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Dipartimento di Agrobiologia e Agrochimica, Università della Tuscia, Viterbo, Italia
Istituto di Produzione Vegetale, Università di Udine, Italia

Characterization of a potassium-stimulated ATPase in membrane fraction isolated from roots of grapevine seedlings

by

Z. VARANINI, G. POLETTINI, R. PINTON and A. MAGGIONI

Caractérisation d'une ATPase stimulée par potassium dans une fraction de membrane isolée de racines de plants de semis de vignes

Résumé : Une fraction microsomale qui possède activité ATPasique dépendant du Mg^{2+} et stimulée par le K^+ a été extraite de racines de plantules de semis de vigne (*Vitis vinifera* L., cv. Verduzzo) au moyen de centrifugation différentielle.

La production des racines a été stimulée par GA_3, et par application de traitements capables de contrôler la contamination microbienne.

Les caractéristiques biochimiques de l'activité ATPasique ont été étudiés et comparées avec les résultats déjà obtenus avec les racines de boutures.

L'addition de choline-Cl, éthanolamine et de glycérol-1-P en plus de BSA, PVPP, EDTA et DTT au milieu d'homogénisation était nécessaire pour enregistrer le composant de l'activité stimulé par K^+.

L'enzyme activé par Mg^{2+} et stimulé par des ions monovalents, montre préférence pour l'ATP comme substrat et un optimum de pH de 6,5 en présence de Mg^{2+} et K^+.

L'efficacité de stimulation des différents ions monovalents suit une séquence semblable à celle enregistrée pour les préparations des racines de céréales, mais très différente comparée à la préparation des racines de boutures.

L'activité ATPasique était inhibée par le vanadate et le DES, alors que le molybdate et l'azide avaient peu ou aucune d'efficacité.

L'activité ATPasique montre une simple cinétique de Michaelis-Menten lorsque l'on augmente la concentration du substrat ATP : Mg et un profil complexe d'une coopérativité négative possible pour la stimulation de K^+.

Les microsomes fractionnés au moyen du gradient de saccharose ont montré un enrichissement de vésicules présumées de plasmalemma à une densité de 1,10—1,15 g ml^{-1}.

Ce paramètre distingue cette fraction de préparations semblables contenant ATPase du plasmalemma obtenues des racines de différentes plantes annuelles.

Key words: seed, root, cell, enzyme, extraction, analysis, potassium, magnesium, cation, additive, acidity.

Introduction

The central role of the plasma membrane ATPase as a biochemical mechanism of energy coupling for plant nutrition has been widely stressed (see LEONARD 1984). It has been shown in both native and reconstituted vesicles (SZE 1985) that this enzyme can utilize energy derived from ATP hydrolysis to pump protons, thus producing the electrochemical gradient required for solute uptake. In spite of the importance of this subject, our knowledge about the characteristics of the plasmalemma ATPase comes from studies on a small number of plant species. Nevertheless, differences between species, mainly in pH optima and sensitivity to monovalent ions (BRISKIN and THORNLEY 1985; SOMMARIN *et al.* 1985), have already been recorded. Moreover, negative effects of divalent cations were observed on microsomal ATPase from fruit tissues (BEN-ARIE and FAUST 1980; LURIE and BEN-ARIE 1983). The study of ATPase systems from plants not widely investigated and from different tissue systems of the same plant will improve

our knowledge about the variability of the biochemical basis of ion movements across membranes. It may also clarify the relationships between membrane enzyme processes and agronomic performance of crops (WIGNARAJAH *et al.* 1985).

We are particularly interested in the mineral nutrition of grapevine and in the study of ion uptake mechanisms of this woody species. As reported in a previous paper (VARANINI and MAGGIONI 1985), roots obtained from woody cuttings of grapevine possess Mg^{2+}-dependent and K^+-stimulated ATPase activity associated with membranous vesicles of the microsomal fraction. It is supposed that this activity is representative of plasmalemma ATPase. It could be shown only by adding to the homogenization medium (HODGES and LEONARD 1974) protectants able to bind phenolic compounds and free fatty acids.

In our experimental conditions (VARANINI and MAGGIONI 1985), roots from woody cuttings could be obtained only within a short period during the year. Furthermore, the amount of root tissue which could be collected from each cutting was scarce and distributed over a quite long time (1—2 g of root tissue in about 40 d). These facts made it practically impossible to get enough material to purify and biochemically characterize the enzyme activity.

Therefore, we tried to obtain roots from grape seeds, with the aim of obtaining roots all year long, as usual for cereals and other annual crops, and in an amount suitable for this kind of research. The present work deals with the biochemical characteristics of ATPase activity of the microsomal fraction of grape roots obtained from germinating seeds. Furthermore, a partially purified plasma membrane fraction was identified in a discontinuous sucrose density gradient. In this paper we show that roots from grape seeds can be obtained by applying treatments that stimulate germination and prevent microbial contamination. Our procedure, similar to that used by ELLIS *et al.* (1983), was developed independently in our laboratory at the same time. By this way it was possible to obtain roots all year long. Seedling roots appeared morphologically different from woody cutting roots, namely shorter and thinner. Therefore, the yield of roots remained quite low, ranging from 10 to 15 mg of fresh root tissue per seed in 16—18 d.

