Influence of the Disorder Bitter Pit on Microsomal Vanadate-Sensitive ATPase Activity from Apple Fruit

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Summary

“Fuji” apple trees on *Malus prunifolia* rootstock showed a higher incidence of bitter pit (a physiological disorder of apple fruit) when ammonium salt rather than nitrate was supplied in the nutrient solution. The number of spots induced by bitter pit was intimately associated with a decrease in the calcium content of the fruits.

Proton transport and ($K^+ + Mg^{2+}$)-ATPase activity were observed in the microsomal fraction of the fruit flesh of apple. Sodium vanadate at 0.1 mM decreased ($K^+ + Mg^{2+}$)-ATPase activity to 8% of the control and inhibited the proton transport. Neither the proton transport nor the ($K^+ + Mg^{2+}$)-ATPase activity was inhibited by nitrate at 50 mM. On a continuous sucrose density gradient the distribution of the vanadate-sensitive ATPase activity was different from those of NADPH-cytochrome c reductase and cytochrome c oxidase activities. In apples seriously affected with bitter pit, proton transport and the vanadate-sensitive ATPase were reduced respectively to 37% and 55% of the levels in healthy fruit, while fruits slightly affected with bitter pit showed a higher activity of the vanadate-sensitive ATPase and a similar proton transport activity to that in healthy fruit.

The relationship between bitter pit disorder and the changes in the microsomal proton transport and vanadate-sensitive ATPase activities is discussed.

Introduction

Bitter pit, a physiological disorder of apple, is now recognized as a calcium-deficient disorder, although an excess of nitrogen, potassium or magnesium increases its incidence (10, 11, 13, 14). We have also shown an intimate relationship between bitter pit and calcium deficiency, and the development of bitter pit-like spots following infiltration with calmodulin antagonists (5). Marinos (8) observed by electromicroscopy of barley tissues that calcium deficiency induced the destruction of tonoplasts and plasmalemma. Several researchers showed that the occurrence of bitter pit increased the K^+ efflux

from the tissues (6, 12, 17). Matsumoto and Yamaya (9) reported that the K^+ -stimulated ATPase activity for putative plasma membranes from cucumber roots and the K^+ uptake in the intact roots were repressed by Ca^{2+} starvation.

These findings prompted us to investigate whether changes in the proton-translocating ATPase are related to the occurrence of bitter pit.

In the present paper the effect of calcium on the occurrence of bitter pit, and the changes in microsomal proton transport and vanadate-sensitive ATPase activity of apple fruit with its occurrence are described.

Materials and methods

Plant materials. Eight-year old “Fuji”

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apple trees on *Malus prunifolia* rootstock were grown in water culture medium using 4 mM ammonium- or nitrate-nitrogen as described previously(5). The apple fruits with or without bitter pit were taken at harvest and stored at 0°C until used. The incidence of bitter pit was determined just before harvest. All experiments described here were done by using mature fruits which were harvested from the water culture field at Morioka Branch, Fruit Tree Research Station.

Preparation of microsomal fraction. Peels, stems and cores were removed from mature apple fruits and the fruit flesh was used in the present experiment. For determining the effect of bitter pit disorder on the microsomal proton transport and ATPase activities, the flesh was cut at the equator and the flesh near the calyx end removed and the healthy flesh near the pitted area was used. The fruit flesh was sliced and homogenized in 2 volume of 0.25 M sucrose, 4 mM DTT, 2 mM EDTA, 0.2% (w/v) bovine serum albumin, 5% (w/v) polyvinylpyrrolidone and 0.2 M Tris-HCl (pH 7.5) with an ice-cold mortar and pestle. After filtering through four layers of gauze, the 6,000×g for 10 min, and 50,000×g for 30 min fraction was prepared by differential centrifugation. The microsomal pellets were resuspended in a small amount of 0.25 M sucrose and 2 mM Tris-2 mM MES (pH 7.0) and used for determining the proton transport and the (K⁺+Mg²⁺)-ATPase activity. The 6,000×g for 10 min and 80,000×g for 30 min fraction, instead of the 50,000 g pellet, was layered onto a continuous sucrose gradient and centrifuged for 2 hr at 80,000×g in a Hitachi RPS 25-2 rotor.

