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INHIBITION OF CHLOROPHYLL DEGRADATION IN STAY-GREEN LANGRA MANGO (*MANGIFERA INDICA* L.) FRUITS

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SUMMARY

Langra mangoes (*Mangifera indica* L.) ripened at tropical temperature (25-30ºC) appear green due to incomplete degradation of chlorophyll even though the fruits are table ripe. About 30% of total chlorophyll was retained and two fold increase in total carotenoids was observed during ripening. Ripening at lower temperature resulted in more loss of chlorophyll and reduced levels of carotenoids. RP-HPLC of the pigments extracted from various stages of ripening indicated accumulation of an unknown Chl derivative (retention time 7.32 min) and pheophytin a (retention time 20.4 min). Soluble protein fraction from peel catalyzed faster degradation of Chl a as compared to Chl b. Screening of the intermediates of enzymatic Chl a degradation by RP-HPLC indicated operation of chlorophyllase pathway. Accumulation of the unknown intermediate was observed when Chl b was used as a substrate with respect to incubation time up to 60 min. However, other polar intermediates were formed and disappeared after 30 min. The results indicated that upon ripening possibly a derivative of Chl b and pheophytin a accumulate giving the fruit a 'stay-green' character.

Key words : **Green-ripe, green-unripe, *Mangifera indica* L., Mg-dechelatase, RFC reductase, stay-green fruits, yellow-ripe.**

INTRODUCTION

Disappearance of green colour is the first visible result of degradation of chlorophyll as a consequence of maturation and ripening of fruits. Chlorophyll breakdown is a regulated process and various enzymes catalyzing the different reactions have been identified. Brown *et al.* (1991) has classified these reactions in to two groups, type I and type II. Type I comprise the reactions catalyzed by the enzymes chlorophyllase, Mg-dechelatase, pheophorbide a oxygenase to opening of tetrapyrrole ring. Type II reactions lead to cleavage and opening of the porphyrin macrocycle. The biochemistry underlying the Chl decomposition during leaf senescence has been elucidated (Hörtensteiner 1999, Takamiya *et al.* 2000). Some of the enzymes have been cloned and studied in more detail (Jacob-Wilk *et al.* 1999, Wüthrich *et al.* 2000). Apart from this, Chl is also degraded by chlorophyll oxidase and peroxidase (Schoch *et al.* 1984, Yamauchi and Watada 1994).

During ripening of fruits disappearance of Chl is normally associated with unmasking of carotenoids and the fruit acquiring bright yellow-red colour. Langra mango and Cavendish banana are commercially important fruits of India. Despite their pleasant pulp colour, flavor and general acceptance, these fruits fail to develop yellow colour due to incomplete degradation of Chl when ripened

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at temperatures above 25-30ºC (Thomas and Janave 1992). These 'green-ripe' fruits affect consumer preference and consequently fetch a lower price. In 'stay-green' Cavendish bananas evidence has been provided for operation of two distinct degradative pathways, chlorophyllase pathway and Chl oxidase pathway (Janave 1997). The soluble enzyme fraction was partially purified and presence of various enzymes are reported (Janave and Sharma 2004). Although degradation of Chl by Chl oxidase was reported earlier (Schoch *et al.* 1984), no information is available on how the product, 13^2-OH-Chl is further metabolized. Also, there are very few reports on how Chl b is metabolized during senescence. In the present paper attempt has been made to understand the mechanism of inhibition of Chl a and Chl b degradation in Langra mango which show similar ripening character as Cavendish banana.

MATERIALS AND METHODS

Mature unripe fruits of mango (cv. Langra) were procured from a local market and stored in perforated polyethylene bags in cardboard boxes in dark for ripening at 25-30ºC and 20ºC. The peel was used for acetone powder preparation and pigment extraction. Acetone powder was prepared at different ripening stages as detailed in the previous paper (Janave 1997). The weighed amounts of the acetone powder were sealed in polyethylene bags and stored at –30ºC until further use. Authentic pigments Chl a, Chlorophyllide (Chlide) a, Chl b, and phenophytin (Pheo) a and b were prepared from spinach leaves as detailed earlier (Janave 1997).