For this reason, it was necessary to arrange a miniature density gradient separation and we could use this technique only to ascertain membrane densities and to confirm the biochemical characteristics of ATPase activity recorded for the unfractionated microsomal fraction.

Preliminary results of this research were presented at the 25th SIFV meeting (S. Margherita Ligure, 1985) and at the 5th FESPP Congress (Hamburg, 1986).

Materials and methods

Plant material

Grape seeds (*Vitis vinifera* L. cv. Verduzzo) were collected from mature clusters, cleaned, desiccated and stored in the dark at 10 °C and 50 % R.H. After soaking in running tap water for 48 h, they were surface sterilized with 8 % (w/v) $Ca(ClO)_2$ for 20 min and treated with concentrated H_2O_2 (30 % v/v) for 4 h. In order to stimulate germination, seeds were treated for 48 h with 1000 ppm gibberellic acid (GA_3). After rinsing, seeds were sown in Petri dishes, between two discs of sterilized filter paper moistened with a solution containing 1 mM $CaSO_4$, 100 ppm Benomyl and 1 mg (4800 USP units) ml^{-1} Nystatin (Sigma) in order to control bacterial and fungal contamination. Antibiotic treatment changed neither the percentage of seed germination nor the growth rate of roots. Seedlings were grown in the dark at 25 °C, 95 % R.H. for 15—20 d.

Isolation of microsomal fraction

Roots of 2—4 cm in length (15—20 g fresh weight) were collected and washed in cold distilled water and homogenized using pestle and mortar according to HODGES and LEONARD (1974), with minor changes suggested by VARANINI and MAGGIONI (1985) for grape roots from woody cuttings. Two different media were used: the first (M1) contained 0.25 M sucrose, 3 mM EDTA, 8 mM dithiothreitol (DTT), 0.1 % (w/v) bovine serum albumin (BSA), 10 % polyvinyl-polypyrrolidone (PVPP), in 25 mM Tris-MES at pH 7.2; the second (M2) was prepared by adding to M1: 4 % (v/v) ethanolamine, 10 mM sodium glycerol-1-phosphate and 4 % (w/v) choline-Cl adjusted to pH 7.2 with 2N H_2SO_4. The root homogenate was squeezed through four layers of cheesecloth and centrifuged for 5 min at 1,000 *g*. The supernatant was collected and the microsomal fraction was obtained by differential centrifugation (13,000—82,500 *g* pellet), as described by HODGES and LEONARD (1974). This was used for enzyme assays and further fractionation.

Sucrose density gradient centrifugation

Because of the scarce quantity of microsomal membrane vesicles obtained from roots of grape seedlings we had to perform sucrose gradient separation in a 5 ml ultracentrifuge tube. The microsomal pellet was resuspended by syringe and a 25-gauge needle 100—200 µl 18 % (w/w) sucrose solution containing 1 mM Tris-MES pH 7.2 and 5 mM DTT, and layered onto a 3.7 ml gradient consisting of 0.5 ml 45 %, 1.7 ml 34 % and 1.5 ml 25 % (w/w) sucrose in 1 mM Tris-MES pH 7.2. After centrifugation for 120 min at 95,000 *g*, vesicles at the interface of sucrose solutions of different density and the pelleted material were collected and used for enzyme assays.

Enzyme assays

ATPase activity was measured by determining released inorganic phosphate (Pi) according to FISKE and SUBBAROW (1925). Aliquots of enzyme preparations (20—40 µg protein) were assayed in 1 ml of solution containing 3 mM ATP (Tris-salt), 33 mM Tris-MES at pH 6.5, 3 mM $MgSO_4$ and 50 mM KCl. The reaction was usually run for 30 min at 37 °C in a shaking water bath, and stopped with 2 ml of 2 % ammonium molybdate in 2N sulfuric acid. Changes in assay conditions are indicated within the results. The vanadate stock solution (5 mM) was prepared according to GALLAGHER and LEONARD (1982) dissolving V_2O_5 in 20 mM NaOH and allowing to stand overnight at room temperature.

Malate dehydrogenase (MDH) activity was measured at 338 nm by following the oxidation of NADH in 2.7 ml of 25 mM tricine-NaOH pH 8.5, 6mM oxalacetic acid and 2 mM NADH.

Trichloroacetic acid precipitable protein was determined by the method of LOWRY *et al.* (1951) using BSA as a standard.

Results

Extraction procedure

Previous results (VARANINI and MAGGIONI 1985) obtained with roots from grape woody cuttings demonstrated that microsomes with measurable activity of K^+-stimulated ATPase could be obtained only when several compounds, able to inhibit hydrolytic enzymes active on polar lipids, were added to the homogenization medium, as first

Table 1

ATPase activity of microsomes from grape seedling roots homogenized with media containing different sets of additives: EDTA, DTT, BSA, PVPP (M1); as M1 plus ethanolamine, glycerol-1-P, choline-Cl (M2)

Activité ATPasique des microsomes des racines de plantules de semis de vigne homogénisées avec les milieux d'extraction contenant des groupes differents d'additifs: EDTA, DTT, BSA, PVPP (M1); comme M1 auquel on ajoute éthanolamine, glycérol-1-P, choline-Cl (M2)

