

Some Studies on the Flabelliferins and Carotenoids of the Fruit Pulp of Palmyrah (*Borassus flabellifer* L)

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Abstract

In studies directed at understanding fully, the options for utilization of palmyrah fruit pulp (PFP), the work reported here was concentrated on flabelliferins (saponins of β -sitosterol) and carotenoids. Flabelliferins, F-11, Fb, Fc and Fd which were purified by solvent-gradient chromatography were subjected to naringinase hydrolyses at pH=4.0 (β rhamnosidase activity). The analysis of products of hydrolysis indicated that the bitter flabelliferin (F-11) had a straight chain carbohydrate moiety of sequence Rha. Glu. Glu. Rha. The antimicrobial flabelliferin F_B, had, as proved by previous studies, a branched carbohydrate moiety with 2-rhamnose terminii linked to glucose, at least one of which had a β anomeric carbon. This was further confirmed by subjecting F_B to the action of fermenting yeast. F_C did not contain β rhamnose anomeric bonds. The two rhamnoses were probably linked. F_D, a diglycoside containing glucose and rhamnose, contained β rhamnose terminus as proved by previous spectroscopic studies. Flabelliferins of PFP were stable to temperatures of less than 75°C over a period of 6 hours but were hydrolysed by fermenting yeast, which results in a lowering of bioactivity flabelliferins in products like wines. The carotenoids of λ_{max} of 443, 427, 470, 400 were shown to be the following carotenoids: α carotene, β -zeacarotene, lycopene, and ζ -carotene. Only the first two named are structurally pro-vit A. Treatment to mimic alcoholic fermentation and recovery of a distilled spirit results in loss of pro-vitamin A carotenoids that become oxygenated and give a deepening of colour. As a result the pigment can only be utilized as a colouring material after fermentation.

Key Words : Palmyrah Fruit Pulp, Flabelliferins, Enzymatic Hydrolysis, Carotenoids, Effect of Heat and Fermentation.

1. Introduction

Palmyrah fruit pulp (PFP) is a vastly underutilized resource with a potential yield of 10-20 ktonnes.⁻¹¹ Over the past decade, efforts had been made to increase utilization. One of the two major potential uses of PFP is fermentation to alcohol, which is feasible² despite an antimicrobial steroidal saponin (flabelliferin-F_B)³. The other major use is expected to arise from debittering (enzymatic hydrolysis) of flabelliferins⁴. PFP has a yellow to orange colour. This was known for sometime⁵ to be due to carotenoids, but the nature of carotenoid (eg whether structurally Pro-vit A or not) was unknown.

The objectives of this study were manifold. (I) To separate and purify flabelliferins. (II) To determine if they are subject to specific enzyme hydrolysis. (III) And if so, if by studying such hydrolytic patterns, determine if evidence could be provided for the structures of the flabelliferins, some of which had been partly⁶ and others fully⁷ characterized by spectroscopic methods. (IV) To determine if the anti-yeast flabelliferin F_B and other flabelliferins can be hydrolysed by a mixed culture of commercial yeast. (V) To determine if heat affects flabelliferin profile (which is important in selecting improved methods of isolation of flabelliferins). (VI) To determine the carotenoid profile of Palmyrah fruit to obtain an indication of pro-vit A activity and (VII) To determine the effect of alcoholic fermentation and alcohol distillation conditions on stability of carotenoids, in order to find out if carotenoids could be isolated and used as a by-product of alcoholic fermentation.

2. Materials and methods

Fruit pulp

The pulp of ripe fresh palmyrah fruits (PFP) collected from Kalpitiya in the North-West of Sri Lanka was provided by Palmyrah Development Board (PDB).

Extraction and desugaring of PFP

Crude flabelliferins were extracted using methanol, petroleum ether, 60-80°C (for cleaning) from carotenoids and acetone and desugared using dry cellulose chromatography⁴.