ATPase assays. ATPase activity was measured at 30°C for 15 min. The standard assay mixture (1 ml) contained 75 mM Tris-MES (pH 7.2), 3 mM MgCl₂, 3 mM ATP-Na₂ (neutralized with Tris), 50 mM KCl, 1 mM NaN₃, 0.1 mM ammonium molybdate and a proper amount of the membrane vesicles. Phosphate was determined by the Fiske-Subbarow method modified by Tashima (16). The (K⁺+Mg²⁺)-ATPase activity was corrected for that of a basal ATPase lacking

K⁺ and Mg²⁺. Vanadate-sensitive ATPase activity is defined as the difference between the activities in the absence and the presence of 0.1 mM sodium vanadate.

Fluorescence assays. Formation of interior acid pH gradients was monitored as the quenching of fluorescence of quinacrine as previously reported by Bennett et al(1). The standard assay mixture (2.5 ml) was composed of 0.25 M sucrose, 3 mM MgCl₂, 50 mM KCl, 1 mM NaN₃, 3 mM ATP-Na₂ (neutralized with Tris), 0.01 mM quinacrine, 60 mM Tris-60 mM MES (pH 7.2) and the microsomal fraction (60-200 µg membrane protein). Fluorescence was measured at 28°C with a Jasco FP-770 spectrofluorimeter at excitation and emission wavelengths of 423 nm and 500 nm, respectively. The reaction medium was continuously stirred with a magnetic bar.

Chemical estimations. The fruit flesh was freeze-dried, milled and used for chemical estimations. The contents of nitrogen, potassium, calcium and magnesium were determined as described earlier(5).

NADPH-cytochrome C reductase and cytochrome C oxidase were assayed spectrophotometrically(7). Protein content was measured by a modified Lowry method(2).

Results and Discussion

Induction of bitter pit by ammonium supply. We previously reported that ammonium supply increased the incidence of bitter pit in apple fruit(5). In this experiment 53.3% of apple fruits were affected with bitter pit at harvest time in the ammonium treatment, while the incidence was only 1.9% in fruit supplied with nitrate (Table 1). The affected fruit had a lower content of calcium and a higher content of nitrogen than the unaffected fruit (Table 2). Interestingly, the symptom of bitter pit was remarkably influenced by the calcium content of the fruit flesh (Table 3). The more seriously the apple fruit was damaged by bitter pit, the lower the calcium content of the fruit flesh (Table 3).

Three types of proton-translocating ATPase have so far been reported in higher

Table 1. Induction of bitter pit by ammonium supply in water culture medium.

Treatment	Incidence of bitter pit (%)	Yield (kg)	Mean fruit weight (g)
Nitrate(4mM)	1.9±2.0 ^z	33.5±9.1	237±14
Ammonium(4mM)	53.3±7.7	20.8±3.0	205±7

^z The results represent the mean±SD from 3 replicate trees.

Table 2. Effect of bitter pit disorder on the contents of nitrogen, potassium, calcium and magnesium in the flesh of mature apple fruit.

	Nitrogen	Potassium	Calcium	Magnesium
	(% dry weight basis)			
Healthy fruit	0.141 ±0.020 ^z	0.413 ±0.038	0.0182 ±0.0019	0.0151 ±0.0027
Affected fruit	0.260 ±0.057	0.363 ±0.042	0.0097 ±0.0004	0.0140 ±0.0015

^z The results represent the mean±SD of 3 replications of 3 apple fruits each.

Table 3. Effect of calcium content of the fruit flesh of apple on the symptom of bitter pit.

Number of spots	Calcium Content (ppm, dry weight basis)
Healthy Fruity	138.0 ^{a z}
1~3	98.3 ^b
4~10	75.9 ^c
>20	61.0 ^d

^z Mean separation within columns by Duncan's multiple range test, 5% level.

plants, that is, plasma membrane-, tonoplast- and golgi membrane-type ATPases(3, 15). Vanadate and nitrate are useful tools to discriminate between them. The plasma membrane-type ATPase activity is vanadate-sensitive and nitrate-insensitive, but the tonoplast-type one is vanadate-insensitive and nitrate-sensitive(15). The golgi membrane-type one is thought to be vanadate-insensitive and less sensitive to nitrate(3). As

Fig. 1. Effect of pH on (K⁺+Mg²⁺)-ATPase activity by microsomal fraction from apple fruit. The results indicate means of 2 replications.

Each of the assay medium contained 0.5mM EGTA, 75mM Tris-MES (pH 7.2), 3mM MgCl₂, 3mM ATP-Na₂, 50mM KCl, 1mM NaN₃, 0.1mM ammonium molybdate and a proper amount of the membrane vesicles.

