

Chlorophyllase in flavedo of (*Citrus sinensis* L.) Osbeck during color development

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Abstract: Ethephon induces an accumulation of chlorophyllase and loss of chlorophyll in flavedo of detached 'Valencia' oranges (*Citrus sinensis* L. Osbeck). Pretreatment with gibberellic acid inhibits both responses to ethephon. Benzyladenine behaves similarly to gibberellic acid but is less effective. However, during natural degreening and regreening of fruit on the tree, chlorophyll increases to a maximum in November, is lost during the winter and reappears during the spring, chlorophyllase levels steadily decline. When gibberellic acid is used to enhance regreening of fruit on the tree, chlorophyllase levels in the flavedo are unaffected.

These results demonstrate that loss of chlorophyll during maturation of citrus fruit is not in response to elevated levels of chlorophyllase and suggest that increases in chlorophyllase do not contribute to the loss of chlorophyll induced by ethephon.

Additional keywords: Chlorophyll, chlorophyllase, Ethephon, gibberellic acid, benzyladenine, degreening, regreening, orange.

INTRODUCTION

From observed increases in levels of chlorophyllase during the respiratory climacteric of apples (Loeny and parterson, 1967, Rhodes and Waltorton, 1967) and bananas it has been suggested that the first step in chlorophyll degradation during fruit ripening is hydrolysis of chlorophyll to chlorophyllide and phytol and that chlorophyll loss during fruit

ripening might be regulated by levels of this enzyme. This hypothesis had been extended to citrus fruit, which are non-climacteric, by observations that ethylene causes increases in levels of chlorophyllase in flavedo of *Citrus microcarpa* (Barmore, 1975), and *C. unshiu* Marc (Shimokawa et al., 1978) while also stimulating loss of chlorophyll.

In contrast to the foregoing observations on fruit ripening chlorophyllase increased in parallel with chlorophyll during greening of peas (Holden, 1961), *Chlorella protothecioides* (Chiba et al., 1967; Ganoza and Macfeeters, 1976) and *Chlorella vulgaris* (Boger, 1965) and declined in parallel with chlorophyll during degreening of *C. protothecoides*. During degreening of *C. vulgaris* there was no apparent change in chlorophyllase levels. During the development of tobacco leaves (Shimizu and Tamaki, 1962) found that chlorophyllase in chloroplasts increased to a maximum preceding chlorophyll accumulation by about 35 days, then fell somewhat in advance of chlorophyll during leaf senescence. Furthermore, during leaf senescence phytol appears to remain esterified to chlorophyll (Park et al., 1973) suggesting that hydrolysis to chlorophyllide and phytol is not an early step in the degradation of chlorophyll.

Thus, an inverse relation between levels of chlorophyllase and chlorophyll seems to be a feature of fruit ripening, but not the development of leaves or *Chlorella*, and while a degradative role has been ascribed for the enzyme in fruit ripening a synthetic role has been otherwise suggested.

Since ethylene does not appear to play a role in the natural degreening of citrus fruit (Apelbaum et al., 1976) the significance of an inverse correlation between chlorophyll and chlorophyllase in response to ethylene is questionable. In view of this, the relation between chlorophyllase and chlorophyll in flavedo of 'Valencia' organs (*Citrus sinensis* L. Osbeck) was investigated during natural degreening and regreening of fruit in the tree. In addition, gibberellic acid and benzyladenine inhibit several ethylene elicited responses of citrus fruit including chlorophyll loss (Goldschmidt et al., 1977) and experiments were performed to determine if these substances inhibit chlorophyll loss by affecting levels of chlorophyllase.

MATERIALS AND METHODS

Orange fruit (*Citrus sinensis* L. Osbeck cv. 'Valencia') were obtained from the University of Arizona Citrus Research Farm at Tempe, Arizona. To follow chlorophyll and chlorophyllase levels in flavedo of fruit during their natural degreening and regreening, one fruit was picked from each of 10 trees periodically and combined for analysis.

