

Zeitschrift: Archives des sciences et compte rendu des séances de la Société
Herausgeber: Société de Physique et d'Histoire Naturelle de Genève
Band: 47 (1994)
Heft: 3: Archives des Sciences

Artikel: Proton transport driven by the plasma membrane ATPase from Spinacea oleracea leaves : biochemical characterization
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DOI: <https://doi.org/10.5169/seals-740192>

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Archs Sci. Genève	Vol. 47	Fasc. 3	pp. 287-296	Décembre 1994
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Communication présentée à la séance du 16 juin 1994

PROTON TRANSPORT DRIVEN BY THE PLASMA MEMBRANE ATPASE FROM *SPINACEA OLERACEA* LEAVES. BIOCHEMICAL CHARACTERIZATION

BY

Jalil BELLAMINE* & Hubert GREPPIN*

ABSTRACT

Proton transport driven by the plasma membrane ATPase from *Spinacia oleracea* leaves. Biochemical characterization. – The plasma membrane of spinach leaves was purified by two phase partitioning and the H⁺pumping activity associated to the purified plasma membrane was characterized. The pH optimum was 6.7 similar to that of the ATPase activity and the substrat specificity was more obvious for ATP with a K_{mapp} of 0.697. The H⁺pumping activity was more or less inhibited by different ATPase inhibitors, and especially by VO₄ with I₅₀ of 160 μM. The SDS-PAGE of the purified plasma membrane proteins revealed the presence of a polypeptide of about 100 kDa which cross-reacted with the polyclonal antibodies against the plasma membrane ATPase of *Arabidopsis thaliana*.

Key-words: *Spinacia oleracea*, Adenosine triphosphatase, H⁺pump.

Abbreviations: ADP, Adenosine diphosphate; ATP, Adenosine triphosphate; DCCD, Dicyclohexylcarbodiimide; DCMU, 3-(3,4-Dichlorophenyl)-1,1-dimethylurea; DES, Diethylstilbestrol; GTP, Guanosine triphosphate; EB, Erythrosine B; PEP, Phosphoenolpyruvate; PP_i, Pyrophosphate. SDS, Sodium dodecylsulfate.

INTRODUCTION

Many important aspects of plant physiology, including growth, development, nutrient transport and stomata movements, seem to be controlled by the H⁺pumping ATPase of plasma membrane (PM) (Serrano,1989). This could be supported by the fact that the early event in the action of growth-modifying pathogens, hormones, and light is an alteration in the PM proton pumping activity (Assmann *et al.*, 1985; Cleland, 1987; Bidwai & Takemoto, 1987).

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In vitro activation of the H⁺pump of the purified PM, by auxins has been demonstrated for tobacco (Barbier-Brygoo, 1989; Santoni *et al.*, 1991), Petunia (François *et al.* 1992) and Spinach (Bellamine *et al.* 1993 a), suggesting that the pump is one of the earliest responses to the phytohormone (Cleland, 1987; Rayle & Cleland, 1992).

The PM H⁺ATPase of higher plants belongs to the P-type family of cation-translocating enzymes and generates an electric potential and pH gradient (H⁺motive force) that drive solute uptake across the PM. That type of enzyme is sensitive to vanadate (Jacobs & Taiz, 1980) and forms a phosphorylated intermediate (Briskin & Hanson, 1992). On the basis of the polypeptide composition and sensitivity to inhibitors, the plant PM H⁺ATPase is readily distinguished from that found in membrane derived from chloroplast, mitochondria and vacuole (Pederson & Carafoli, 1987). The plant PM H⁺ATPase was well characterized by biochemical and electrophysiological techniques, and the purified enzyme contains a single polypeptide of about 100 Kda (Grouzis *et al.*, 1990; Becker *et al.*, 1993). Based on molecular approach, this enzyme presents many transmembrane segments (Harper *et al.* 1989).

The genes of different bacterial, fungal, animal and plant ATPases of this family have already been cloned and sequenced (Serrano, 1988; Harper *et al.* 1989). The predicted amino acid sequence of *Arabidopsis thaliana* H⁺ATPase is more closely related to fungal and protozoan H⁺ATPase than bacterial K⁺ATPases or to animal (Na⁺/K⁺)- (H⁺/K⁺)- and Ca²⁺ ATPases (Pardo & Serrano, 1989).

In this study we reported a biochemical Characterization of the PM H⁺ATPase from spinach leaves. The plasma membrane was purified by phase partitioning and characterized as in a previous work (Bellamine *et al.*, 1993 a).