Homogenizing media	ATPase activity (μmol Pi/mg prot · h) in the presence of		
	None	3 mM Mg^{2+}	50 mM $K^{+1)}$
M1	3.9	9.5	9.7
M2	3.9	9.9	13.6

[1]) In the presence of 3 mM Mg^{2+}.

indicated by SCHERER and MORRÉ (1978). Therefore, in the initial experiments, two solutions (M1 and M2) containing different additives were tested as homogenizing media for roots from grape seedlings. ATPase activities of microsomes extracted with both methods were compared. Table 1 shows that the two preparations behave similarly in terms of activity in the absence and in the presence of Mg^{2+}, both being stimulated by the divalent cation in a similar way. However, when assayed in the presence of both Mg^{2+} and K^+, only the microsome preparation obtained after homogenization with all preservative compounds added (M2 medium) showed significant K^+ stimulation. Therefore, the possibility of recording the K^+ effect on ATPase seems to be dependent on the presence in the grinding medium of choline, glycerol-1-phosphate and ethanolamine, which are thought to limit activity of hydrolases on polar lipids of membranes. In subsequent experiments, the complete (M2) medium was constantly used in order to obtain preparations with measurable levels of monovalent ion stimulation.

Table 2

Phosphohydrolase activities of microsomes from grape roots with various substrates · K^+-ATPase was calculated as explained in Fig. 1

Activités phosphohydrolasiques des microsomes des racines de vigne avec des substrats différents · K^+-ATPase était calculé comme expliqué dans la Fig. 1

Substrate 3 mM	Phosphohydrolase activity (μmol Pi/mg prot · h)		
	3 mM Mg^{2+}	50 mM $K^{+1)}$	K^+-ATPase
ATP	10.3	14.3	4.0
ADP	4.4	4.7	0.3
GTP	8.5	10.3	1.8
CTP	4.8	4.7	—
UTP	5.7	5.3	—
PNPP	4.3	4.4	0.1

[1]) In the presence of 3 mM Mg^{2+}.

Effect of pH

Mg^{2+}-dependent and Mg^{2+} + K^+-stimulated ATPase activities of the microsomal fraction were measured in the pH range 5—9, using Tris-MES and Tris-HEPES buffered assay solutions. Optimum pH of ATPase activity in the presence of Mg^{2+} was between 6.5 and 7.0, whereas in the presence of both Mg^{2+} and K^+ it was at pH 6.5 (Fig. 1). Optimum pH for K^+ stimulation was at pH 6.0. The pH values recorded and the shift in the two activity peaks are in accordance with those generally observed for plant plasma membrane ATPases (LEONARD 1984). Activities and K^+ stimulation at pH

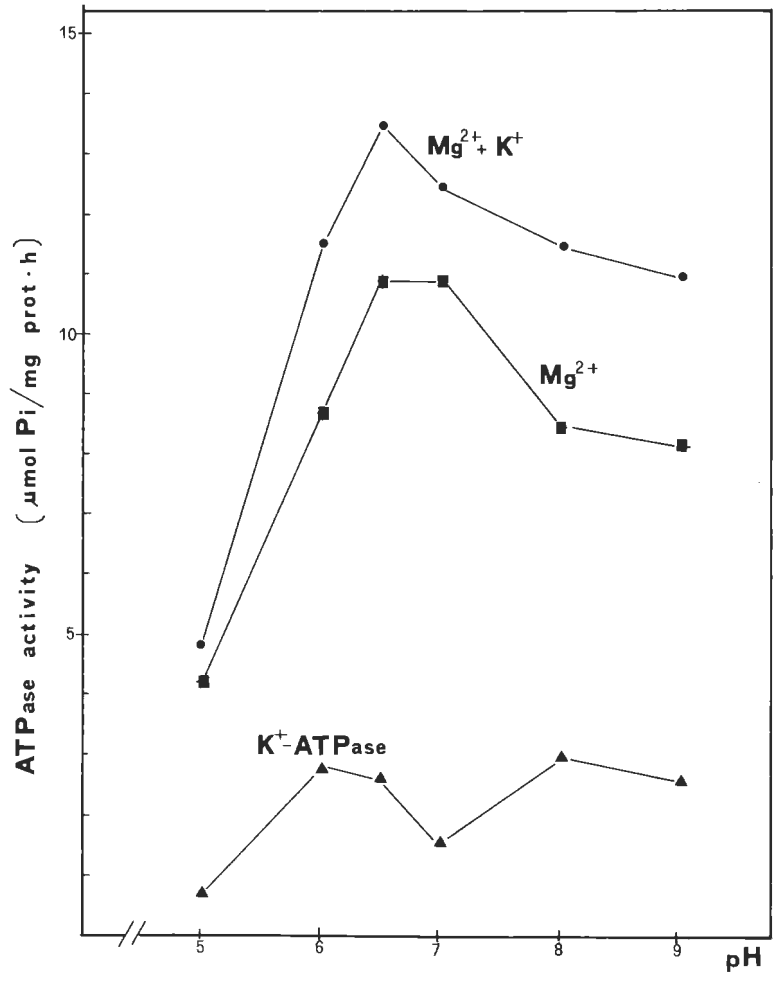

Fig. 1: Effect of assay pH on ATPase activity associated with microsomes from grape seedling roots. K^+-ATPase activity was calculated by subtracting activity in the presence of Mg^{2+} from activity in the presence of Mg^{2+} + K^+.