Gibberellic acid (GA_3) enhanced regreening was investigated by selecting 6 pair of mature orange - colored fruit on 6 trees and twice dipping one member of each pair into 1.44 mM GA_3 in 0.02% Triton X-100 for 30 seconds. Treated and control fruit were left on trees for 39 days, then harvested and chlorophyll and chlorophyllase contents determined.

To determine the effects of GA_3 and benzyladenine (BA) on ethephon induced chlorophyllase increases, 320 mature green fruit collected from 20 trees on November 11, were treated with ethephon or ethephon in combination with 0.23 mM GA_3 or 0.35 mM BA. The fruit were twice immersed for 30 sec. in solutions of growth regulator containing 5% ethanol and 0.02% Triton X-100, followed about 3 hrs later with a 25 μ l droplet of 64 mM ethephon applied to the pedicel scar. Control fruit were dipped in similar solutions without growth regulator. Each treatment included 80 fruit stored in darkness at room temperature. Samples of 20 fruit each were taken for chlorophyll and chlorophyllase determinations after 1, 2, 4 and 6 days. A group of 20 untreated fruit were used to establish initial conditions.

Determination of chlorophyll in peels. Chlorophyll was estimated in vivo by measuring the difference in light absorption by intact fruit at 675 and 735 nm with a Bausch and Lomb Model 340 spectrometer fitted with an integrating sphere reflectometer (Jahn, 1970 and Jahn and Young, 1976) One measurement was made on each of 20 fruit in the equatorial region and the values obtained were averaged. Total chlorophyll was estimated by multiplying this average value by a surface area calculated from average length and width assuming a prolate spheroid. The reflectometer is sensitive to low levels of chlorophyll and is especially useful in estimating chlorophyll in degreened and regreened fruit.

Acetone powder preparation. After estimating chlorophyll content, flavedo were removed with a potato peeler, combined, weighed, cut into small slices, and then homogenized in 100% acetone with a Sorvall Omni Mixer for one minute. The resulting slurries were filtered through a Buchner funnel, washed with excess acetone and dried at room temperature. The resulting coarse powders were stored at - 18 C until assayed for chlorophyllase.

Extraction of chlorophyllase. Chlorophyllase was extracted from 0.300 g of each acetone powder with 20 ml of 100 mM potassium phosphate buffer (pH 7) containing 0.4 M sodium chloride and 0.5% Triton X-100, after first grinding with quartz sand in a chilled mortar and pestle. Homogenates were then centrifuged for 10 minutes at 20200g and 4°C. Residues were discarded and supernatants allowed to stand for 75 minutes at 4 C before assaying for chlorophyllase.

Preparation of chlorophyll substrate. Chlorophyll was extracted from fresh spinach leaves with methanol and partially purified by precipitation with dioxane as described by Iriyama et al. (1974). Chlorophyll a was separated from chlorophyll b by partitioning between petroleum ether and 97% methanol. Chlorophyll a in petroleum ether was then dried with anhydrous sodium sulfate and stored at - 18 C.

Aqueous solutions of chlorophyll a were prepared by layering petroleum ether solutions over 100 mM sodium citrate buffer (adjusted to give a final assay pH of 5.0) containing 0.25% Triton X-100 and then removing the ether by vacuum distillation.

Chlorophyllase assay. Optimum pH for assaying chlorophyllase has been reported to lie between 5.5 and 8.7 depending upon source of enzyme and assay procedure (Barmore, 1975; Ighinose and Sasa, 1973, Mcfeeters et al., 1971; Rhodes and Wooterton, 1967 and Terpstra, 1974). Optimum pH under our conditions was found to be 5.0.

Chlorophyllase in extracts was estimated by determining the relative amount of chlorophyllide a generated at pH 5.0 when 0.5 ml of extract (pH 7) was incubated 20 min with 3 ml of chlorophyll a in sodium citrate buffer (pH 4.4). At zero time and after 20 min, 1.5 ml of reaction mixture was added to a centrifuge tube containing 10 ml. of a 1:2 (v/v) mixture of acetone and hexane and 0.5 ml of 300 mM tricine buffer

adjusted so that the pH at this point was 9 ± 0.3 . After vigorous shaking, two phases were separated by centrifugation at 1,500 g for 5 min. Chlorophyllide a was estimated in the lower phase by its absorbance at 663 nm.

