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Research Note

Production and Purification of Cyclosporin A from Fermentation Broth of *Tolypocladium inflatum*

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Purification of cyclosporin A (CyA), which is a specific and potent immunosuppressive produced by some fungi as a secondary metabolite, has been accomplished by chromatography techniques in this research. After submerged incubation of the mould *Tolypocladium inflatum* in the production medium, the fermentation mixture was extracted by ethylacetate and underwent column liquid chromatography to purify CyA. In the first step, silica gel-40 and ethylacetate/isopropanol (95:5, v/v) were applied as stationary and mobile phases, respectively. The obtained fractions were assayed for the existence of lipids by the phosphovanilin method. The bioassay method measured the amount of cyclosporins in the fractions. For further purification, the fractions verified to have relatively pure CyA were pooled and submitted to the second step of the column liquid chromatography on Sephadex LH-20 resin. All the fractions obtained were tested by CyA bioassay and thin layer chromatography. HPLC and IR (infrared) spectrometry confirmed the purity and identity of the product. The amount of total cyclosporin produced was 73 mg/L, including 71% cyclosporin A, which reached 98% after purification steps.

INTRODUCTION

Cyclosporins, a family of neutral, highly lipophilic, cyclic 11-amino acid peptides, are produced by some filamentous fungi, such as *Tolypocladium inflatum*, as secondary metabolites. Twenty-five different natural forms of cyclosporin have been described as cyclosporin A to Z [1,2]. Eleven non-polar amino acids take part in the structure of cyclosporins. Beside the nonpolarity of the amino acids, there are some N-methyl substitutions in cyclosporin rings, making them very lipophilic [3]. Cyclosporin A, also specified as CyA, was originally discovered as an antifungal compound due to its inhibitory effects on the growth of some fungi such as *Aspergillus niger* and *Neurospora crassa*. Its excellent effects as a powerful and specific immunosuppressive agent were discovered later [4]. CyA is very potent in preventing graft rejection in post organ transplantation and is also used in the treatment of some autoimmune diseases [3,5]. Furthermore, cyclosporins are being applied as research tools in the field of molecular cell biology. Hence, they have participated in discoveries related to regulatory and other roles of particular proteins in prokaryotic and eukaryotic cells, such as rotamases [6,7], calcineurine [8], P-glycoproteins [9] and certain transcription factors [10-12]. Another interesting application of cyclosporins is in research related to the HIV-1 virus life cycle, due to their inhibitory effects on the virion assembly [5,13-16].

Cyclosporins are hydrophobic compounds and can be extracted from culture by non-polar solvents [17,18]. CyA is the most important member of the cyclosporin family and should be isolated from other cyclosporins that may be present in the fermentation mixture. Cyclosporins differ in their structures, at one or more of the eleven amino acids [2] showing different levels of hydrophobicity which could help to be separated by methods based on the hydrophobicity of molecules, such as partition chromatography.

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The purification of cyclosporins A, B and C from the fermentation broth of the fungus *T. inflatum*, using liquid chromatography techniques is presently reported. This may be the first detailed report of cyclosporin A isolation from the lipid content of the fermentation extract.

MATERIALS AND METHODS

All of the chemicals and materials were purchased from MERCK, unless otherwise specified.

Microorganisms

The producing and test strain fungi applied in this study were obtained from the Persian Type Culture Collection, registered as *Tolypocladium inflatum* PTCC 5253 and *Aspergillus niger* PTCC 5013, respectively.

Inoculum Development

The initial culture and growth of the two strains were carried out as follows: PTCC 5253 and PTCC 5013 were raised on agar slants in the dark at 27°C utilizing MYA (Malt Yeast extract Agar) and PDA (Potato Dextrose Agar) media, respectively. After completion of growth, the slants were maintained at 4°C.

Shake Flask Fermentation

In order to produce cyclosporins by submerged fermentation, 200 μl of seed inoculum containing 1×10^7 conidia of *T. inflatum* PTCC 5253 was added into 500-ml Erlenmeyer flasks containing 100 ml of SSM medium [= Semi-Synthetic Medium: glucose 5% (w/v), Bactopeptone 1.0% (w/v), KH_2PO_4 0.5% (w/v), KCl 0.25% (w/v)] [18] adjusted to pH 5.7 and incubated at 27°C in a gyratory shaker incubator (Clim-o-Shake, IRC-1-7) at 200 rev/min for 14 days.

