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Increase in the Permeability of Tonoplast of Garlic (*Allium sativum*) by Monocarboxylic Acids

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Immersion of intact aged garlic (*Allium sativum*) cloves in a series of 5% weak organic monocarboxylate solutions (pH 2.0) resulted in green color formation. No color was formed upon treatment with other weak organic acids, such as citric and malic acids, and the inorganic hydrochloric acid under the same conditions. To understand the significance of monocarboxylic acids and their differing function from that of other acids, acetic acid was compared with organic acids citric and malic and the inorganic hydrochloric acid. The effects of these acids on the permeability of plasma and intracellular membrane of garlic cells were measured by conductivity, light microscopy, and transmission electron microscopy. Except for hydrochloric acid, treatment of garlic with all three organic acids greatly increased the relative conductivity of their respective pickling solutions, indicating that all tested organic acids increased the permeability of plasma membrane. Moreover, a pickling solution containing acetic acid exhibited 1.5-fold higher relative conductivity (∼90%) as compared to those (∼60%) of both citric and malic acids, implying that exposure of garlic cloves to acetic acid not only changed the permeability of the plasma membrane but also increased the permeability of intracellular membrane. Exposure of garlic to acetic acid led to the production of precipitate along the tonoplast, but no precipitate was formed by citric and malic acids. This indicates that the structure of the tonoplast was damaged by this treatment. Further support for this conclusion comes from results showing that the concentration of thiosulfinates [which are produced only by catalytic conversion of *S*-alk(en)yl-L-cysteine sulfoxides in cytosol by alliinase located in the vacuole] in the acetic acid pickling solution is 1.3 mg/mL, but almost no thiosulfinates were detected in the pickling solution of citric and malic acids. Thus, all present results suggest that damage of tonoplast by treatment with monocarboxylates such as acetic acid may be the main reason for the greening of garlic.

KEYWORDS: Garlic; tonoplast; permeability; monocarboxylic acids; plasma membrane; greening

INTRODUCTION

Garlic (*Allium sativum*) has been used as a food and as a spice and in herbal remedies in many parts of the world for more than 4000 years. Garlic-containing remedies are one of the most common herbal drugs in Europe, Asia, and North America (*1*). Many health properties of garlic are attributed to its organosulfur compounds, particularly thiosulfinates [R−S−S(O)−R′]. Diallyl thiosulfinate (allicin) is the most abundant compound, accounting for 60−80% of the overall thiosulfinates formed by the interaction of the enzyme alliinase with the non-protein amino acid *S*-allyl-L-cysteine sulfoxide (alliin) (*2*, *3*). Alliinase is found in vacuoles and is thereby physically separated from its natural substrate alliin, which occurs in the cytosol (*4*, *5*). Only upon injury of the garlic cloves does it come into contact with alliin, suggesting that the alliinase/alliin system may be a primitive plant defense system.

Garlic is processed in various forms such as powder, granules, puree, minced paste, and oleoresin. During processing, greening is a major concern because it limits commercial utilization and reduces economic value (*6*, *7*). Green pigments corresponding to the greening are considered to be secondary metabolites in garlic. Interestingly, this greening is desirable and required for the traditional homemade Chinese "Laba" garlic ("Laba" means the eighth of December in the Chinese lunar calendar), which has a long history in folklore (*8*). Laba garlic is usually prepared by soaking garlic in vinegar for more than a week during winter. Because of its green color and unique taste as compared to normal garlic, it is usually eaten with dumplings to celebrate Chinese New Year in northern China. An aging process of fresh garlic is necessary for the formation of the green pigments. Garlic is harvested in September and stored in a dry area for several months. Simulation of the greening of Laba garlic was carried out in the laboratory by soaking aged garlic in 5% (v/v, pH ∼2.0) acetic acid solution (*8*). In contrast, under the same conditions, the green pigments were not produced when garlic was immersed in inorganic acid and other weak organic acids

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such as citric acid and malic acid. This opens the question as to the role of acetic acid in the green color formation of intact garlic cloves.

