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Detection and analysis of carotenoid pigments in pummelo (*Citrus grandis* cv. Melomas) fruit peel using reverse-phase high performance liquid chromatography

[Pengesanan dan analisis pigmen carotenoid di dalam kulit buah limau bali (*Citrus grandis* cv. Melomas] dengan menggunakan 'reverse-phase high performance liquid chromatography']

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Key words: *Citrus grandis*, carotenoid biosynthesis pathway, lutein, α- and β-carotene, lycopene

Abstract

A total of 21 carotenoid pigments were separated from the 3-month saponified pummelo *Citrus grandis* cv. Melomas peel using a C-30 reverse-phase high performance liquid chromatography (RP-HPLC) column. Lutein was the major yellow pigment (xanthophyll) detected, followed by β- and α-carotenes while lycopene, zeaxanthin and isolutein were detected as minor pigments only. Lutein was also the major pigment detected throughout all the developmental stages until maturity while lycopene and zeaxanthin could only be detected as minor pigments in this 3-month-old fruit peel. Analysis showed that there was a progressive decrease in the total carotenoid content during fruit development. The failure to form orange peel colour could probably be due to the absence of orange pigments, β-cryptoxanthin and zeaxanthin, during the ripening stage.

Introduction

The maturation of most citrus fruits including the Satsuma mandarin (*Citrus unshiu* Marc.) is accompanied by a series of biochemical changes, affecting colour, texture, accumulation of sugars and reduction of acids (Ikoma et al. 2001; Lee and Castle 2001). One of the peculiar features among these changes is the synthesis and accumulation of a large amount of carotenoid (colour) pigments in the plastids located in various parts of the fruits, primarily found in the cells of flavedo (peel) and juice vessicles.

The first step in the carotenoid biosynthesis pathway is catalyzed by the key enzyme, phytoene synthase *(psy)* which converts two molecules of geranylgeranyl pyrophosphate (GGPP) (C_{20}) into phytoene (C_{40}) via the intermediate prephytoene pyrophosphate (PPPP) as shown in *Figure 1* (Daito et al. 1975; Cunningham and Gantt 1998). Subsequently, the colourless compound, phytoene, is converted into yellow (zeta-carotene), orange (neurosporene) and red (lycopene) carotenoids by four sequential desaturations of phytoene through the introduction of the conjugated double bonds (Daito et al. 1975; Bartley and Scolnik 1995; Cunningham and Gantt 1998). In plants and algae, these steps are catalyzed by two enzymes, phytoene

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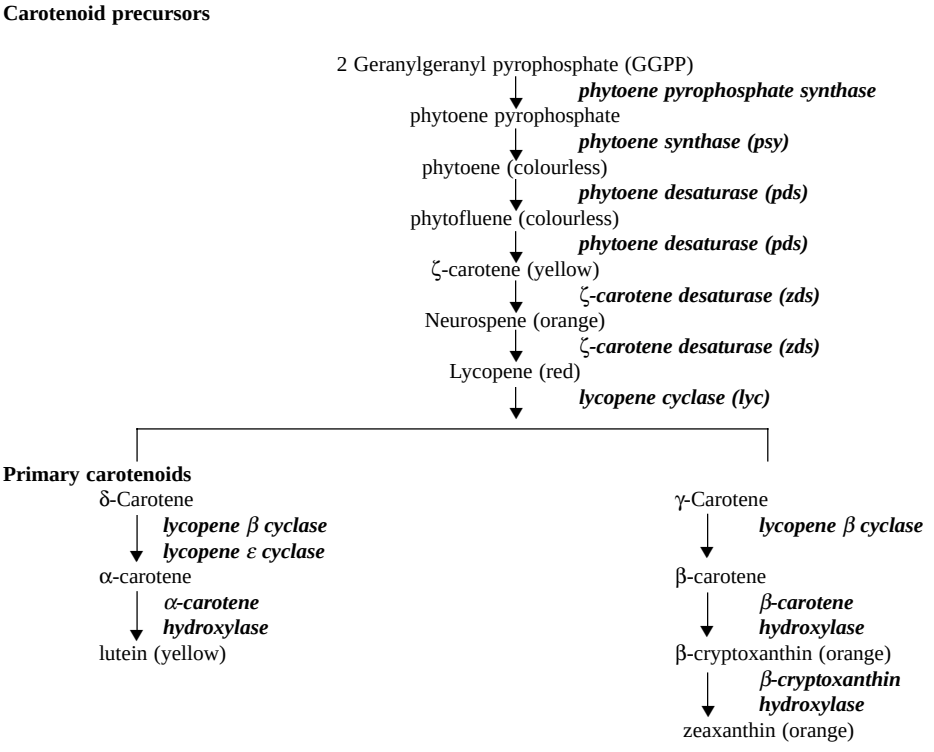

