Phytochemistry, Vol 22, No 1, pp 49–52, 1983
Printed in Great Britain

0031-9422/83/010049-04$03 00/0

CHARACTERIZATION OF PLASMALEMMA ATPase FROM APPLE FRUIT

SUSAN LURIE and RUTH BEN-ARIE

Division of Fruit and Vegetable Storage, Agricultural Research Organization, The Volcani Center, P O Box 6, Bet Dagan 50250, Israel

(*Revised received* 17 *May* 1982)

Key Word Index—*Malus sylvestris*, Rosaceae, apple fruit, plasmalemma ATPase, cation effects

Abstract—The ATPase found in the 80 000*g* pellet of apple fruit showed a pH optimum of 6, was inhibited by divalent cations and stimulated by monovalent cations The enzyme was specific for ATP and inhibited by diethylstilbestrol and dicyclohexylcarbodiimide, while unaffected by oligomycin and the uncoupler SF 6874 The K_m for ATP was 0 48 mM, V_{max} 1 2 μmol Pi/mg protein/min Mg^{2+} was a competitive inhibitor to ATP, K_i 0 7 mM As the apple ripened from preclimacteric to postclimacteric, the ATPase activity increased more than two-fold

INTRODUCTION

Plasmalemma ATPases, requiring Mg^{2+} and stimulated by monovalent cations such as K^+ and Na^+, have been found in all plant material examined [1, 2] They are thought to play a role in monovalent cation and proton transport into and out of the cell [1, 2] An exception to this general picture was found in strawberries, where divalent cations such as Mg^{2+} and Ca^{2+} inhibited rather than stimulated the ATPase of the 80 000 *g* fraction [3] The pattern of inhibition and ATPase activity changed during ripening of the strawberries [3] This inhibition by divalent cations is in contradiction to the finding that in other plant tissues the substrate of the plasmalemma ATPase is Mg–ATP [4, 5] It was of interest, therefore, to determine whether other fruit plasmalemma ATPases were similar to that of strawberries Thus, work was carried out in an attempt to characterize the plasmalemma ATPase from apple fruit

RESULTS

ATPase activity was found in all particulate fractions prepared from apple tissues—1000, 13 000 and 80 000 *g*, but not in the supernatant Most of the activity in the 80 000 *g* pellet was concentrated on the plasma membrane, as shown by further purification on a sucrose density gradient (Fig 1) ATPase activity was concentrated in the 36% sucrose while acid phosphatase activity was high at the top and bottom of the gradient and low in this region Cytochrome *c* oxidase activity was only found at the very bottom of the gradient (data not shown), indicating minimal mitochondrial contamination The ATPase activity is at a similar density (36–38% w/w sucrose) as that identified as the plasmalemma with other plant material [2, 4, 11]

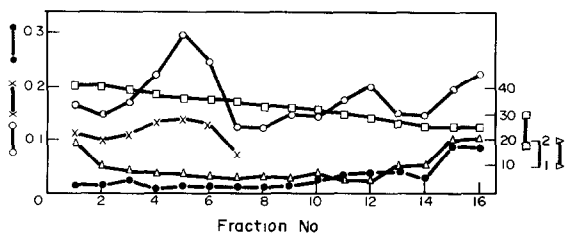

Fig 1 Sucrose density gradient fractionation of the 80 000 *g* pellet % sucrose (□), protein (μg/reaction) (△), ATPase (A_{655}) $-Mg^{2+}$ (○), $+Mg^{2+}$ (×), acid phosphatase (A_{410}) (●)

ATPase activity in the 80 000 *g* fractions from apples at different stages of ripening showed an increase as the apples ripened (Table 1) The greatest increase occurred from the climacteric peak to the post-climacteric stage The effect of ions on ATPase activity also changed as the fruit ripened Inhibition by Mg^{2+} increased as the climacteric rise began and was greatest at the climacteric peak Inhibition by Ca^{2+} also increased as the climacteric began but showed no maximum at the climacteric peak K^+ enhancement of the ATPase was greatest in preclimacteric apples and declined as the climacteric rise began

