

**Aspects of the binding of acyclic carotenoids to
flabelliferins from palmyrah fruit pulp.**

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Abstract

The fruit pulp of palmyrah (*Borassus flabellifer*) contains flabelliferins which are glycosides of β -sitosterol. Naturally bound to flabelliferins is an *UV* active compound, phytofluene, which affects biological activity. This *UV* active compound shows intense blue fluorescence and can be dissociated from flabelliferins.

The objectives of this study were to determine the main carotenoid binder to flabelliferin-II (F-II, a tetraglycoside) in a sample of PFP from Kalpitiya, Sri Lanka by HPLC and some of the chemical features of this complex (F-II+ carotenoid binder).

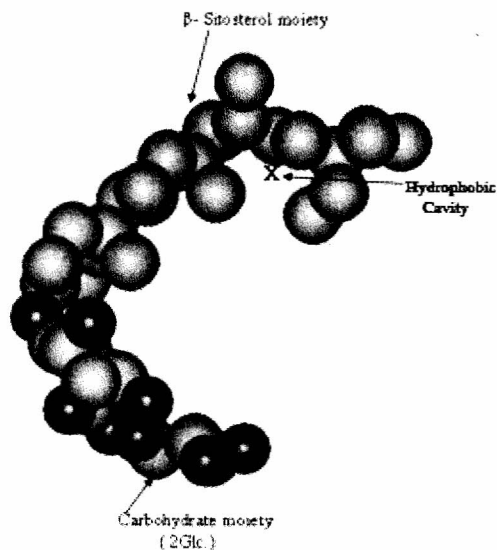
HPLC studies showed that the major binder to F-II in a sample of PFP from Kalpitiya was phytofluene.

No correlation was found between the stoichiometry of binding of phytofluene and F-II ($r^2= 0.406$) as well as for Fb ($r^2=0.007$) indicating that the binding ratios of phytofluene with F-II or Fb are not constant in different PFP samples tested.

Computational calculations ($-\Delta\Delta H_f / \text{KJ mol}^{-1}$) suggest that binding is a less stable between F-II and phytofluene compared to the other flabelliferins tested. This was probably due to phytofluene distorting the conformation of the carbohydrate moiety of F-II. Computer modeling provided confirmatory evidence for this assumption.

Key Words : Palmyrah, *Borassus Flabellifer*, Flabelliferins

Graphical abstract



2Glc.2Rha

Geometry optimized structure of Flabelliferin-II Glc.=Glucose, Rha.=Rhamnose

Introduction

Palmyrah is a palm growing in the arid zones of South and South-East Asia. The main bitter principle of palmyrah fruit pulp has been identified as a steroidal saponin tetraglycoside (flabelliferin-II) containing two glucose and two rhamnose residues¹.

It has long been recognized that all flabelliferins (glycosides of β -sitosterol) of palmyrah fruit pulp (PFP) are hydrophobically associated with a compound that gives fluorescence in UV light². This UV active compound can be separated by isopropanol: methanol (1:1) in

chromatotron³ or by a toluene: methanol gradient in MPLC². The presence of hydrophobic binder lowers inhibition of intestinal ATPase action and promotes anti-bacterial action⁴.

The UV active complex in palmyrah flour contained 3 components namely a UV active compound, flabelliferin (F_B or F_C) and amine/ amino acid. At the time of this study the nature of the UV active compound had not been characterized and it was tentatively identified as being the carotenoid, phytoene by Bandara⁵. Computer modeling studies showed that phytoene and phytofluene are the only carotenoids that form stable complexes with β -sitosterol⁶.

The objectives of this study were to determine:

1. The main carotenoid binder to F-II in a sample of PFP from Kalpitiya, Sri Lanka by HPLC studies.
2. The ratio of binding of UV active binder to the major flabelliferins in 10 morphologically different collections of PFP from fruits collected from different locations of Sri Lanka.
3. Heat of formation of UV active binder to F-II (flabelliferin-II, a tetraglycoside) and other flabelliferins.
4. To develop a model for understanding the binding of F-II with UV active complex using a computer modeling.
5. The association of phytofluene and F-II in an *in-vitro* study.

Materials and Methods

Palmyrah fruit pulp

Palmyrah fruits were collected from Kalpitiya (collection I, collection II, collection III, collection IV) Hambantota, Jaffna, Kotahena (collection I, collection II, collection III) and Mannar of Sri Lanka. Objective no.2 was achieved by using above 10 collections. For objective no. 3, a bulk sample of PFP was obtained from Kalpitiya. PFP extracted as described previously⁷.

Extraction of flabelliferin mixture from PFP

The flabelliferin mixture was extracted by the methods described previously⁷ using methanol followed by de-carotenising with petroleum ether (60-80°C). The sugars were separated to a great extent from flabelliferins by extracting with acetone. The acetone was evaporated in a rotary evaporator (45°C). A dry cellulose column chromatography was used to further separate flabelliferins from any reducing sugar⁷.