Figure. 1. Carotenoid biosynthesis pathway. (Daito et al. 1975; Bramley 1985; Bartley and Scolnik 1995; Cunningham et al. 1996; Cunningham and Gantt 1998; Ikoma et al. 2001)

desaturase *(pds)* and zeta-carotene desaturase (*zds*).

It has been suggested that the cyclization of the lycopene cyclase enzyme into β- and/or ε-cyclases causes the carotenoid biosynthesis pathway to branch to produce either δ- or γ-primary carotenoids from lycopene initially (Cunningham et al. 1996). It has also been reported that the presence of lycopene-β-cyclase alone further converts γ-carotene to β-carotene which is then hydroxylated to β-cryptoxanthin and zeaxanthin (orange pigments) by β-carotene hydroxylase as in the case of the Satsuma mandarin peel. (Ikoma et al. 2001). On the other hand, the presence of both lycopene β- and ε-cyclases convert δ-carotene to α-carotene which is then hydroxylated to lutein (yellow pigment) by α-carotene hydroxylase (Cunningham et al. 1996). These desaturation and cyclization reactions were found to occur within the plastids and are catalysed by integral membrane enzymes (Bramley 1985).

Most citrus fruits which accumulate a variety of carotenoids change from green to orange during ripening as observed in Satsuma mandarin (Daito et al. 1975; Stewart 1977). This rapid accumulation of carotenoids, particularly β-cryptoxanthin and zeaxanthin, takes place concomittantly with a decrease of chlorophyll (Giuliano et al. 1993; Ikoma et. al. 2001). However, the colour of the local Malaysian pummelo fruit peel, *Citrus grandis* cv. Melomas, remained mainly green to yellowish green until maturity without any change to orange.

Therefore, this study was carried out to investigate the fluctuations of the carotenoid composition in the peel of this local citrus fruit at different stages of fruit development. From these preliminary findings, we hope to suggest a possible pathway for carotenoid biosynthesis in the peel of this local variety

and also to explain the lack of orange colour development during the ripening stages. This understanding of the pathway may help us alter the peel colour through metabolic engineering for commercial or nutritional values.

Materials and methods

Plant materials

Flowers were tagged on pummelo plants cultivated at the MARDI Station, Jelebu (Negeri Sembilan, Malaysia) in July 2001. The fruits (*C. grandis* cv. Melomas) were collected monthly for five consecutive months until maturity. The flavedo *(*peel) was separated from other parts of the fruit, weighed, immediately frozen in liquid nitrogen and stored at –80 °C for further analysis.

Analysis of carotenoid pigments by RP-HPLC

A sample of 10 g of frozen peel was homogenized in acetone:methanol solution (7:3 solution containing 0.1% 2,6-di-*tert*-butyl-*p*-cresol) and partitioned into diethyl ether. The pigments were extracted as described by Ikoma et al. (2001) with modifications. The extracts were dissolved in methyl tertiary butyl ether (MTBE): MeOH (50:50) and evaporated to 4 ml by flushing with nitrogen gas. An aliquot 20 µls was applied to a C-30 column (YMC Inc., Kyoto, Japan, 5 µm, 250 mm x 4.6 mm r.d.) (Russell et al. 1996) and HPLC was performed with a Waters model 2690 using a Waters 996 PDA detector. The ternary gradient mobile phase elution was used as described by Sanders et al. (1994) at a flow rate of 1 ml/min. The eluent was monitored at 450 nm and the peaks identified by comparison with commercial standards for lutein, α- and β-carotenes and lycopene. Some of the peaks were identified based on published UV-visual absorption spectra while other peaks have not been identified yet due to unavailability of commercial standards.