The pH optimum for ATPase with no ions present was found to be 6 (Fig 2) There was another small peak in activity at pH 8 5, but with much lower activity than the acid ATPase In the presence of 3 mM calcium chloride or magnesium chloride the pH optimum was broader, extending from pH 5 to 6 or 6 5, and the activity was lower Mg^{2+} inhibited ATPase activity to a greater extent than did Ca^{2+} In the presence of ions, the peak at pH 8 5 was not observed

The effect of divalent and monovalent cations is shown in Tables 2 and 3 Mn^{2+} and Ca^{2+} had little effect at 1 mM but inhibited similarly to Mg^{2+} at 3 mM The inhibitory effect of Ca^{2+} was variable for unknown reasons Sometimes inhibition was much less than that of Mg^{2+} at 3 mM and sometimes it was equal to Mg^{2+} Fe^{2+} and Cu^{2+} both inhibited ATPase more than Mg^{2+} At 3 mM the color of the copper sulphate solution interfered with the ATPase assay To see whether the inhibitory effect of Cu^{2+} and Fe^{2+} was due to SO_4^{2-}, the effects of calcium chloride vs calcium sulphate, magnesium chloride vs magnesium sulphate were examined It was found that both forms of Ca^{2+} and Mg^{2+} inhibited equally The inhibition caused by Ca^{2+} and Mg^{2+} together was additive (Fig 3) Even at high concentrations (10 mM) of Ca^{2+} and Mg^{2+}, the addition of more Mg^{2+} or Ca^{2+} further enhanced inhibition

Monovalent cations enhanced the ATPase activity

Table 1 Effect of ripening on ATPase activity in 80000 *g* pellet from apple fruit

Stage of ripening	Experiment 1 (Sp act)	Experiment 2 (Sp act)						
		Control	3 mM	$MgCl_2$	3 mM	$CaCl_2$	50 mM	KCl
Pre-climacteric	14 6	12 6	9	(71%)	12 1	(94%)	17 7	(140%)
Mid-climacteric	21 7	16 5	8 2	(49%)	14 1	(86%)	20 9	(126%)
Climacteric peak	22 9	15 9	6 5	(40%)	14 1	(88%)	19 2	(120%)
Post-climacteric	35 2	26 7	14 6	(54%)	23 1	(86%)	33 9	(126%)

Specific activity is μmol Pi released/mg membrane protein/hr Percent of activity compared with absence of ions (control) is in parentheses

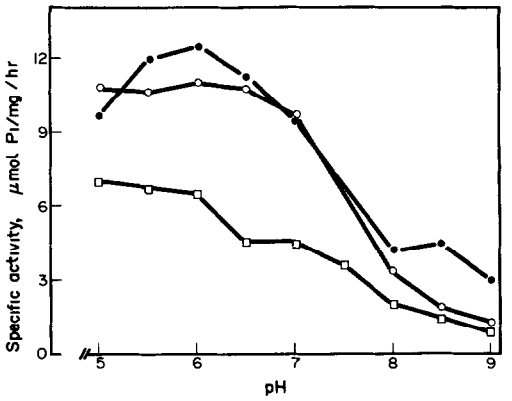

Fig 2 pH curve of ATPase No ion addition (○), 3 mM $CaCl_2$ (●), 3 mM $MgCl_2$ (□)

Table 2 Effect of divalent cations on ATPase activity from the 80000 *g* pellet of apple fruits

Compound	% of control	
	1 mM	3 mM
$MnCl_2$	105	47
$CaCl_2$	97	52
$MgCl_2$	85	45
$FeSO_4$	65	38
$CuSO_4$	30	—

Table 3 Effect of monovalent ions on ATPase activity from 80000 *g* pellet of apple fruits

Compound (50 mM)	% of control	
	$-MgCl_2$	$+3$ mM $MgCl_2$
KCl	138	118
NaCl	138	108
Choline Cl	168	138

(Table 3). In the absence of Mg^{2+}, K^+ and Na^+ were equally stimulating, but choline was more effective than either In the presence of Mg^{2+}, stimulation was less, but still present

