

A Rapid Technique for Plant Peptides Isolation Based on their Polarity by Using BIOTAGE Isolera One Flash Chromatography

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ABSTRACT

Several years of experience performing columns chromatography have been condensed into simple guidelines useful for translating thin layer chromatography (TLC) results into either isocratic- or gradient-flash chromatography. The present study describes the use of such protocol with a silica Biotage SNAP KP-Sil 10 g cartridge (21 mm × 55 mm, 50 μm particle size), to purify, in a single step, four (4) peptides from crude proteins extract of germinated *Amaranthus hybridus* seeds. The best solvent system used was a combination of n-hexane (non-polar solvent B from 5% to 0% final concentration) and a mixture of polar solvents (solvent A) composed of acetonitrile, n-butanol, glacial acetic acid and water in 0.9:2:1:1 ratio, respectively. The elution was done in normal-phase with a linear gradient. All the flash chromatography procedure took at most twenty-five minutes (25 min), and less than a liter (750 mL) of total solvents was used. The purified peptides named AhP_A, AhP_B, AhP_C and AhP_E showed apparent homogeneity on TLC plates, and purification yields of about 11.15%, 10.38%, 14.50% and 17.25%, respectively. This innovative technique provides an efficient alternative to researchers' in peptides purification and participates in reducing the waste of solvents, gels, time and thus, money.

Keywords: *Amaranthus hybridus* seeds; Germination; Normal-phase flash chromatography; Peptides purification; TLC

INTRODUCTION

Chromatography is a biophysical technique that enables the separation of mixtures into their solute or constituent parts by distribution them into mobile and stationary phases. It is the only technique mainly use for the purification of molecules of scientific interest prior to their detailed study regarding structural and functional properties [1,2]. The review of literature in this field has shown that, due to the structural complexity of most of biological molecules already studied, the chromatographic methods used for their isolation generally differ [3]. So, it's not daring to assert that each molecule has a particular way of purification. For example, each isoform of α-mannosidase contributing to the expression of the α-mannosidase activity of the digestive fluid of *Rhynchophorus palmarum* larvae, although coming from the same material, has its own protocol of purification [4], as it is the case of isoforms of the phosphatase activity of *Largenaria siceraria* seed extract [5].

Proteins and peptides purification are of great importance in to acquire knowledge about structural and functional properties and to foretell their potential applications. Regarding peptides, they generally are purified by following proteins separation techniques. Thus, several typically used methods for peptides separation are available, in a series of purification steps. Those are ammonium sulphate precipitation, separation through reverse-phase chromatography based on hydrophobicity, ion-exchange chromatography based on charge, gel permeation separation based on molecule size, shape and density, affinity chromatography, SDS PAGE and immunoblotting etc [6]. However, despite the existence of all these techniques, the search for innovative methods is still relevant. In this respect, new purification protocols exploiting specific, effective and robust methods, as well as the use of new devices and materials are expected to guide the future of proteins and peptides purification area [7,8].

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Apart from its main purpose of separation, applying a satisfactory chromatographic technique should be necessarily achieved within a suitable time interval. So, efficient methods and high-performance devices have been developed to that end. Among these devices, the Biotage Isolera One chromatographic system (flash chromatograph) which is a key silica gel system efficiently used for rapid purification of drugs based on their polar or non-polar properties [9,10]. We became familiar with this equipment in 2018 during a six-month postdoctoral research fellowship on bioactive molecules of pharmaceutical interest at JSS College of Pharmacy, INDIA. The present report highlights a one-step purification process of four peptides by translating their thin layer chromatography (TLC) separation characteristics into a linear gradient flash chromatography system.

MATERIALS AND METHODS

Materials

The plant material used in this study was dried seeds of a tropical herbaceous orphan plant commonly known as pigweed (*Amaranthus hybridus* Linneaus). The silica TLC plates used for peptides identification and all the organic solvents applied as mobile phase during the purification process were purchased from MERCK, Germany. The BIOTAGE Isolera One chromatography device used was manufactured from BIOTAGE India Private Limited, MUMBAI, India.