RESULTS AND DISCUSSION

In order to determine when chlorophyll was accumulated and lost it was found necessary to estimate the total chlorophyll content of the fruit peels by using the product of measured values of chlorophyll per unit area and estimated average surface areas. This was because the fruit were enlarging until late December and even though the amount of chlorophyll in the fruit was increasing the chlorophyll per unit area was decreasing.

Chlorophyll accumulated in the fruit peels until late November then abruptly began to disappear (Figure 1). By February (1979) and March (1978) the fruit has lost all or nearly all their chlorophyll, but from March and especially in June, (1978 and 1979) chlorophyll again accumulated in the fruit. By the end of June (1978, 1979) the fruit peels had recovered one third to one half the chlorophyll lost during November and December, although the increase in chlorophyll per unit area was somewhat less. This 'regreening' is characteristic of some citrus fruit, most notably 'Valencia' oranges, and is thought to be caused by the return of warm weather in the spring (Caprio, 1956).

Levels of chlorophyllase extractable from these same fruit were high on September 1st and declined sharply through September and October. This was during a period when chlorophyll was accumulating. By November the decline in chlorophyllase had slowed, and throughout the remainder of the study-period, chlorophyllase levels slowly decreased with some slight fluctuations. Thus, the relationship between chlorophyll and chlorophyllase under these conditions differs from that previously observed for citrus fruit. Initially, chlorophyll was accumulating while chlorophyllase was declining, but during the period that chlorophyll was lost there was no marked increase in chlorophyllase levels, which would be expected on the basis of results obtained by Barmore (1975) and Shimokawa et al. (1978).

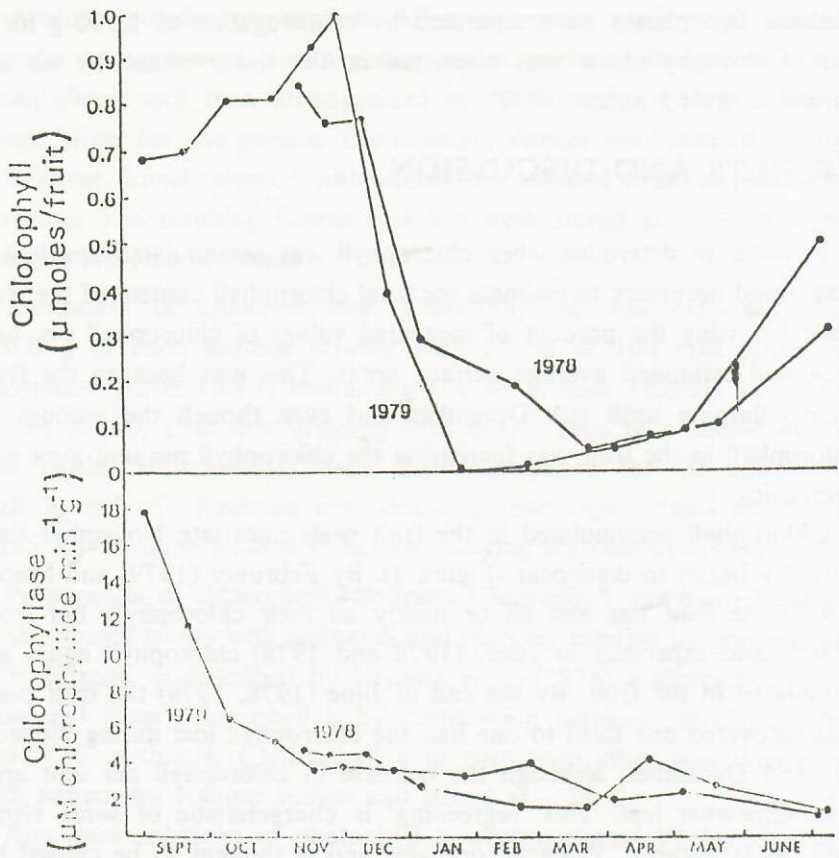


Fig. 1: Chlorophyll and chlorophyllase changes in flavedo of 'Valencia' orange fruit during 2 seasons.

