pH range 3-10). 155 g zucchini hypocotyl was used. The pooled fractions (ca. 4.8 m*l*) were desalted and concentrated 40 times and 40 μ*l* was used for the IEF. After sedimentation of gel particles the supernatant (2 m*l*) was concentrated 10 times and 50 μ*l* was used for the enzyme test.

vity of the acid phosphatase.

Furthermore, the midpoint of activity was eluted at about 400 mM NaCl and did not overlap with any of the other ATP-hydrolysis activities. The fractions of solubilized vesicles obtained from the ion-exchange chromatography were sampled into 4 groups (0-100, 100-300, 300-500, 500-600 mM NaCl) and further characterized.

Fractions from 300-500 NaCl were pooled and isoelectrically focussed. The pI-value of acid phosphatase was 5.4 (Fig. 3). Further ATP-hydrolysis activity was detected in fractions eluted at 500-600 mM NaCl. Using IEF these proteins were further characterized. Near pI 4.7 the strong bands with ATP-hydrolysis activity was found (data not shown).

The marked fractions containing most of the proteins were focused on a gel with a pH-range 4-6. There were 2 peaks of ATP hyrolysis activity, namely at pI 4.75 and pI 5.3 (Fig. 4). For comparison the entire solubilized proteins from membrane vesicles (the second supernatant) were isoelectrically focused directly in a pH range of 4-6 and ATP-hydrolysis and acid phosphatase activities were assayed (Fig. 5). The first maximum of ATP-hydrol-

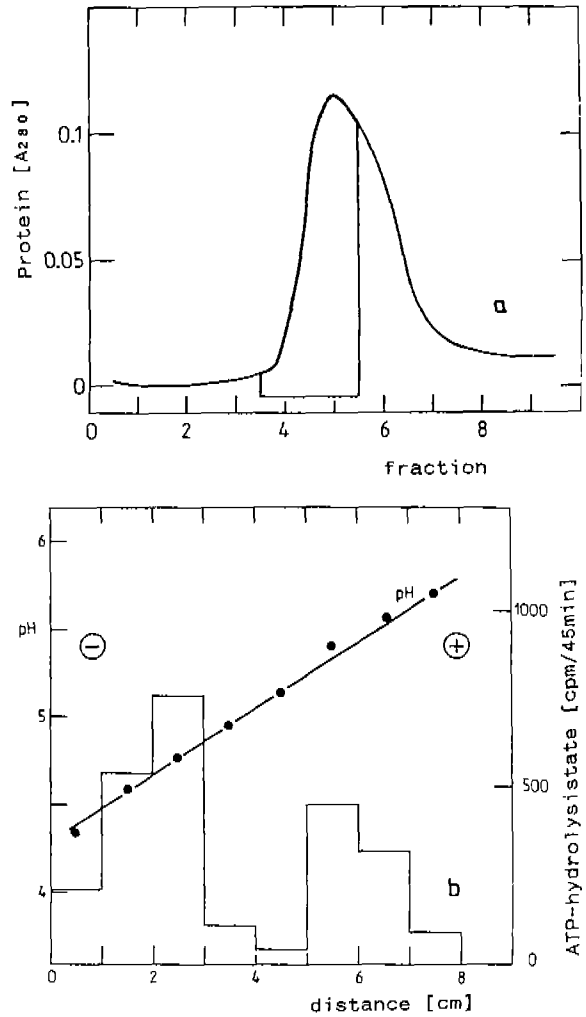

Fig. 4. (a) The protein profile after gel filtration from fractions eluted at 500-600 mM NaCl from Sephadex G-100. 150 g zucchini hypocotyl were used. The pooled fractions were concentrated to 1 m*l* and filtrated. (b) Distribution of the ATP-hydrolysis activity from the IEF with narrow pH range (pH 4-6). The marked fractions (ca. 4 m*l*) were desalted, concentrated 40 times and 40 μ*l* was used for IEF. After sedimentation of gel particles the supernatant (2 m*l*) was concentrated 10 times and 30 μ*l* was used for the enzyme tests.