Germination process

The germination process has consisted both in determining the best imbibition time of seeds and its influence on the germination. 200 g of dried *Amaranthus hybridus* seeds were cleaned thoroughly with tap water and 50 g weighted for initial proteins quantification. The rest of the washed seeds were soaked at $28 \pm 2^\circ\text{C}$ in 1:2 (w/v) proportion of distilled water for 6 hours. During this imbibition time period, two samples of the moisturized seeds were withdrawn after 3 and 6 hours respectively, and scattered across two different Petri dishes wallpapered with watered tissue paper. The germination process was carried out at ambient temperature ranged from 16°C to 20°C for up to 72 hours (March 2018, Ooty-Tamil Nadu, INDIA). For the monitoring of the proteins profile during the germination process, 50 mg of seeds samples were taken both during the period of imbibition and germination. Sampling times are determined as follows: 3 hours and 6 hours for the wetting period (imbibition), and 24 hours, 48 hours and 72 hours for the germination period. Seeds were considered to have germinated when up to 2 mm radicle emergence was noticed [11].

Proteins extraction and quantification

At the indicated sampling times, proteins extracts were obtained by grinding seeds with Milli Q water in a mixed proportion of 1:1 (w/v) using an electric blender (PREETHI Silver). The crushed solution was sonicated for 10 min and centrifuged at 5,000 rpm for 1 hour with a REMI R-8C digital centrifuge. The supernatant was filtered through a $0.45 \mu\text{m}$ filter and proteins

were quantified by following the [12] method using the Folin & Ciocalteu's clinical reagent (SD Fine-Chem Limited, Mumbai) and egg albumin (0.5 mg/mL) as the standard protein. Peptides concentrations were determined in UV wavelength of 214 nm using a SHIMADZU UV 1700 UV-Vis Spectrophotometer. The extract containing both highest proteins and peptides concentration was freeze-dried at -80°C with an ESQUIRE BIOTECH SKL-12N Freeze Dryer, and the lyophilized powder was stored at 0°C for further analysis.

Determining the best solvent selectivity using thin layer chromatography (TLC)

The first step in successful flash purification has consisted in improving the retention factors (R_f) of targeted peptides by increasing their silica contact time then maximizing the chances of flash chromatography resolution. So, before flash purification, the crude protein extract was run with different solvents system on TLC Silica gel 60 F₂₅₄ plates (Merck KGaA, Germany). Separated peptides were detected by spraying the chromatographic plates with 1 gram of ninhydrin in pyridine (95 mL) and glacial acetic acid (5 mL), and their spots were visualized by heating at 100°C for 5 min. The retention factor of each compound was determined by dividing its travelled distance by that of the solvent front. The solvent system that eluted peptides in R_f values ranged from 0.15 to 0.40, and with largest resolution (ΔCV) between the different compounds was selected for flash chromatography. CV values are given by the inverses of R_f values.

Peptides purification using flash chromatography

Peptides were submitted to a normal phase flash purification procedure using a silica Biotage KP-Sil cartridge. This is the most frequently used silica for flash purification features with a high surface area (500 m²/g), moderate porosity (60 Å), a tight uniform particle distribution (40-63 μm) with neutral pH and low metals content. The silica Biotage SNAP KP-Sil 10 g cartridge (21 mm × 55 mm, 50 μm particle size) was mounted on a fully automated flash chromatography instrument (BIOTAGE ISOLERA ONE). The system was equipped with an expanded fraction collector bed and dual wave length UV-V is detectors. For the purification, the lyophilized protein powder was dissolved in the mobile phase to a final concentration of 50 mg mL⁻¹. Then, 1.5 mL of the resulted solution were loaded onto the cartridge samplet and dried at Lab temperature ($19 \pm 2^\circ\text{C}$). The elution process was done at a flow rate of 12 mL/min and 15 mL of fraction were collected per tube by UV absorbance of 214-215 nm. All the chromatographic procedure was performed using a linear gradient solvent system. The elution started by equilibrating the column with 2 CV of 5% of n-hexane, the non-polar solvent B in 95% of polar solvents (Solvent A). The solvent B was prepared with respective volume ratios [0.9:2:1:1] of acetonitrile, n-butanol, glacial acetic acid and Water. Then, the cartridge was eluted with 20 CV of the mobile phase starting from 5% to 0% of solvent B (95% to 100% of solvent A).