Extraction

A 50-ml sample of whole culture was added to an equal volume of ethylacetate in a 500-ml polypropylene centrifuge bottle (Beckman), stoppered tightly and extracted overnight on a desktop gyratory shaker (GFL 3015) at 200 rev/min and room temperature. Subsequent to centrifugation (1500 g, 15 minutes), 40 ml of the upper organic layer was removed and evaporated at room temperature by blowing air onto it.

Cyclosporin Analysis

The dried organic layer was dissolved in 1 ml of HPLC-grade acetonitrile, filtered through a 0.45 μm Milipore microfilter and analyzed by bioassay, Thin-Layer Chromatography (TLC) and High-Performance Liquid Chromatography (HPLC). In addition, the identity and purity of CyA after column chromatography, were assessed by HPLC and IR methods.

In the bioassay, 200 μl of a homogeneously suspended 5×10^7/ml conidia solution of *A. niger* PTCC 5013 was spread on the surface of the solidified 40 ml of PDA medium, in 150-mm diameter plates. Then, 35 μl of CyA authentic standard solutions and 35 μl of dissolved samples were poured in 8-mm diameter wells, previously bored out of the plates. After 72 hours of incubation at 27°C, the inhibition zones were measured and used for cyclosporin measurement.

For the TLC analysis, 5 μl of dissolved sample, CyA authentic standard and CyA oral solution (Sandimmune™, Novartis) were applied on heat-activated silica gel-60 aluminum sheets. The sheets underwent chromatography in a solvent-saturated TLC tank containing a 95:5 (v/v) mixture of ethylacetate and isopropanol for approximately 2 hours and, finally, developed in iodine vapor.

HPLC analyses of the samples were carried out on the Knauwer HPLC system. The system consisted of a model K1009 pump, a RH77251 injector, a temperature control module and oven and a K2600 absorbance detector, set at 210 nm, attached to a model V7566 integrator. A 10 μl filtered sample was injected and separation was performed on a reverse-phase column (250×4 mm, Eurospher™ 100 C-18 5μ), prewarmed at 81°C. Samples were eluted isocratically with acetonitrile-methanol-water (40:30:30, v/v/v) as the mobile phase, setting the flow rate at 1.5 ml per minute.

Column Chromatography

Two successive steps of column chromatography were performed to achieve CyA purification. In the first, 0.5 ml of dissolved sample were laid on the top of a glass column (12 × 600 mm) filled with silica gel-40 powder. The sample was eluted isocratically with a 95:5 (v/v) mixture of ethylacetate-isopropanol as the mobile phase. One hundred and fifty fractions of 3 ml were collected by a model 2110 BioRad fraction collector and assayed for the presence of lipids using the phosphovaniline method [19], as well as the presence of cyclosporins using bioassay and TLC methods.

In the second chromatography step, the fractions evidenced to contain CyA were pooled and concentrated. A 0.5 ml of this sample underwent liquid chromatography via a glass column (18 × 250 mm) containing Sephadex LH-20 resins (Pharmacia). Throughout the elution, the polarity of the mobile phase decreased continuously by applying a linear gradient of methanol (100%) to ethylacetate (100%). All of the 40 collected fractions were evaluated by bioassay, TLC, and HPLC

methods. The fractions containing CyA were pooled and checked for purity and identity by HPLC and IR (Philips PU9600) methods.

RESULTS AND DISCUSSION

Fungal Growth

The *T. inflatum* PTCC 5253 used in this research developed spherical pellets, 1-1.5 mm in diameter, after 14 days in fermentation flasks. Under microscope, hyaline and septate mycelia, with occasional short conidiophores bearing terminal bottle-like conidium-generating cells, were seen. A moderate number of round to oval conidia, $2 - 4 \times 2$ μm were observed. This mycellial morphology is the same as reported in the literature for *T. inflatum* [17,20-22].

Cyclosporin Production

The volumetric amount of total cyclosporin produced in the submerged culture of *T. inflatum* was equal to 75 mg/l, consisting of at least three cyclosporin components. The microbial cyclosporin production so far reported in literature shows a lot of variation, due to differences in the fungal strains, media, growth and recovery conditions [23-28]. In order to verify that the fungal metabolites under investigation were cyclosporins, authentic CyA standard and CyA oral solutions were used as references. The IR absorption patterns of purified CyA and authentic standard were the same and both agreed with published data [17]. A major peak was found to move along with the CyA standard in all of the described chromatography techniques.