Solvent gradient chromatography

The purified flabelliferins F-II, FB, FC, FD⁶ were separated from a crude desugared flabelliferin mixture using solvent gradient chromatography using ratio of ethylacetate: methanol from 3:1 to 1:1 (100ml each) after flushing with dichloromethane (100ml) and hexane (100ml) on a silica gel column (column length 32cm, diameter 2.5cm)

Removal of fluorescent compound

The fluorescent compound which co-chromatographed with all flabelliferins was separated by TLC using 1-butanol: ethanol: ammonia (7:3:4), after conducting MPLC using gradients of the solvent system toluene → methanol (0%, 0.78%, 3.125%, 6.25%, 12.5%, 25%, 50%, 100% methanol in toluene). Preparative TLC was conducted (Silica gel G₆₀ on plate of 1.5mm thickness) in order to remove the fluorescent compound while monitoring the flabelliferin spots using anisaldehyde spray reagent and fluorescence before spraying using a UV light (366nm).

Effect of yeast on F_B

Brown sugar (1g) was dissolved in water (6ml). To this was added pure F_B in 1 ml of water (F_B was spectroscopically pure and provided by courtesy of Ms. Dharshika Ariyasena), A control with no F_B was also used. To each solution was added a mixed culture of bakers' commercial yeast (1mg). This was allowed to stand at ambient temperature (29-32°C) for fermentation.

Enzyme hydrolysis by naringinase.

Purified flabelliferins (F-11, F_B, F_C, and F_D) were incubated separately with 1 mg naringinase (ex. *Penicillium decumbens* from Sigma, St Louis, Mo. USA) in a total volume of 3ml at 40°C at pH=4.0 (acetate buffer 0.05M) for 0, 3 6h and overnight (20h). Aliquots (5μl) were spotted at each time indicated above on silica G₆₀ plates and TLC conducted by using 1-butanol: ethanol: ammonium hydroxide (sp gr 0.88) in the ratio of (7:3:4).

Identification of flabelliferin hydrolyates.

Standards of aglycone, F-11, FB, rhamnose and glucose were available. The nature of hydrolysis products were identified as triglycosides, diglycosides or monoglycosides. This was deduced from past studies of this group^{6,7 and 8} using R_f values.

Effect of heat on flabelliferins

Crude flabelliferins were isolated by dry cellulose chromatography and separated by TLC (see above) after the following treatment of PFP. (a) Control (b) Heated at 75⁰ C for 6 hours (c) Autoclaved (121⁰C), 1050Pa for 20 min and (d) fermenting (c) (18h) with bakers' yeast.

MPLC Separation of carotenoids

(a) The carotenoids were separated by MPLC using either (1) a gradient of the solven system, hexane→ dichloromethane 0%, 0.78%, 3.125%, 6.25%, 12.5%, 25%, 50%, 100% dichloromethane in hexane or (11) using a gradient of the solvent system hexane → dichloromethane 25%, 50%, 100% dichloromethane in hexane and dichloromethane→ ethylacetate 0%, 3.13%, 6.25%, 12.5%, 50%, 50%, 100% ethylacetate in dichloromethane. The latter procedure was used after treating with the fermented, heated PFP at 100⁰ C for 45 min. The carotenoids, which separated, were analysed by uv-visible absorption spectra (Shimadzu, Japan, double beam scanning spectrophotometer, model UV 1601) and identified through visible spectroscopic data available in a literature data base⁹ and standards (Sigma, St Louis. Mo. USA)

Method of quantification of carotenoids

The volume of each carotenoid solution was measured and the concentration of carotenoids was calculated by the method given below⁹.

$$X (\mu\text{g}) = A.y (\text{ml}). 106/A^{1\% \text{ 1cm}} 100$$

X = weight or concentration of carotenoid.

Y = Volume of solution that gives an absorbance of A at a specific wavelength

$A^{1\% \text{ 1cm}}$ = Absorption coefficient of carotenoid in the solvent used.