MATERIALS AND METHODS

Plant Material

Spinach (*Spinacia oleracea*, cv.Nobel) plants at the vegetative stage were grown in a phytotron for 4 weeks under non inductive short days (SD) of 8 h light (8:00 am to 4:00 pm; 400 $\mu\text{mol m}^{-2} \text{s}^{-1}$) and 16 h dark (4:00 pm to 8:00 am). The temperature was set at $20 \pm 1^\circ\text{C}$ and the relative humidity at $80 \pm 5 \%$ during the light period and $60 \pm 5 \%$ during the darkness.

Preparation of microsomal vesicles

Crude microsomal vesicles preparation and PM purification were as described by Bellamine *et al.* (1993 a).

ATPase activity

The plasmalemma ATPase activity was measured at 37°C as described by Bellamine *et al.* (1993 b).

Proton pumping activity

The initial rate of quinacrine fluorescence quenching was utilized to measure the plasmalemma proton translocation activity as described by Bellamine *et al.* (1993 a).

Protein determination

Membrane aliquots were diluted 20 fold with cold water and centrifuged at 96,100 x g for 30 min at 5 °C. The pellet was resuspended in cold water (160 µl) and proteins were determined using Bio-Rad solution. 50 µl of protein suspension were diluted in 750 µl of water and 200 µl of Bio-Rad solution were added. The obtained solution was mixed and incubated at room temperature for at least 5 min. The OD at 595 nm was measured and proteins were quantified using BSA as the standard.

SDS PAGE and Western blot

Purified plasma membrane vesicles were washed in a solution containing 10 mM Tris, 20% glycerol, 1 mM EDTA Na₂, 1 mM PMSF, 50 mg chymostatin and adjusted to pH 6.7 with HCl. Equivalent to 90 µg of proteins were pelleted by centrifugation at 96,500 g for 30 min at 5°C (40,000 rpm, Beckmann T50 rotor). The resulting pellet was resuspended in a sample buffer, as described by Laemmli (1970), supplemented by 1 mM PMSF and 50 mg/ml Chymostatin. After the SDS polyacrylamide gel electrophoresis (SDS PAGE), the peptides were transferred to a nitrocellulose sheet as described by Towbin *et al.* (1979), and revealed using antibodies to the central part of *Arabidopsis thaliana* plasmalemma ATPase (gift from R. Serrano). Immunodetection in western blot was made with TBS as a basic medium. Immune serum was diluted to 1/1000 and the second antibody was diluted 1/2000 (Anti-Rabbit IgG (whole molecule) alkaline phosphatase conjugate provided from sigma). The phosphatase alkaline substrates were NBT and BCIP.

RESULTS AND DISCUSSION

pH effect on ATP dependent H⁺pumping activity and on ATP hydrolase activity

The H⁺pumping and the phosphohydrolyzing activities were tested as described in Materials and Methods according to the pH of the reaction medium (Fig. 1). Both activities showed a maximum at pH 6.7 described for other plant species (De Michelis & Spanswick, 1986; Olivari *et al.*, 1992).

Vanadate effect on H⁺pumping activity

The sodium orthovanadate, a poison well known to inhibit plant PM H⁺ATPase, inhibits also the H⁺pumping activity of spinach leaves PM. Figure 2 shows the kinetic of inhibition by vanadate of the initial rate of H⁺pumping measured as described in

