Ph.D. Thesis

Purification of fungal secondary metabolites by centrifugal partition chromatography

Gábor Endre

Supervisor

Szekeres András Ph.D.

Senior research fellow

DOCTORAL SCHOOL OF BIOLOGY

Department of Microbiology

Faculty of Science and Informatics

UNIVERSITY OF SZEGED

2022

1. Introduction

Secondary metabolites are not vital but play an important role in the life of a microbe, fungus. These compounds are produced in the ideal state of growth, and also can be broken down by the organism.

Aspergillus species produce a wide variety of these secondary metabolites. Probably the most examined of these is the group of aflatoxins (AFs). These chemicals were named after *Aspergillus flavus*, the fungi that were isolated from. Their mutagenic, teratogenic, and carcinogenic properties were revealed, hence strict limit levels were imposed in the EU. These restrictions imply the regular quality assurance of food and feed and toxicological studies in Europe, therefore the demand for pure AFs as reference material is high. Numerous publications exist on the isolation and purification of AFs, including thin layer chromatography (TLC), chromatographic methods including silica gel and alumina, and HPLC separations as well. But the application of novel liquid-liquid chromatographic techniques could result in faster and cheaper production of these toxins.

Ergometrine (ErgM) is produced by mainly *Claviceps* species. Most *Claviceps* species can be found in equatorial climates, but *Claviceps purpurea* infects crops and grasses in Mediterranean and temperate climates as well. ErgM and its variants are used in medicine, therefore the demand for the pure form by the pharmacological industry is high. It can be synthesized, but the isolation and purification can also be carried out from the overwintering sclerotium of the fungus, utilizing liquid-liquid chromatography as the separation technique.

Centrifugal partition chromatography (CPC) belongs to the preparative separation techniques, where two, immiscible liquid phases are used to partition the different components of a mixture. The differences in the partition coefficients of the compounds result in separation during the elution. The stationary phase is immobilized by a constant amount of centrifugal force, while the mobile phase is pumped through it, resulting in droplets with high specific surface area, achieving better distribution. Since the two-phase can be assembled from several solvents, this separation technique offers a wide variability, fast and robust separations, with high efficacy.

In this work the method development for the purification of AFs and ErgM by CPC is described in detail, as well as the scale-up of the separation of AFs.

2. Aims

This work aimed to determine AF and ErgM production of isolated endophytic fungi and to develop novel liquid-liquid purification methods for the compounds. Furthermore, to carry out effective scale-up of the separations, and also to verify the quality and quantity of the achieved final products.

Regarding AF purification the main objectives are,

- Screening for an AF producer *Aspergillus* strain,
- Large scale cultivation of the selected strain,
- Effective, large-scale extraction of the produced AFs,
- CPC method development for the separation of AFs, possibly to separate them in one run,
- Scale-up of the separation, to achieve maximum yield,
- Verify the quality and purity of the final products.

In the case of ErgM purification, the main objectives are,

- Screening for an ErgM producer *Claviceps* strain,
- Large scale cultivation of the selected strain,
- Effective, large-scale extraction of the produced ErgM,
- CPC method development for the separation of ErgM,
- Verify the quality and purity of the final product.

3. Results

Screening for an AF producing *Aspergillus* strain

13 *Aspergillus* strains were cultivated on 11 different culture media, to have their AF production capabilities measured. To achieve this, the produced AFs were extracted from all the solid and liquid cultures, the AF content was qualified and quantified by a novel HPLC-MS/MS technique, and absolute AF concentrations of the extracts were determined, first in the literature. It was revealed that on solid culture media, four isolates produced all the four main AFs, while *A. parasiticus*, SZMC 2473 produced the toxins in the highest amounts (348 mg/kg) when it was cultivated on wheat. On liquid media, the same four isolates produced all the AFs, and the same fungus (SZMC 2473) produced them in the highest concentration (40 mg/l) when

it was cultivated on Bio vegetable cocktail. It was concluded, that for further experiments *A. parasiticus*, SZMC 2473 will be utilized, due to its high AF production capability.