To date, some weak organic acids including acetic acid have been widely applied in the preservation of foods and beverages (*9*). Because these weak organic acids are generally more toxic to microorganisms at low pH, it is assumed that the antimicrobial activity of these acids is the result of an increased proportion of undissociated molecules. Undissociated weak acids diffuse passively into the microbial cell until an equilibrium is established across the membrane. Inside the cell the acids dissociate due to the more neutral pH and release protons, leading to a decrease of intracellular pH, which interferes with normal metabolic pathways. Weak acids cause several strong changes in intracellular processes: for example, in nutrient and ion transport, membrane structure, and fatty acid and phospholipid composition, as well as in protein synthesis (*10*, *11*). Similarly, weak organic acids, especially monocarboxylates, traverse the membrane of plant cells in their corresponding undissociated forms, resulting in different physiological effects (*12*−*14*). To date, there is little information available on the effect of these weak organic acids on plant organelles, especially on the intracellular membrane.

In the present study, we provide evidence to show that, except for inorganic hydrochloric acid, all tested weak organic acids significantly increase the permeability of the plasma membrane of garlic cells. However, unlike citric and malic acids, monocarboxylates such as acetic acid further enhance the permeability of the tonoplast. This may be an important reason for the green color formation of intact aged garlic immersed in solutions of 5% monocarboxylic acids rather than in solutions containing citric and malic acids.

MATERIALS AND METHODS

Chemicals. All solvents/chemicals used were of analytical grade or purer. Acetic, *n*-propionic, *n*-butyric, *n*-valeric, *n*-caproic, citric, malic acid, benzoic, sorbic, and hydrochloric acids were purchased from Beijing Chemistry Co. (Beijing, China). 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) and L-cysteine were purchased from Sigma Chemical Co. (Beijing, China). The solutions of L-cysteine (6.0×10^{-4} M) and DTNB (3.0×10^{-4} M) were both freshly prepared in sodium phosphate buffer, and DTNB was stored in a brown reagent bottle at 4 °C after preparation.

Plant Material. Garlic bulbs were obtained from a local market of China Agriculture University. They were harvested in September 2005 and aged by storing at 4 °C for more than 15 days.

Preparation of the Pickling Solution of Different Monocarboxylic Acids. To 5% organic acid pickling solutions were added ∼5.0 g or 5.0 mL of different monocarboxylic acids added to 95 mL of ddH_2O with stirring at room temperature, respectively. The pH of the solutions was adjusted to 2.0. Because of the low water solubility, *n*-caproic, sorbic, and benzoic acids cannot be dissolved completely in H_2O at pH 2.0. Insoluble parts were filtered, and the filtrate was directly used as pickling solutions. The final concentrations of these three organic acids were measured as ∼2−3%.

Conductance Measurement. Garlic cloves (∼50 g) were soaked in 30 mL of 5% (v/v) acetic, 5% citric, 5% malic, and 5% hydrochloric acid solutions at pH 2.0 for different lengths of time (1, 2, 3, 4, 5, 6, or 7 days). Pickled garlic cloves were taken from the solution, rinsed three times with distilled water, and chopped into 3-mm-thick slices. One batch of sliced garlic (2.0 g) was placed in a test tube containing 30 mL of ddH_2O and then was put in the incubator at 25 °C for 1 h with shaking at 170−185 rpm for 1 h. The conductivity, L_1, of the filtered solution was measured. In parallel, another batch of sliced garlic (2.0 g) was placed in a test tube containing 30 mL of ddH_2O, which was boiled for 10 min and cooled to room temperature. The conductivity of the filtrate was determined, L_0. The relative conductivity (L_R) for each sample was defined as $L_R = L_1/L_0 \times 100\%$. Each point represented the average of three independent measurements. All conductance measurements were carried out with a DDS-12A digital conductivity meter (Shanghai Dapu Instrument Co., Shanghai, China), using a dip-type conductivity cell containing platinum electrodes.