Effet du pH sur l'activité ATPasique des microsomes des racines de plantules de semis de vigne. L'activité K^+-ATPasique avait été calculée par la sustraction de l'activité en présence de Mg^{2+} de l'activité en présence de Mg^{2+} + K^+.

8.0—9.0 can be attributed to mitochondrial particles contaminating the microsomal preparation.

Substrate specificity

Table 2 shows that in the presence of Mg^{2+} ATP was clearly the preferred substrate. This preference was even more evident when the K^+-stimulated component of the activity was considered. In this case, with the exception of GTP, the specificity was almost absolute.

Effects of cations

Among the divalent cations (Table 3), the highest capacity of stimulating ATPase activity was shown by Mg^{2+} followed by Mn^{2+} at a slightly lower level, whereas Zn^{2+} and Ca^{2+} were almost without effect. The capacity of K^+ to further stimulate enzyme activity seems to be strictly related to the presence of Mg^{2+}, as shown by over 70 % reduction of the K^+ effect when Mn^{2+} or Zn^{2+} replaced Mg^{2+}, as well as the absence of K^+ stimulation when Ca^{2+} was used. Stimulation of ATPase activity above the Mg^{2+}-dependent level was caused by each one of the monovalent cations tested (Table 4). However, the highest level of stimulation was obtained in the presence of K^+, the effect being slightly lower in the presence of Rb^+ and NH_4^+. Much lower levels of stimulation were obtained with Na^+, Cs^+, or Li^+.

Table 3

Effect of divalent cations on ATPase activity of microsomes from grape roots · K^+-ATPase represents the activity in the presence of K^+ and divalent ion minus activity in the presence of divalent ion alone · Values in parentheses are percentages

Effets des cations divalents sur l'activité de l'ATPase des microsomes des racines de vigne · Le K^+-ATPase représente l'activité en présence de K^+ et du ion divalent moins l'activité en présence seulement du ion divalent · Les valeurs entre parenthèses sont des pourcentages

Divalent cation 3 mM	ATPase activity (μmol Pi/mg prot · h)			
	3 mM Mg^{2+}	50 mM K^+[1])	K^+-ATPase	
None	4.1	—	—	
$MgSO_4$	10.8	14.5	3.7	(100)
$MnSO_4$	9.9	11.0	1.1	(30)
$ZnSO_4$	5.5	6.3	0.8	(22)
$CaSO_4$	4.3	4.4	0.1	(3)

[1]) In the presence of 3 mM divalent cation.

Inhibitors

The effects of inhibitors of plasma membrane ATPase were studied by utilizing the microsomal fraction (Table 5). K^+-ATPase activity was 80 % inhibited by 50 μM vanadate while the inhibition exerted by 100 μM DES was about 50 %. DCCD (10 μm) and 2-IB (0.25 mM) caused a 25—30 % inhibition. Sodium azide (1 mM), an inhibitor of mitochondrial ATPase, reduced enzyme activity by 29 %. Sodium molybdate (1 mM) had no effect, indicating the absence of unspecific phosphatase activity in grapevine microsome preparations. When the effects of sodium azide and vanadate were assayed at pH 9.0, which is the optimum pH of mitochondrial ATPase, the inhibition caused by the first compound increased to 54 %, while that of vanadate decreased to 35 %

Table 4

Effect of monovalent cations on ATPase activity of microsomes from grape roots · Values were calculated by subtracting activity in presence of 3 mM $MgSO_4$ from activity in presence of Mg^{2+} plus monovalent ion · Values in parentheses are percentages

Effect des cations monovalents sur l'activité ATPasique des microsomes des racines de vigne · Les valeurs sont le résultat de la soustraction de l'activité en présence de 2 mM $MgSO_4$ de l'activité en présence de Mg^{2+} avec le ion monovalent · Les valeurs entre parenthèses sont des pourcentages

Cation 50 mM	Ion stimulated ATPase (µmol Pi/mg prot · h)	
KCl	3.5	(100)
RbCl	3.1	(89)
NH_4Cl	2.7	(77)
NaCl	2.0	(57)
CsCl	1.7	(49)
LiCl	1.5	(43)

Table 5

Effect of inhibitors on K^+-ATPase activity associated with microsomes from grape seedling roots · K^+-ATPase activity was calculated as indicated in Fig. 1

Effet des inhibiteurs sur l'activité de K^+-ATPase des microsomes des racines des plantules de semis de vigne · K^+-ATPase a été calculée comme expliqué dans la Fig. 1

Treatment		K^+-ATPase (µmol Pi/mg prot · h)	Inhibition (%)
Control		4.6	
Vanadate	50 µM	0.6	87
NaN_3	1 mM	3.3	28
Na_2MoO_4	1 mM	4.6	0
Control EtOH	2 % (v/v)[1])	4.3	
DES	100 µM[1])	2.1	51
DCCD	10 µM[1])	3.2	26
2-IB	0.25 mM[1])	3.1	28

[1]) Membrane preparations were incubated for 10 min at 2 °C with inhibitors prior to ATPase assay.

(Table 6). Therefore we can conclude that most of ATPase activity shown at pH 6.5 can be attributed to plasmalemma ATPase.