RESULTS

Germination process

The proteins and peptides concentrations determined during the imbibition and germination periods were relatively converted into percentages and presented in Figure 1a and 1b. The results showed that six hours (6 hours) of dried seeds imbibition represent the best time period to obtain the highest proteins and peptides concentration (100%) at the end of germination. After three hours of imbibition, a maximum value of 83% (Figure 1b) representing peptide concentration was obtained.

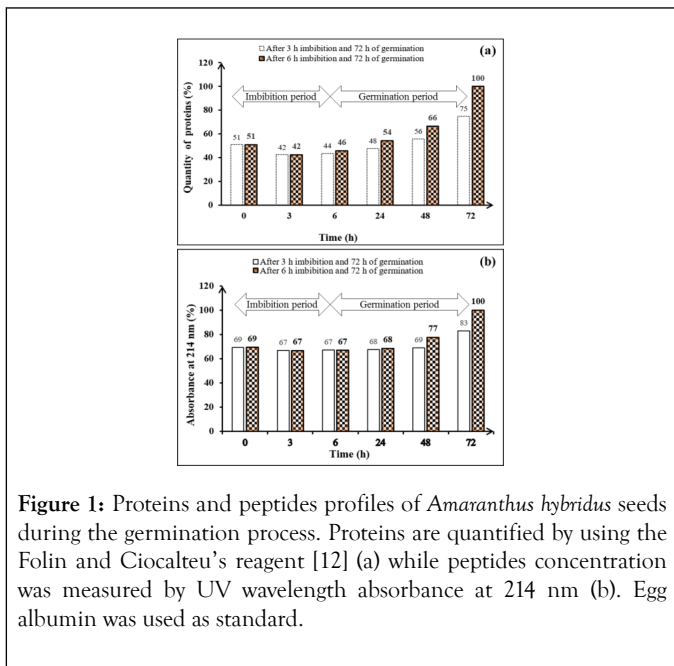


Figure 1: Proteins and peptides profiles of *Amaranthus hybridus* seeds during the germination process. Proteins are quantified by using the Folin and Ciocalteu's reagent [12] (a) while peptides concentration was measured by UV wavelength absorbance at 214 nm (b). Egg albumin was used as standard.

Thin layer chromatography (TLC)

Based on the trial and error method, we have optimized the solvent system after migration and visualization of peptides spots onto TLC plates (Figure 2). The best solvent system was a composed of 0.1 mL of n-Hexane (non-polar solvent B) and a mixture of polar solvents (solvent A) made by Acetonitrile, n-Butanol, Glacial Acetic Acid and Water in the ratio of 0.9:2:1:1, respectively. This mobile phase enabled an efficient separation of peptides by increasing their retention factor (R_f) values and improving the resolution of the variation of calculated column volume (ΔCV) as shown in Table 1. R_f values were within the optimal range from 0.10 to 0.40 as were ΔCV values ≥ 0.7 .