3. Results

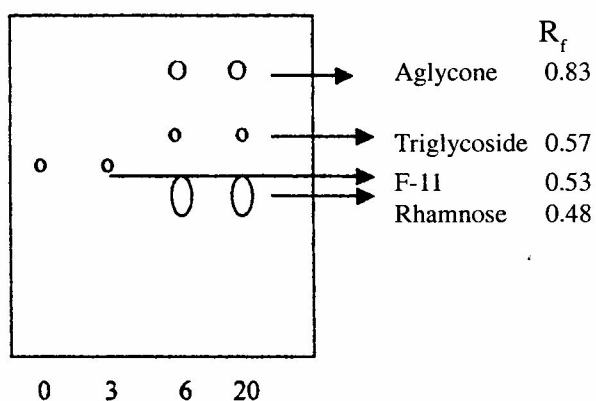
Purification of flabelliferins

The flabelliferins purified by solvent gradient chromatography give the following R_f values in butanol: ethanol: ammonium hydroxide (sp. gr. 0.88) (7:3:4) (Table 1).

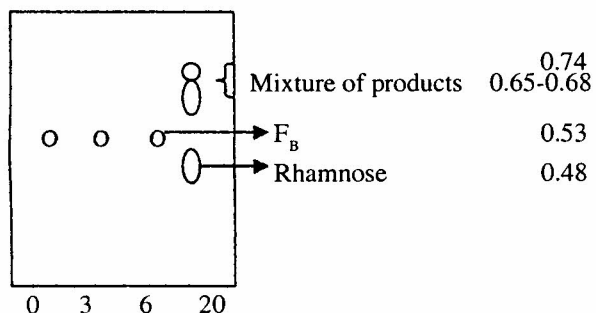
Table 1: R_f values of purified flabelliferins.

	R_f
F-11	0.50
FB	0.53
FC	0.57
FD	0.64

F-11 was hydrolysed by naringinase yielded spots for aglycone (0.83) and a triglycoside (0.57) and rhamnose (0.48).

**Fig 1 : Hydrolysis of F-11 (diagrammatic)**

Fc (R_f 0.57) was not hydrolysed by naringinase. F_B on the other hand was hydrolysed to give rhamnose (R_f 0.48) and a mixture of derivatives probably mono and diglycosides.

**Fig 2: Hydrolysis of FB (Diagrammatic).**

F_D on naringinase hydrolysis yields a monoglycoside (R_f 0.74) and rhamnose (R_f 0.48) see fig 3.

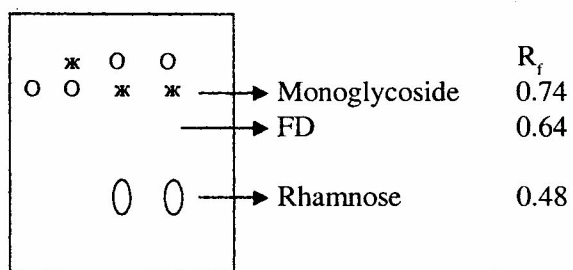


Fig 3: Hydrolysis of F_D (Diagrammatic)

* - disappearing

Yeast hydrolysis

F_B was hydrolysed slowly to yield a monoglycoside (R_f 0.74), diglycoside (R_f 0.63 and 0.68), residual F_B (R_f 0.53), rhamnose (R_f 0.83) (Table 2)

Table 2: Products of yeast hydrolysis of F_B .

	R_f
Monoglycoside	0.74
Diglycoside 1	0.68
Diglycoside2	0.63
Residual F_B	0.53
Rhamnose	0.48
Aglycone	0.83

Effect of heat and fermentation on flabelliferins

Heating to 75° C for 6 hours resulted the no decomposition of flabelliferins however autoclaving and fermentation caused changes.

Light Blue ↓ ✱	Light Blue ↓ ✱	Dark Blue ↓ ○	Dark Purple ↓ ○	R _f
○	○	○	○	0.83
○	○	○	○	0.75
			Light purple { ○	0.72
○	○	○	○	0.67
○	○	○	○	0.64
○	○	○	○	0.58
○	○	○	○	0.55
○	○	○	○	0.53
○	○	○	○	0.47
A	B	C	D	

Fig 4: Effect of heat and fermentation on flabelliferins (Diagrammatic)

*_: disappearing

A_:Control

B_: Heated at 75° C for 6 hours

C_: Autoclaved (121°C) 15 min.

