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ATPase IN RIPENING STRAWBERRIES

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Key Word Index—*Fragaria ananassa*; Rosaceae; strawberry; ATPase; maturity; calcium and magnesium inhibition.

Abstract—ATPase was found in 1000 *g*, 13 000 *g* and 80 000 *g* fractions from strawberry fruits. The optima pH for ATPase was the same (i.e. 6) for the 3 fractions, which also showed similar substrate specificity. However, the enzyme associated with the 80 000 *g* fraction showed the highest affinity for ATP and the maximum V_{max}/K_m value. As the fruit ripened, from the green to dark-red stage, ATPase activity in the 80 000 *g* fraction increased more than three times. The ATP content of the fruit pulp, which was high at the green stage, decreased as the fruit matured and ripened. Na^+ and K^+ slightly stimulated enzyme activity associated with the 1000 *g*, 80 000 *g* and soluble fractions, whereas, Ca^{2+} and Mg^{2+} inhibited the enzyme activity in all fractions. However, the extent of inhibition due to divalent cations lessened as the fruit ripened.

INTRODUCTION

ATPases (EC 3.6.1.5) have been studied in plants chiefly in roots and transport tissues, in an attempt to establish a role for these enzymes as energy transducers in ion transport, as found in animals and bacteria [1, 2]. These ATPases have generally been located in the plasma membrane [3–6], but activity has also been found in cell walls [7–9], with no clear correlation to ion transport. Another energy transducing role for ATPase in plants has been demonstrated in photophosphorylation coupling in chloroplasts [10]. All these ATPase preparations have been shown to be stimulated by either univalent or divalent cations (generally Na^+, K^+, Mg^{2+} and Ca^{2+}). The endogenous levels of these cations and the equilibrium between them are known to be of importance in controlling the ripening and senescence of fruits, but the mechanism and interactions involved are not clear [11]. To our knowledge, the pattern of ATPase activity in ripening fruits (both climacteric and non-climacteric) is unknown. An attempt was therefore made to determine ATPase in subcellular fractions of strawberry fruits at different stages of ripening, and to characterize the enzyme with regard to the effects of univalent and divalent cations. Furthermore, an attempt has been made to relate the ATPase activity to endogenous ATP levels in the fruit.

RESULTS

Endogenous ATP levels in two strawberry cvs declined markedly as the fruit matured from the pink to the dark-red stage; however, the levels in the soft, small and fast-ripening Everbearing seedling were much lower than in the large, firm Earliglow cv (Table 1). Correspondingly, assay of total ATPase in homogenates from fruit at 5 stages of maturity, showed an increase in activity in both cvs throughout ripening, but chiefly from the green to the pink stage. The sp. act. in the Everbearing seedling was much higher than in the Earliglow, largely due to the higher protein content of the latter (420 and 700 μg/g fr.wr, respectively).

In order to determine in which compartment of the cell the rise in ATPase activity occurred during ripening, the subcellular distribution of ATPase activity was examined. Three subcellular fractions were prepared by differential

Table 1. Endogenous ATP levels and total ATPase activity in strawberries at different stages of maturity

Stage of maturity	ATP nmol/g fr. wt		ATPase μmol/g fr. wt./hr		ATPase μmol Pi/mg protein/hr	
	Everbearing	Earliglow	Everbearing	Earliglow	Everbearing	Earliglow
Green	3.6	34.5	4.2	4.1	9.5	4.5
White	9.6	32.2	6.3	5.1	17.8	6.1
Pink	6.4	35.0	9.5	6.3	23.2	11.5
Red	0.2	28.0	11.1	9.3	24.6	14.0
Dark-red	0.6	14.3	12.2	12.4	27.5	22.7

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centrifugation and their homogeneity was examined by EM. The micrographs showed that the 1000 *g* residue predominantly contained cell walls, but some still had some plasmalemma fragments attached. The 13 000 *g* residue was composed mainly of mitochondria and mitochondrial membranes, but other unidentified membranes could also be seen. The PACP stain [12] for plasmalemma did not stain this fraction. The 80 000 *g* residue was heavily stained by the PACP stain, but there was a considerable amount of membrane fragments that were not stained.