Light Microscopy Analysis. Similar portions taken from the middle of all tested garlic cloves were removed for light microscopy analysis after the garlic cloves were immersed in 5% acetic, citric, malic, and hydrochloric acids at pH 2.0 for 7 days followed by three washes with ddH_2O. The cut parts were fixed in 40% formaldehyde, 10% glacial acetic acid, and 50% ethanol (FAA) for at least 2 days, followed by dehydration in a series of *tert*-butylethanol and ethanol. Then they were embedded in paraffin for sectioning in a rotary microtome. The sections (10−12 μm) were stained with safranine and fast green as previously described (*15*). A Wild Heerbrugg optical microscope model M20EB equipped with a camera was used for observations and photomicrographs.

Transmission Electron Microscopy (TEM) Analysis. For studying the microstructure of the garlic cells with a transmission electron microscope, garlic cloves, which had been immersed in H_2O or 5% (v/v) acetic acid, 5% citric acid, or 5% malic acid at pH 2.0 for 7 days were sectioned into 2-mm cubes. Resulting sections were fixed in 3% glutaraldehyde (0.1 M sodium phosphate buffer, pH 6.8) for 4 h prior to fixation in 1% osmium tetroxide (OsO_4, same buffer) for 4 h. Rinsing occurred twice, 45 min after the first fixation step and 12 h after the second fixation step, in 0.1 M sodium phosphate buffer, followed by dehydration in a series of graded ethanol (30, 50, 70, 90, and 100%) and anhydrous acetone. Subsequently, the samples were embedded in Spurr's resin at room temperature. Ultrathin sections were contrasted with lead citrate and uranyl acetate, followed by examination in a transmission electron microscope (Hitachi H-600, Japan) at 100 kV.

Measurement of the Concentration of Total Thiosulfinates in Pickling Solution. Garlic cloves (100 g) were taken from pickling solutions (100 mL) after soaking in ddH_2O or 5% acetic, 5% citric, or 5% malic acids at pH 2.0 for 7 days. The pickling solution was centrifuged to remove any particle, and then supernatant (1 mL) was mixed with 99 mL of 50 mM sodium phosphate buffer (pH 6.9), containing 1 mM EDTA. Subsequently, the solutions were filtered, and total thiosulfinates were quantified spectrophotometrically with a UV spectrophotometer TU-1901 (Beijing Spectroscopic Analysis Co.) as described previously (*16*). Briefly, a fresh excessive L-cysteine solution was added to the crude garlic extracts. The resulting solution was allowed to stand for 10 min at room temperature followed by the addition of DTNB solution. All solutions of DTNB, L-cysteine, crude garlic extract, and buffer were mixed in equal volumes. This solution produces an absorbance at 412 nm as A_1 with a mixture of DTNB/crude garlic extract/buffer (1:1:2, v/v/v) as a blank. In parallel, a mixture of DTNB/cysteine/buffer (1:1:2, v/v/v) was used as control and gave an absorbance at 412 nm as A_2 with a mixture of DTNB/buffer (1:3, v/v) as a blank. The content of thiosulfinates in garlic was calculated according to the formula of $0.5 \times (A_2 - A_1) \times f \times \epsilon$, where f is the dilution factor and $\epsilon = 14150\ M^{-1}\ cm^{-1}$ (*16*).

RESULTS

The intact aged garlic cloves turned green 1 day after they were soaked in 5% acetic acid at pH 2.0 (a condition similar to the preparation of Laba garlic in Chinese folklore), and the green color became deeper with time and reached a maximum by day 7 (**Figure 1**). A very similar process was likewise observed with other monocarboxylic acids such as propionic, butyric, valeric, capric, which have structures similar to that of acetic acid (**Table 1**), indicating that these organic weak acids had the same function as acetic acid for the greening of garlic. Green color in garlic cloves was also formed upon immersion of garlic cloves in the most widely used food preservatives including benzoic acid and sorbic acid, but it was significantly lighter (**Table 1**). In contrast, under the same conditions, no color was generated when the aged garlic cloves were immersed in citric, malic, oxalic, or hydrochloric acids.

