On the Colorless Polyenes of Sweet Potatoes

(*Ipomoea batatas*)

Yūichirō TOMITA

(*Laboratory of Animal Nutritional Chemistry*)

In the biosynthesis of triterpenes, squalene (**A**) the C_{30}, precursor of steroids, is formed from the condensation of two C_{15} farnesyl pyrophosphate molecules[1,2]. By analogy with this reaction, the C_{40} precursor of the tetraterpene carotenoids would be lycopersene (**B**), condensed out of two C_{20} units.

Grob et al[3,4] reported the results endorsing this view. On the other hand, some investigators[5~10] reported to have failed in detecting lycopersene in carotenogenic systems.

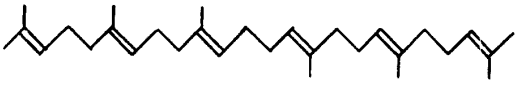
In the course of an investigation of the process of carotenoid biosynthesis in sweet potatoes [11,12], the identification of the colorless polyene components was necessitated in the sweet potatoes. Purcell[13], in 1962, reported the existence of phytoene(**C**) and phytofluene (**D**) in Goldrush variety, but did not refer to lycopersene.

The present investigation was carried out to identify the colorless polyenes of sweet potatoes, especially the lycopersene.

Materials and methods

Kyushū-28 variety of sweet potato, was grown at the Kagoshima University "Toso farm" harvested November 11, 1963, and analysed immediately.

Purification of solvents. Commercial petroleum ether was distilled over solid potassium hydroxide to remove any traces of acid and nonvolatile residue. The 40-60°C fraction



(A) Squalene

(B) Lycopersene

(C) Phytoene

(D) Phytofluene

was used for extracting and chromatographing the crude pigment preparation. Ethanol and Methanol were refluxed with zinc and sodium hydroxide, and then distilled. Ethyl ether was allowed to stand over sodium hydroxide pellets for three days before distillation. Acetone was distilled before use.

Chromatographic adsorbents. Magnesia for chromatography was prepared, by thorough mix, in equal quantities (by weight) of magnesium hydroxide and Hyflo Super Cel. Alumina, suitable for the chromatographic adsorption (Merck), was deactivated with water, and then, used in the final separation of colorless polyenes.

Silica gel G "according to Stahl for thin layer chromatography" was obtained from Merck.

Authentic samples. Squalene was bought from Wakō pure chemicals Ind.. Phytoene and phytofluene were extracted from tomatoes and were purified.

Extraction of carotenes. Raw sweet potatoes were weighed, peeled and blended with acetone. The mixture was filtered, by suction, through filter paper on a Buchner funnel. The residue was returned to the blender and extracted again with acetone-petroleum ether (1 : 1, v/v). The mixture was filtered and washed, on the filter, with the acetone-petroleum ether mixture. This extraction process was repeated until the filtrate turned colorless. All of the filtrates were combined in a separatory funnel. Two distinct phases were formed by adding water of about 10 to 20% of the used acetone. The bottom phase was drained into another separatory funnel and extracted with ethyl ether. The ethyl ether phase was combined with petroleum ether epiphase and the mixture was washed with water to remove the acetone. This solution was dried over anhydrous sodium sulfate, and concentrated, under reduced pressure, to a small volume in a nitrogen atmosphere. The concentrated solution was saponified with equal volume of 5% potassium hydroxide in ethanol solution. After one night, the formed lower phase was drained off, diluted with about five volumes of water and extracted with ethyl ether. The ethyl ether was added to unsaponifiable extract and the mixture was washed to remove alkali. The total extract was dried by filtering through anhydrous sodium sulfate on a glass filter and then was evaporated to dryness under reduced pressure. The resulting lipid was then redissolved with petroleum ether and was washed three times with equal volume of 90% aqueous methanol. The methanolic extracts were discarded. The remaining epiphase was washed several times with water, dried over anhydrous sodium sulfate and concentrated under reduced pressure into a small volume.

Upon refrigeration of this solution at −20°C for 2 days, crude carotenes, sterol crystals and waxy substance were precipitated. The filtrate was filtered and concentrated further, and then, the additional substances were removed.

Chromatographic separations. The petroleum ether solution of the carotenoids extracts was chromatographed on a magnesia column, and the chromatogram was developed with a mixture of petroleum ether-acetone (19 : 1, v/v).