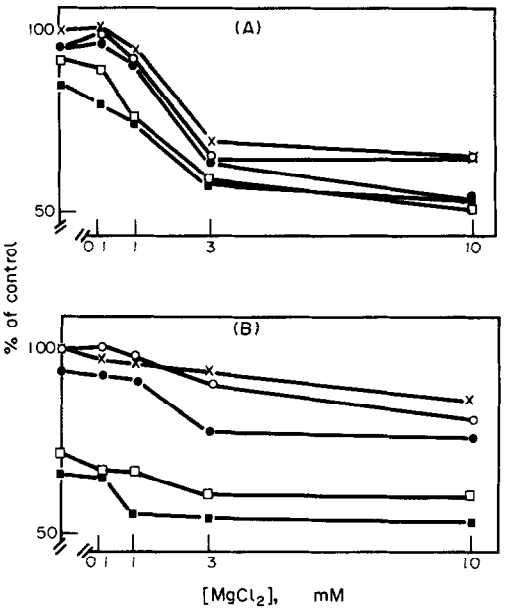

Fig 3 Effect of varying concentrations of $CaCl_2$ and $MgCl_2$ on ATPase activity (A) Changing Mg^{2+} concentrations with a fixed Ca^{2+} concentration no Ca^{2+} (×), 0 1 mM Ca^{2+} (○), 1 mM Ca^{2+} (●), 3 0 mM Ca^{2+} (□), 10 mM Ca^{2+} (■) (B) Changing Ca^{2+} concentrations with a fixed Mg^{2+} concentration no Mg^{2+} (×), 0 1 mM Mg^{2+} (○), 1 mM Mg^{2+} (●), 3 0 mM Mg^{2+} (□), 10 mM Mg^{2+} (■)

The ATPase of the 80000 *g* fraction showed a high specificity for ATP (Table 4) ADP as a substrate gave only 15% of the activity of ATP The best alternate substrate was GTP, which had 38% of the activity of ATP

ATPase activity was inhibited by compounds known to inhibit plasmalemma ATPase (Table 5) Oligomycin, a mitochondrial ATPase inhibitor was ineffective, as was

Table 4 Specificity of ATPase from 80000 *g* pellet of apple fruits

	Specific activity (μmol/mg protein/hr)	% activity with ATP
ATP	22 8	100
ADP	3 4	15
GTP	8 8	38
CTP	5 3	23
UTP	1 2	5

Table 5 Effect of inhibitors and uncouplers on ATPase (80 000 *g* pellet)

Compound	Concentration	Specific activity (μmol Pi/mg protein/hr)	% of control
None	—	8 0	100
Oligomycin	5 g/ml	8 0	100
	10 g/ml	7 9	99
Diethylstilbestrol	0 2 mM	4 2	52
N,N'-Dicyclohexyl-carbodiimide	0 05 mM	5 25	65
	0 1 mM	3 75	47
SF 6874	0 002 mM	7 8	97
	0 005 mM	8 1	101

the uncoupler SF 6874 The two compounds, diethylstilbestrol and dicyclohexylcarbodimide, which are plasmalemma ATPase inhibitors, were effective, although at higher than normal concentrations Diethylstilbestrol is generally used at 0 1 mM and dicyclohexylcarbodimide at 0 01 mM [9–11]

Michaelis–Menten kinetics were performed in the presence and absence of Mg^{2+} Apparent K_m and V_{max} for the enzyme were $4\,8 \times 10^{-4}$ M ATP and 1 2 mol Pi/mg protein/min, respectively The K_i for Mg^{2+} was 7×10^{-4} M Mg^{2+} was found to be a competitive inhibitor If the substrate was assumed to be Mg–ATP as in other plant plasmalemma ATPases and the data were plotted in a Michaelis–Menten plot, one should obtain a straight line for the different concentrations of free Mg^{2+}, as in ref [5] Calculating the concentrations of free ATP, free Mg^{2+} and Mg–ATP by using the dissociation constant 0 05 mM [12], the data were plotted for three different concentrations of free Mg^{2+} (Fig 4) At all concentrations Mg^{2+} interfered with the reaction The substrate for this ATPase is apparently free ATP and not Mg–ATP