D_: Autoclaved + Fermented (18h)

Carotenoids

Separation of carotenoids by MPLC and quantification 9 showed that there were 4 main carotenoids (Table 3). Fermentation 18h and distillation (100° C, 45min) done in order to mimic the conditions of producing a distilled spirit produced vast changes in carotenoid profile (Table 4).

Table 3: Carotenoids of PFP

	$\lambda_{\max}$	Y/(ml)	Absorbance	Concentration $\mu\text{g} \cdot \text{g}^{-1}$
α - carotene	443	13.5	0.081	8.1
zeta-carotene	400	26	0.04	8.1
Lycopene	470,444	25	0.092	13.3
β -zeacarotene	427	46.5	0.025	11.0

Y:- Volume of solution that gives an absorbance of A at a specific wavelength.

Table 4: Effect of fermentation and heat on carotenoids

	$\lambda_{\max}$	Y/(ml)	Absorbance	Concentration $\mu\text{g} \cdot \text{g}^{-1}$
Not identified	427	25	0.068	-
Lycopene	470,444	25,	0.068	8.6
Canthaxanthin	466	40	0.055	20
Bixin	456	26	0.03	3.6
Not identified	405	64	0.04	-

Y:- Volum of solution that gives and absorbance of A at a specific wavelength.

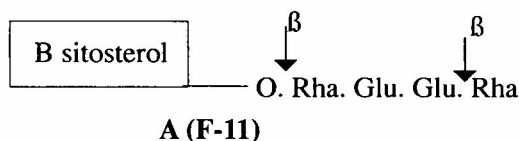
- cannot be calculated.

4. Discussion

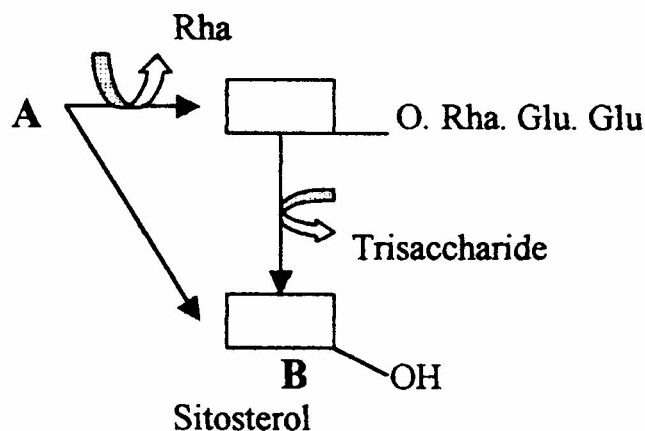
The results show that the flabelliferins can be isolated pure by first using solvent gradient chromatography followed by MPLC and preparative TLC. The last two steps are needed to separate a fluorescent impurity, which bind flabelliferins in propotion to their concentration¹⁰.

On subjecting the flabelliferins to naringinase hydrolysis at pH 4.0 (specific β rhamnosidase activity) the following conclusions were reached.

Flabelliferin -11 (F-11), the bitter flabelliferin⁴ which inhibits the Na/K⁺ pump¹¹ and reduces weight gain of ICR mice¹² and which according to Nikawala⁶ had a molecular weight of 1030 and a terminal rhamnose in its carbohydrate moiety of 2 glucoses and 2 rhamnoses⁴ underwent hydrolysis to yield: (1) rhamnose and the aglycone (β sitosterol)⁷. This shows that both rhamnoses are β . Since the structure of the carbohydrate moiety is straight chained (as no M-162 peak was seen in previous FAB-MS studies^{4,6}) its partial structure is consistent with (A).

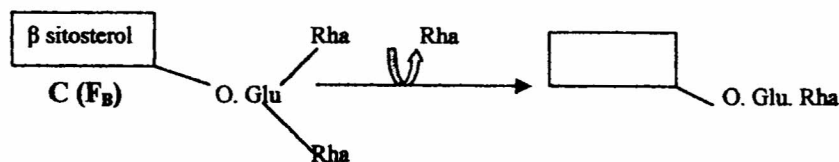


On hydrolysis with F-11 produces rhamnose, a trisaccharide and the aglycone (B) (previously identified) by the way of the following reaction.