Chromatography was conducted in darkness and the development was observed intermittently with the use of an ultraviolet lamp and of a flashlight, because for the prevention of oxidation it is important to protect the pigment from being exposed to light for a long time. The column chromatogram is shown in Fig. 1. The fluorescent zone was eluated, washed several times with water, desiccated with anhydrous sodium sulfate, evaporated to dryness under reduced pressure with gentle heating, and rechromatographed on an

alumina column (deactivated with water to Brockmann grade II) for further separation.

The column was developed with solution of increasing concentration of ethyl ether and acetone in petroleum ether, as described in the following; 120 *ml* of petroleum ether only, 100 *ml* of 1% ethyl ether, 100 *ml* of 2% ethyl ether, 500 *ml* of 4% ethyl ether, 150 *ml* of 5% ethyl ether, 100 *ml* of 2% acetone, 60 *ml* of 5% acetone and 120 *ml* of 10% acetone in petroleum ether.

The eluates were collected in 5 *ml* fractions. Each fractionated solution was evaporated to dryness under reduced pressure, redissolved in petroleum ether, each absorbancy being determined at 286 *mμ* and 348 *mμ*, each absorption spectrum being measured, too.

Spectroscopic examination. Absorbancy and absorption spectra were measured by Shimadzu QB-50 spectrophotometer.

The extinction values ($E^{1\%}_{1cm}$) used for quantitative determination were 850 (286 *mμ*) for phytoene[14] and 1200 (348 *mμ*) for phytofluene[15] in petroleum ether.

Thin layer chromatography of colorless polyene groups.

The chromatogaphy and staining were employed after the Davies et al method[9].

Results and Discussion

The location of the lowest colorless zone in magnesia column chromatogram was indicated by faint violet and yellow-green fluorescence when the column was irradiated with ultraviolet ray (Fig. 1). The eluate of diffused faint violet fluorescence zone was combined with eluate of yellow-green fluorescence zone, but the latter clung tenaciously to the zone of carotene and was not separated completely. Therefore, this eluate contained a

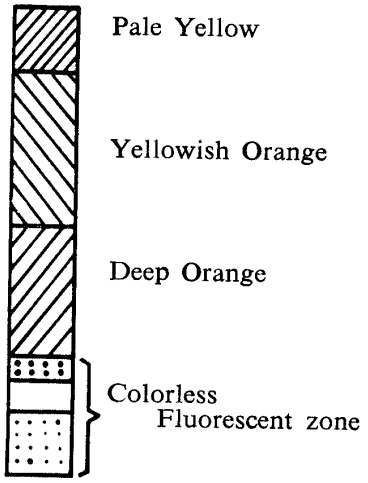


Fig. 1. Column chromatogram of the epiphasic pigments of sweet potato (Kyushū-28 variety)

small quantity of carotene and colored yellow. The eluate containing this colorless polyene was evaporated to dryness under reduced pressure with gentle heating, and then treated with digitonine in order to remove the impurities, sterols, but no digitonide precipitate was found. This may be due to the fact that almost all of sterols in the pigment extract were removed as precipitates when stored at −20°C, and the remaining sterol stayed in column. This sterol-free solution was rechromatographed on alumina column which had been washed previously with petroleum ether. The chromatogram was developed with the solvents as described above.

Two hundred and twenty fractions, each of 5 *ml*, were collected, evaporated to dryness, redissolved in petroleum ether to measure absorbancies at 286 and 348 *mμ*, and each fraction with the same developer was combined to Group I-VIII, and each group was chromatographed on a thin layer of silica gel G.

Petroleum ether used as solvent and developer for chromatography, must be purified by redistillation, because the residue left after distillation contains some impurities. Davies et al[9] found that components of the residue left after distillation of light petroleum ran with Rf values 0.7, 0.4, 0.3, 0.15, and 0.0 on the thin layer of silica gel G, and stained with iodine, and then synthetic lycopersene also ran with Rf 0.3. However, the lycopersene can be distinguished from the petroleum impurity by the examination under ultraviolet ray and antimony trichloride color reaction. Similar impurities were contained in the ethyl ether and acetone, so these were redistilled, too.

The Rf values, were obtained by thin layer chromatography with petroleum ether for developer, and they are shown in Table 1. When a chromatoplate was exposed to iodine in an airtight chamber, the unsaturated terpenoid hydrocarbons were revealed as brown spots against white background.