Enyzme kinetics

The Mg^{2+}-dependent and K^+-stimulated ATPase activities of microsomes from roots of grape seedlings were tested in the concentration range $3 \cdot 10^{-4}$ — $5 \cdot 10^{-3}$ M of the Mg : ATP substrate complex. It showed a Michaelis-Menten saturation curve (Fig. 2) with apparent K_m of 0.48 mM in the presence of Mg^{2+} and 0.53 mM in the presence of both Mg^{2+} and K^+. The addition of K^+ affected also the V_{max} which increased from 13.5 to 16.8 µmol Pi/mg prot·h.

Table 6

Effect of vanadate and NaN_3 at pH 6.5 and 9.0 on K^+-ATPase activity associated with microsomes from grape seedling roots · K^+-ATPase was calculated as indicated in Fig. 1 · Values in parentheses are percentages of inhibition

Effet du vanadate et du NaN_3 à pH 6,5 et 9,0 sur l'activité K^+-ATPasique associé aux microsomes des racines de plantules de semis de vigne · K^+-ATPase a été calculée comme expliqué dans la Fig. 1 · Les valeurs entre parenthèses sont les pourcentages d'inhibition

Treatment	K^+-ATPase (µmol Pi/mg prot · h)			
	pH 6.5		pH 9.0	
Control	4.0	(0)	3.9	(0)
Vanadate 50 µM	0.7	(82)	2.5	(35)
NaN_3 1 mM	2.8	(30)	1.8	(54)

The effect of different concentrations of K^+ on the K^+-stimulated ATPase activity is shown in Fig. 3. The pattern obtained did not fit a simple Michaelis-Menten saturation curve, but rather a negative cooperativity model as confirmed by the Eadie-Hofstee plot of data.

Purification on discontinuous sucrose gradient

Microsomal vesicles from different subcellular membranous systems can be separated on the basis of their densities (HODGES and LEONARD 1974). This method was applied to localize plasma membrane vesicles on a 3.7 ml discontinuous sucrose density gradient (25/34/45 % w/w). Membrane vesicles of different origin were tentatively identified both by measuring K^+-ATPase and MDH activities and by testing the effects of vanadate, sodium azide and KNO_3 on the ATP-phosphohydrolase activity. As shown in Table 7, fractionation through the sucrose gradient allowed separation of a conspicuous pellet of low K^+-ATPase specific activity. All fractions of defined density at the interfaces of sucrose solutions had K^+-ATPase specific activity higher than that of unfractionated microsomes. However, the greatest enrichment was at the 25/34 % sucrose interface. Vesicles with vanadate sensitive Mg^{2+} + K^+-ATPase activity were also present in all fractions, but the highest level was at the 25/34 % interface. In this fraction, scarce inhibition of ATPase activity by sodium azide and KNO_3 indicated little contamination due to mitochondrial and tonoplast ATPase membranes. MDH activity showed two peaks of enrichment in the 18/25 % and 34/45 % fractions and a lower level in the 25/34 % fraction. Therefore, it is possible to consider the 25/34 % fraction as a partially purified preparation of plasma membrane vesicles.

Discussion

Among the biochemical characteristics of plasma membrane ATPase, the stimulation of enzyme activity by K^+ at neutral pH, though variable and dependent on species, should be considered a marker for the plasmalemma (QUAIL 1979). Recently, it has been shown that the K^+-stimulated component of ATPase activity in purified preparations of plasma membrane can be very low or even absent (YOSHIDA *et al.* 1983; BRISKIN and THORNLEY 1985). Therefore, it is still unclear how the ATPase acts and whether different mechanisms are present in different plant species and tissues (BEN-ARIE and FAUST 1980; LURIE and BEN-ARIE 1983). However, differences in the enzyme characteristics,