Phosphatase (ATPase) activity was present in the 3 particulate fractions (1000 *g*, 13 000 *g* and 80 000 *g* residues), and only a little activity was found in the 80 000 *g* supernatant, the protein content of which was very low. The greatest affinity for ATP and the highest sp. act. for ATPase was found in the 80 000 *g* residue (Table 2). The optimum pH for activity in all fractions was 6 with very little activity at pH 9.

The subcellular distribution of ATPase activity at different stages of maturity is shown in Fig. 1. The most significant increase in activity with advancing maturity occurred in the 80 000 *g* fraction, which in the dark-red fruit was more than 3 times the activity of this fraction in green fruit. From the green to the pink stage of maturity activity in the 1000 *g* and 13 000 *g* fractions increased *ca* three times and two times respectively, but thereafter remained more or less constant. The apparently increased activity in the soluble fraction was due in part to a 50% decline in the protein content of this fraction (from 82 μg/ml in green fruit to 39 μg/ml in dark-red fruit). The protein content of the 1000 *g* and 13 000 *g* fractions remained unchanged as the fruit ripened (630 μg/ml and 270 μg/ml, respectively). In the 80 000 *g* fraction there was even an increase in protein content as the fruit ripened from 82 μg/ml in green fruit to 112 μg/ml in dark-red fruit.

None of the enzyme preparations showed an exclusive specificity for ATP, and a number of nucleoside, tri- and diphosphates were equally hydrolysed (Table 3).

Cation effects on ATPase

Univalent ions. K^+ and Na^+ both caused stimulation of ATPase, chiefly for the enzyme in the soluble fraction, and less so for that in the 1000 *g* and 80 000 *g* fractions. The 13 000 *g* fraction did not respond to either ion in the incubation media. (An example of K^+ stimulation is presented in Fig. 2.)

Table 2. Characterization of the ATPase in different subcellular fractions extracted from ripe strawberry fruits

Subcellular fraction	Total soluble protein (mg/fraction)	ATPase activity			
		Optimum pH	V_{max} μmol Pi/mg/hr	K_m (mM)	V_{max}/K_m
1000 *g* residue	13.50	6.0	14.5	0.27	5.37×10^4
13 000 *g* residue	4.80	6.0	27.0	0.21	12.86×10^4
80 000 *g* residue	1.68	6.0	32.3	0.15	21.5×10^4

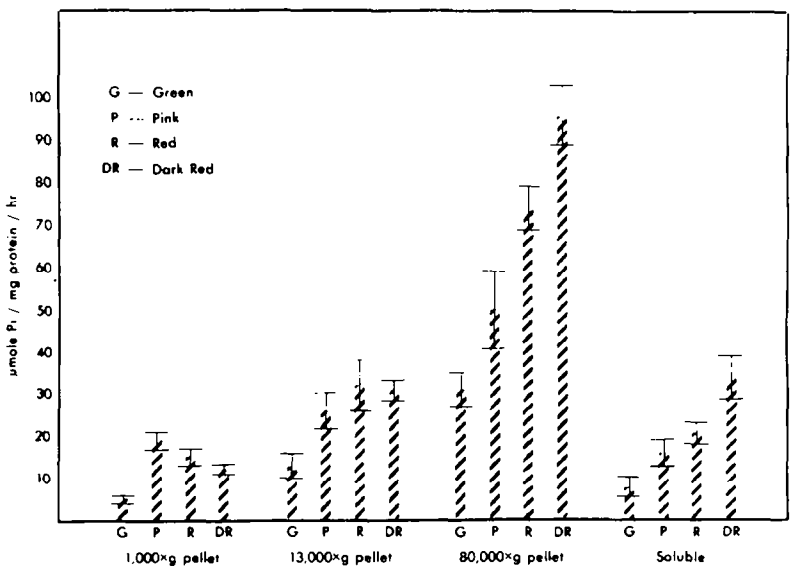

Fig. 1. The effect of stage of maturity of strawberry fruits on the ATPase activity of subcellular fractions (Everbearing seedlings). Bar: ± s.e.