Results and discussion

Changes in peel colour during fruit development

The colour of the peel remained green until the third month of fruit maturation in the pummelo *(Plate 1)* and gradually changed to greenish yellow between four and five months of fruit maturation and ripening stages. This colouration could most probably be due to a reduction of chlorophyll and accumulation of the yellow pigment, lutein. The peel did not turn orange as in the case of the Satsuma mandarin and this could be due to the absence of β-cryptoxanthin and zeaxanthin. (Daito et al. 1975; Stewart 1977; Guiliano et al. 1993; Ikoma et al. 2001).

Fluctuation of carotenoid pigments during fruit development

RP-HPLC analysis of the saponified peel of *C. grandis* cv. Melomas showed that more than 21 peaks were separated from the different stages of fruit development as shown in *Figure 2*. However, only four of the peaks, lutein (peak no. 13), α-carotene (peak no. 18), β-carotene (peak no. 19) and lycopene (peak no. 21) were identified, based on comparison, to commercial standards as shown in detail in the enlarged chromatogram for the 3-month developmental stage in *Figure 3*. In

Plate 1. Different stages of pummelo fruit development in ***Citrus grandis*** *cv. Melomas, Bottom row from left: 1, 1.5, 1.75 and 2 months developmental stages. Top row from left: 3, 4 and 5 months developmental stages*

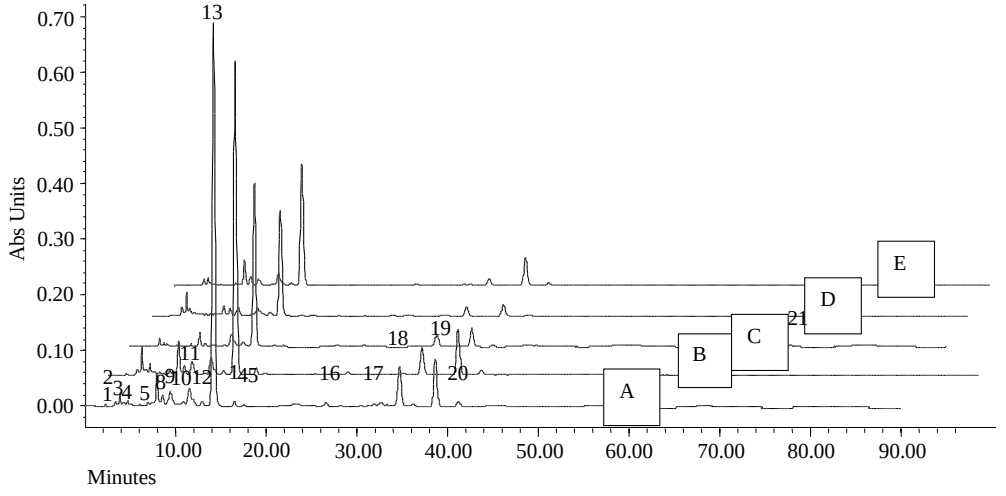

Figure 2 . RP-HPLC profiles of saponified carotenoids in the peel of ***Citrus grandis*** *cv. Melomas at 1, 2, 3, 4 and 5 months: A, B, C, D and E respectively. Detection at 450 nm. Abs Units: Absorbance units. 1-21: peak numbers*

addition, zeaxanthin (peak no. 14), isolutein (peak no. 15) and neoxanthin (peak no. 5) were tentatively identified based on UV-visible absorption spectra from published data as shown in *Table 1*. Other pigments such as β-cryptoxanthin and the colourlesss carotenoid precursors, phytoene and phytofluene could not be identified due to unavailability of commercial standards and published data. Some peaks (1–6) at positions earlier than that of lutein (peak 13) are believed to be epoxylated carotenoids but these were not identified.