Figure 1. Green color formation of aged garlic immersed in 5% acetic acid at different times (**a**, 0 days; **b**, 1 day; **c**, 2 days; **d**, 3 days; **e**, 4 days; **f**, 5 days; **g**, 6 days; **h**, 7 days). No color was formed when the aged garlic was soaked in H_2O, citric acid, malic acid, oxalic acid, phosphoric acid, or hydrogen chloride for 1 week.

Table 1. Relationship between pK_a of All Kinds of Acids and Green Color Formation of Garlic

acid	pK_1	pK_2	pK_3	color
acetic acid	4.76	N/A	N/A	green
n-propionic acid	4.87	N/A	N/A	green
n-butyric acid	4.82	N/A	N/A	green
n-valeric acid	4.82	N/A	N/A	green
n-caproic acid	4.83	N/A	N/A	green
sorbic acid	4.76	N/A	N/A	slightly green
benzoic acid	4.19	N/A	N/A	slightly green
citric acid	3.13	4.76	6.40	none
malic acid	3.46	5.05	N/A	none
oxalic acid	1.27	4.27	N/A	none
hydrogen chloride	N/A	N/A	N/A	none

It is worth noting that the green pigments diffused out of the garlic cloves into the pickling solution with 5% acetic acid; this phenomenon was easily observed after 5 days, suggesting that acetic acid might increase the permeability of the plasma membrane of garlic. To test this idea, the relative conductivity of all pickling solutions was measured at different times (**Figure 2**). It was observed that, except for the pickling solution prepared with hydrochloric acid, the relative conductivity of the other three pickling solutions increased with time. However, the acetic acid pickling solution reached a maximum on the fifth day, but the pickling solutions containing both citric and malic acids reached their maximum on the third day. More importantly, the pickling solution of acetic acid exhibited the largest relative conductivity (~90%) among all of the samples, whereas the pickling solutions of both citric and malic acids had lower and nearly identical relative conductivities at ~60%, a result implying that treatment with acetic acid may disrupt the organelle(s) within the garlic cells, leading to more electrolyte released to the bulk solution.

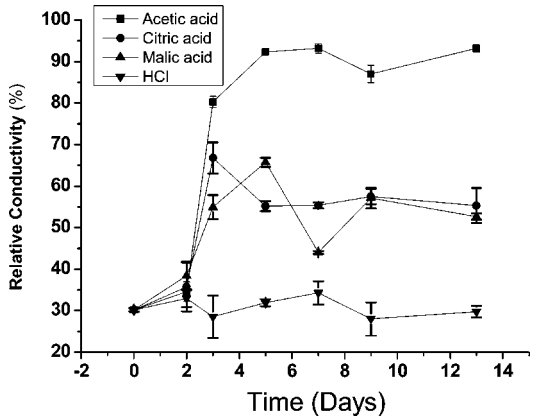

Figure 2. Effects of acetic, citric, malic, and hydrochloric acids on the permeability of garlic membrane. Garlic cloves were soaked in 5% acetic, citric, malic, and hydrochloric acids at pH 2, and then their corresponding relative conductivity was measured. Each value is the average of three measurements. Vertical bars represent the standard deviation.