Up to this point the results are similar to those obtained with tobacco leaves (Shimizu and Tamaki, 1962), however, there was no increase in chlorophyllase before or during the period of fruit regreening.

Both gibberellic acid (GA_3) and benzyladenine (BA) inhibit several ethylene elicited responses of citrus fruit (Goldschmidt et al., 1977) and it was felt that if, as suggested chlorophyll loss was due to elevated levels of chlorophyllase then GA_3 or BA might prevent those increase in

chlorophyllase. On the other hand, if both substances prevented chlorophyll loss without reducing chlorophyllase levels, than the involvement of that enzyme in chlorophyll degradation in citrus fruit would be questionable. It was found that GA_3 and BA do indeed reduce both increases in chlorophyllase and losses of chlorophyll in ethephon treated fruit.

Mature green fruit treated with ethephon as a source of ethylene (Abeles, 1973) lost chlorophyll more quickly than control fruit (Figure 2)

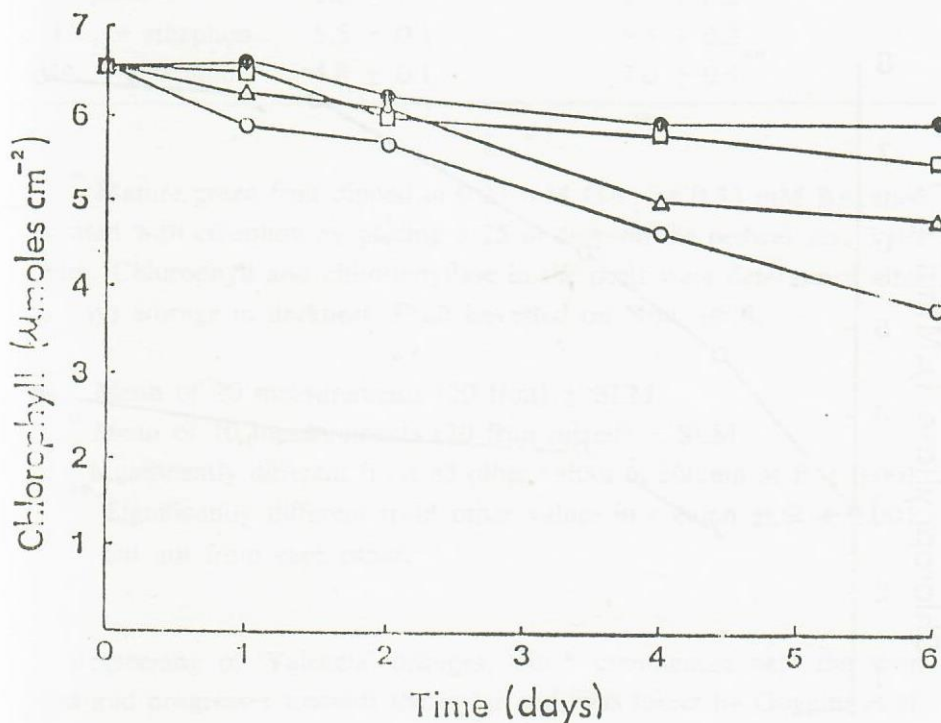


Fig. 2. Loss of chlorophyll from 'Valencia' orange fruit during 6 days storage in darkness (●), from similar fruit treated with ethephon (○), and from fruit treated with ethephon after pretreatment with GA_3 (□) and BA (Δ). (fruit harvested on Nov. 1978).

and this accelerated loss of chlorophyll was accompanied by increased levels of chlorophyllase (Figure 3). Loss of chlorophyll was largely prevented by pretreating the fruit with 0.23 mM GA_3 and somewhat less so by pretreating with 0.35 mM BA. Similarly, GA_3 markedly reduced the increase in chlorophyllase induced by ethephon (Table 1), and BA was less effective than GA_3 . Chlorophyll and chlorophyllase levels are inversely correlated ($r = -0.976$); results quite consistent with several earlier studies. However, similar effects of GA_3 were not observed on regreening fruit.