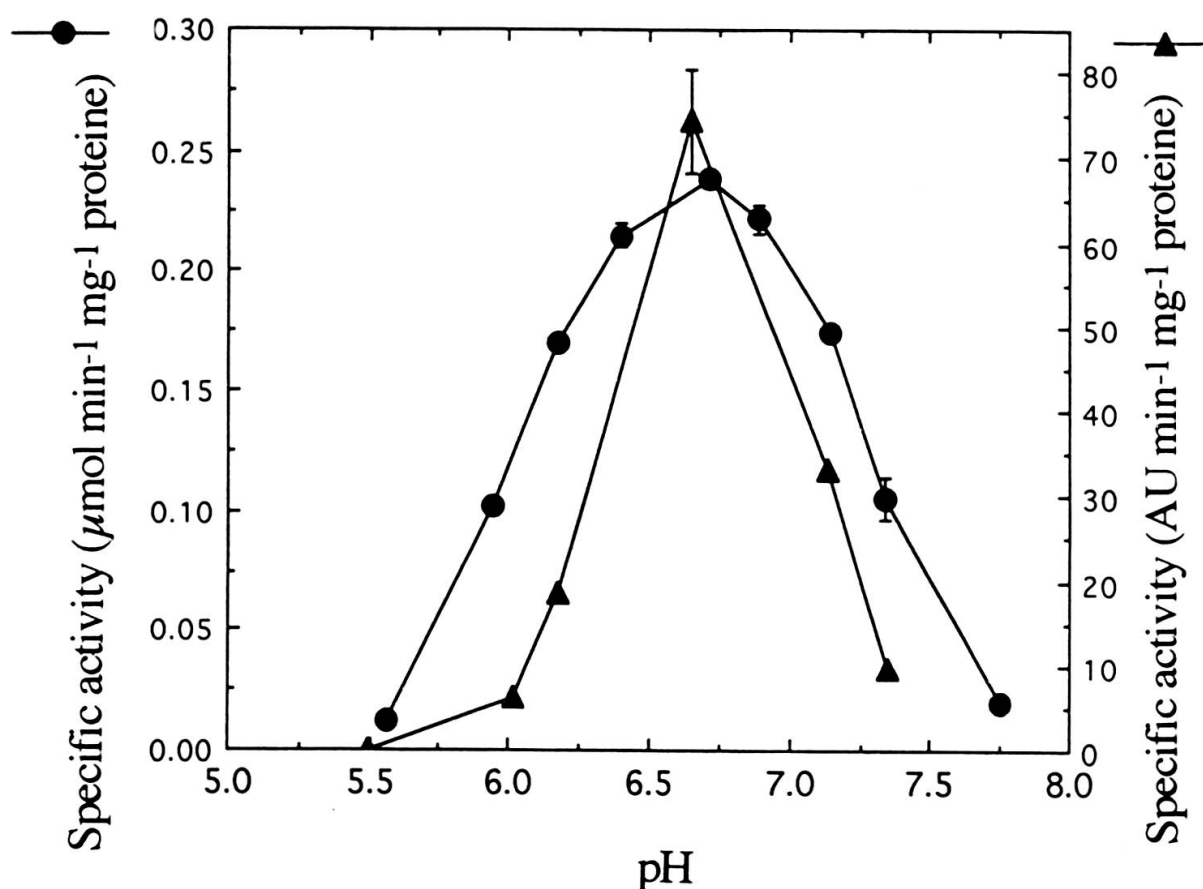


FIG. 1.

pH effect of the reaction medium on the ATP hydrolase and H⁺pumping activities of the purified plasma membrane as described in Materials and Methods. The pH of the medium was adjusted by varying the proportions of BTP and Mes (50 mM final concentration).

Materials and Methods. The inhibition increases with vanadate concentration over the whole concentration range tested. The concentration of vanadate required to inhibit the H⁺ATPase activity by 50% (I_{50}) was about 160 μ M, higher than that found for the same enzyme in other plants (Rasi-Caldogno *et al.*, 1985; De Michelis & Spanswick, 1986; Olivari *et al.*, 1992). However, the I_{50} value of vanadate depends on the plant species and on the experimental conditions for the same plant specie (Rasi-Caldogno *et al.*, 1985; De Michelis & Spanswick, 1986).

Substrat specificity of the H⁺pumping activity

Table 1 shows that the H⁺pumping requires rather ATP as source of energy than other phosphorylated substrats tested. The apparent Michaelis-Menten constant ($K_{m,app}$) for ATP was 0.697, similar to that obtained for the same enzyme in other plant species (Rasi-Caldogno *et al.*, 1985; De Michelis & Spanswick, 1986).