Fig. 2. Distribution of vanadate-sensitive ATPase, cytochrome c oxidase and NADPH-cytochrome c reductase activities on a continuous sucrose gradient of the microsomal fraction from apple fruit. Vanadate-sensitive ATPase (●), cytochrome c oxidase (■), NADPH-cytochrome c reductase (□).

Table 4. Effect of chemicals on the microsomal (K^+ + Mg^{2+})-ATPase activity from apple fruit.

Treatment	Activity (nmol/g FW/min)	(%)
Control	16.3	100
+Na ₃ VO ₄ , 0.1 mM	1.3	8
+Gramicidine D, 2 μ M	19.9	122
+KNO ₃ , 50 mM ^z	14.9	91

The results represent means of 2 replications.

^z Potassium nitrate was added instead of potassium chloride.

Table 4 shows, microsomal (K^+ + Mg^{2+})-ATPase from apple fruit was decreased to 8% of the control by adding 0.1 mM sodium vanadate, but little affected by nitrate at 50 mM. The addition of gramicidin D at 2×10^{-3} mM increased the ATPase activity to 122% of the control, indicating that the microsomal fraction contained tightly-sealed vesicles(1). The optimum pH was around 7.0 (Fig. 1).

The microsomal fraction from apple fruit was fractionated by continuous sucrose density centrifugation (Fig. 2). The major peak of the vanadate-sensitive ATPase activity was at 1.11 g/cm³, while the major peaks of the cytochrome c oxidase activity, a marker for mitochondria, and the NADPH-cytochrome c reductase activity, a marker for endoplasmic reticulum, were at 1.14 g/cm³ and ~1.04 g/cm³, respectively.

These findings indicate that the vanadate-sensitive ATPase described here has similar characteristics to the plasma membrane-type proton-translocating ATPase, except that the distribution of this ATPase activity showed a lower density peak than that (1.13~1.17 g/cm³) of the previously characterized plasma membrane-type ATPase activity(15). We observed that the peak of the vanadate-sensitive ATPase activity was shifted to 1.19 g/cm³ by homogenizing in a buffer solution containing 4 mM MgSO₄ followed by a continuous sucrose density centrifugation with 4 mM MgSO₄ (data not shown). Apple fruit has quite a low magnesium content as compared to any other part of the apple tree (4). The lower density of this ATPase activity might be due to its low magnesium content.

Table 5. Distribution of the vanadate-sensitive ATPase activity in the homogenate fractionated by differential centrifugation.

Fruit ^z	Vanadate-sensitive ATPase (nmol/g FW/min)		
	6,000 $\times$ g pellet	6,000—50,000 $\times$ g	Supernatant
Exp. 1			
Healthy	7.1(26%) ^y	12.7(47%)	7.4(27%)
Affected	7.0(30%)	10.1(44%)	5.9(26%)
Exp. 2			
Healthy	6.7(24%)	13.1(46%)	8.4(30%)
Affected	7.4(29%)	11.3(45%)	6.4(25%)

The affected fruits had 7–10 spots.

^z used after being stored at 0°C for about 3 months.

^y indicate the % basis of the total activity in each fruit.

Fig. 3. Proton transport by microsomal fraction from apple fruit. Vanadate and nitrate were added at 0.1 mM and 50 mM, respectively. The arrow indicates the addition of gramicidine D at 2 μ M.

Microsomal proton transport. The microsomal fraction from apple fruit catalysed ATP-dependent quenching of quinacrine fluorescence (Fig. 3), which indicates the formation of an acid interior pH gradient as previously reported in red beet(1). The proton-transporting activity reached a steady state after about 15 min. Both microsomal proton transport and the microsomal ATPase were inhibited by sodium vanadate but not by nitrate. This proton transport seems to be controlled through the vanadate-sensitive ATPase activity.

Effect of bitter pit disorder on the activities of microsomal proton transport and vanadate-sensitive ATPase. Table 5 shows the distribution of vanadate-sensitive ATPase

Fig. 4. Effect of bitter pit disorder on micromal vanadate-sensitive ATPase activity from apple fruit which was stored at 0°C for 2–3 weeks.

