JNSF 30-1&2-55

J. Natn. Sci. Foundation Sri Lanka 2002 ***30****(1&2): 55-60*

DIRECT ISOLATION OF FLABELLIFERINS OF PALMYRAH BY MPLC

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(Received: 30 December 2001 ; accepted: 04 July 2002)

Abstract: The flabelliferins (steroidal saponins) of palmyrah are of importance due to their significance in food as well as their bioactivity. There is a large number of flabelliferins in palmyrah fruit pulp (PFP) and their separation is a challenge. Crude flabelliferins had been isolated from PFP by methanol extraction, petroleum ether cleaning and acetone extraction followed by dry cellulose chromatography for desugaring. Flabelliferins from crude mixtures have been separated by a number of techniques namely: flash chromatography, selective solvent extraction, solvent gradient column chromatography, the chromatotron and medium pressure liquid chromatography (MPLC). The large amounts of solvent used and the considerable time taken for the final separation raise questions regarding the possibility of losses of flabelliferins during this tedious procedure. A technique based on direct MPLC (without methanol extraction, petroleum ether cleaning, acetone extraction and dry cellulose chromatography) was worked out to separate not only the flabelliferins in their pure state but also the carotenoids and the free sugars in PFP. This has the advantage of not subjecting materials to heat and the usage of lesser amounts of chemicals. In addition, it is less time consuming and gives yields 2.4 fold that of the indirect method of isolation. Further, this technique is applicable to any PFP sample regardless of its flabelliferin profile.

Key words: Dilution factor, flabelliferin profile, medium pressure liquid chromatography (MPLC), pre-adsorption, solvent gradient.

INTRODUCTION

Steroidal saponins from palmyrah were first reported by Jayaratnam[1] who identified a monoglucoside and a monorhamnoside of spirost-5en-3βol. Isolation was achieved using dried palmyrah fruit pulp (PFP), first extracted with petroleum ether (40-60 °C) and chloroform in a Soxhlet extractor followed by methanol extraction and acetone extraction in which acetone fractions were concentrated and cooled in an ice water bath to obtain a white solid.

The bitter flabelliferin, F-II of PFP from Kalpitiya, Sri Lanka, was isolated and identified as a tetraglycoside of the same aglycone and also a tetraglucoside, F-I.[2] Here the crude flabelliferins were obtained using PFP, first by methanol extraction followed by petroleum ether cleaning, acetone extraction and dry cellulose chromatography. Crystals of the two flabelliferins were obtained by crystallization from a methanol extract on addition of acetone.

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Nikawala and co-workers followed the same procedure for isolation of crude flabelliferins[3] but found that crystalization did not work on a complex mixture of flabelliferins obtained from PFP of Hambantota, which contained 4 flabelliferins: F-II, F_B-antimicrobial flabelliferin, F_C and F_D inactive flabelliferin.[4]

Further studies showed that there are large variations in flabelliferin profiles depending on sources of PFP[5] and, therefore, a universally applicable technique for the isolation was needed. This was successfully done by solvent gradient chromatography (using the chromatotron and the silica gravity column) and MPLC.[6, 7, 8]

However, obtaining crude flabelliferins from PFP and then their separation by different techniques was time consuming and a waste of large amounts of solvents to obtain very little flabelliferins. It was clearly shown that the acetone extraction step causes a significant loss in total flabelliferins.[5] The flabelliferins isolated are subjected to high temperatures at some point during the isolation procedure in order to evaporate different solvents. This may cause structural changes.

The objective of this study was to find a better method for the isolation of flabelliferins from PFP, which involves fewer steps, lesser amounts of solvent, and gives large quantities of flabelliferins with no involvement of heating steps.

METHODS AND MATERIALS

Fruits and the fruit pulp: Ripe palmyrah fruits were collected from Anamaduwa, Ampara and Mannar, Sri Lanka. PFP was extracted manually with distilled water to the ratio of 1:1 (v/v).

Pre-adsorption of the sample: PFP was crushed in a blender and strained through a sieve to remove the fibre. The pulp (50g) was mixed with 75g of silica gel (Merck) using a spatula. Large particles formed in mixing were broken into smaller particles using the spatula. The mixture was allowed to stand for two hours in order to dry. The material then resembled a yellow dry silica gel. Any large particles remaining were separated using a 300 micron sieve and mixed back after breaking into smaller particle sizes with the help of the spatula.

MPLC: The MPLC apparatus of Baeckstrom with Separo columns, FMI pump (model QD O SSY), 10 ml min^{-1} and up with a pressure not exceeding 12 bars, air tight mixing chamber (gradient mixture), and the solvent reservoir were used.

(a) *Column packing:* After inserting the first piston and lightly tightening it, the filter disk was placed with the help of the packing rod. The column was clamped vertically. Using an ordinary funnel with a long stem, it was filled with a continuous stream of silica gel (30 mm id column to a length of 50 mm in order

to separate a sample of 62.5g of the pre-adsorbed sample). The pre-adsorbed sample was filled the same way on top of the silica bed. The column was removed from the clamp and lightly tapped against the tabletop to allow the captured air pockets to rise and leave. The column was also tapped horizontally with a rubber hose. After placing the filter disk, the second piston was inserted and lightly tightened. The column bed was compressed using a "Quick Grip Vise" and finally the two pistons were firmly tightened at both ends. When the column was clamped vertically to the MPLC system, the end which had the pre-adsorbed sample was at the bottom.