Separation of flabelliferin -II

Selective solvent extraction was carried out to remove small molecular weight flabelliferins (F_D and F_E)². The water fraction, which contained FB and F-II, was subjected to preparative TLC². The F-II band was scraped and dissolved in methanol: water (1:1) to obtain F-II with the binder.

HPLC studies on UV active carotenoids bound to F-II

Flabelliferin -II with the binder was introduced into a reverse phase C₁₈ column (symmetry 3.9×150mm) and eluted with methanol: acetonitrile: tetrahydrofuran (35:58:7) on HPLC Waters model (2487, Dual λ absorbance detector) from USA. The eluent was scanned with UV light at 280 nm and 330nm. Relative retention times of peaks were recorded. The process was repeated collecting fractions of 0.5 ml around the eluent volume corresponding to retention times. The fractions were evaporated and spotted on TLC plates observed under UV light and sprayed with anisaldehyde² to detect the carotenoids and flabelliferins respectively.

Determination of binding ratio of carotenoid binder to F-II and F_B in 10 samples of PFP

Pre-prepared TLC plates after spotting were run in butanol:ethanol:ammonia (B:E:N) =7:3:4¹. F-II and F_B spots were scanned using Shimadzu densitometer (CS-9301 PC, dual wave length, Flying spot scanner) at 280 nm for fluorescence followed by a

Pharmacia Biotech Computerized densitometer at 500 nm for intensity for flabelliferins (after spraying with anisaldehyde). A graph was plotted for area under the curve (AUC) for F-II (at 500 nm) and for UV binder (at 280 nm) vs AUC. A similar graph was plotted for F_B.

Computer modeling study

Molecular Mechanics (MM+) and subsequent Parametric Method 3 (MP3) semi-empirical computational calculations (Hyperchem[®] Molecular Modeling and Computational Software, Registered version 4.2) were employed to investigate the stability⁸ and heats of formation values of F-II, F_B, F_C, F_D with the acyclic UV active compound (phytofluene) were calculated.

Attempts to reconstitute the adduct

Phytofluene (0.24 μmol) isolated by open column chromatography⁶ was incubated in the dark for 24 h with F-II (0.05 μmol) and the mixture was separated using Silica gel G₆₀ and developed in butanol:ethanol:ammonia. The plates were observed under UV light and sprayed with anisaldehyde².

Results

HPLC studies on UV active carotenoids bound to F-II

HPLC charts obtained at 280nm and 330 nm are shown in figure I and II respectively. A sharp peak appeared at 280nm and 330nm at relative retention time 1.10 minute. This peak corresponded to standard phytofluene. The small shoulder present in the sharp peak gave the free F-II spot on TLC. A small peak that appeared just before the phytofluene peak did not show as a steroid spot in TLC. Therefore it cannot be phytofluene- β sitosterol complex and most likely to be phytoene. It was evident from TLC that HPLC solvent system had removed F-II from phytofluene.

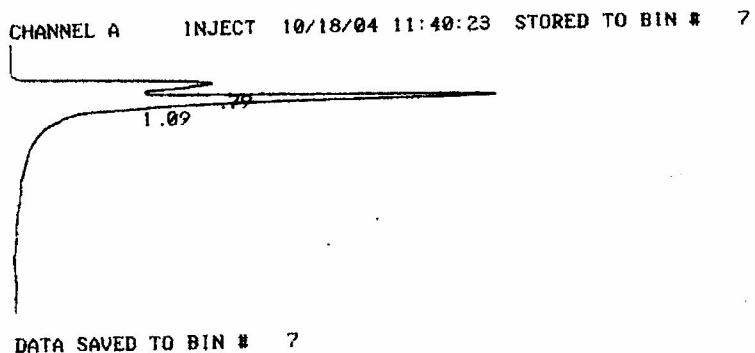


Figure 1. HPLC chart record for the UV compound at 280nm. The retention time of standard phytofluene is 1.10min.

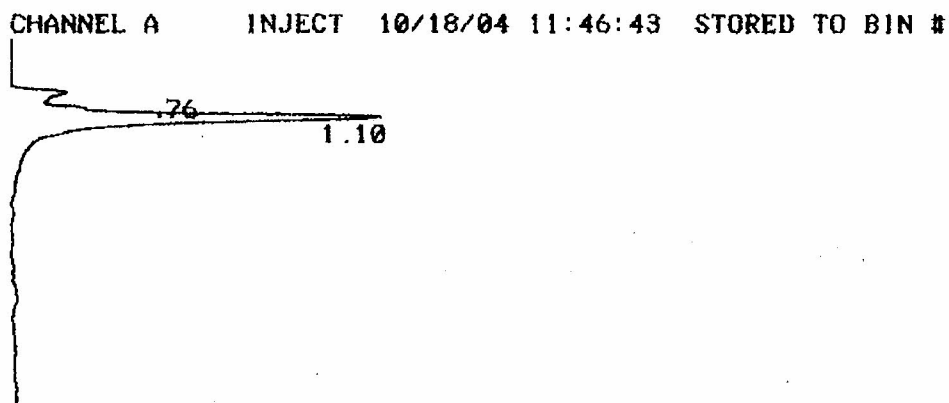


Figure 2. HPLC chart record for the UV compound at 330nm.