Pigments from the peel (20 g) in triplicate after removing adhering pulp were extracted four times with 60 ml of acetone : hexane mixture (60 : 75). A pinch of $NaHCO_3$ was added to prevent Chl from pheophytinization. The filtrate was diluted with distilled water and separated in a separating funnel. The hexane epiphase containing all the pigments was washed with distilled water, dried over anhydrous Na_2SO_4, volume was made up and an aliquot was used for pigment analysis. An aliquot (1 ml), was evaporated by a stream of N_2, dissolved in 1 ml of spectroscopy grade diethyl ether and total chlorophylls, Chl a, Chl b and total carotenoids content was determined spectrophotometrically by using the equations of Lichtenthaler (1987).

Another aliquot from hexane layer was evaporated by stream of N_2, dissolved in HPLC grade methanol. The pigments after passing through 0.22 µ GVWP filter were analyzed by JASCO HPLC system. The sample was loaded into a 100 Aº Enertsil ODS RP C-18 column (4.6 ID x 250 mm) with an autoinjector and eluted with HPLC grade methanol isocratically. The operation conditions set were, flow rate 1 ml min^{-1}, detection at 425 nm and results were analyzed by using Borwin chromatography software.

The soluble enzyme from acetone powder was extracted as detailed earlier (Janave 1997). To 40 ml 0.02 M HEPES buffer (pH 7.2) containing 0.1 M NaCl, 10% glycerol and insoluble 2% polyvinyl polypyrrolidone (PVPP), 2 g acetone powder was added and stirred on magnetic stirrer at 4ºC in dark for 2 h. The slurry after passing through muslin cloth was centrifuged at 27,000 x g for 10 min. The supernatant was used as soluble enzyme. Protein content was determined by the Bradford dye binding (Bradford 1976) using bovine serum albumin as a standard.

The disappearance of Chl *a* and Chl *b* was measured as enzyme activity as detailed in the previous paper (Janave 1997). Reaction mixture (1.5 ml) in 30% acetone contained 70mM phosphate buffer (pH 7), 10µM substrate (Chl *a* or Chl *b*) in acetone and 0.5 ml enzyme and incubated at 25ºC for specified time intervals. The reaction was stopped by 0.1 ml of 1 N NaOH followed by addition of 3ml of acetone/hexane mixture (2/4, v/v). The contents were vigorously vortexed until emulsion formation, allowed to stand for 10 min and centrifuged at 3000 × g for 5 min. The reaction mixture after separating in acetone/hexane mixture (2/4, v/v), absorbance of hexane layer at 663 mm and 642 nm for Chl a and b, respectively was recorded. Concentrations of Chl a and Chl b were determined by employing extinction coefficients 94.5 (Chiba *et al.* 1967) and 56.2 (Schoch and Ihl 1998) respectively. The aqueous acetone layer was used for HPLC analysis after extraction with diethyl ether. Ether layer was evaporated with a stream of N_2, dissolved in HPLC grade methanol and analyzed by HPLC as detailed under pigment preparation section. For control experiment, authentic chlorophylls were treated with reaction mixture components without enzyme under identical conditions.

RESULTS AND DISCUSSION

Langra mangoes remain green at table-ripe stage showing about 32% and 22% retention of total chlorophylls when ripened at 25-30ºC and 20ºC respectively (Table 1). At 'Green-unripe' stage, the concentrations of Chl a and Chl b were 11.37 mg and 5.1 mg $100g^{-1}$, respectively and upon ripening about 67-80% loss was observed at both the temperatures. However, at 20ºC the Chl content at ripe stage was much lower than that in fruits ripened at room temperature indicating that Chl degradation is inhibited at higher temperature. Total carotenoids increased by about two folds in 'RT-ripe' and 1.6 folds in '20ºC -ripe' mangoes. Ripening at lower temperatures is known to affect carotenoid synthesis in Alphonso mango (Thomas and Janave 1975).