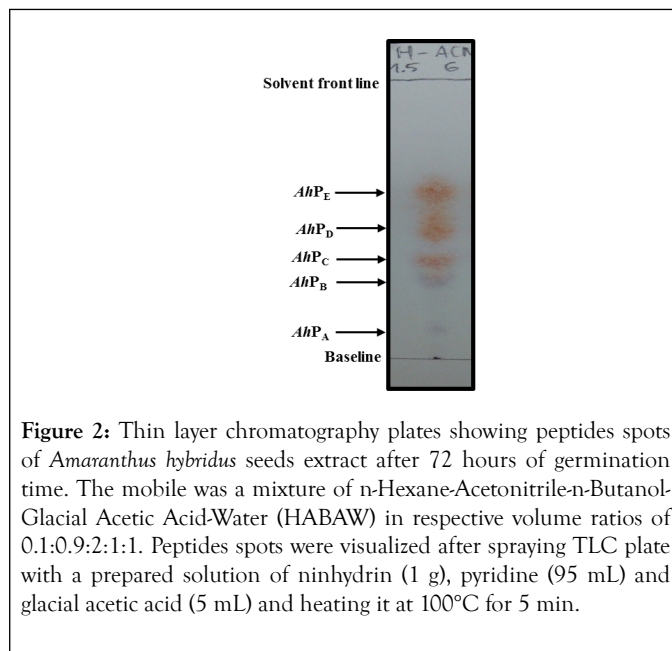


Figure 2: Thin layer chromatography plates showing peptides spots of *Amaranthus hybridus* seeds extract after 72 hours of germination time. The mobile was a mixture of n-Hexane-Acetonitrile-n-Butanol-Glacial Acetic Acid-Water (HABAW) in respective volume ratios of 0.1:0.9:2:1:1. Peptides spots were visualized after spraying TLC plate with a prepared solution of ninhydrin (1 g), pyridine (95 mL) and glacial acetic acid (5 mL) and heating it at 100°C for 5 min.

Table 1: Translating peptides TLC frontal ratios (R_f) to column volumes (CV) and calculation of adjacent compounds resolution (ΔCV) for flash chromatography.

Peptide	Stained peptide front (cm)	Mobile phase front (cm)	R_f	CV (mL)	ΔCV
AhP _A	0.8	7.7	0.10	9.6	6.2
AhP _B	2.3	7.7	0.30	3.4	0.6
AhP _C	2.8	7.7	0.36	2.8	0.7
AhP _D	3.7	7.7	0.48	2.1	0.5
AhP _E	4.7	7.7	0.61	1.6	0

AhP_x: *Amaranthus hybridus* Peptide_x, R_f : Retention factor, CV: Column volume; The solvent system was HABAW (n-Hexane-Acetonitrile-nButanol-Glacial Acetic Acid-Water) in proportion 0.1:0.9:2:1:1 respectively.

Flash chromatography

By transferring TLC plates R_f and ΔCV values into the Biotage Isolera One device program, four (4) peptides were eluted by starting with the less polar (spot A on TLC plate) to the more polar one (spot E). Figure 3 introduced flash chromatogram profiles of the four purified peptides namely AhP_A, AhP_B, AhP_C and AhP_E. The peptides showed apparent homogeneity on TLC plate (Figure 4) with purification yields varying from 10.38% (AhP_A) to 17.25% (AhP_E).

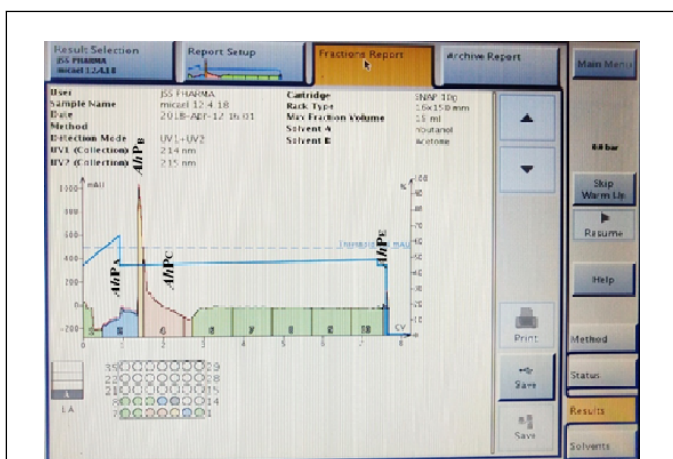


Figure 3: Normal phase purification of peptides from germinating seeds of *Amaranthus hybridus*. Elution was done using linear gradients composed of a mixture of polar solvents (solvent A) and n-Hexane (non-polar solvent B) starting from 5%. solvent A was composed of Acetonitrile, n-Butanol, Glacial Acetic Acid and Water in the ratios of 0.9:2:1:1 (v/v/v/v). Wavelengths absorbance at 214 nm and 215 nm shown.