(b) *Creating the gradient:* Hexane (200 ml) was passed through the column in order to separate the carotenoids in the pre-adsorbed sample into the silica layer.

The gradient was in the order of hexane → ethyl acetate→ methanol, with a dilution factor of 0.5 and number of dilution being 7 at each step from one solvent to the other. For example: from ethyl acetate → methanol, 0%, 0.78%, 1.562%, 3.125%, 6.25%, 12.5%, 25%, 50%, 100% ethyl acetate in methanol. 100 ml of each dilution was passed through the column up to ethyl acetate and thereafter 150 ml ethyl acetate added. This was followed by the ethyl acetate: methanol gradient. The fraction number was noted whenever a new solvent composition was introduced to the system. Nine (9) ml fractions were collected. TLC was conducted to monitor the separations. Fractions were pooled depending on the flabelliferins present in each fraction (ex: fraction 1, 2, 3, 4, 5, 6 and 7).

(c) *Further separation:* This step was carried out mainly to effect the separation among the flabelliferins, as some fractions obtained from the previous step (crude separation) contained more than one flabelliferin.

Hence for each particular fraction, separation was designed to have a unique solvent gradient. This was usually done starting from the solvent composition of the fraction before the particular fraction to the solvent composition of the fraction after that fraction, making use of the fraction numbers and the relevant solvent compositions noted down in the previous step. For example, to separate fraction number-4, the gradient commenced from the solvent composition at which fraction number-3 (ex: 12.5% ethyl acetate in methanol) was isolated and completed at the solvent composition at which fraction number-5 (ex: 25% ethyl acetate in methanol) was isolated.

The dilution factor and the number of dilutions depended on the number of flabelliferins present in each fraction. The number of dilutions needed was proportional to the number of flabelliferins in the mixture. Nine (9) ml fractions were collected. Tlc was conducted to monitor the separation. Samples were pooled depending on the separation.

(d) *Purification of flabelliferins:* This step of purification was carried out mainly to isolate each flabelliferin from the UV-active impurity, which overlapped exactly on top of the flabelliferin spots on TLC. Solvent gradients from toluene → methanol with a dilution factor of 0.5 and the number of dilutions 6 (0%, 0.78%, 1.562%, 3.125%, 6.25%, 12.5%, 25%, 50% and 100%). Nine (9) ml fractions were collected. TLC was conducted to monitor the separation. Samples were pooled depending on the separation.

TLC: TLC was conducted on pre-prepared Silica gel G_{60} plates (Merck) using n-butanol, ethanol and aqueous ammonia (Sp. Gr. 0.88) to the ratio of 7:2:5.

RESULTS AND DISCUSSION

As hexane passed through the column, the carotenoids in the pre-adsorbed sample eluted to the silica layer, and appeared as a dark red band. As the gradient was increased with ethyl acetate, it gave rise to five (5) orange and yellow bands separating out the carotenoids. This showed that the carotenoids of PFP were a mixture of at least five compounds. As the gradient moved towards ethyl acetate one by one, these bands eluted from the column and at 100 % ethyl acetate all the carotenoids were removed from the sample.

As the gradient is created from ethyl acetate to methanol, 6 flabelliferins (F_F, F_E, F_B, F_C, F_D and F-II) were eluted out of the column. Few fractions contained some of F_B and F_C together and also F_B and F-II together. From the further separations done on these fractions, F_B, F_C and F-II were isolated. The final separation step using toluene and methanol removed the UV-active impurity from the flabelliferins, which overlapped on top of each flabelliferin. This fluorescent impurity appeared to bind flabelliferins in proportion to their concentration unaffected by the sugar moiety.[9]

The sample contained 6 flabelliferins monitored by TLC. The total flabelliferins obtained from 50 g of PFP by this method was 52.4 mg, which is 2.4 times more flabelliferins than the total flabelliferins obtained from the same sample by the previous method (22.2 mg)[3]. Quantities of each flabelliferin, isolated by this method are given below.

(1) F_F 5.3 mg (flabelliferin monoglucoside M.W. 576)

(2) F_D 12.8 mg (flabelliferin diglucoside M.W. 722)

(3) F_E 3.2 mg (flabelliferin diglucoside M.W. 734)

(4) F_B 14.2 mg (branched flabelliferin triglycoside M.W. 868)

(5) F_C 0.6 mg (Linear flabelliferin triglycoside M.W. 868)

(6) F-II 6.2 mg (flabelliferin tetraglycoside M.W. 1030)

The technique provides an easy method of isolating the bioactive flabelliferins: F_B[10] and F-II[11], where isolation for structural elucidation was necessary. It is now known that F_B is a saponin of β-sitosterol with a carbohydrate moiety comprising two rhamnoses linked α 1,2 and α 1,4 to the glucose and the β glucose linked by the anomeric carbon to the sterol nucleus.[12]

This technique is also an easy method of isolating carotenoids from PFP, with the possibility of use as a pro-vitamin A supplement or as a food colorant.

Acknowledgement

The authors thank IPICS, Sweden for grant SRI:07 and the National Science Foundation, Sri Lanka for grant RG/99/C/3.

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