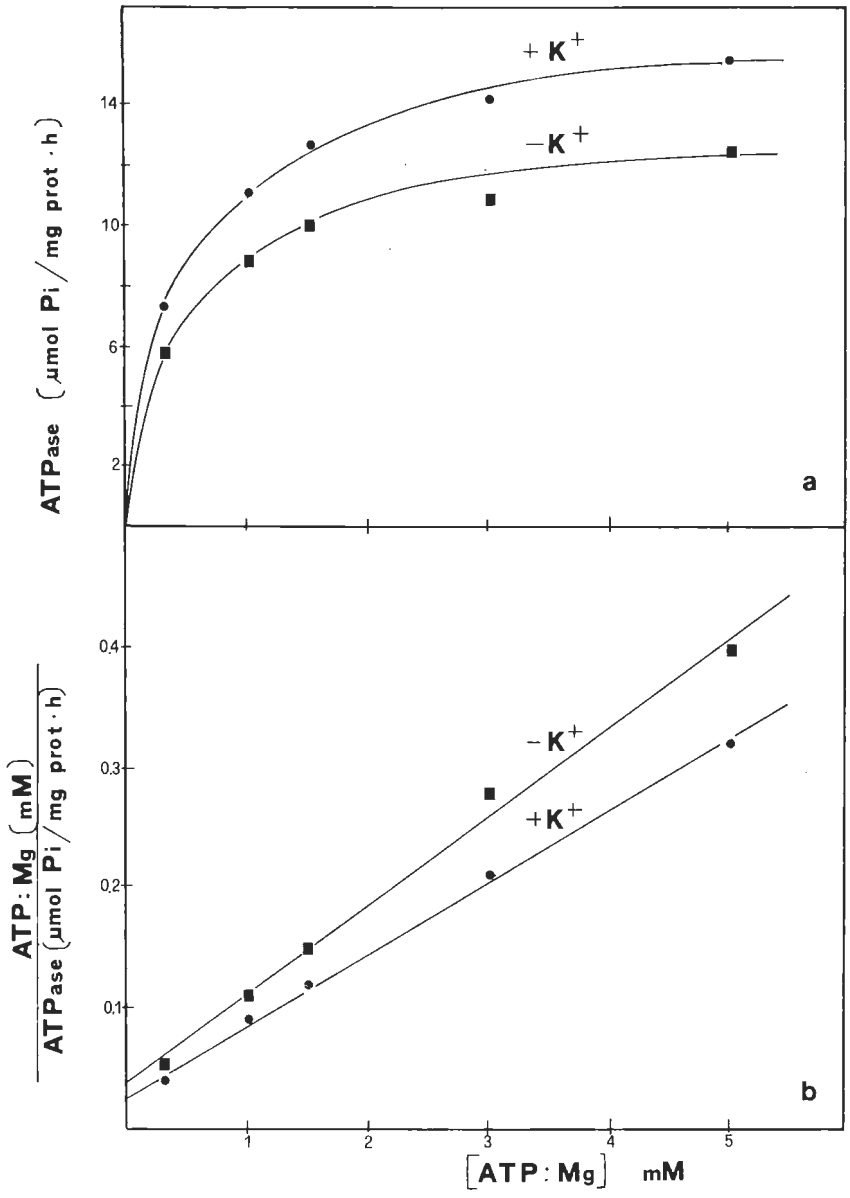

Fig. 2: a) Microsomal ATPase activity of grape seedling roots as a function of Mg : ATP concentration. — b) Data plotted according to linear transformation of Michaelis-Menten equation: $s/v = K_m/V_{max} + (1/V_{max})s$ as recommended by Dowd and Riggs (1965). — Kinetic constants, calculated by linear regression analysis, were K_m = 0.53 mM, V_{max} = 13.5 µmol Pi/mg prot · h in the absence of K^+, and K_m = 0.48 mM, V_{max} = 16.8 µmol Pi/mg prot · h in the presence of 50 mM K^+.

a) Activité ATPasique microsomale des racines de plantules de semis de vigne en fonction des concentrations du Mg : ATP. — b) Les données ont été élaborées selon la transformation linéaire de l'équation de Michaelis Menten: $s/v = K_m/V_{max} + (1/V_{max})s$ comme recommandé par Dowd et Riggs (1965). — Les constantes cinétiques calculées par une analyse de régréssion linéaire étaient K_m = 0,53 mM, V_{max} = 13,5 µmol Pi/mg prot · h en absence de K^+ et K_m = 0,48 mM, V_{max} = 16,8 µmol Pi/mg prot · h en présence de 50 mM K^+.

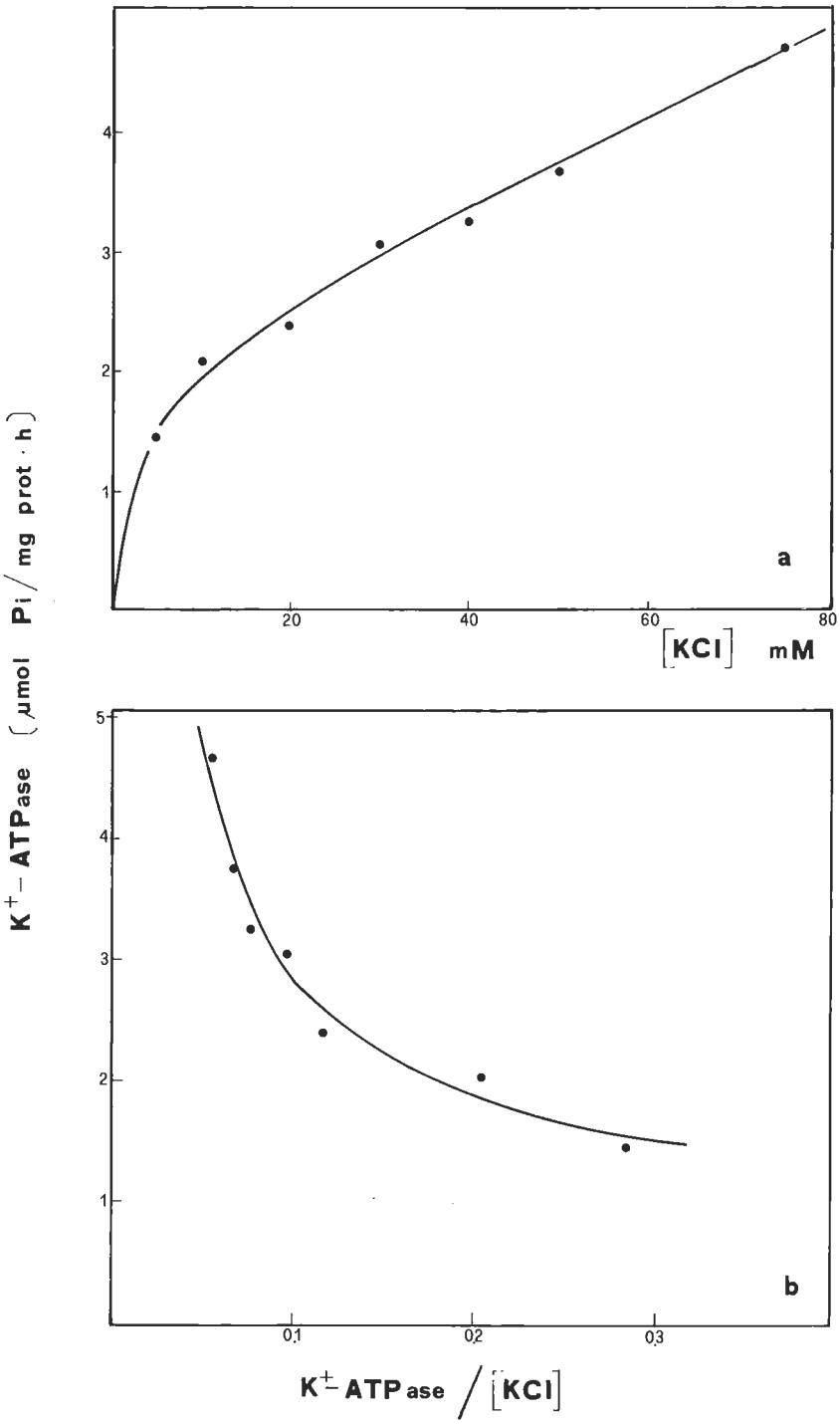
K+ – ATPase (µmol Pi / mg prot · h)
4
3
2
1
20
40
60
80
[KCl] mM
a
5
4
3
2
1
0,1
0,2
0,3
K+ ATPase / [KCl]
b