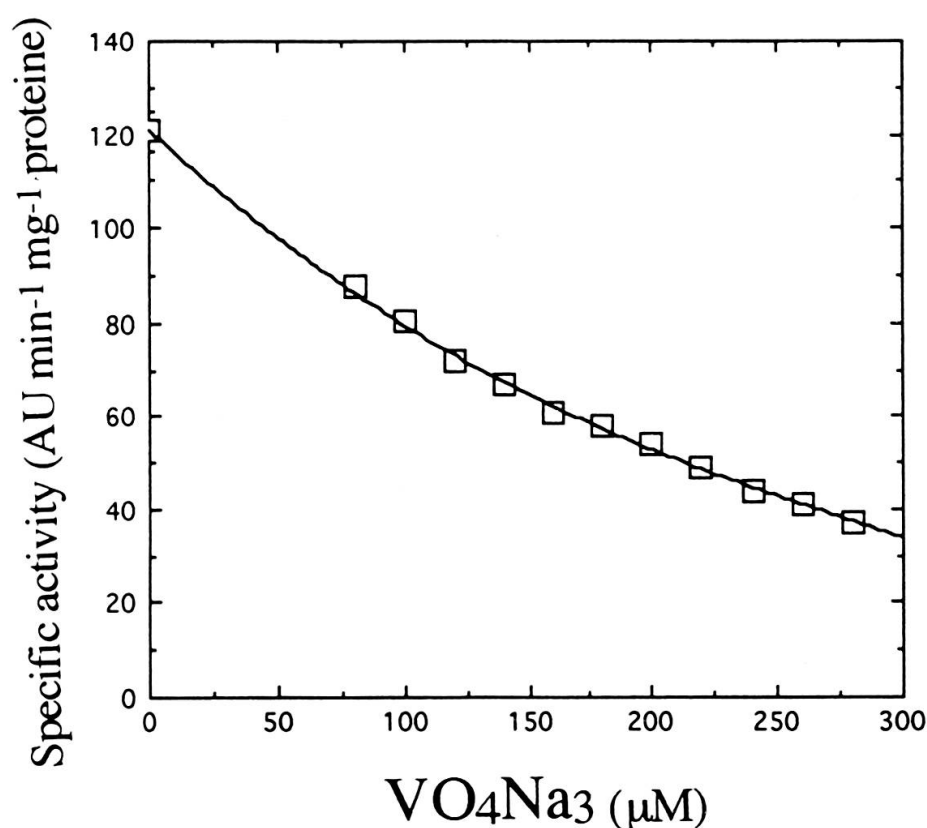


FIG. 2.

Effect of increased vanadate concentration on the ATP dependent H^+ pumping activity of the purified plasma membrane. The reaction was started by addition of 1.5 mM Mg ATP, after a 10 min pre-incubation at room temperature in presence of indicated concentration of vanadate.

TABLE 1.

Effect of some phosphorylated substrats on H^+ pumping activity of the purified plasma membrane as described in Materials and Methods. The reaction was started by addition of 1.5 mM of the substrat, and the activities were expressed in $\text{AU min}^{-1} \text{mg}^{-1} \text{protein} \pm$ standard deviation. All the substrats were sodium salt except the PEP which was tricycloammonium salt. The reaction medium contained 5 mM MgSO_4 . Values between brackets represent the activities in the presence of various substrats as a percent of the activity in presence of ATP.

Substrate	Initial rate of substrat-dependent quenching of quinacrine fluorescence ($\text{AU min}^{-1} \text{mg}^{-1} \text{protein}$)
ATP	61.3 ± 4.7 (100)
GTP	4.0 (6.5)
PPi	13.7 (22.3)
ADP	16.3 (26.6)
PEP	10.3 (16.8)

Effect of some inhibitors on H⁺pumping activity

H⁺pumping was tested in presence of Ca²⁺ channel antagonist (verapamil), animal Na⁺/K⁺ ATPase inhibitor (ouabain), plant ATPase inhibitors (EB, DES, VO₄, DCCD), and photosynthetic electron transport inhibitor (DCMU).

The obtained results indicated that the plant ATPase inhibitors, inhibited the H⁺pump activity of spinach leaves with more effective inhibition obtained for VO₄ and DCCD than for EB or DES. Some experimental problems with these later inhibitors have limited the study of their effect on the H⁺ATPase according to their concentration.

The ouabain had almost no effect on the H⁺pump suggesting that the secondary structure of the animal Na⁺/K⁺ATPase is different from that of the spinach H⁺ATPase. Surprisingly DCMU inhibited also the enzyme by about 50% at the indicated concentration. This could support the mechanism of the H⁺ transport by the ATPase proposed by Briskin *et al.* (1992).

The verapamil known as a plant Ca²⁺ channel antagonist inhibited strongly the H⁺ATPase of spinach leaves. This result could explain, in the context of the acid growth theory, the inhibition of the elongation rate of spinach petiole as described by Malatyal (1990).

TABLE 2.

Effect of some inhibitors on the H⁺pumping activity of the spinach leaves plasma membrane. The plasma membrane was incubated in the reaction medium in the presence of the inhibitors for 10 min and the reaction was started by addition of 1.5 mM Mg ATP. The activities in the presence of various inhibitors were expressed as the percent of the activity of the control (without inhibitors). Stock solutions of DCCD and DES were prepared in ethanol and the specific activities were compared to the activity of the control assay containing 1% ethanol which is very similar to that of the control without ethanol.