RP-HPLC of pigments extracted from mango peel is shown in Fig. 1. It was observed that an unknown pigment (retention time (Rt) 7.32 min) and pheophytin a (Rt 20.4 min) were accumulated and very slowly degraded during ripening. Comparison of the area of the peak of unknown pigment revealed that it was degraded only 35% and 55% in 'RT-ripe' and '20ºC-ripe' mangoes respectively (Table 1). Pheophytin loss was about 63-74% in the two stages of ripening studied. These mangoes in unripe stage are very sour due to high acidity thereby resulting in conversion of Chl to pheophytins. As pheophytins are not on direct route in enzymic Chl degradation (Janave 1997, Vicentini *et al.* 1995), its accumulation may be resulting in 'stay-green' character. Very small amounts of Chl a (Rt 15.27 min) and Chl b (Rt 9.67 min) were detected (Fig. 1). This is in contrast to the spectrophotometric data of Chl a and Chl b levels reported in Table 1. This may be due to the degradation products of Chl exhibit almost similar spectral characteristics as that of parent chlorophyll a or b. Degradation of Chl a and Chl b by soluble enzyme

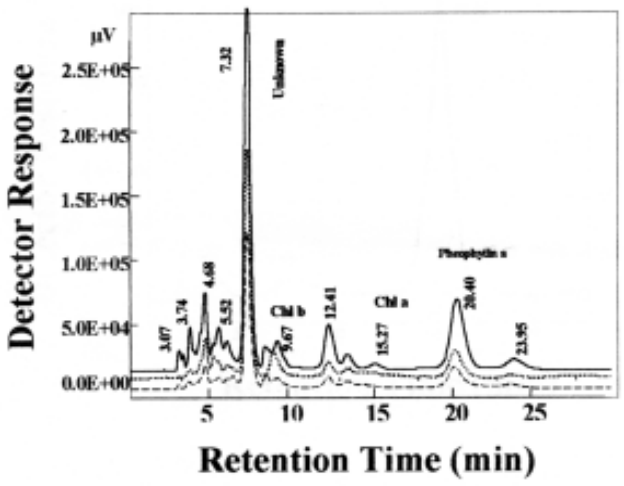

Fig. 1. RP-HPLC separation profile of pigments of Langra mangoes at (-) Green-unripe, (…) Green-ripe and (—) Yellow-ripe stages. The pigments after phase separation were subjected to RP-HPLC using JASCO HPLC system as detailed under Materials and Methods. The figures on the heads of peaks indicate retention time.

Table 1. Changes in pigment content during ripening of Langra mangoes at room temperature (28-32ºC) and 20ºC.

Ripening stage	Total Chl* (mg $100g^{-1}$)	Chl a* (mg $100g^{-1}$)	Chl b* (mg $100g^{-1}$)	Total carotenoids* (mg $100g^{-1}$)	#Unknown pigment (Rt 7.32 min) (Area µV)	#Pheophytin a (Rt 20.4 min) (mg $100g^{-1}$)
Green-unripe	16.48 (100)	11.37 (100)	5.1 (100)	3.63 (100)	6589737 (100)	2.67±0.57 (100)
RT-semiripe	10.19 (38.2)	7.23 (36.5)	2.96 (41.9)	4.70 (129.7)	4872839 (26±1.4)	3.58±0.25 (134.6)
RT-ripe	5.29 (67.9)	3.81 (66.5)	1.49 (70.9)	6.93 (191.3)	4291820 (35±0.3)	0.97±0.11 (63.5)
20ºC -semiripe	9.06 (45.0)	5.91 (48.0)	3.15 (38.2)	4.27 (117.9)	6401414 (2.9±0.1)	2.53±0.3 (5.0)
20ºC-ripe	3.59 (78.2)	2.33 (80.0)	1.27 (75.2)	5.67 (156.3)	2940524 (55±1.2)	0.71±0.1 (73.8)

*The pigments were extracted and determined spectrophotometrically as detailed in Materials and Methods. The data is average of triplicate samples from three different experiments.