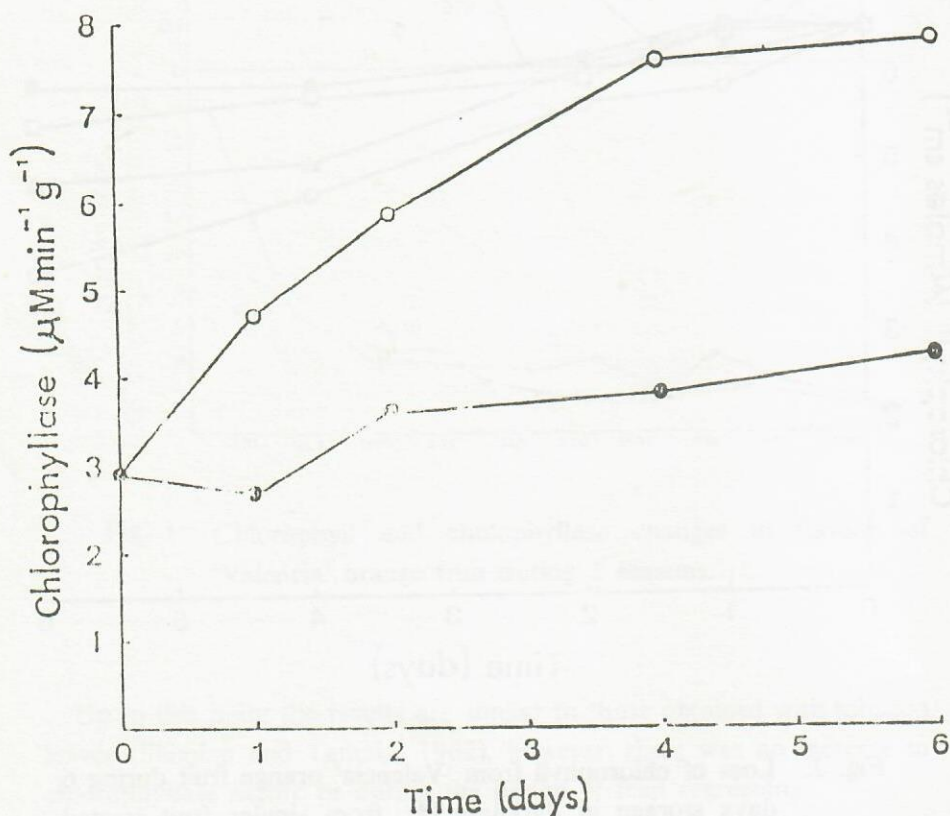


Fig. 3. Chlorophyllase in flavedo of 'Valencia' orange fruit during 6 days storage in darkness (●) and in flavedo of similar fruit treated with ethephon (○). (Fruit harvested on Nov. 1978).

Table 1. Changes in extractable chlorophyllase and chlorophyll levels in response to 6 days treatment with ethephon and ethephon following treatment with GA₃ or BA.^a

Treatment	Chlorophyll (nmoles/cm ²)	Chlorophyllase (um/min/g)
initial	6.6 ± 0.1 ^b	3.8 ± 0.3 ^c
control	6.0 ± 0.2 [*]	4.8 ± 0.2 ^{**}
ethephon	3.8 ± 0.1	7.5 ± 0.2
GA ₃ + ethephon	5.5 ± 0.1	5.5 ± 0.2 ^{**}
BA + ethephon	4.8 ± 0.1	7.0 ± 0.4

^a Mature green fruit dipped in 0.23 mM GA₃ or 0.35 mM BA, then treated with ethephon by placing a 25 ul drop on the pedical scar 3 hrs later. Chlorophyll and chlorophyllase in the peels were determined after 6 days storage in darkness. Fruit harvested on Nov. 1978.