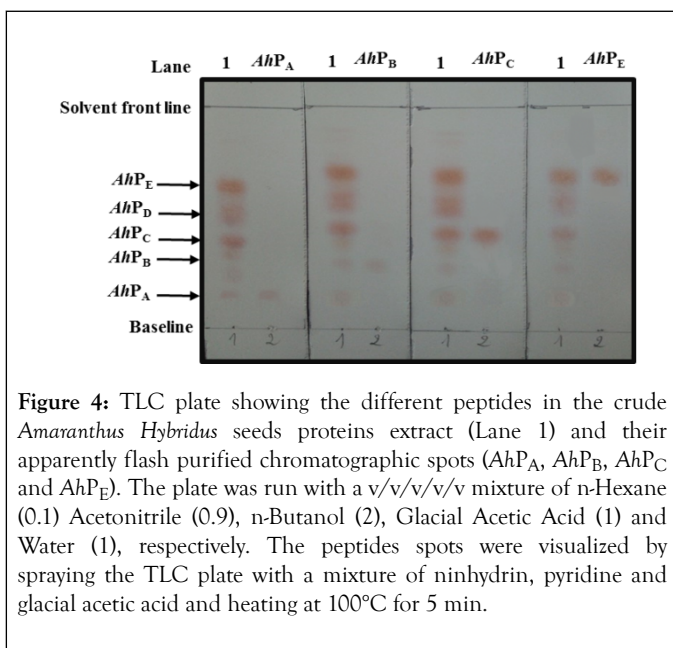


Figure 4: TLC plate showing the different peptides in the crude *Amaranthus Hybridus* seeds proteins extract (Lane 1) and their apparently flash purified chromatographic spots (AhP_A , AhP_B , AhP_C and AhP_E). The plate was run with a v/v/v/v mixture of n-Hexane (0.1) Acetonitrile (0.9), n-Butanol (2), Glacial Acetic Acid (1) and Water (1), respectively. The peptides spots were visualized by spraying the TLC plate with a mixture of ninhydrin, pyridine and glacial acetic acid and heating at 100°C for 5 min.

DISCUSSION

The purification of proteins and their peptide subunits has been an important asset in determining their structural conformations and elucidating their [13,14]. To date, most of the chromatographic techniques used for isolation target their intrinsic properties such as size and shape, ion charge and hydrophobicity. Although they have the advantage of achieving satisfactory purity, most of the described protocols are solvents, gels and time consuming. Also, they are considered to be laborious and therefore difficult to implement [15,16]. All these drawbacks have prompted the implementation of innovative techniques in terms of procedures and equipment. One is BIOTAGE Isolera One Flash chromatography system, a medium pressure liquid purification devices separating efficiently and

rapidly polar and non-polar compounds mainly used in pharmacy [17].

In the present work, a medium pressure chromatography technique using a silica Biotage SNAP KPSil 10 g cartridge as a separation column has been used for the purification of peptides from crude proteins extract of *Amaranthus hybridus* germinated seeds by targeting their polarity properties. It has begun with a separation onto thin layer chromatography plates which allowed us both to visualize a clear separation of the peptides and therefore to select the appropriate solvent system. Then, frontal ratios (R_f) data were converted in column volume (CV) in terms of volume of solvent necessary for the elution of each molecule. Finally, the differences between the volumes of solvents (ΔCV) necessary for the elution of adjacent peptide were programmed in the flash chromatography device for one step separation [10].

In most of the work related to the purification of peptides, the separation is done by reverse-phase chromatography [18,19]. The credit therefore goes to our work for using a normal-phase chromatography on silica gel column to allow efficient separation of peptides with interesting purification yields of 11.15%, 10.38%, 14.50% and 17.25 % for peptides AhP_A , AhP_B , AhP_C and AhP_E , respectively. The renewed interest granted in recent years to peptides as bioactive molecules in the regulation of a number of metabolic disorders (obesity, diabetes and hypertension etc.) that afflict humanity make them attractive [20-22] and therefore give prior choice to this technique as an asset for peptides purification.

The present work lays the foundations for a new peptide purification technique taking advantage of their polar or non-polar characteristics. This is an innovative technique that should be explored because it has the advantage of efficient purification and significantly reducing the consumption of solvents, gels and especially time.

CONCLUSION

The current study has optimized a solvent system to isolate, in a single chromatographic step, four peptides with different polarities from *Amaranthus hybridus* seed by using a normal-phase flash chromatography which is a novel approach. As a result, the future studies in this field prompts in the use of this technique for rapid purification of related molecules prior to their extensive characterization for potential application.

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CONFLICTS OF INTEREST

None.

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