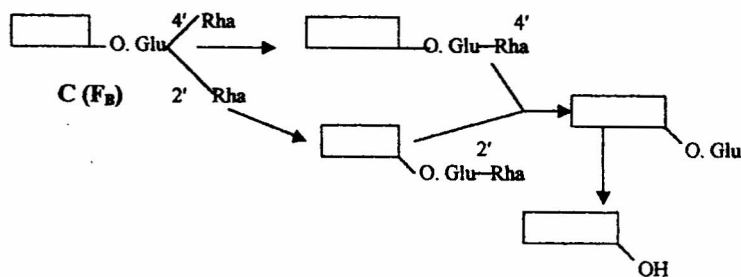
Thus only the linkage position of sugars in F-11 needs to be worked out. The structure of F_B is known (Ariyasena, 2001)⁷. It has a triglycoside carbohydrate moiety with a branched chain with glucose attached to the steroid nucleus and rhamnose linked to the glucose in 2' and 4' positions. This is in conformity to past studies, which identified the aglycone as β sitosterol (M.W 414)⁷ with a M.W of 868 (2 Rha, 1 Glu) with rhamnose terminus⁶.

Naringinase hydrolysis gave rhamnose and diglycoside, but no aglycone.



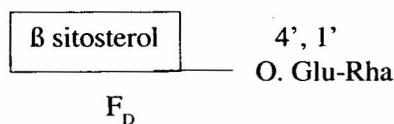
This shows that one rhamnose is and one β . Hydrolysis with yeast was slow (showing much remnant F_B) but yielded 2 diglycosides rhamnose and the aglycone. Since yeast may contain both α and β glycosidases the nature of bonding (α/β) is not possible to deduce, however the presence of two diglycosides prove the branched structure proposed earlier.⁷

Possible scheme



Flabelliferin C (F_C) known to have a M.W. of 868⁶ i.e β -sitosterol+ 2 Rha and Glu with rhamnose terminus is inactive unlike its isomer F_B ⁶. Naringinase could not hydrolyse it, indicating that the rhamnose anomeric bonds are α and not β . Spectroscopic studies are needed to work out the carbohydrate linkage positions of F_C .

Flabelliferin D (F_D) M.W, 722⁶ is a diglycoside, (1 Rha, 1 Glu) rhamnose terminus⁶ determined previously⁷ to be



F_D was hydrolysed by naringinase to yield rhamnose and a monoglycoside. Thus confirming past work⁷.

Heating below 75⁰ C had no effect on flabelliferin profile but slight changes were obtained on autoclaving (121⁰C). Therefore one would not expect normal laboratory isolation conditions to change flabelliferin structure. Fermentation, however, showed hydrolysis of flabelliferins. This was

not surprising as pure F_B too was hydrolysed. This is important, as destruction of bioactive flabelliferins would be favourable in a product like a wine.

The common PFP has a $\lambda_{\max}$ of 426-428⁷ using these samples the following carotenoids were identified. α -carotene ($8.1 \mu\text{g.g}^{-1}$) and β -zeacarotene ($11.0 \mu\text{g.g}^{-1}$) (Pro-vitamin A structures) ζ -carotene ($8.1 \mu\text{g.g}^{-1}$), lycopene ($13.3 \mu\text{g.g}^{-1}$), (non pro-vitamin A structures).

Since fermentation is one likely end use of PEP, it has decided to determine carotenoid content after mimicking the conditions of making an alcoholic spirit. The idea was to find out if carotenoid could be utilized as a by-product of fermentation.

Results showed that the carotenoid profile changed in the case of in the case of most of the carotenoids, originally present. These were destroyed giving a number of compounds two of which were identified as the oxygenated carotenoids: canthaxanthin and bixin. It is concluded that after fermentation the carotenoids cannot be extracted for use as a pro-vit A base. However (since its colour had intensified and deepened) use as a colouring agent was more feasible. That is either as a food colour or the colouring of a toothpaste based on PFP (Nikawala, 2001, personal communication) here the anti-microbial flabelliferin is only partially destroyed. Which is an advantage for a toothpaste

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