CPC separation of AFs

After the selection of the organism, it was cultivated in large scale. In one batch 4.5 l of liquid culture was prepared and was inoculated. After the fermentation, the mycelia was filtered off, and the produced AFs were extracted in a three-step extraction method. In one batch approximately 500 mg of crude extract could be gained.

This crude extract was used for the method development of the centrifugal partition chromatographic (CPC) separation. For the method development, the "best solvent" method was utilized, and altogether 63 solvent systems were tested. It was resulted, that the most suitable solvent system would be toluene/acetic acid/water = 30/24/50.

After instrumental optimization, with this composition, for the first time in the literature, the separation and purification of the four main AFs in one run was successfully achieved. It was revealed, that the system is stable and the separation is repeatable. With this procedure, from 90 mg crude AF mixture, 81 mg pure AFs could be gained, with a purity above 97% and the recovery around 90%.

Scale-up of the AF separation

To achieve the maximum capacity of the CPC system, the separation of AFs was scaled-up. This scale-up procedure is unique in the literature and has never been reported before. In the linear scale-up method the maximum loading capacity was determined. The solvent system can be saturated with a concentration of 25 mg/ml of crude AF extract. The instrument has a 10 ml injection loop, therefore the maximum separated AF amount can be 250 mg on a 250 ml column.

After the maximum loading concentration was determined, the maximum injected volume was determined. Experiments were carried out with the built-in pump of the system, and the maximum loading capacity turned out to be 25 ml (10% of V_c), that is 625 mg of crude AF extract. Therefore on a 250 ml column in one run altogether 463 mg of pure AFs could be gained.

In the next step, the separation was also scaled up to a 1000 ml column. Taking the linear scale-up model into consideration, the parameters of the separation were quadruplicated, while, the centrifugal force in the system stagnated. With this method, 100 ml of the saturated solvent

system could be injected into the system. That is 2.5 g crude AF mixture that results in 1.74 g of pure AFs, with the purity above 98% and the recovery around 70% in one chromatographic run.

Screening for an ErgM producer *Claviceps* strain

16 *C. purpurea* isolates were cultivated in defined *Claviceps* broth to test their ErgM producing capabilities. After the cultivation, the produced ErgM was extracted from the culture media, and the ErgM content was quantified by a novel HPLC-FLD method. It was revealed that the most ErgM (33 mg/ml) was produced by the strain *C. purpurea*, SZMC 25562. This isolate was used in further large-scale cultivations to gain as much crude ErgM for further experiments as possible.

CPC separation of ErgM

After the selection of the producer strain for these experiments, the fungus was cultivated in a large scale. In one batch, 9 liters of culture media was prepared and inoculated. After the fermentation, a two-step extraction procedure was carried out and approximately 450 mg of crude ErgM extract was gained.

This product was used to develop a liquid-liquid chromatographic method for the purification of ErgM. From three ethers, ten alcohols, and water in two compositions, altogether 60 solvent systems were tested and **diethyl-ether/*iso*propanol/water = 40/20/40** was selected. With the chosen ternary system a 45-minute-long separation was developed and ErgM was separated from the impurities found in the crude mixture. In one run 100 mg of the mixture was injected into the system and around 70 mg ErgM was gained. The stability and repeatability of the system were also revealed to be appropriate. With this novel centrifugal partition chromatographic method from 450 mg crude extract, 312 mg pure ErgM could be gained with the exceptional purity above 98% and the recovery of around 70% with the consumption of only three liters of solvents that is 40% water.

4. Materials and methods

4.1. Fungi and cultivation

13 selected *Aspergillus* isolates were cultivated on 11 different culture media to test their AF production capability.

For the cultivation of 16 selected *Claviceps purpurea* isolates defined *Claviceps* broth was utilized.

4.2. Solvent systems

For the purification of AFs altogether 63 solvent systems were tested and **toluene/acetic acid/water = 30/24/50** was selected.

For the purification of ErgM, 60 solvent systems were tested and **diethyl-ether/*iso*propanol/water = 40/20/40** was selected.