Table 3. Substrate specifity of fractions from ripe 'Earliglow' strawberries

Nucleoside Substrate*	Nucleoside phosphatase activity (μmol Pi/mg protein/hr) 1000 g residue	13 000 g residue	80 000 g residue
ATP	10.8 (100)	16.6 (100)	45.8 (100)
CTP	8.8 (82)	13.9 (84)	34.6 (76)
GTP	8.3 (77)	12.7 (76)	36.4 (80)
XTP	8.0 (74)	12.4 (75)	37.0 (81)
UTP	7.5 (69)	12.7 (76)	29.2 (64)
ADP	9.3 (86)	15.9 (96)	40.0 (87)
IDP	9.6 (89)	17.4 (105)	47.2 (103)
XDP	6.2 (56)	9.0 (54)	26.0 (57)

* All substrates were sodium salts. Incubation media: 3.0 mM substrate, 35 mM Tris–MES buffer, pH 6 and 0.1 ml enzyme fraction containing 33, 19 and 16 μg protein, respectively, for each fraction, in a total volume of 1 ml.

Numbers in parentheses are the percentage of activity compared to ATP.

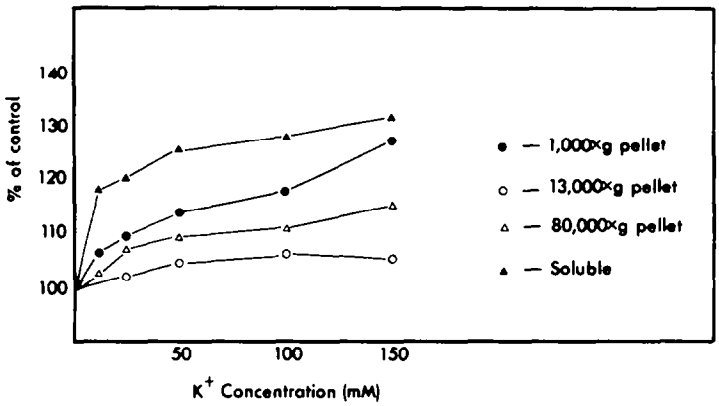

Fig. 2. Stimulation of ATPase activity by KCl in subcellular fractions from ripe strawberries (Everbearing seedlings).

Table 4. The concentration of $MgCl_2$ and $CaCl_2$ which caused 50% inhibition of ATPase activity in subcellular fractions extracted from ripe strawberry fruits

Subcellular fraction	$CaCl_2$ (mM)	$MgCl_2$ (mM)
0–1000 g × 10 min residue	25.0*	9.2
1000–13 000 g × 10 min residue	10.5	6.2
13 000–80 000 g × 30 min residue	12.6*	6.8
80 000 g × 30 min supernatant	—	12.0

* Calculated by extrapolation.

Divalent ions. Both Mg^{2+} and Ca^{2+} inhibited ATPase in all the fractions (Table 4). Mg^{2+} was more potent and the enzyme associated with the 13 000 g and 80 000 g fractions was the most susceptible. ATPase in the soluble fraction was the least sensitive to both ions. The point to notice was a change in the sensitivity of the enzyme extracts to divalent cation inhibition as the fruit matured. Figure 3 shows that as the fruit matured from green to dark-red, the ATPase activity in the 80 000 g fraction became less sensitive to inhibition by each of the cations.

Combined ion effects

Ca^{2+} and K^+. The inhibitory effect of Ca^{2+} on ATPase was alleviated in the presence of K^+ (Fig. 4). Increasing K^+ concentrations to 0.1 M even resulted in some stimulation of activity at Ca^{2+} concentrations up to 1.5 mM. However, at 3 and 6 mM, Ca^{2+} inhibition was not entirely overcome even at this high K^+ level.

Ca^{2+} and Mg^{2+}. The inhibitory effects of Ca^{2+} and Mg^{2+} were additive at 1.5 mM concentrations of each ion and equalled the effect of 3 mM Ca^{2+} alone (Fig. 5). The inhibition obtained in the presence of 3 mM of each ion was not quite additive, but equalled the amount of inhibition caused by 6 mM Ca^{2+}. No additional inhibition could be obtained by adding $CaCl_2$ to 3 mM $MgCl_2$, at which level inhibition was almost maximal, with only a slightly greater inhibition at 6 mM. As the fruit ripened, enzyme activity in the 80 000 g fraction appeared to become generally more

