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A novel water soluble carotenoid derivative from palmyrah (*Borassus flabellifer* L) fruit pulp

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Abstract

A water soluble carotenoid derivative is reported for the first time. The source was the fruit pulp of palmyrah (*Borassus flabellifer* L). Using the normal carotenoid extraction procedure the yellow pigment was found to be unextractable into petroleum ether and diethyl ether. Subjecting the freeze dried water extract to naringinase hydrolysis resulted in the release of glucose and small amount of rhamnose as detected by thin layer chromatography (Tlc). Enzymic hydrolysis resulted in the pigment becoming petroleum ether soluble. The spectrum was typical of carotenoids. All parameters measured changed on enzymic hydrolysis *viz.*, λ_{max} from 413, 443, 479 to 401, 426, 462; R_f on Tlc on 5% methanol in toluene from 0 to 0.89; high performance liquid chromatography (HPLC) retention time in the carotenoid solvent system (acetonitrile : methanol : tetrahydrofuran 58 : 35 : 7) from 4 to 28 min; while the water soluble carotenoid had no epoxy groups, after hydrolysis, in the epoxy-furanoid rearrangement and fuming HCl test gave two epoxy groups which was compatible with the R_f values on Tlc and the retention time on HPLC. The product configuration was *trans*. Since the enzymic hydrolysis used was to split glycosidic bonds, the parent aglycone is likely to have had two hydroxy groups, which during or after glycosidic cleavage resulted in the formation of two epoxy groups. Since the raw material (palmyrah fruit pulp) contains several underivatised carotenoids, glucose and rhamnose as well as glycosyl and rhamnosyl transferases, which normally act on β-sitosterol to form glycosides, it is hypothesized that the precursor carotenoid acts as a substrate analog of β-sitosterol. The water soluble derivative or synthetic analogs could have commercial applications as a food colour in the beverage industry.

Key words: Carotenoid; Water soluble; Glycoside; Palmyrah; *Borassus flabellifer*

Introduction

Water soluble carotenoids have not been reported before. Palmyrah (*Borassus flabellifer* L) is a palm that grows in the arid regions of Asia[1]. Its fruit pulp is yellow and has many traditional uses[2]. The carotenoids of palmyrah fruit pulp (PFP) have been reported previously[3,4]. Although those studies noted a yellow pigment not extractable into 1% diethyl ether in petroleum ether (Pathberiya, 2004; Wijemanna, 2005, personal communication) the researchers did not persue it. As the water soluble pigment was found to have a typical carotenoid spectrum, a study was performed to determine if this was indeed a carotenoid and if so what moiety can be present so as to give it extensive hydrophilicity.

Materials and Methods

Fruits:

Palmyrah fruits were collected after optimum ripening from the Hambantota district in the South of Sri Lanka selecting the main morphological type of fruit (type II B)[5] which comprised 80 – 90% of the population from varying eco-locations *viz.,* sea shore, on arid hillocks away from the sea and near paddy fields. Six fruits were selected and subjected to the normal carotenoid extraction procedure[6].

Extraction:

The extraction procedure separated the normal hydrophobic carotenoids in 1% diethyl ether in petroleum ether (bp 60-80 °C). As a marked yellow colour remained, the percentage diethyl ether was progressively increased to 100% diethyl ether. The yellow colour remained unextracted. From the six fruits 500 mL acetone, water extract was collected. This showed a typical three peaks carotenoid spectrum with λ_{max} 413, 443 and 479 nm with a reverse phased HPLC retention time of 4 min in acetonitrile:methanol:tetrahydrofuran (58:35:7) solvent system and remained at the origin on thin layer chromatography (Tlc) in 5% methanol in toluene.

Concentration:

The extract, which contained acetone and water, was flushed with nitrogen to remove acetone and the water soluble yellow solution was freeze dried in 50 mL portions.

Enzymatic hydrolysis:

The following enzymes were initially used; amyloglucosidase (EC No:3.2.1.3) at pH 4.5 and naringinase at pH 4 (for β-rhamnosidase action) and 5.25 (for β-glucosidase action). The naringinase preparation is known to be contaminated with α-rhamnosidase. The freeze dried extract was dissolved in 1.5 mL of distilled water and divided into 0.5 mL portion to which enzymes and 0.1 M acetate buffer of appropriate pH were added and the extracts were incubated

at 37 °C and 50 °C for naringinase and amyloglucosidase actions respectively. Aliquots of 1 µL and 5 µL were spotted at zero time, 3h, 6h, and overnight (14 h) together with rhamnose, glucose and sucrose standards and hydrolysates spiked with the above sugars on Tlc pre-prepared plates (glass) (Merck, Germany) and run in the solvent system of n-butanol:ethanol:NH_4OH (specific gravity 0.88) 7:3:4[7] and sprayed with anisaldehyde[7]. R_f values were calculated.