4.3. Separation and purification

With the selected solvent systems, CPC separation s were optimized and carried out on a 250 ml column. In the case of AFs, the scale-up of the separation to a 1000 ml column was also carried out.

4.4. Product analysis

Analytical techniques used to verify product purity: HPLC-UV, HPLC-FLD, HPLC-MS/MS, and HPLC-OHRMS

5. Summary

In this work, the separation and purification of fungal secondary metabolites were described. For the separation a not-so-commonly used technique, centrifugal partition chromatography was used with great success.

Within our research, the following results were established

- 13 *Aspergillus* isolates were cultivated, and their AF production capability was successfully determined with a novel HPLC-MS/MS method.
- The *A. parasiticus*, SZMC 2473 isolate was selected due to its high AF producing capability and was successfully cultivated in large-scale conditions, and the produced AFs were extracted in a three-step extraction method.
- With the gained crude AFs, first time in the literature, a novel CPC separation was developed, and the separation of the four main AFs was achieved with the solvent system **toluene/acetic acid/water = 30/24/50**.

- First time in the literature, the linear scale-up of the separation was achieved in three steps, therefore the capacity of the CPC system was maximized. In one run, approximately 1.7 g AFs can be gained, with the purity above 98% and with around 70% recovery.
- 16 *C. purpurea* isolates were cultivated and their ErgM producing capability was determined by a novel HPLC-FLD method.
- *C. purpurea*, SZMC 25562 was selected as it was the organism that produced the most ErgM. The fungus was cultivated in large-scale conditions and the produced ErgM was extracted in a two-step extraction method.
- With the gained crude ErgM, a novel CPC method was successfully developed and the ErgM was separated from the impurities with the solvent system **diethyl-ether/*iso*propanol/water = 40/20/40**. From the injected 100 mg crude extract, approximately 70 mg pure ErgM could be gained in one run, with the purity above 98% and the recovery around 70%.

6. Összefoglalás

A dolgozatban gombák által termelt másodlagos metabolitok elválasztását és tisztítását tárgyaljuk. Az elválasztásra a centrifugális megoszlásos kromatográfiát alkalmaztuk.

Kutatásunk során az alábbi eredményeket értük el

- 13 Aspergillus izolátumot tenyésztettünk, majd egy újonnan fejlesztett HPLC-MS/MS módszerrel sikeresen meghatároztuk a gombák által termelt négy fő AF mennyiségét.
- A legtöbb AF-t az A. parasiticus, SZMC 2473 izolátum termelte, ezért ezen organizmust nagyléptékű tenyésztésbe vontuk be, és az általa termelt nagy mennyiségű AF-t egy háromlépéses extrakciós módszerrel nyertük ki.
- Az így nyert nyers kivonatot egy, az irodalomban még ismeretlen és újdonságnak számító CPC módszer fejlesztésére és beállítására használtuk fel. Az elválasztásra a **toluol/ecetsav/víz = 30/24/50** összetételű oldószer rendszert használtuk.
- Ezen elválasztás lineáris léptéknövelését is sikeresen hajtottuk végre. Az irodalomban egyedülálló léptéknövelés végrehajtásával maximalizáltuk a kromatográfiás rendszert, így egyetlen elválasztással akár 1,7 g tiszta AF-t is nyerhetünk. A végtermékek tisztasága minden esetben meghaladja a 98%-ot 70%-os visszanyerés mellett.
- 16 *C. purpurea* izolátum ErgM termelő képességét is megvizsgáltuk. A termelt ErgM mennyiségét egy új HPLC-FLD módszerrel határoztuk meg.

- A legtöbb ErgM-t a *C. purpurea*, SZMC 25562 számú izolátum termelte, melyet nagy léptékben tenyésztettünk. A termelt ErgM-t egy kétlépéses extrakció során nyertük ki a tápoldatból.
- Az így nyert kivonatot felhasználva egy, az irodalomban ismeretlen CPC elválasztást fejlesztettünk ki az ErgM tisztítására a **dietil-éter/*izo*propanol/víz = 40/20/40** összetételű oldószerrendszert felhasználva. Egy elválasztás alkalmával a rendszerbe injektált 100 mg nyers kivonatból összesen 70 mg ErgM nyerhető 98% feletti tisztasággal 70%-os visszanyerés mellett.