Table 7

Distribution of protein and enzyme activities in fractions obtained after centrifugation of microsomes on discontinuous sucrose density gradient · Control activity was assayed in the presence of 3 mM $MgSO_4$ and 50 mM KCl · Inhibitor-sensitive components of ATPase activity are indicated by the difference between the control activity and the activity in the presence of 50 µM vanadate (ΔVan) or 50 mM KnO_3 (ΔKNO_3) or 1 mM NaN_3 (ΔNaN_3)

Distribution de la protéine et des activités enzymatiques dans les fractions obtenues à la suite de la centrifugation des microsomes dans un gradient discontinu de densité de saccharose · L'activité de contrôle a été examinée en présence de 3 mM $MgSO_4$ et 50 mM KCl · Les composants de l'activité ATPasique sensibles aux inhibiteurs sont représentés par la différence entre l'activité de contrôle et l'activité en présence de 50 µM vanadate (ΔVan) ou 50 mM KNO_3 (ΔKNO_3) ou 1 mM NaN_3 (ΔNaN_3)

	Microsomes	Gradient fractions			
		18/25	25/34	34/45	Pellet
ATPase activity (µmol Pi/mg prot · h)					
Control	10.6	14.8	17.5	13.2	8.1
K^+-ATPase	2.5	4.2	5.8	3.1	1.3
ΔVan	5.8	7.9	13.2	10.1	3.8
ΔKNO_3	1.3	2.5	1.4	1.7	0.8
ΔNaN_3	2.6	1.2	1.8	3.3	2.0
MDH activity (µmol NADH ox/mg prot · h)	21.9	32.4	21.6	46.2	14.7
Protein (mg/fraction)	2.3	0.2	0.3	0.3	1.1

depending on either the extraction procedures or the homogenizing media, have been found (Du Pont and Hurkman 1985; Varanini and Maggioni 1985).Our results emphasize the importance of this. In fact, the K^+-stimulated component can be shown only when the enzymatic activities hydrolizing membrane phospholipids are inhibited by the appropriate additions to the homogenizing medium. It is also noteworthy that different sources of grape roots require different conditions in order to preserve K^+ stimulation. In fact, membrane preparations from seedling roots require compulsorily choline-Cl, ethanolamine and glycerol-1-P besides EDTA, PVPP, DTT and BSA, the last four compounds being sufficient to preserve activity in membrane preparations from roots of woody cuttings (Varanini and Maggioni 1985). Therefore, components and additions to the extraction medium must be carefully tested when the separation of membrane fractions with K^+-ATPase activity is pursued.

The biochemical characteristics of ATPase in the microsomal fraction of roots from grape seedlings are similar to those generally found in plasmalemma fractions of

Fig. 3: a) Influence of KCl concentration on K^+-ATPase activity of microsomes from roots of grape seedlings. K^+-ATPase was calculated as indicated in Fig. 1. — b) Eadie-Hofstee plot of data presented in (a).

a) Influence des concentrations du KCl sur l'activité K^+-ATPasique des microsomes des racines de plantules de semis de vigne. K^+-ATPase était calculée comme déjà expliqué dans le Fig. 1. — b) Elaboration de Eadie-Hofstee des données présentées en (a).