Isolation and characteristics of free carotenoid:

Based on the result of the above experiment, the following conditions were chosen. The water soluble solution (50 mL) was freeze dried and to it added distilled water (0.5 mL), 0.02 M acetate buffer of pH 4 (0.5 mL) and naringinase (5 mg). This was incubated for 3h in nitrogen atmosphere at 30°C, following which, 0.3 mL of 0.1M acetate buffer of pH 5.25 was added, flushed with nitrogen and re-incubated at 30°C for 3h. The carotenoid was extracted with petroleum ether (10 mL). The extraction was tested on HPLC[8] Tlc[6] and chemical and spectral tests[6] to characterize the carotenoid.

Results

On extracting using the normal procedure, the yellow pigment remained in the water-acetone layer and was not extractable in petroleum ether (100%), diethyl ether (100%) and ratios of the two solvents. Removal of acetone by nitrogen flushing gave a water soluble yellow pigment with the characteristics λ_{max} value of a carotenoid (Table 1). The mean estimated quantity was 7.1 mg.$100g^{-1}$ dry weight. Concentration by freeze drying and subjecting to amyloglucosidase action produced no sugars and the yellow Tlc spot remained at the origin. Naringinase had fully hydrolysed >92% of the derivative (from HPLC data) at pH 4 (3h) and pH 5.25 (3h). The balance (low retention time) was probably water soluble intermediates. Tlc of the hydrolysate resulted in the detection of glucose and a much smaller amount of rhamnose as determined by spiking with standards on Tlc. Carotenoids were not present in the water layer after hydrolysis. The main product extracted into petroleum ether also contained traces of phytoene and phytofluene. The product was a *trans* carotenoid whose HPLC retention time was 28 min and R_f on Tlc was 0.89. It contained two epoxy groups (epoxy-furanoid shift = 26 nm). Its HPLC and Tlc positions and the fuming HCl test confirmed epoxides but no hydroxyl group (Table 1).

Table 1: Characteristics of water soluble carotenoid

Before hydrolysis	After hydrolysis
λ_{max} Values:	
413, 443, 479	401, 426, 462
HPLC retention time:	
4 min	28 min
R_f on Tlc:	
0 (Remained at the origin)	0.89
HCI vapour test:	
Negative to epoxy group	Positive to di-epoxy groups
Epoxy-furanoid rearrangement:	
Negative to epoxy group	Positive to di- epoxy groups
Iodine catalysed *cis-trans* isomerisation: Could not test as water soluble carotenoid is not soluble in petroleum ether	Positive to *trans* configuration

Discussion

Palmyrah fruit pulp (PFP) has many hydrophobic carotenoids and xanthophylls[4]. This is the first report of a water soluble carotenoid derivative. The finding can have commercial implications as water soluble yellow natural colours of high colour intensity are rare, if any.

The study showed that the carotenoid was derivatised by mainly glucose and some rhamnose. A paradox in the study was that for glycosidic links to form hydroxy groups must be present. This was seen in the product. On the contrary two 5,6 epoxide groups were found to be present on removing sugars. It is hypothesized that while the glycosidic moiety was present it was attached to hydroxy groups and that either during or after the enzymic cleavage two 5,6 epoxides were formed. This could result during the mechanism of glycosidic enzymic hydrolysis which proceeds through charged centers or due to the free hydroxy groups that form being unstable and cyclisation occurs to form epoxides.

The enzyme preparation used for successful hydrolysis was naringinase, which at pH 4 has β-rhamnosidase activity and at pH 5.25 has β-glucosidase activity. Amyloglucosidase has α-glucosidase activity and could not hydrolyse the glycoside. Therefore the configuration of the anomeric carbon of glucose was β. The rhamnosidase activity of naringinase is β but is known to be contaminated with α-rhamnosidase[9,10] and therefore a conclusion on the configuration of the rhamnose anomeric carbon is not possible.

A question arises as to why these glycosylation reactions occur only in PFP. It appears more than a coincidence that PFP has been shown to contain glucose and rhamnose[11] and glycosyl transferases that link α-rhamnose and β-glucose to β-sitosterol in many ways to form the plethora of 16-20 glycosides (flabelliferins) of PFP[9]. It is therefore conceivable that the hydroxy carotenoid may have been acting as an analog to β-sitosterol. Judging from the solubility of flabelliferins (27-C), to be soluble, the glycosidic moiety should have at least four saccharides. The presence of traces of phytoene and phytofluene in the petroleum ether extract of the hydrolysate cannot be due to chemical conversion and unless there is some hydrophobic interaction with the carotenoid section of the derivative, this cannot be explained.

Conclusion

A natural carotenoid glycoside has been located in PFP, which could have implications in the food industry.

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