ysis activities (pI 4.7) had a ratio of 2 : 1 between ATP-hydrolysis and acid phosphatase activity, in contrast to the second maximum (pI 5.1) with a ratio 1 : 1. The first maximum, therefore might be another phosphatase with an optimal pH-value different from the previously known acid phosphatase. The shift of pI value of the acid phos-

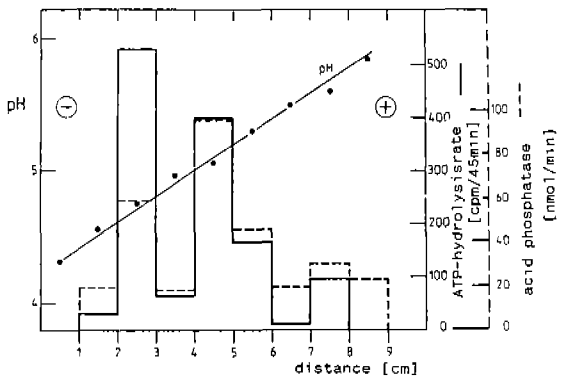

Fig. 5. Distribution of ATP-hydrolysis and acid phosphatase activity of the solubilized proteins from isolated membrane vesicles after separation by IEF in a pH range 4-6. Membrane vesicles were isolated from 160 g zucchini hypocotyls and the proteins solubilized. The solubilized proteins (ca. 24 m*l*) were concentrated 80 times and 40 μ*l* were used for IEF. After sedimentation of gel particles the supernatant (2 m*l*) was concentrated 10 times and the activity test was performed using 30 μ*l* for ATP-hydrolysis activity and 100 μ*l* for acid phosphatase activity.

phatase from 5.3 to 5.1 could be due to a disturbance of the pH gradient by high salt.

Crossreaction of zucchini protein with polyclonal antibodies against Ca^{2+}-ATPase from human erythrocytes. In plants Ca^{2+} is a physiologically important regulator. The level of Ca^{2+} in the cytosol is very low (0.1-1 μm) (Macklon, 1983). However, in the vacuoles the Ca^{2+} concentration reach values above 0.1 mM (Williamson and Ashley, 1982). Ca^{2+} transport is achieved by a Ca^{2+}/H^{+} antiport system. Furthermore, Ca^{2+} is transported by a Ca^{2+}-ATPase in the plasma membrane, ER (Gross, 1982; Zocchi and Hanson, 1983; Schumaker and Sze, 1985) and tonoplast (Gross, 1982) respectively.

Several ATPases have been identified and classified using antibodies against known ATPases (Oleski and Benett, 1987; Bowman *et al.*, 1986).

After separation by ion-exchange chromatography the fractions were pooled into 4 groups as described above, desalted and used in the ELISA test. Fractions that were elutriated at 300-500 NaCl cross reacted strongly with the antibodies (Table 1).

At least one protein from these pooled fractions cross-reacted with the antibodies against Ca^{2+}-ATPase from human erythrocytes. The source of this protein is not clear. Whether this protein is one of Ca^{2+}-ATPases from zucchini is an open question.

Table 1. Cross-reaction of polyclonal antibodies against Ca^{2+} ATPase from human erythrocytes with proteins from the pooled fractions after seperation with DEAE-ion-exchange chromatography

Sample[1]	Relative reactivity[2]
Control	−
pooled fractions from	
0-100 mM NaCl elution	+
100-300 mM NaCl elution	+
300-500 mM NaCl elution	+++
500-600 mM NaCl elution	+

[1]125 g zucchini hypocotyl were used. After separation with ion-exchange chromatography the pooled 4 fractions were desalted, concentrated into ca. 1 m*l* and each 200 μ*l* used for the ELISA test.

[2]The relative reactivity was estimated as 0.1 OD of the absorption difference between 490 and 600 nm.

Purification of two types of ATPases. When the pool of fractions from 0-300 mM NaCl elution was separated by Sephadex G-150 superfine the separation was improved as compared to the separation on Sephadex G-100, but in the void volume there were some ATPase-activities. In the pooled fractions only very low activities of alkaline phosphatases were detected and no correlation between the profiles of ATPase and alkaline phosphatase activities could be found (data not shown).