TLC analysis of the cyclosporin content of the extracted material showed three bands (Figure 1), the fastest one with equal R_f to authentic CyA standard and the major band of cyclosporin oral solution. The latter was the largest in quantity.

In almost all of the reports, cyclosporins A, B and C have been introduced as the major metabolites of *T. inflatum*. The only difference among cyclosporins A, B and C is in the second amino acid residue, i.e. aminobutyric acid, alanine and threonine, respectively [2]. Therefore, these three cyclosporins move very close to each other in chromatography procedures [24]. Threonine that is an alcoholic amino acid is more hydrophilic than alanine, therefore, CyC is more polar than CyB and shows a lower R_f value in normal phase chromatography. Based on these data, it could be assumed that the two other bands in lane 1 were related to CyB (the middle band) and CyC. However, it was preferred to call them bands b and c, considering the fact that we did not have access to CyB and CyC authentic standards (Table 1).

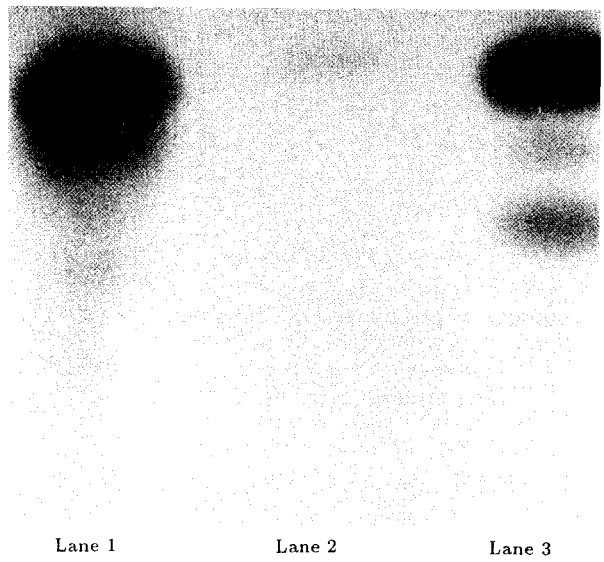

Figure 1. TLC separation of hydrophobic metabolites of *T. inflatum* PTCC 5253. (Lane 1: extracted sample, Lane 2: CyA authentic standard, Lane 3: CyA oral solution.)

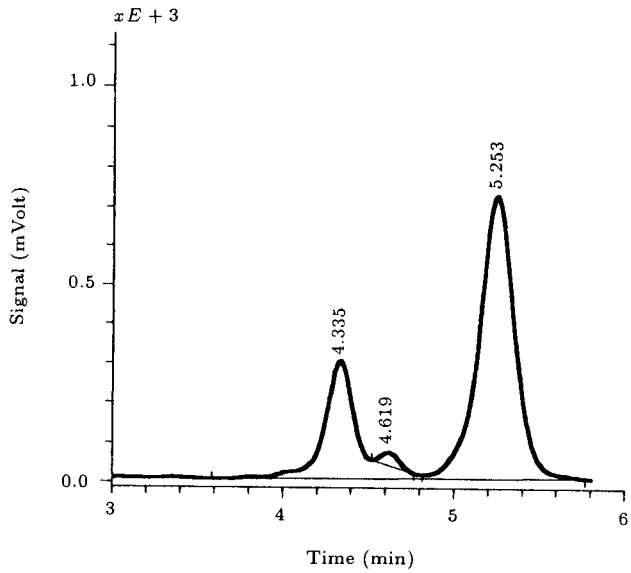

Figure 2. Chromatogram from HPLC analysis of *T. inflatum* PTCC 5253 metabolites (the numbers above the peaks show retention times).

Figure 2 shows the HPLC chromatogram of the extracted sample, which is qualitatively consistent with TLC results.

The amount of CyA was about 71% of the total cyclosporins. The other cyclosporins, probably CyC and CyB, which were called peaks c and b, respectively, were of less contribution to the cyclosporin content of the fermentation mixture (Table 2).

Cyclosporin Purification

The results of silica gel-40 column chromatography of the extracted sample are illustrated in Figure 3. Most

Table 1. R_f values of three bands detected by TLC.

Cyclosporin	R_f Value
A	0.91
Band b	0.86
Band c	0.81

Table 2. Cyclosporin production by *T. inflatum* PTCC 5253 measured by HPLC.