Inhibitors	Percent of the specific activity to the control
Control	100
Verapamil 100μM	23
Ouabain 100μM	93
DCMU 100μM	47
Erythrosin B	
1μM	91
2.5μM	67
DES	
30μM	85
100μM	63
Na ₃ VO ₄	
20μM	95
200μM	49
2000μM	23
DCCD 500μM	22

SDS PAGE and Western blot

SDS-PAGE of the purified PM of spinach leaves revealed after Coumassie brilliant blue G250 staining, a large number of bands of different molecular weight, particularly a polypeptide of about 100 kDa (fig. 3 A) which corresponds in molecular mass to the PM ATPase. This polypeptide cross-reacted with the polyclonal antibodies directed against the PM ATPase of *Arabidopsis thaliana* (arrow), kindly given by Prof. R. Serrano (fig. 3 B). On the same blot we can observe a contaminating band of lower molecular weight which probably resulted from proteolysis of the 100 kDa polypeptide.

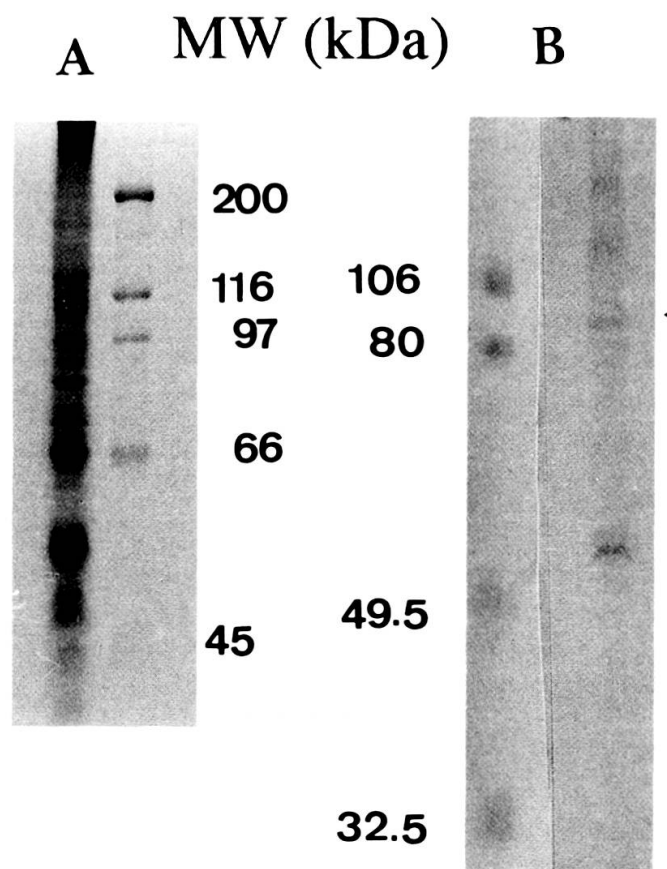


FIG. 3.

A. SDS polyacrylamide gel electrophoresis of the purified plasma membrane (90 μ g protein). The polypeptides were stained by Coumassie brilliant blue G250. **B.** immunoblot analysis of the purified plasma membrane. The arrow indicates the polypeptide of about 100 kDa. The experimental procedure was described under Materials and Methods.

ACKNOWLEDGMENTS

We would like to thank Professor R. Serrano from Department of Biotechnology (Valencia, Spain) for his kind gift of polyclonal antibodies against PM ATPase of *Arabidopsis thaliana*.

RÉSUMÉ

La membrane plasmique des feuilles d'épinard a été purifiée par partition de phase et l'activité pompe à proton associée à la membrane plasmique caractérisée. Elle présente un pH optimum à 6.7 similaire à celui de l'activité ATPase, et une spécificité du substrat plus évidente pour l'ATP avec un $K_{m_{app}}$ de 0.697. L'activité de cette pompe est plus ou moins inhibée par différents inhibiteurs des ATPases, et spécialement par le vanadate avec un I_{50} de 160 μ M. L'électrophorèse sur gel de polyacrylamide, des protéines de la membrane plasmique purifiée, a révélé la présence d'une bande à 100 kDa qui a réagit avec les anticorps polyclonaux anti ATPase de la membrane plasmique de *Arabidopsis thaliana*.

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