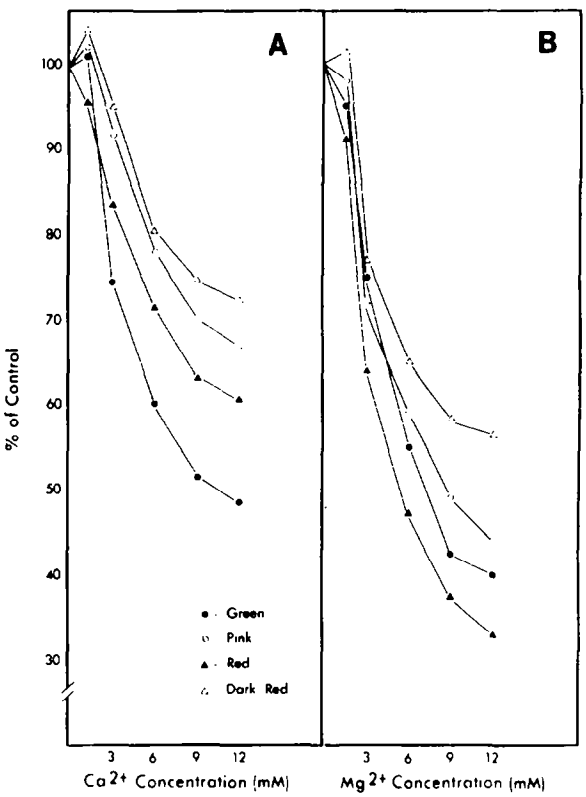

Fig. 3. Effect of Ca^{2+} (A) and Mg^{2+} (B) in the incubation medium on ATPase activity in the 80 000 g fraction from strawberries at different stages of maturity (Everbearing seedlings).

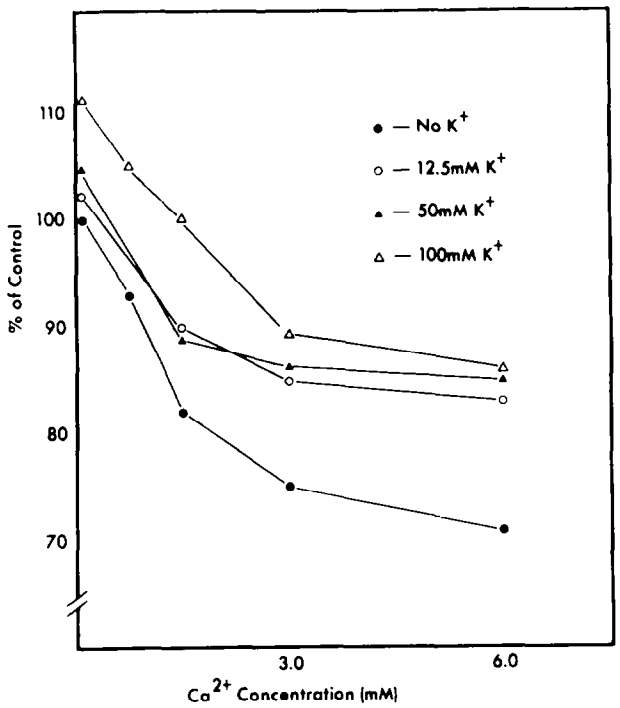

Fig. 4. Combined effects of $CaCl_2$ and KCl on ATPase activity in the 80 000 g fraction from ripe strawberries (Everbearing seedlings).