Determination of the binding ratio of phytofluene to F-II and F_B

No correlation between the stoichiometry of carotenoid binder and F-II ($r^2= 0.406$) as well as for F_B ($r^2=0.007$) was found in 10 different collections of PFP used.

Computer modeling study

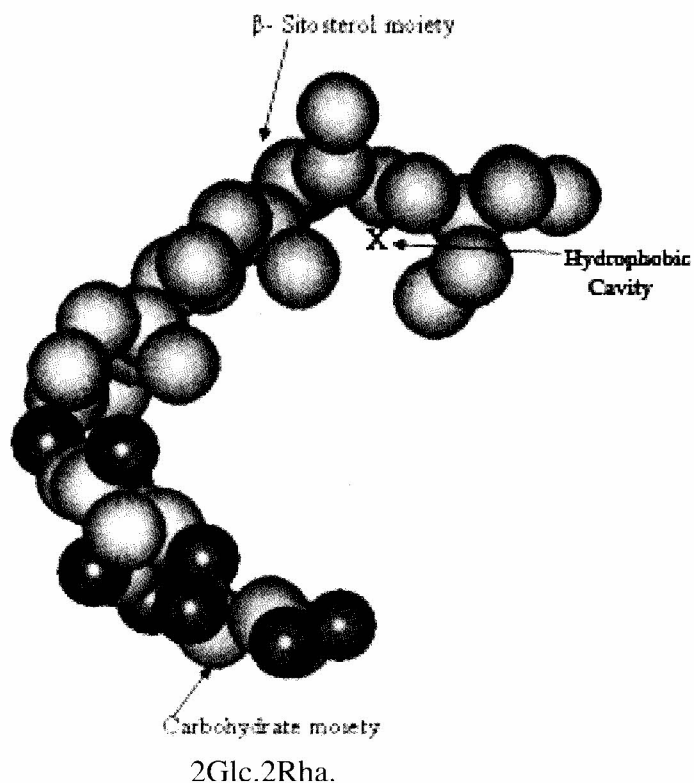


Figure 3. Geometry optimized structure of F-II. Phytofluene lies in the cavity of the β -sitosterol molecule. Phytofluene molecule is shown as “X”.

Glc.= Glucose, Rha.=Rhamnose

Geometry optimized model diagram of F-II is given in Figure 3. It showed that there is a well-defined cavity in the β -sitosterol molecule. According to previous studies the most possible conformation of phytofluene (an acyclic carotenoid) is to lie in this cavity at right angles to the β -sitosterol molecule⁸.

Table I. Calculated heat of formation values

Associated Complex (Flabelliferin type+ phytofluene)	$\Delta\Delta H_f / \text{KJ mol}^{-1}$
F-II Tetraglycoside (MW 1030)	647062
F _B Triglycoside (MW 868)	65
F _C Triglycoside (MW 868)	61
F _D Diglycoside (MW 722)	69

As shown in the Table I, the relative heat of formation value obtained for F-II+ phytofluene associated complex is extremely high when compared with associated complexes of some other flabelliferins. This may result in an unstable configuration for this complex and lead to a huge distortion of carbohydrate moiety, which is the active moiety of F-II. This could explain why the presence of phytofluene binding F-II reduces the ATPase activity of the adduct⁴.

Association of F-II to phytofluene

No re-association was observed.

Discussion

Results show that the binder in PFP (for F-II) in the bulk sample from Kalpitiya is mainly phytofluene, as judged by retention data on HPLC and UV absorbance spectrum. The β sitosterol –phytoene or phytofluene complex has been shown to be most stable among all carotenoids of palmyrah fruit pulp by computer modeling studies⁸. Therefore by elimination, the smaller peak appeared in HPLC could only be due to phytoene. It has been shown that phytoene is the prominent carotenoid binder in palmyrah flour⁵. This is not so in the PFP sample studied. The heat of formation and molecular modeling shows that there is a major distortion of the carbohydrate moiety of F-II unlike the other flabelliferins. This distortion could explain why the binding of phytofluene reduces the ATPase activity of F-II⁴. Whether this inhibition is an all or none-effect where only bound molecules are inhibited or proportionate inhibitory effect on the molecule is yet unknown. The inability to re-associate phytofluene with F-II is not surprising due to the high ΔH_f . The question of how is it formed in

nature then arises. Perhaps the carbohydrate moiety of F-II is biosynthesized after phytofluene complexes with β -sitosterol. The absence of correlation between binding of phytofluene to F-II and F_B in different samples can be attributed to be mainly due to the possible differences of content of phytoene and phytofluene in different samples of PFP. As heats of formation of phytoene or phytofluene - β -sitosterol complex are similar, they would be competitors to the F-II and F_B binding sites. As the relative concentrations of the two carotenoids are different in different samples thus would result in the low F-II: phytofluene and F_B : phytofluene correlation⁹.

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