(A): Healthy fruit (Control), the nitrate treatment; (B): unaffected fruit, the ammonium treatment; (C): affected fruit, less than 5 spots; (D): affected fruit, more than 20 spots. Each bar expresses the mean $\pm$ SD ($n=5$ except for D, $n=3$).

among $6,000 \times g$ precipitate, $6,000 \sim 50,000 \times g$ (microsomal fraction) and the supernatant from healthy and affected fruits. The affected fruit used in this experiment had 7 to 10 spots. The microsomal fractions of the healthy and the affected fruits recovered 46~47% and 44~45%, respectively, of the total vanadate-sensitive ATPase activity, demonstrating that the recovery of this activity may be little changed whether apple fruit is affected with bitter pit or not. In the presence of gramicidin D at 2×10^{-3} mM vanadate-sensitive ATPase activity from the affected fruit was increased to 113% of the control (data not shown). This suggests that the ATPase might reside in sealed membrane vesicles, although the membranes from the affected fruit might be less able to form tightly-sealed vesicles than those from the healthy fruit.

Apple fruits affected with bitter pit were divided into two groups. One is fruits seriously damaged by bitter pit which have more than 20 large deep spots. The other is slightly affected fruits which have less than 5 small spots. When apple fruit was seriously affected, the vanadate-sensitive ATPase activity was remarkably lowered to 55% of the control (Fig. 4). Fruit slightly affected, however, had a higher activity of this

Fig. 5. Effect of bitter pit disorder on microsomal proton transport from apple fruit which was stored at 0°C for 2–3 weeks.

(A), (B), (C), (D) and the bars are the same as shown in Fig. 3.

ATPase than the control. Figure 5 shows the effect of bitter pit on quenching after 20 min of quinacrine fluorescence, which expresses the ability of the microsomal vesicles to form a pH gradient, with the inside acidic. Fruit seriously affected with bitter pit showed a lower capability for this formation, while slightly affected fruit had a very similar capability to the control. As Figures 4 and 5 show, the unaffected fruits obtained from trees treated with ammonium-nitrogen had slightly higher activities of the proton transport and the vanadate-sensitive ATPase than the healthy fruits.

The unaffected fruits tend to contain a higher nitrogen and a lower calcium content than the healthy fruit(5). In the present study the effect of calcium on the changes of proton-translocating ATPase activity with the occurrence of bitter pit is not separated from that of nitrogen. At the first stage of decrease in calcium content and/or increase in nitrogen content of apple fruits, the proton-translocating ATPase activity described here seems to be stimulated. Then this ATPase activity may be drastically decreased. A proton pump catalysed by a $(K^+ + Mg^{2+})$ -ATPase associated with the cell membranes is now considered to be the primary transport process in higher plants(15). The occurrence of bitter pit was followed by the increase in K^+ efflux from apple tissues(6, 12, 17), suggesting that the affected fruit may have

"leaky membranes". Thus the cell membranes of the affected fruit would have a higher energy demand to keep a normal transport system in the cells. That might be a reason for the temporary rise in the vanadate-sensitive ATPase activity followed by the development of bitter pit. Furthermore, it is tempting to think that the metabolic function of cell membranes in the pitted area had been disturbed before bitter pit occurred.

Unfortunately, we failed to find the tonoplast-type ATPase activity from apple fruit in spite of the fact that more than 80% of the cell volume is occupied by vacuoles(18). It will be necessary to consider the transport system in the apple tonoplast to elucidate the role of vacuoles in this disorder.

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リンゴ果実のバナデート感受性 ATPase 活性に 及ぼすビターピット発生の影響

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摘 要

リンゴ樹を水耕栽培することによって人為的にビターピットを発生させた(5)．‘ふじ’のビターピット発生程度は、果肉中のカルシウム濃度の減少と密接に関係していた．

リンゴ果実のミクロゾーム画分にはバナジン酸ナトリウムで阻害され、硝酸カリウムで阻害されない(K^+ + Mg^{2+})-ATPase が存在した．また、蛍光物質であるキナクリンを用いてプロトンの輸送活性を調べたところ、本ミクロゾーム画分には ATPase と同様の性質を持っ

たプロトン輸送活性が存在した．即ち、リンゴ果実には原形質膜に由来する H^+ -ATPase が存在すると考えられた．

ビターピット発生の重症果では、バナデート感受性 ATPase 並びにプロトン輸送活性の著しい低下が認められた．このことから、ビターピットの発生する果実の原形質膜では、正常な物質輸送を低下させるような変化を生じていることが推察された．