7. Required publications

Publications in refereed journals

Endre, G.; Hegedüs, Zs.; Turbat, A.; Škrbić, B.; Vágvölgyi, Cs.; Szekeres, A.; Separation and purification of aflatoxins by centrifugal partition chromatography, *Toxins*, *11*, 309 – 315, **2019**. IF: 3.531

Bartal, A.; Hunkár, H.; **Endre, G.**; Vörös, M.; Vágvölgyi, Cs.; Szekeres, A.; Purification of surfactin compounds produced by a *Bacillus subtilis* strain, *Acta Biol. Szeged.*, *64*, 121 – 128, **2020**. IF: 0.439

Summarized impact factor: 3.970

Conference abstracts

Endre, G.; Hercegfalvi, D.; Nagy, B.E.; Papp, D.A.; Varga, M.; Vágvölgyi, Cs.; Szekeres, A.; Purification of ergometrine from *Claviceps purpurea* isolates by centrifugal partition chromatography. In: A Magyar Mikrobiológiai Társaság 2020. évi Nagygyűlése és a XIV. Fermentációs Kollokvium: 10, **2020**.

Endre, G.; Nagy, B.E.; Hercegfalvi, D.; Papp, D.A.; Varga, M.; Vágvölgyi, Cs.; Szekeres, A.; Investigation of aftatoxin production of *Aspergillus* species, In: A Magyar Mikrobiológiai Társaság 2020. évi Nagygyűlése és a XIV. Fermentációs Kollokvium: 10 – 11, **2020**.

Endre, G.; Hegedüs, Zs.; Škrbić, B.; Vágvölgyi, Cs.; Szekeres, A. Linear scale-up of aflatoxin separation by centrifugal partition chromatography, In: Book of Abstracts. 21st Danube-Kris-Mures-Tisza (DKMT) Euroregional Conference on Environment and Health, 35 – 35, **2019**.

Endre, G.; Hegedüs, Zs.; Varga, M., Vágvölgyi, Cs.; Szekeres, A.; Separation of aflatoxins by centrifugal partition chromatography, In: 16th Wellmann International Scientific Conference: Book of Abstracts, 40 – 41, **2018**.

Endre, G.; Hegedüs, Zs.; Varga, M.; Vágvölgyi, Cs.; Szekeres, A.; Aflatoxinok elválasztása centrifugális megoszlásos kromatográfiával, In: Elválasztástudományi vándorgyűlés, 2018: végleges program, előadás és poszterkivonatok, 44, **2018**.

Endre, G.; Hegedüs, Zs.; Varga, M.; Vágvölgyi, Cs.; Szekeres, A.; Separation of aflatoxins by centrifugal partition chromatography, In: Proceedings of the 24th International Symposium on Analytical and Environmental Problems, 41 – 42, **2018**.

8. Other publications

Publications in refereed journals

Minh, L.T.; Huynh, T.; **Endre, G.**; Szekeres, A.; Fülöp, F.; Szakonyi, Zs.; Stereoselective synthesis and application of isopulegol-based bi- and trifunctional chiral compounds, RSC Adv., *10*, 38468 – 38477, **2020**. IF: 3.361

Marik, T.; Tyagi, C.; Balazs, D.; Urban, P.; Szepesi, A.; Bakacsy, L.; **Endre, G.**; Rakk, D.; Szekeres, A.; Andersson, M.A.; Salonen, H.; Druzhinina, I.S.; Vagvolgyi, Cs.; Kredics L.; Structural diversity and bioactivities of peptaibol compounds from the *Longibrachiatum* clade of the filamentous fungal genus *Trichoderma*, *Front. Microbiol.*, *10*, 1434 – 1472, **2019**. IF: 4.236

Kasi, PB.; Borics, A.; Varga, M.; **Endre, G.**; Molnar, K.; Laszlo, L.; Kotormán, M.; Grapefruit seed extract inhibits the formation of amyloid-like fibrils by trypsin in aqueous ethanol, *Nat. Prod. Commun.*, *13*, 1437 – 1440, **2018**. IF: 0.554