Table 1. Rf values and results of color reaction in silica gel G thin layer chromatography of authentic and sweet potato's colorless polyene samples.

Sample	Color	Color with $SbCl_3$*	Rf*
Squalene	Colorless	Pink	0.4
Phytoene	〃	Brown	0.2
Phytofluene	〃	Dark grey-brown	0.1
Group III	〃	Pink	0.4
IV	〃	Brown	0.2
VII	〃	Dark grey-brown	0.1
VIII	Yellow	Dark grey	0.0

* Synthetic lycopersene is stained pink, having Rf 0.3[9]

This method is sensitive, and Davies et al[8] found that as little as 0.05 *μg* of squalene, lycopersene and phytoene could be detected, and these hydrocarbons could also be revealed as several colors by spraying the plate with a saturated solution of antimony trichloride in chloroform and by heating the plate at 110°C for 15-20 min., but this reagent, though less sensitive, had the advantage that different color reactions were given with different hydrocarbons. These Rf values varied sometimes, although a chamber saturated by solvent vapor was employed, but authentic samples of squalene, phytoene and phytofluene were always chromatographed on the same plate. The average values of Rf are shown

in Table 1.

Authentic squalene, phytoene and phytofluene ran with Rf values 0.4, 0.2 and 0.1 on the thin layer of silica gel G, and were stained with iodine vapor. In eight separated groups, four groups of III, IV, VII and VIII were stained with iodine, and ran with Rf values 0.4, 0.2, 0.1 and 0.0, respectively. When thin layer chromatogram was developed with petroleum ether, Group VIII was colored yellow, because of the existence of small quantities of carotene, while the others were colorless.

Upon irradiation with an ultraviolet ray, an authentic phytofluene and Group VII showed a pale blue white fluorescence. The faint whitish violet fluorescence was apparent only when the chromatoplate carried a high concentration of phytoene, but the fluorescence was very difficult to be recognized, and it disappeared under low concentration. The whitish violet fluorescence corresponding to the phytoene could not be observed in these groups. Group VIII appeared as a dark spot at the original point.

When the chromatoplate was treated with antimony trichloride and then heated, it revealed several colored spots (Table 1). Revealed colors of authentic polyenes were almost identical with the colors that Davies et al found; pink spots of squalene and Group III, brown spots of phytoene and IV, dark gray-brown spots of phytofluene and VII, and dark gray spot of VIII. Davies et al[9] found that synthetic lycopersene stained pink by spraying antimony trichloride, and had Rf 0.3, and revealed the position corresponding to that between squalene and phytoene. Grob et al[3] reported that the Rf value of lycopersene was 0.44 ± 0.02 and squalene was 0.30 ± 0.01 when the chromatoplate had been developed with cyclohexane. When these polyene groups were chromatographed on the plate and

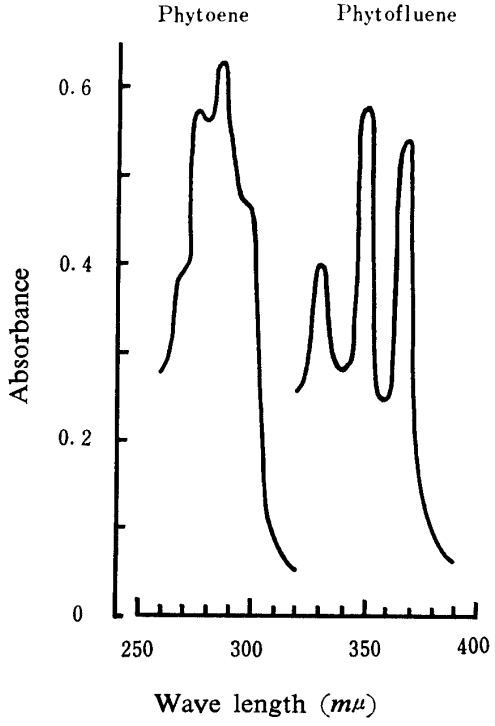


Fig. 2. Absorption spectra of phytoene and phytofluene from Kyushū-28 variety of sweet potato. (in petroleum ether)

developed with cyclohexane, no Rf value higher than that of squalene was obtained.