Figure 4 shows that lutein was the major carotenoid detected in the peel throughout the developmental stages, decreasing from about 110 mg/100 g fresh weight at one month to about 30 mg/100 g fresh weight at four months and then increased slightly again to about 40 mg/100 g fresh weight at five months. In comparison, the α- and β-carotenes were present in lower amounts, slightly less than 20 mg/100 g fresh weight at one month and decreasing to less than 5 mg/100 g fresh weight at 4 months. α-carotene continued to decrease to almost no synthesis at all towards five months fruit development while β-carotene increased slightly again to about 10 mg/100 g fresh weight. Lycopene was not detected at all until after 2 months of fruit development and in very low amounts peaking to only about 5 mg/100 g fresh weight at 3 months and then declining to almost no synthesis at all towards four and five months of fruit development.

As ripening stages of the fruit increased, all four pigments, lutein, β-carotene, α-carotene and lycopene progressively decreased, together with other unknown carotenoids towards the fourth month of fruit development while only lutein and β-carotene increased slightly again at the 5 months ripening stage. This was accompanied by colour changes of the peel from intense green to light green and finally to yellowish green as observed in the 5 months fruit ripening stage.

These changes in peel colour could be due to a steady decrease in the total chlorophyll content and increase in the carotenoid content as the fruit begins to mature as reported in the Satsuma mandarin peel (Ikoma et al. 2001). Such clear differences in the carotenid composition depending on the expression of carotenoid biosynthetic genes have been obtained from experiments on tomato leaves and flowers