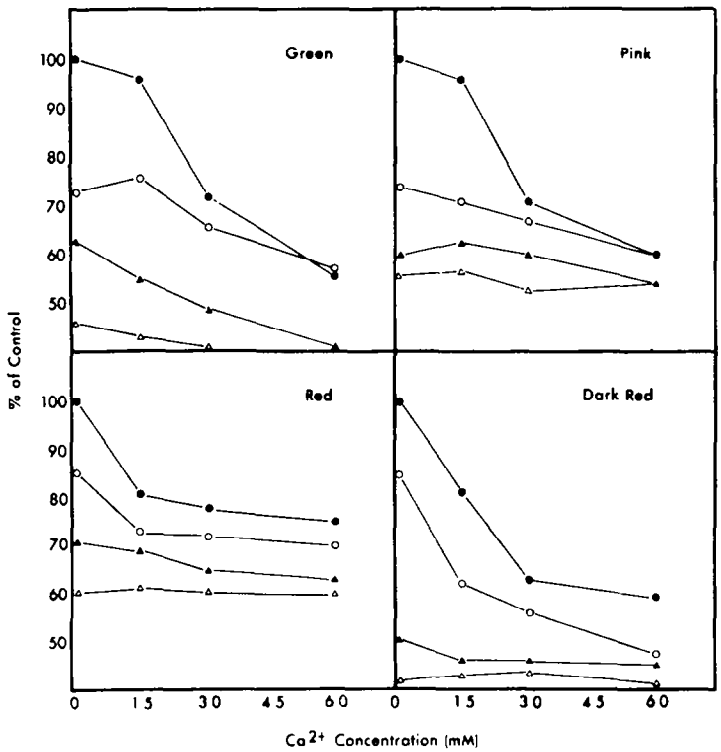

Fig. 5. Combined effects of $CaCl_2$ and $MgCl_2$ in the incubation medium on ATPase activity in the 80 000 g fraction from strawberries at different stages of maturity (Everbearing seedlings). $MgCl_2$ concentrations: 0 (●), 1.5 (○), 3.0 (▲), and 6.0 (△) mM.

resistant to the inhibitory effects of Mg^{2+} and Ca^{2+} either alone or in combination (Figs. 3 and 5).

DISCUSSION

Nucleoside triphosphatase activity was found associated with particulate subcellular fractions of strawberry fruit cells. ATP and ADP were the best substrates for the enzymes, followed by a series of di- and triphosphates, differing only slightly from the substrate specificity described for an ATPase from maize scutellum [6]. However, the enzyme preparations used were not homogenous or pure and the presence of other phosphatases, apart from ATPase, is probable. The same type of distribution of ATPases with similar pH optima, substrate specificity and K_m values have been reported in roots [13, 14], hypocotyls [15], leaves and petals [16]. Although the enzymic activity in the 3 particulate fractions from strawberry fruit cells showed considerable uniformity in enzyme characteristics, such as pH optima, substrate specificity and sensitivity to inorganic ions, there were marked differences in the affinity of the fractions for the substrate ATP. The 80 000 *g* enzyme had greatest affinity for ATP and seemed physiologically more active, as determined by the highest V_{max}/K_m value. Additionally, the ATPase associated with this fraction showed the largest increase in activity as the fruit ripened from the green-immature to the overripe-dark-red stage.

Stimulation of enzyme activity by cations was obtained only with K^+ and the most responsive ATPase was that associated with the 1000 *g* residue and the 80 000 *g* supernatant. Enzyme preparations from all fractions were inhibited by the divalent cations Mg^{2+} and Ca^{2+}, but here the 1000 *g* residue and supernatant enzymes were the least sensitive.

The inhibitory effect of low Mg^{2+} concentrations was surprising. In most systems studied, Mg^{2+} stimulates ATPase activity and has been regarded as a requisite for ATPase assay, even though basal ATPase activity, without additional Mg^{2+} was not always measured [5, 14]. Inhibition of ATPase activity by Mg^{2+} at concentrations below 10 mM has, however, been reported for cell-wall and nuclei preparations from subcellular fractions from maize root tips [13] and for solubilized preparations from mung bean hypocotyls [15]. The remaining membrane-bound enzyme was still stimulated by Mg^{2+}. In strawberry fruits both the particulate and soluble fractions contained Mg^{2+}-sensitive ATPase, which was also inhibited by Ca^{2+}. Although Ca^{2+} inhibition was also shown by Edwards and Hall [13] with cell-wall and nucleus ATPase from maize root tips, this was the exception rather than the rule. Ca^{2+} has been shown to stimulate ATPase activity in different subcellular fractions from plants animals and bacteria [1]. The inhibition of ATPase from strawberry fruit tissue by Ca^{2+}, the inhibition of solute leakage from strawberry slices by Ca^{2+} salt solutions (unpublished data), and the known physiological effects of Ca^{2+} on fruit ripening, quality and storage disorders [17], suggest that the proposed effects of Ca^{2+} in maintaining membrane integrity [18] might be connected to its inhibitory effect of ATPase activity, thereby reducing energy potential in that particular component of the cell and thus regulating metabolic activity. Ca^{2+} has recently been found to inhibit banana ATPase and to affect the Arrhenius plots of ATPase (Majmudar, Mattoo and Modi, personal communication). However, as Mg^{2+} appears to have the same effect on ATPase, it should be expected to have similar physiological effects. The effect of Mg^{2+} on membrane integrity has not been well defined, and the often-mentioned antagonistic effect of Ca^{2+}–Mg^{2+} is only in respect to certain metabolic disorders. The changing sensitivity of membrane-bound ATPase to cations with ripening could be regarded as evidence for a change in membrane integrity.