^b Mean of 20 measurements (20 fruit) ± SEM

^c Mean of 10 measurements (20 fruit mixed) ± SEM

^{*} Significantly different from all other values in column at P < 0.001

^{**} Significantly different from other values in column at P < 0.001, but not from each other.

Regreening of 'Valencia' oranges, which commences near the stem end and progresses towards the stylar end, was found by Goggins et al. (1960) to be enhanced by application of potassium gibberellate. In our experiments, enhancement of regreening with GA₃ was not accompanied by a reduction in extractable chlorophyllase (Table 2).

Thus, our observations confirm that under certain conditions loss of chlorophyll from citrus flavedo is accompanied by increased levels of extractable chlorophyllase and conservation of chlorophyll by substances such as GA₃ also reduces the amount of chlorophyllase found in the flavedo. However, these results were only obtained when the loss of

Table 2. Changes in levels of extractable chlorophyllase in response to GA₃ application to regreening 'Valencia' oranges^a.

Treatment	Chlorophyll (nmoles/cm ²)	Chlorophyllase (um/min/g)	
	equator	stem end	
initial	1.8 ± 0.3 ^b *	2.6 ± 0.4 ^b *	2.11 ± 0.6 ^c
control	3.9 ± 0.1	6.0 ± 0.3	3.10 ± 0.12
GA ₃ (1.44 mM)	5.5 ± 0.3	6.5 ± 0.1	2.80 ± 0.12

^a Mature orange colored fruit were treated with GA₃ 1979, then left on the tree for 39 days at which time chlorophyll and chlorophyllase in the peel was determined.

^b Mean of 12 measurements (6 fruit) ± SEM

^c Mean of 10 measurements (6 fruit, mixed) ± SEM

* Significantly different than other values in column at P < 0.001.

chlorophyll was induced by ethephon just as the observations of Barmore (1975), Shimokawa et al. (1978) and Purvis (1980) were obtained using ethylene to induce degreening of citrus fruit.

In contrast there were no such relationships observed between levels of chlorophyll and extractable chlorophyllase during degreening and regreening of 'Valencia' oranges on the tree, in the absence of applied ethylene or ethephon. Nor were reduced levels of chlorophyllase observed in fruit that had been treated with GA₃ to enhance regreening.

It appears then that elevated levels of chlorophyllase are associated with chlorophyll loss when induced by ethylene. For ripening apples and bananas this is endogenously produced ethylene, but for citrus fruit it is not, and such relationships between elevated chlorophyllase levels and chlorophyll loss observed in citrus flavedos are artifacts induced by treatment with ethylene. Chlorophyll loss from citrus flavedo does not follow as a consequence of elevated chlorophyllase levels, and perhaps

even in ripening bananas and apples the correlation between chlorophyll loss and elevated chlorophyllase is coincidental.

In the various studies previously mentioned, emphasis was placed upon relation between extractable chlorophyllase levels and chlorophyll levels without regard for the relation between chlorophyllase levels and rates of changes in chlorophyll. For instance, even though Shimokawa et al. (1978) noted an increase in chlorophyllase while chlorophyll declined in flavedo of *Citrus unshiu* Marc., rates of chlorophyll loss remained unaffected during periods in which chlorophyllase levels increased 133%. Furthermore, highest rates of chlorophyll loss were observed early when chlorophyllase levels were increasing most rapidly, not when chlorophyllase levels were highest. Similarly, in experiments reported here the rate of chlorophyll loss did not increase as chlorophyllase accumulated in response to ethephon nor was there any correlation between rates of change in chlorophyll levels with changes in chlorophyllase levels during chlorophyll loss or accumulation in flavedo of fruit on trees. This lack of any relation between rates of chlorophyll change and chlorophyllase levels, whether chlorophyll is accumulating or declining, casts further doubt upon any causal relation between the two. Changes in chlorophyll content does not appear to be regulated by the abundance of chlorophyllase whether the enzyme is acting synthetically or degradatively. This, of course, does not rule out changes in the enzymes in vitro activity regulating chlorophyll loss or synthesis.

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