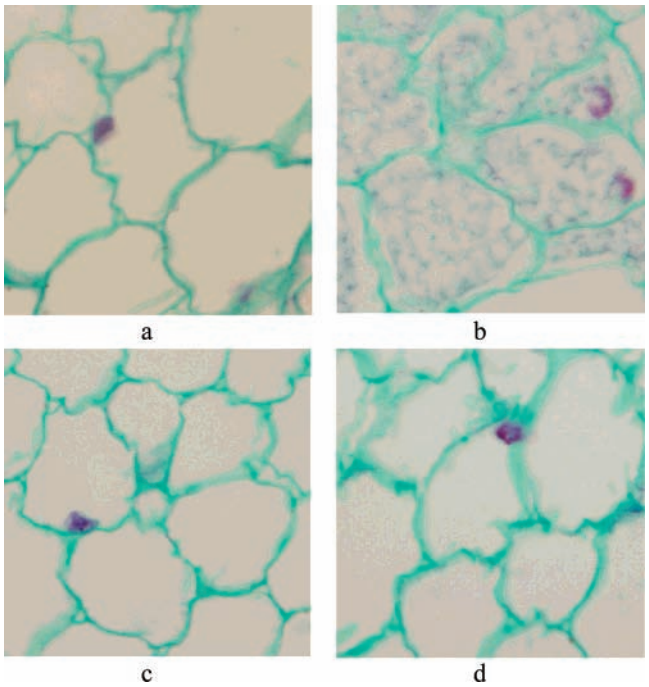

Figure 3. Light micrographs of 10-μm sections of garlic without treatment (**a**) and of garlic treated with 5% acetic (**b**), citric (**c**), and malic (**d**) acids at pH 2.0 for 1 week. All samples were stained with safranine and fast green and magnified 100 times.

Light microscopy was used to assess the membrane changes of garlic upon treatment with 5% acetic, 5% citric, 5% malic, or 5% hydrochloric acids at pH 2.0 (**Figure 3**). With safranine and fast green dyes, the nucleus and plasma membrane of garlic were easily distinguished red and green color, respectively, in all samples. Similar to the control (garlic cloves without treatment, **Figure 3a**), the garlic cells showed almost no change and nearly the same morphology upon treatment with citric (**Figure 3c**) and malic (**Figure 3d**) acids, respectively, and are shaped like a tear drop. Likewise, exposure of garlic cloves to hydrochloric acid resulted in nearly the same effect on the garlic cells as the two organic acids (data not shown). In contrast, after treatment with 5% acetic acid (pH 2.0), the garlic cells underwent a pronounced change characterized by the production of noticeable precipitate within the cell, indicating that acetic acid caused great damage to certain organelles.

To find what organelle was damaged by acetic acid, TEM was used to observe the microstructure of garlic cells that had been immersed in acetic, citric, or malic acids for 7 days (**Figure 4**). The internal organization was hardly discerned by TEM for the control sample. Compared to the control sample (**Figure 4a**), there was no obvious change made by incubation of garlic with either 5% citric or 5% malic acids (pH 2.0) for 1 week (**Figure 4c**,**d**); this is consistent with the above observations obtained with light microscopy (**Figure 3c**,**d**). The effect of

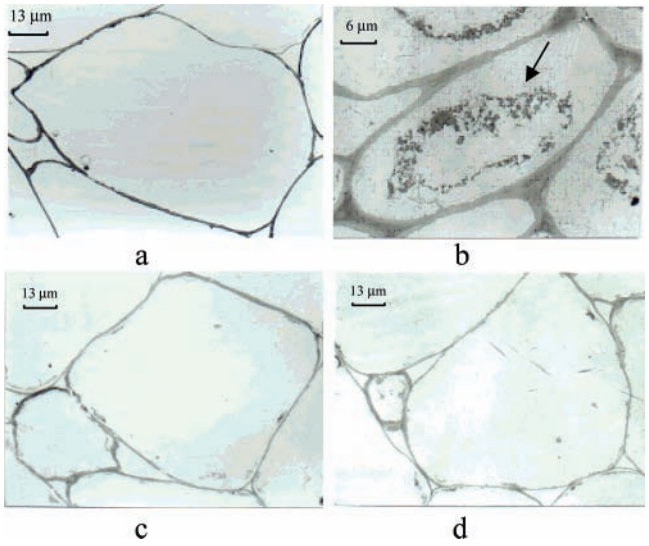

Figure 4. Transmission electron micrographs of sections of garlic without treatment (**a**) and of garlic treated with 5% acetic (**b**), citric (**c**), and malic (**d**) acids at pH 2.0 for 1 week.