The relatively slight stimulation of ATPase by univalent ions might indicate that, if there is active ion transport in fruit cells, it is not regulated in the same manner as has been visualized for ion uptake by roots [2]. Moreover, K^+ stimulation of ATPase in root tissues has generally been found to be most extensive in the presence of Mg^{2+} [19], but in strawberry fruit extractions, it was not even measurable in the presence of Mg^{2+}, which, as mentioned above, inhibited ATPase activity. Also in maize scutellum preparations Na^+ and K^+ gave much greater stimulation of ATPase, when no divalent cation was added [6]. In the strawberry preparations high concentrations did, however, alleviate the extent of inhibition by divalent ions at lower concentrations.

EXPERIMENTAL

Plant material. Strawberry fruits (*Fragaria ananassa* Duch.) were harvested at different stages of maturity from seedlings of Everbearing plants grown in the greenhouse or from field plots of Earliglow cv. The criterion used for maturity was fruit color, the following stages being defined: (1) green—immature fruit weighing above 5 g, turning to light green; (2) white–light green turning to white with small spots of pink beginning to appear; (3) pink—at least 50% of the fruit pink; (4) red—fruit entirely red, ripe for commercial harvest; (5) dark-red—fruit still firm, but too ripe for commercial harvest.

Enzyme extraction and assay. Washed, chilled fruit (50 g) were extracted in a mortar and pestle with 150 ml homogenizing medium (0.25 M sucrose, 25 mM Tris–MES, 3 mM EDTA, 5 mM DTT, pH 7.2). The fruit was extracted either fresh or after being powdered under liquid N_2 and stored at −70°. Fractions sedimenting at 1000 *g* (10 min), 13 000 *g* (10 min), 80 000 *g* (2 × 30 min) and the 80 000 *g* supernatant were collected. The residues were resuspended in the homogenizing medium and assayed for ATPase activity [14]. Unless otherwise mentioned the standard reaction mixture consisted of 3 mM ATP (Boehringer Mannheim)—Tris salt in 35 mM Tris–MES buffer, pH 6 in a final vol. of 1 ml. After 30 min incubation at 38° the reaction was terminated by addition of 1 ml 1% (w/v) ammonium molybdate in 2 N H_2SO_4. Inorganic phosphate was determined by the method of ref. [25]. Proper controls with aliquots of boiled enzyme were run with each assay. Protein was determined by the method of ref. [21].

ATP determination. Fruit pulp (5 g) were homogenized with 7 ml cold 10% TCA. The homogenate was centrifuged for 10 min at 10 000 *g* and the residue resuspended in 3 ml 10% TCA and centrifuged as above. The supernatants were combined and brought to pH 5 by repeated extractions in cold H_2O-saturated Et_2O. The Et_2O was evapd and an aliquot of the aq. phase (*ca* 10 ml) was diluted (1:20) in 25 mM $MgSO_4$ and 25 mM HEPES buffer, pH 7.5. ATP was determined with luciferin-luciferase (Du-Pont) [22].

Electron microscopy. The residues obtained by centrifugal fractionation were embedded in 4% Nobel agar, fixed in cold 4% (w/v) glutaraldehyde for 2 hr and post-fixed overnight in 2% OsO_4. Following dehydration in an EtOH series and propylene oxide, the specimens were embedded in Spurr's low viscosity embedding medium [23]. The ultra thin sections cut from

the blocks were mounted on gold grids and stained either with lead acetate or with periodic acid–chromic acid–phototungstic acid (PACP), proposed as a specific stain for plasmalemma [12]. Micrographs were taken with a Phillips 200 electron microscope.

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