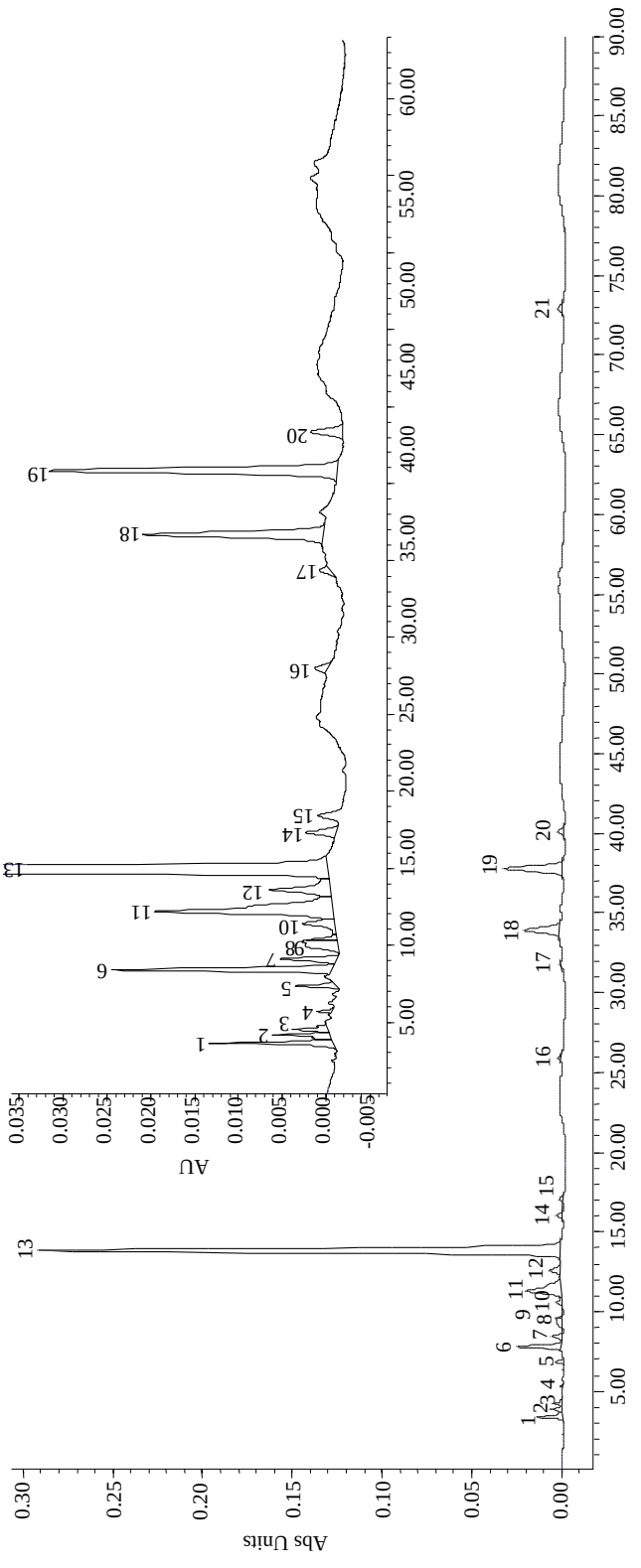

*Figure 3. RP-HPLC chromotogram for saponified carotenoid in pummelo (**Citrus grandis** cv. Melomas) peel of 3-month fruit stage. YMC C_{30} carotenoid column (4.6 x 300 mm, 5 µm). Chromatographic conditions are given in the text. Detection at 450 nm. Peaks are numbered and identified in Table 1. Abs Units: Absorbance units*

Table 1. Spectral characteristics used for carotenoid identification separated from 3-month fruit stage the C-30 RP column

Peak no.	Carotenoid	RT (min)	Observed Peak (nm)			Literature Peak (nm)			Ref
			I	II	III	I	II	III	
1	unknown	3.4		364.3					
4	unknown			397					
5	neoxanthin*	6.98		441.5		418	441	470	7
6	unknown	7.945	413.8	436.7	466.8				
7	unknown	8.34	411.4	436.7	463.2				
8	unknown	9.1		421	447.5				
9	unknown	9.55		439.1	466.8				
10	unknown	10.87	406.5(S)	429.4	458.4				
11	unknown	11.53	413(S)	436.7	463.2				
12	unknown	12.96	416	439.1	462				
13	lutein	14.21	420	442.7	471.7	424.5	445.5	471.5	9
14	zeaxanthin*	16.52		449.9	477.7	427.5	449.5	476.5	9
15	isolutein*	17.56		440.3	466.8	418.5	439.5	467.5	9
	unknown	23.02		439.1	468				
	unknown	23.56		441.5	471.7				
16	unknown	26.61		441.5	472.9				
18	α-carotene	34.72	421	443.9	472.9	424.5	445.5	473.5	9
	unknown	36.24		441.5	465.6				
19	β-carotene	38.657	424.6	449.9	477.7		450.5	478.5	9
20	unknown	41.18		446.3	472.9				
21	lycopene	72.93	445.1	471.1	504.3	444	472	502	8

Chromatograhic retention time (RT) (in min) and the wavelength (nm) of some selected peaks from Figure 3; *tentative identification
Ref: references; 7 = Ikoma et al. (2001), 8 = Lee and Castle (2001), 9 = Ronen et al. (1999)