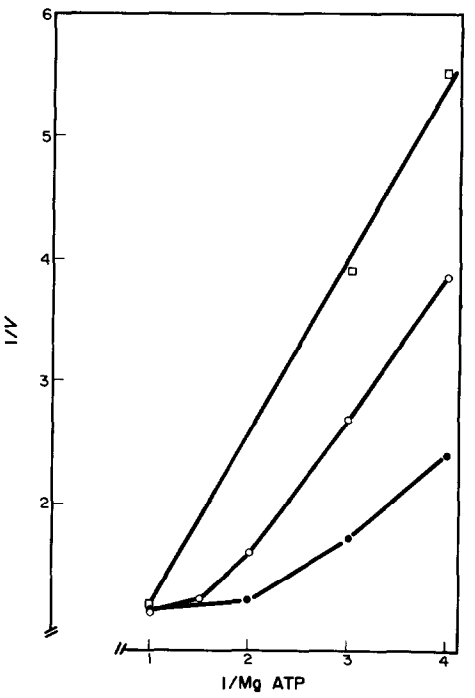

Fig 4 Reciprocal plot of reaction velocity and substrate (Mg–ATP) concentration at different fixed concentrations of free magnesium 1 mM free Mg^{2+} (●), 2 mM free Mg^{2+} (○), 5 mM free Mg^{2+} (□)

DISCUSSION

It was found that, as in strawberries, apple fruit contains a plasmalemma ATPase which is inhibited by divalent cations, and the activity of which increases as the fruit ripens This rise in activity could be expected in climacteric fruit as a reflection of the increase in energy demand accompanying fruit ripening However, as was also found in the non-climacteric strawberry during the phases of maturation and ripening, it is probably not directly connected with fruit respiration, but with other aspects of fruit ripening, such as changes in membrane permeability or integrity [18]

The enzyme preparation (80 000 *g* pellet) was not fully homogeneous and the presence of other phosphatases, apart from this ATPase, is possible An increase in soluble acid phosphatase in apple tissues has been shown to accompany the climacteric rise in respiration [19], but the fact that the enzyme was connected chiefly to a specific membrane portion of the microsomal fraction, as shown by the sucrose density gradient, indicates minimal contamination from this source

The characteristics of the ATPase, other than the divalent cation inhibition, were similar to those found in roots [13], hypocotyls [14], leaves and petals [15] The pH optimum, substrate specificity, K_m and V_{max} and inhibitor action were all within the ranges found in the literature The stimulatory effect of monovalent cations in the absence of divalent cations was higher than that found in strawberries and also within the range reported for plasmalemma ATPases from other plant organs, although in the latter case the stimulation was in addition to that caused by Mg^{2+} The fact that choline showed the best stimulation is surprising in view of the postulated role of this ATPase in ion transport across the plasmalemma and the fact that choline is transported much more slowly than either K^+ or Na^+ [16] However, this has been found to be true for ATPase from roots as well [16] and it may be that in the isolated plasmalemma fraction larger binding sites are exposed than are available in the intact membrane

The ATPase from apple fruit was inhibited by all the divalent cations tested In the case of Mg^{2+} it was shown

to be a competitive inhibitor to ATP This has been found in other systems [4, 17] but only at high concentrations in excess of 10 mM It appears that ion transport in fruits is not regulated in the same manner as has been found in other plant organs Monovalent cations may be transported by the plasmalemma ATPase, but this uptake will be inhibited rather than stimulated by the presence of divalent cations

EXPERIMENTAL

Plant material Apples (*Malus sylvestris* cv Calville de San Sauveur) were harvested at the preclimacteric stage and kept in cold storage until use To determine the stage of ripeness, the fruit was removed from storage to 20°, and ethylene and CO_2 were monitored daily for individual fruits held in 0 5 l jars, through which a CO_2-free air-stream passed Ethylene was measured in a Packard GLC equipped with an activated alumina column and a flame ionization detector, CO_2 was measured in a Packard GLC equipped with a Porapak column and a thermal conductivity detector