In order to distinguish between ATPases from the tonoplast and the plasma membrane, the sensitivities against nitrate and o-vanadate were tested.

Tonoplast H^{+}-ATPase is reported to be sensitive to nitrate. Isolated tonoplast-H^{+}-ATPases from *Hevea* and *Saccaromyces* are inhibited to about 70% (Marin *et al.*, 1985) and H^{+}-ATPase from corn to about 80% at 50 mM KNO_3 (Mandala and Taiz, 1985).

ATPase from the tonoplast is a relatively large protein (400-520 kDa) (Bowman and Bowman, 1986; Bowman *et al.*, 1985) composed of several subunits (Monolson *et al.*, 1985). The protein that eluted at 0-100 mM NaCl was run through a Sephadex G-200 column which separates globular proteins in the range of 5-600 kDa.

ATPase-activities were inhibited to 40-60% by 50 mM NO_3^{-} (Fig. 6). The rather low inhibition by NO_3^{-} could be explained by a contamination with a vanadate-sensitive ATPase which could have elutriated the nitrate-sensitive ATPase. The molecular weight was determined as 115-120 kDa with gel filtration (data not shown).

This value was smaller than total sum of the known subunits. Purified proteins could be a complex of 2-3 sub-

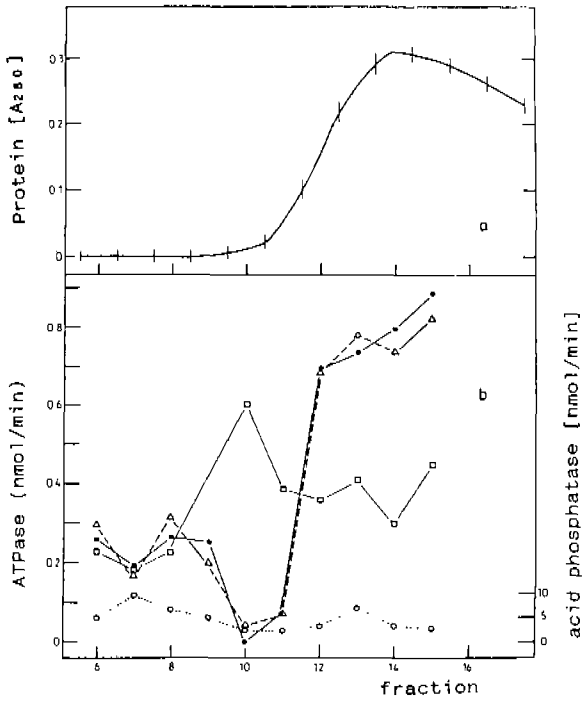

Fig. 6. Gel filtration of proteins which were eluted at 0-100 mM NaCl in the DEAE-ion-exchange chromatography with Sephadex G-200. Starting material was 125 g. (a) Protein profile. (b) ATPase- and acid phosphatase-activity. Each fraction (ca. 1.2 m*l*) was concentrated with dry Sephadex G-25 4 times and 50 μ*l* used for the enzyme test. ATPase-activity: control (closed circles), with 100 μM vanadate (open triangles), with 50 mM KNO_3 (open squares). Acid phosphatase activity: open circles.