The unknown pigment (Rt 7.32 min) and pheophytin A were determined by RP-HPLC. Nature of unknown pigment is not known, hence change in area is compared. Pheophytin a concentration is calculated by running known concentrations of the authentic pigment. Figures in parenthesis indicate per cent change in respective pigments as compared to that at green-unripe stage as control.

Table 2. Degradation of Chl a and Chl b by soluble enzyme fraction from acetone powder of Langra mangoes at three stages of ripening.

Ripening Stage	Volume (ml)	Protein (µg g^{-1} fw)	Chl a* hydrolyzed (nmol)	Chl b* hydrolyzed (nmol)
Green-unripe	60	1335	222.0	123.2
RT-semiripe	60	1170	301.2	289.2
RT-ripe	60	2038	328.0	106.4
20ºC-semiripe	60	1512	234.0	278.4
20ºC-ripe	60	2539	177.0	100.9

*The assay of Chl a and Chl b was carried out as detailed in Materials and Methods and the data is average of three different experiments. The total activity is expressed as nmol of Chl hydrolyzed/oxidized 30 min^{-1} $2g^{-1}$ acetone powder.

revealed that chl a degradation was faster than that of Chl b (Table 2). Chlorophyll b degradation was about half of that when Chl a was used as substrate. Activity of Chl a breakdown was much higher in 'Rt-ripe' mangoes than that in '20ºC-ripe' mangoes. The total protein content of green-ripe mango increased by 1.5 fold, whereas, in fruits ripened at 20ºC, about 2 fold increase was observed.

Fig. 2 shows the RP-HPLC profile of degradation products of Chl a catalyzed by soluble enzyme fraction. Almost similar trend was observed when the reaction was carried out for 5-60 min, hence, data for 15 min incubation is shown. Most of the polar intermediates eluting between the Rt of 1.79 min to 4.04 min indicate operation of chlorophyllase pathway. As was the case in Cavendish banana (Janave 1997), not a single intermediate of oxidase pathway was detected. However, the intermediates (Rt 7.18 and 8.06 min) possibly may be formed by the oxidative enzymes as revealed by their longer retention time.

Fig. 3 shows the profile of HPLC separation of enzymatic degradation products of Chl b with respect to time of incubation. Apart from the dephytylated polar

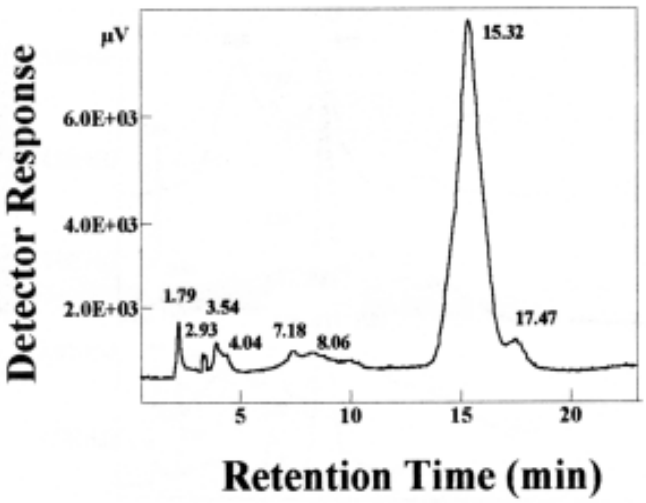

Fig. 2. RP-HPLC separation of Chl a degradation products catalyzed by soluble enzyme from Green-ripe Langra mangoes after 15 min of incubation. The assay of Chl a degradation was carried out as described in Materials and Methods. The pigments in acetone phase were subjects to HPLC. Peak eluting at Rt of 15.32 min is unutilized Chl a, the substrate.