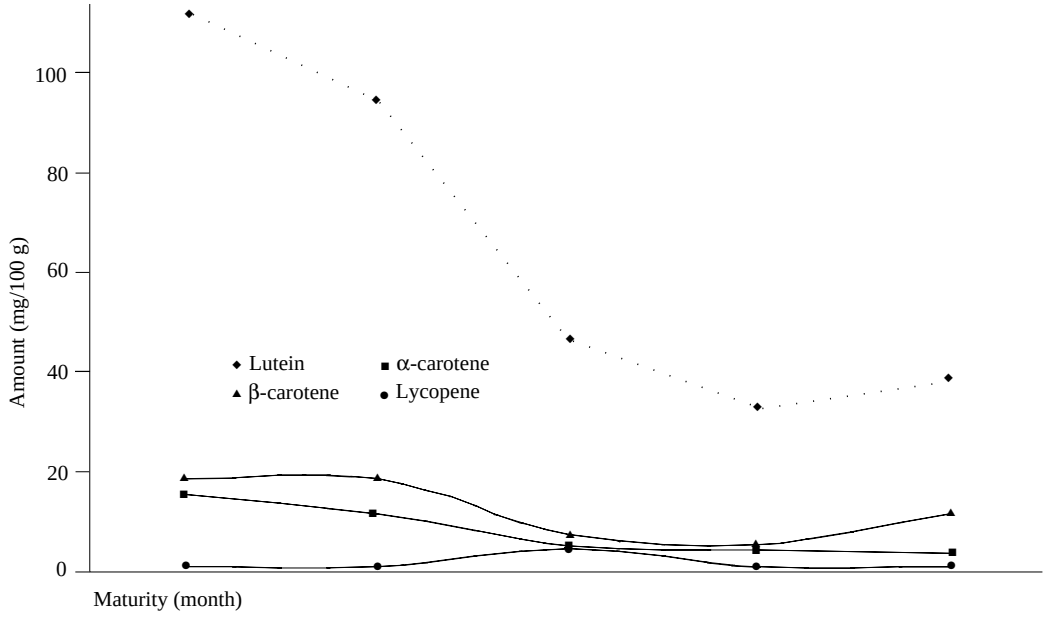

Figure 4. Changes in the carotenoid pigment composition of pummelo peel during fruit ripening. Quantification was done using HPLC

(Ronen et al. 1999) and in the thylakoid membranes and stroma of plants and algae (Cunningham and Gantt 1998) where two types of the desaturase/cyclase complexes were reported (β,β complex to produce β-carotene and β,ε-complex to produce α-carotene).

Even though lutein progressively decreased towards fruit maturation and ripening in pummelo, the peel still changed ultimately to yellow because lutein was still the major carotenoid that was being synthesized in the peel throughout the developmental stages compared to the other carotenoids. This could be due to the presence of both the lycopene β- and ε-cyclases and the following α-carotene hydroxylase enzymes producing the yellow pigment that may be active in the pummelo fruit peel throughout the developmental stages. In contrast to this, only the lycopene β-cyclase and the following β-carotene hydroxylase enzymes may be actively functioning in the peel of the mature Satsuma mandarin fruit to give the orange colour (Ikoma et al. 2001).

Conclusion

The biosynthesis of xanthophylls having a β,ε-carotene structure such as lutein, becomes particularly dominant than that of the xanthophylls having a β,β-carotene structure, such as β-cryptoxanthin in the peel of maturing fruits of *C. grandis* cv. Melomas. These findings suggest that the carotenoid biosynthesis in the Malaysian pummelo peel may follow the alternative branching pathway to give the yellow (lutein) pigment instead of the orange (β-cryptoxanthin and zeaxanthin) pigments as shown in *Figure 1*. Further analysis on the pigmentation of the peel and detailed analysis of the enzymes and their activities will have to be thoroughly investigated to complete this study and understand the carotenoid biosynthesis pathway in the peel of this local citrus fruit.

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Abstrak

Sebanyak 21 pigmen carotenoid telah diasingkan dari kulit buah limau bali *Citrus grandis* cv. Melomas di peringkat tumbesaran 3 bulan selepas berbunga dengan menggunakan kolum "C-30 reverse-phase high performance liquid chromatography (RP-HPLC)". Lutein telah dikenal pasti sebagai pigmen kuning (xanthophyll) utama. Ini diikuti oleh β- dan α-carotene manakala lycopene dan zeaxanthin ialah pigmen surih sahaja. Lutein juga didapati sebagai pigmen utama di kulit buah ini pada setiap peringkat tumbesaran buah sehingga matang, manakala lycopene dan zeaxanthin hanya dikesan dalam kuantiti yang kecil di peringkat tumbesaran 3 bulan sahaja. Analisis menunjukkan kandungan kesemua carotenoid menurun secara progresif semasa tumbesaran buah. Kulit buah limau bali kekal dengan warna hijau atau kuning-kehijauan di peringkat matang tanpa berlaku pertukaran ke warna oren. Kegagalan penukaran ke warna oren mungkin kerana ketiadaan kandungan pigmen oren, β-cryptoxanthin dan zeaxanthin, di peringkat buah matang.

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