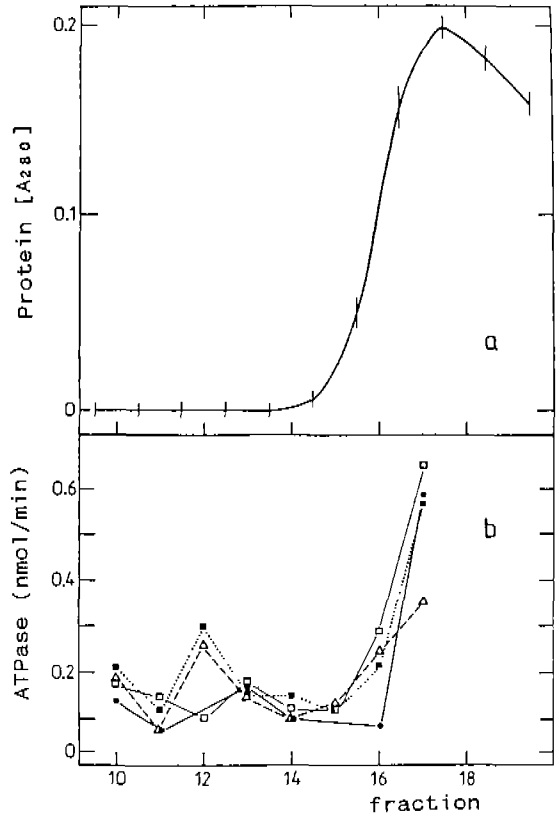

Fig. 7. Gel filtration with Sephadex G-150 superfine of proteins which was eluted at 100-300 mM NaCl in the DEAE-ion-exchange chromatography. Starting material was 170 g. (a) Protein profile. (b) ATPase-activity. Each fraction (ca. 1.1 m*l*) was concentrated with dry Sephadex G-25 3 times and 50 μ*l* used for the enzyme test. Control (closed circles), addition of 100 μM vanadate (open triangles), 100 μM molybdate (closed squares) and of 50 mM KNO_3 (open squares).

units which contains at least a ATP-hydrolyzing subunit.

In addition, an ATP-hydrolysis activity was found which was stimulated by KNO_3. This activity could be found reproducibly in different gel filtration assays (data not shown).

The inhibition by o-vanadate is one of the characteristics of plasma membrane-bound H^+-ATPases. At 50-100 μM vanadate the plasma membrane-bound ATPase from bean root and tomato root is inhibited up to about 90% (Kasamo, 1987; Anthon and Spanswick, 1986).

The molecular weights of known plasma membrane-bound ATPases are in the range of 90-104 kDa (Anthon and Spanswick, 1986; Vera and Serrano, 1982; Briskin and Thornley, 1985).

For isolation of plasma membrane-type ATPase fractions that were eluted at 100-300 mM NaCl were pooled and separated further on Sephadex G-150 superfine, which has a separation range of 5-150 kDa (Fig. 7). In this range a fraction was found in which ATP hydrolysis activity was inhibited by 40% in the presence of 100 μM vanadate but not inhibited by 50 mM KNO_3 or 100 M molybdate, the latter being a specific inhibitor of acid phosphatase (Gallagher and Leonard, 1982). The low inhibition by vanadate might result from a contamination by a different ATPase or it might be caused by the solubilization process. Glycerol was reported to be used during solubilization of plasma membrane-bound ATPase to preserve the maximal activity of the enzyme (Benett and Spanswick, 1983; Kasamo, 1986). The molecular weight of proteins collected during gel filtration with Sephadex

G-150 superfine was about 95 kDa which corresponds well with the known molecular weight of other plasma membrane bound H^+-ATPases.

Further study is required to confirm that the ATPases in the present work are identical with the proton translocating ATPases. The localization of these proteins should also be tested.

적 요

Zucchini 하배축에서 ficoll density gradient를 사용해서 light membrane vesicle를 획득하고 이를 Triton X100로 용해하였다. Ion-exchange chromatography, gel filtration chromatography 및 isoelectic focusing 등의 복합적인 순화방법을 통해서 3가지의 ATP-hydrolysis에 관여하는 효소를 부분적으로 순화하였다. 이들은 각각 vanadate에 의하여 억제되고 nitrate에 영향을 안 받는 원형질막의 ATPase, nitrate에 의하여 억제되고 vanadate에 영향을 안 받는 tonoplast막의 ATPase 및 phosphatase의 활성을 가지나 acid phosphatase와는 다른 pI값을 갖는 효소들이었다. DEAE-ion-exchange chromatography로 분획하여 사람의 적혈구의 Ca^{2+}-ATPase에 대한 polyclonal antibody와 crossreact하는 단백질을 얻었다.

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