Absorption spectra of Group III and VII are shown in Fig. 2. These absorption-spectra-curves corresponded to phytoene (286, 275 *mμ*) and phytofluene (367, 348, 332 *mμ*), respectively, and both of these were due to the positive color reaction of Carr-Price and Liebermann-Burchard test. The eluted quantities of these two components from alumina column are shown in Fig. 3, and 153 *μg* of phytoene and 80.4 *μg* of phytofluene were contained in 1 *Kg* of raw materials.

On the basis of these data, it seemed reasonable to assume that sweet potato contains squalene, phytoene and phytofluene but not lycopersene. In addition, Group II contained an unknown component having an absorption maximum at 277 *mμ* in petroleum ether, but this was not stained in iodine vapor and antimony trichloride, and did not have any fluorescence under ultraviolet ray, either. On this component, further study seems to be necessary.

Grob et al[3,4] reported the enzymatic synthesis of geranyl-geranyl pyrophosphate and the

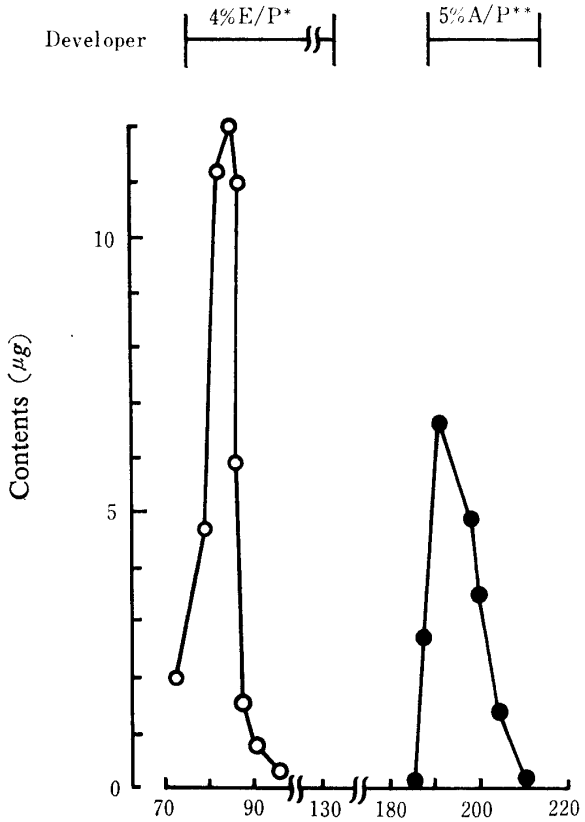


Fig. 3. Phytoene (○, Group IV) and phytofluene (●, Group VII) obtained from colorless polyene fraction of 1 *kg* of sweet potato (Kyushū-28 variety), separated on an alumina column.
* : 4% ethyl ether in petroleum ether
** : 5% acetone in petroleum ether

conversion of this compound into lycopersene by enzyme preparations from *Neurospora crassa*; furthermore, they reported the presence of lycopersene in cultures of *Neurospora crassa* grown in the presence of diphenylamine, a specific inhibitor of the biosynthesis of carotenoids. However, some objections to this were reported too, and in higher plants, such as maize and carrot-root, no lycopersene could be detected by Mercer et al[10].

In view of the above facts the most reasonable conclusion to be drawn from the available data is that the results obtained are different from those of Grob et al and lycopersene is not the first C_{40} compound formed. Furthermore, although it is suggested that phytoene is formed as the first C_{40} compound which is followed by sequential dehydrogenation to carotenes, Purcell et al [16,17] concluded, on the basis of the incorporative experiment of mevalonate-2-C^{14} in tomato's carotenogenic system, that phytoene could not be a general precursor for other carotenes, and also that phytoene is not a pool precursor for lycopene. Karunakaran et al[18] reported that *Neurospora crassa*, when grown in continuous culture, produced phytoene, and the phytoene formed and accumulated during the colorless growth-phase was an end product and not a precursor of the carotenoid pigments.

Further investigation will be necessary to find a working conclusion of this problem.

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Summary

The colorless polyenes were detected in Kyushū-28 variety of sweet potato by absorption-spectrum-determination, by color reaction and silica gel G thin layer chromatography which was preceded by chromatography on magnesia column and rechromatography on alumina column.

Squalene, phytoene and phytofluene, except lycopersene, were detected. One *Kg* of raw materials contained 153 μg of phytoene and 80.4 μg of phytofluene.

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