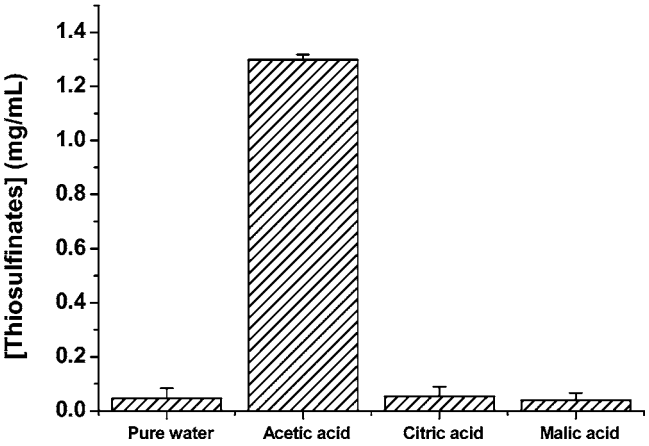

Figure 5. Thiosulfinates concentration in pickling solutions when garlic was soaked in H_2O (**a**) or in solutions containing 5% acetic acid (**b**), 5% citric acid (**c**), or 5% malic acid (**d**) at pH 2.0. The concentration of thiosulfinates was measured on the basis of 100 g of garlic cloves in a 100 mL volume of the above pickling solution.

hydrochloric acid on garlic cells was similar to that of citric and malic acids (data not shown). In contrast, garlic cells underwent both morphological and microstructural changes after acetic acid treatment under the same conditions; they appeared to be much smaller and kidney-shaped as compared to the irregular structure for the control, the pyriform structure for the malic acid sample, and the square-like structure for the citric acid sample (**Figure 4**). These morphological and microstructural changes have been reproducible. The most noticeable change of garlic treated with acetic acid was manifested by precipitate formed along the tonoplast, coinciding with the observation with light microscopy showing that precipitation occurred only upon treatment with 5% acetic acid (**Figure 3b**).

Thiosulfinates result from the interaction of alliinase located in the vacuole with its substrates, *S*-alk(en)yl-L-cysteine sulfoxide(s), which are found in cytosol (*5*). To further confirm whether the tonoplast of garlic is disrupted by treatment with acetic acid, the concentration of thiosulfinates in the pickling solutions containing ddH_2O alone or 5% acetic acid, 5% citric acid, or 5% malic acid was measured (**Figure 5**). The concentration of thiosulfinates was determined on the basis of 100 g of garlic cloves in a 100-mL volume of the above pickling solutions. The acetic acid pickling solution exhibited the highest concentration of thiosulfinates, 1.3 mg/mL, whereas almost no thiosulfinate can be detected with the other three solutions made with ddH_2O alone, citric acid, and malic acid under the same condition, further supporting the conclusion that the permeability of the tonoplast is greatly increased by treatment with acetic acid but not with the other three acids.

DISCUSSION

This study reinforced and extended previous work (*8*) in finding that treatment of intact garlic cloves with acetic acid led to a great increase in the permeability of the tonoplast (**Figure 2**). As a result, the substrates in the cytosol pass through the tonoplast into the vacuole, where they are catalytically converted by alliinase to thiosulfinates and then react with amino acid to produce a pyrrole compound, ultimately resulting in the formation of the green pigments (**Figure 1**) (*20*). Another possibility which cannot be excluded is that alliinase located in the vacuole traversed the tonoplast to the cytosol, where it catalytically converted its substrates to thiosulfinates (**Figure 5**), which subsequently reacted with amino acid to finally produce green pigments. Because the pH optimum for activity of alliinase in garlic is acidic, 4.5−6.5 (*17*), and the vacuole is also acidic with a pH in the range of 4.5−6.0 in many plant species (*18*), whereas the cytoplasm is slightly alkaline (pH 7.4−7.5), the first possibility is more favorable. Consistent with this idea, it has been well-established that alliinase and thiosulfinates are necessary for the formation of the green color of garlic and that thiosulfinates are precursors of the green pigments (*8*, *19*−*22*). Thus, increasing the permeability of the tonoplast of garlic represents the first step, or at least an early event, in the formation of the green pigments.