Paragi, G.; Kupihár, Z.; **Endre, G.**; Guerra, C.F.; Kovács, L.; The evaluation of 5-amino- and 5-hydroxyuracil derivatives as potential quadruplex-forming agents, *Org. Biomol. Chem.*, *15*, 2174 – 2184, **2017**. IF: 3.423

Summarized impact factor: 11.564

Conference abstracts

Turbat, A.; **Endre, G.**; Rakk, D.; Vágvölgyi, Cs.; Szekeres, A.; Determination of indole-3-acetic acid biosynthetic pathways in fungal endophytes, *Acta Microbiol. Imm. H.*, *68*, Supplement 1, 115, **2021**.

Papp, D.A.; **Endre, G.**; Fujkin, K.; Varga, M.; Vágvölgyi, Cs.; Szekeres, A.; Investigation of fatty acid profiles from *Aspergillus* strains, In: A Magyar Mikrobiológiai Társaság 2020. évi Nagygyűlése és a XIV. Fermentációs Kollokvium: 26 – 27 **2020**.

Hegedüs, Zs.; **Endre, G.**; Borbély, B.; Fujkin, K.; Vágvölgyi, Cs.; Szekeres, A.; Potential implementation of liquid-liquid chromatography for preparative purification of ochratoxin A, In: A Magyar Mikrobiológiai Társaság 2020. évi Nagygyűlése és a XIV. Fermentációs Kollokvium: 13, **2020**.

Hegedüs, Zs.; **Endre, G.**; Škrbić, B.; Vágvölgyi, Cs.; Szekeres, A.; Optimization of culture conditions for ochratoxin production of *Aspergillus* species, In: 17th Wellmann International Scientific Conference: Book of Abstracts: Agriculture Without Borders, 32 – 33, **2019**.

Endre, G.; Papp, D.A.; Marik, T.; Kredics, L.; Varga, M.; Vágvölgyi, Cs.; Szekeres, A.; Determination of valine and leucine isomers in peptaibols, In: 16th Wellmann International Scientific Conference: Book of Abstracts, 115 – 116, **2018**.

Volford, B.; Rakk, D.; **Endre, G.**; Škrbić, B.; Vágvölgyi, Cs.; Szekeres, A.; Purification of secondary metabolites from ferment broth of endophytic fungi of *Taxus baccata*, In: 16th Wellmann International Scientific Conference: Book of Abstracts, 98 – 99, **2018**.

Endre, G.; Takács, I.; Varga, M.; Balogh, K.; Mézes, M.; Vágvölgyi, Cs.; Szekeres, A.; Fokhagyma illóolaj vizsgálata GC-MS technikával, In: Elválasztástudományi vándorgyűlés, 2018: végleges program, előadás és poszterkivonatok, 76, **2018**.

Endre, G.; Marik, T.; Kredics, L.; Varga, M.; Vágvölgyi, Cs.; Szekeres, A.; Determination of valine and leucine isomers in peptaibols, In: Proceedings of the 24th International Symposium on Analytical and Environmental Problems, 283 – 285 **2018**.

Takács, I.; Pósa, A.; Szekeres, A.; **Endre, G.**; Gyémánt, Gy.; Új HPLC eljárás az alfa-amiláz aktivitás és gátlás mérésére, In: Proceedings of the 23rd International Symposium on Analytical and Environmental Problems :496 – 500, **2017**.

Endre, G.; Rakk, D.; Varga, M.; Vágvölgyi, Cs.; Szekeres, A.; Optimization of a solvent system for purifying aflatoxins with liquid-liquid chromatography, *Mikológiai Közlemények-Clusiana*, *56*, 91 – 93, **2017**.

Declaration

I declare that the contribution of Gábor Endre was significant in the listed publications and the doctoral process is based on the publications listed. The results reported in the Ph.D. dissertation and the publications have not been used to acquire any PhD degree previously and will not be used in the future either.

Szeged, 11. 04. 2022.

……

Szekeres András Ph.D.