Component	Retention Time (min)	% of Total
CyA	5.253	70.65
Peak b	4.619	1.63
Peak c	4.335	27.72

of the lipids eluted from the column in the first 40 fractions. CyA appeared in fractions 105 to 110 with a sharp peak, followed by other cyclosporins. There were no lipids in fractions containing cyclosporins. All three isolated cyclosporins inhibited the growth of *A. niger*, on bioassay agar plates. CyA purification has received very little attention in the research literature [17,29-32] and there is almost a lack of detailed report on separation of cyclosporin, isolation of lipids from medium and/or producing fungi. Silica gel can adsorb polar groups of lipids to its silanol moieties. Utilizing silica gel-40 in this study, instead of silica gel-60 which has been employed by some researchers [17,29], leads to superior separation of cyclosporins, presumably due to its smaller mean pore size.

The Sephadex LH-20 resin used in the second step of chromatography shows hydrophobic properties. Thus, in the reverse phase chromatography system applied here, CyA eluted slower than more hydrophilic cyclosporins. The linear decrease in polarity of the mobile phase helped in better separation of CyA from other impurities. HPLC analysis of the pooled fractions with CyA is depicted in Figure 4.

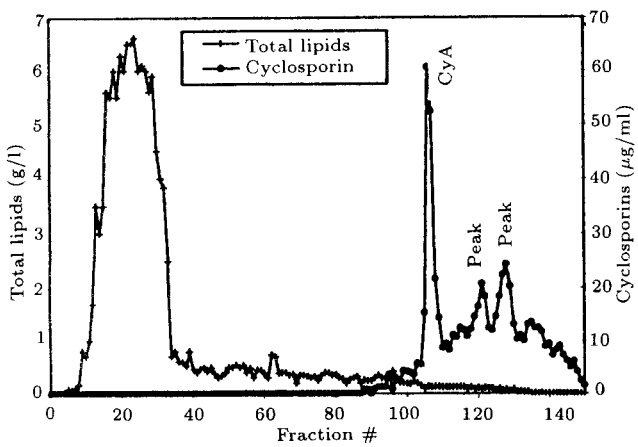

Figure 3. Chromatogram of extracted fermentation mixture from silica gel-40 column chromatography.

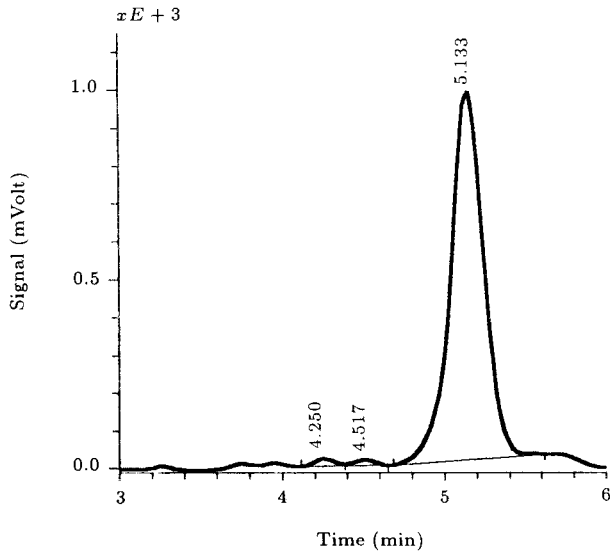

Figure 4. HPLC analysis of purified CyA after Sephadex-LH 20 chromatography (the numbers above the peaks show retention times).

Table 3. Percents of Cyclosporin components after purification measured by HPLC.

Component	Retention Time (min)	% of Total
CyA	5.133	97.99
Peak b	4.517	0.94
Peak c	4.250	1.07

It is evident that there are very small peaks related to other cyclosporins and their amounts have been reduced considerably. The relative concentrations of the three cyclosporins after purification can be observed in Table 3.

In the present study, an attempt was made to carry out CyA separation from impurities such as medium and fungal lipids and other cyclosporins. The final purified sample showed no lipids, but showed negligible amounts of other cyclosporins that could be isolated by further chromatography steps. The purity of CyA increased from 70.65% in the extracted sample to 97.99%, which is higher than the purity of some commercial products supplied for research purposes.

CONCLUSIONS

The experiments in this paper have been directed towards achieving CyA purification from the fermentation broth of the mould *Tolypocladium inflatum*. Considering the very special molecular structure of cyclosporins, many of the protein and peptide purification methods are not applicable for them. Nonetheless, their differences in polarity can be the bases for separation as recruited in this work. Meanwhile, it is possible to exclude lipids from extracted cyclosporins

by ordinary techniques of lipid omission, such as column chromatography on silica gels.

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