other plant roots. The microsomal ATPase of grape roots from seedlings is stimulated by monovalent and divalent ions in sequences of preference in accordance with those found for oat roots (SZE and HODGES 1977). Concerning divalent ions, the obtained sequence is the same as the one shown by microsomes obtained from woody cutting roots. The stimulatory effect of K^+ above the level of ATPase activity reached in the presence of divalent cations is also similar, with the only exception of the effect recorded in the presence of Mn^{2+} which is high in the preparation from woody cuttings (84 % of the activity with Mg^{2+}) and quite low in that from seedlings (only 30 % of the activity with Mg^{2+}). The difference between the two preparations is much more evident when we consider the effect of monovalent cations. In fact, the sequence of stimulation for woody cuttings ATPase was completely different from that recorded in this work. Microsomal ATPase from grape seedlings can discriminate among alkali ions in the same way as *in vivo* roots can discriminate selectively for transport of the same cations (SZE and HODGES 1977). This can be considered as an indication of correct functioning of ATPase, without membrane changes able to modify properties of membrane enzyme.

Optimum pH is around 7 for Mg^{2+}-dependent activity and 6.0 for that stimulated by K^+, which differs from the preparations obtained from woody cuttings characterized by a peak of K^+-stimulated ATPase at pH 6.5 and a very high raise of activity at more alcaline pH, probably caused by mitochondrial contamination. The enyzme is strongly inhibited by vanadate and DES, which are considered specific inhibitors of plasma membrane ATPase of plants, and shows a sharp preference for ATP as a substrate. DCCD and 2-IB showed somewhat lower inhibition than reported for other species (BRISKIN and POOLE 1983; MAGGIONI *et al.* 1984). This can be interpreted either as different susceptibility of grapevine root membranes to these compounds or as an effect of different isolation procedures, which can produce different results even with preparations from the same plant tissue (O'NEILL and SPANSWICK 1984).

Other properties of the membrane ATPase isolated from grapevine roots are very similar to those of other plants: namely, the K_m and V_{max} with Mg : ATP as a substrate and the kinetics of K^+ stimulation. The latter shows a complex pattern which better fits the negative cooperativitiy model (SEGEL 1975), the concentration range tested being too limited to identify multiple phases according to HÅVARSTEIN and NISSEN (1981). This result is sharply different from that observed with two woody cutting preparations showing a biphasic pattern with two saturable phases. It will be interesting to develop this point in future research considering the effects of extraction procedures and membrane integrity on the kinetics of K^+ stimulation of ATPase activity.

In sucrose density gradient the plasma membrane ATPase of cereal roots peaks at sucrose concentration between 34 and 45 %, corresponding to a density of 1.15—1.20 g ml^{-1} (LEONARD 1984). Other plants, however, showed to peak at lower densities (soybean 1.14 g ml^{-1} (HENDRIX and KENNEDY 1977), red beet 1.12 g ml^{-1} (BRISKIN and POOLE 1983)). Moreover, it is known that the density of plasma membrane vesicles changes depending on the vegetative status of the plant tissue (POOLE *et al.* 1984). Grapevine microsomes exhibited the maximum peak of K^+-stimulated activity in the 25—34 % sucrose fraction corresponding to a density of the supposed plasmalemma vesicles of 1.10—1.15 g ml^{-1}. This fraction shows a lower level of MDH activity, almost no inhibition in the presence of sodium azide and the strongest inhibition by vanadate. Therefore, it can be considered a partially purified preparation of grape plasmalemma vesicles.

In conclusion, the microsomal fraction from roots of grapevine seedlings possesses an ATPase activity similar to that found in plasmalemma-enriched fractions from roots of herbaceous plants.

Summary

A microsomal fraction possessing Mg^{2+}-dependent and K^+-stimulated ATPase activity was extracted by differential centrifugation from roots of grape seedlings (*Vitis vinifera* L. cv. Verduzzo).

Roots yield from grape seeds was stimulated by means of GA_3 and further improved by treatments able to control microbial contamination.

The biochemical characteristics of ATPase activity were studied and compared with those previously reported for roots produced by grape woody cuttings.

The presence of choline-Cl, ethanolamine and glycerol-1-P in addition to BSA, EDTA, PVPP and DTT in the homogenizing medium was obligatory in order to record the K^+-stimulated component of activity.

The enzyme was activated by Mg^{2+}, further stimulated by monovalent ions and showed strong preference for ATP as the substrate and optimum pH at 6.5 in the presence of both Mg^{2+} and K^+. The effect of different monovalent ions followed a sequence similar to that found in cereal roots preparations, but very different with respect to that recorded for preparations from roots of grape woody cuttings.

K^+-ATPase activity was inhibited by vanadate and DES whereas molybdate and azide had no or scarce effect. ATPase activity showed a simple Michaelis-Menten saturation with increasing ATP : Mg concentration, and a complex pattern of possible negative cooperativity for K^+ stimulation.

Microsomes fractionated using sucrose density gradient showed enrichment in plasmalemma vesicles at 1.10—1.15 g ml^{-1} density.

This parameter differentiates this fraction from similar preparations containing plasmalemma ATPase obtained from roots of various annual plants.

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Z. VARANINI
Dipartimento di Agrobiologia e Agrochimica
Università della Tuscia
Via S. Camillo de Lellis
01100 Viterbo
Italia

G. POLETTINI
R. PINTON
A. MAGGIONI
Istituto di Produzione Vegetale
Università di Udine
P. le Kolbe 4
33100 Udine
Italia