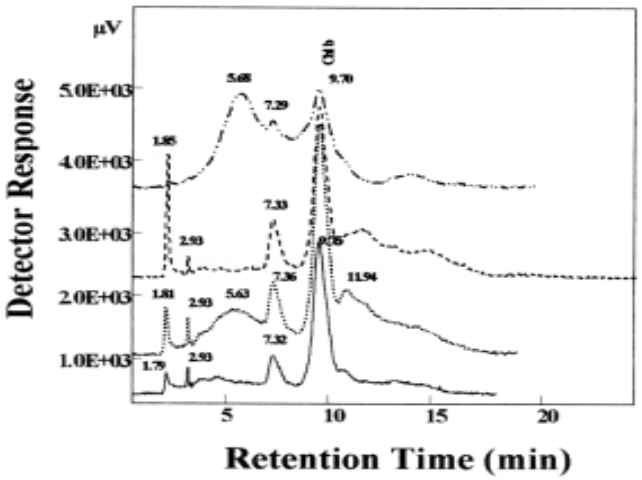

Fig. 3. RP-HPLC separation of Chl b degradation products catalyzed by soluble enzyme from Green-ripe Langra mangoes with respect to time of incubation [(-) 5 min, (...) 15min, (- - -) 30min, (-···-) 60 min]. The assay of Chl b degradation and separation of products by phase separation was carried out as described in Materials and Methods. The pigments in acetone phase were subjected to HPLC. Peak eluting at Rt of 9.7-9.75 min is unutilized Chl b, the substrate.

intermediates eluting between Rt 1.79-4.64 min, an unknown intermediate (Rt 7.33-7.36 min) was constantly formed up to 60 min of incubation. This product showed identical retention time to that of unknown pigment accumulated during ripening (Fig.1) Since the nature of the intermediate is not known at present, no attempts were made to quantitate its concentration. Another intermediate eluting at Rt 5.63-5.68 min was observed at 15 min and 60 min of incubation, the nature of which is not known at present. This intermediate might be a later step after the chlorophyllide b or pheophorbide b formation as it is detected after longer time of incubation. The results indicate Chl b is also decomposed by the chlorophyllase pathway. Further studies are essential to explore whether the product (Rt 7.36 min) is formed by the chlorophyllase pathway or the oxidase pathway.

The inhibition of Chl degreening responsible for 'stay-green' character of Cavendish bananas and Langra mangoes is an intriguing problem. Yah *et al*. (1998) also observed that Kent variety of mango remained green upon ripening and treatment with ethephon resulted in development of yellow-red colour. These fruits ripen, giving soft and edible pulp at tropical temperature with green peel. In Cavendish bananas, Blackbourne *et al*. (1990) have suggested the inhibition may be due to the retention of thylakoid membranes and loss of electron transport capacity due to non-functional Chl. In non-yellowing senescent leaves of *Festuca pratensis* Huds Bf 993, Vicentini *et al*. (1995) have reported that the thylakoids were deficient in pheophorbide a oxygenase, thereby resulting in senescence-induced-deficiency. Apart from this no reports are available to understand the mechanism of inhibition of Chl degreening on enzymatic basis. In the present paper, attempts have been made to study the enzymes at different stages of ripening in Langra Mango.

RP-HPLC analysis of pigments (Fig. 1) at different stages of ripening indicated accumulation of unknown intermediate (Rt 7.32 min) and pheophytin a (Rt 20.4 min). RP-HPLC pattern of enzymatic Chl b degradation (Fig. 3) showed constant formation of an hitherto unknown intermediate (Rt 7.33-7.36 min) which is identical with the Rt of accumulated unknown pigment during ripening (Fig. 1). These results suggest this intermediate could be a derivative of Chl b. The accumulation of pheophytin a and the unknown derivative of Chl b during ripening of Langra mango could be responsible for the stay-green character of this fruit.

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