Enzyme extraction and assay 50 g of fruit were washed, peeled, de-seeded, cut into 1 cm pieces, and ground in a blender in 75 ml of medium (0 25 M sucrose, 50 mM Tris unbuffered, 3 mM EDTA, 1 mM DTT) for four 15-sec bursts The homogenate was strained through four layers of cheese cloth and the pH was adjusted to 7 2 with 1 M NaOH This was then centrifuged at 13 000 *g* for 15 min and the supernatant was centrifuged at 80 000 *g* for 45 min The pellet was resuspended in 10 mM Tris–MES, pH 7 2, 1 mM DTT and stored at 4° until use

When sucrose density gradient centrifugation was done, the above pellet was resuspended in 1 ml 22% sucrose, 0 1 mM $MgCl_2$, 1 mM Tris–HCl, pH 7 This was layered on to a linear density gradient of (25–45%, w/w) sucrose in a soln containing 0 1 mM $MgCl_2$, 1 mM Tris–HCl (pH 7) After centrifuging for 120 min in a SW 27 Spinco rotor (Beckman Instruments) at 26 500 rpm, fractions (1 2 ml) were collected and assayed for protein, ATPase activity and refractive index Cytochrome *c* oxidase activity, which is marker for inner mitochondrial membranes, and acid phosphatase, which is mainly associated with the tonoplast, were also assayed on some gradients [6, 19]

The ATPase assay was that of ref [6] The standard reaction mixture consisted of 3 mM ATP–Tris salt in 35 mM Tris–MES buffer, pH 6, in a final vol of 1 ml Membrane protein (*ca* 20 μg) was added to start the reaction After 20 min incubation at 38° the reaction was terminated by addition of 1 ml 1% (w/v) ammonium molybdate in 2 N H_2SO_4 Pi liberated was determined by the method of ref [7] Proper controls with aliquots of boiled enzyme were run with each assay Protein was determined by the method of ref [8]

Acknowledgements—We thank Shoshana Har-El for her technical assistance This research was supported by a grant from the United States–Israel Binational Agricultural Research and Development Fund (BARD) This is a contribution from the Agricultural Research Organization, The Volcani Center, Bet Dagan, Israel, No 297-E, 1981 series

REFERENCES

1 Hall, J L (1973) in *Ion Transport in Plants* (Anderson, W P, ed) p 11 Academic Press, London
2 Hodges, T K (1976) in *Encyclopedia of Plant Physiology* (Luttge, U and Pitman, M G, eds) Vol 2, p 260 Springer, Heidelberg
3 Ben-Arie, R and Faust, M (1980) *Phytochemistry* **19**, 1631
4 Balke, N E and Hodges, T K (1975) *Plant Physiol* **56**, 83
5 Lindberg, S, Hansson, G and Kylin, A (1974) *Physiol Plant* **32**, 103
6 Hodges, T K and Leonard, R T (1973) in *Methods in Enzymology* (Colowick, S P and Kaplan, N O, eds) Vol 4, p 392 Academic Press, New York
7 Fiske, C N and SubbaRow, Y (1925) *J Biol Chem* **66**, 375
8 Bradford, M M (1976) *Analyt Biochem* **72**, 248
9 Leonard, R T and Hodges, T K (1973) *Plant Physiol* **52**, 6
10 Balke, N E and Hodges, T K (1977) *Plant Sci Letters* **8**, 305
11 Beffagna, N, Marre, E and Cocucci, S (1979) *Planta* **146**, 387
12 Phillips, R C, George, P and Rutnam, R J (1969) *J Biol Chem* **244**, 330
13 Edwards, M L and Hall, J L (1975) *Protoplasma* **78**, 321
14 Dasamo, K and Yamaki, T (1974) *Plant Cell Physiol* **15**, 507
15 Lin, W, Wagner, G J, Siegelman, W H and Hind, G (1977) *Biochim Biophys Acta* **465**, 110
16 Ratner, A and Jacoby, B (1973) *J Exp Botany* **24**, 231
17 Lindberg, S (1976) *Physiol Plant* **36**, 139
18 Sacher, J A (1973) *Annu Rev Plant Physiol* **24**, 197
19 Rhodes, M J C and Wooltorton, L S C (1967) *Phytochemistry* **6**, 1