Exposure of garlic to all tested organic acids significantly increased the relative conductivity of pickling solution, which indicates the increase of the permeability of garlic cell membrane. On the contrary, treatment with ann inorganic acid such as hydrochloric acid lacked this property (**Figure 2**). These results demonstrate that these weak organic acids entered into garlic cells by their corresponding undissociated forms, whereas hydrochloric acid as a strong electrolyte was not able to cross the membrane due to its complete dissociation. Support for this idea stems from the calculation according to the Henderson−Hasselbach equation (eq 1), indicating that much more than 50%

$$\mathrm{pH} = \mathrm{p}K_a + \log[A^-]/[\mathrm{HA}] \quad (1)$$

of these organic acids exist in their undissociated forms in the pickling solutions at pH 2.0, which is much lower than their pK_a values (**Table 1**). In eq 1 A^- and HA are the dissociated and undissociated species, respectively. The present observation is also in accord with previous results showing that organic acids such as formic, acetic, propionic, butyric, and glutaric increased the permeability of barley roots and red rice by their corresponding undissociated forms (*13*, *14*). It has been suggested that changes in the composition of the membrane lipids are closely associated with changes in permeability (*23*).

Interestingly, there is a pronounced difference in the change of permeability between different garlic treatments: acetic acid and other acids including citric, malic, and hydrochloric; namely, a larger increase in the membrane permeability is achieved with acetic acid than with the other three acids, indicating that more electrolyte is released from inside garlic cells to the bulk solution of acetic acid as compared to other bulk solutions containing

citric, malic, and hydrochloric acids. This result implies that organelles inside the garlic cells are broken by treatment with acetic acid but not with the other three acids. Consistent with this idea, precipitation occurred around the tonoplast only with garlic samples treated with 5% acetic acid but not with the other three acids as shown by both light microscopy and TEM (**Figures 3** and **4**). Further support for this conclusion stems from the measurement of thiosulfinates indicating that the concentration of thiosulfinates was much greater in the acetic acid pickling solution than in the other pickling solutions of the three acids (**Figure 5**).

These results raise an interesting question as to why acetic acid has such a distinct effect on the garlic cells. Our studies found that garlic also turned green upon its exposure to 5% acetic acid over the pH range from 2.0 to 7.0, whereas it remained unchanged upon treatment with citric and malic acids even at low pH values such as 1.0 or at pH 7.0 (data not shown), indicating that incubation medium pH has no effect on the permeability of the tonoplast. Thus, the change of the permeability of the tonoplast depends on the structure of the organic acids irrespective of the amount of undissociated forms of the weak organic acids inside the garlic cells. Coinciding with this conclusion, other monocarboxylic acids such as propionic, butyric, valeric, and caproic likewise cause garlic to become green, but other dicarboxylates, succinate and oxalate, do not. Therefore, it seems that only monocarboxylic acids have the ability to increase the permeability of the tonoplast. Previous results provide a reasonable explanation of the above observation that both citric and malic acids are compartmentalized in the large central vacuole (*24*−*26*), especially because they are intermediates of the citric acid cycle occurring in the mitochondrion. Thus, plant cells must have evolved a system responsible for the transportation of citric and malic acids between the vacuole and the mitochondrion and for their regulation inside the cell. This system might prevent citric acid and malic acid from damaging the tonoplast. In contrast, garlic cells presumably lack such a system controlling the concentration of monocarboxylates, consequently disrupting the tonoplast and inducing a series of unidentified reactions to ultimately produce the green pigments.

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