STRATEGIES FOR ENHANCED PRODUCTION AND RECOVERY OF CYCLOSPORINE.

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ABSTRACT

An investigation was made on the improved production and recovery of the new and potent immunosuppressive peptide, cyclosporine (cyclosporin A, Cy A) from a glucose-adapted isolate of the fungus T. [illegible]adium inflatum. Among the amino acid members in the Cy A molecule, L-leucine and especially L-valine had a strong positive effect on production of Cyclosporine. When other constituent amino acids were added with L-valine, negative effects were observed. Optimal time of L-valine was 48 hrs. In the case of semisynthetic medium and its optimal amount was 4 grams/liter (g/l). Considerable improvements in the recovery of this drug were achieved by modifying the available extraction procedure with the addition of surfactant, or with alkali addition followed by moderate heat treatment.

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INTRODUCTION

Since the discovery of the immunosuppressive properties of Cyclosporin A by Borel in 1972, extensive clinical studies have been carried out due to the impressive actual and potential benefits of its use in transplant surgery, autoimmune disease and parasitic disease control. However, very little research has been published on the production of this novel drug through fungal fermentation. Some morphological characteristics of the producer fungus Tolypocladium inflatum have been described by Gams (1971).

Cyclosporin A, a cyclic peptide, is composed of eleven amino acids, some of which are unusual and methylated. Although there are several other natural cyclosporin analogs of Cyclosporin A, major products in fermentation are Cyclosporin A and C (Kobel & Traber, 1982). Agathos et al. (1986) and Agathos and Lee (1987) have addressed microbiological and process characteristics of the Cyclosporine A fermentation with a view towards a better understanding of the factors that may contribute to optimal drug production.

In this present work, it was observed that this strain can produce Cyclosporin A in a significant amount when some amino acids were used as precursors. But some amino acids inhibit the production of Cyclosporin A when used as precursors. Because Cyclosporin A is an intracellular compound, recovery process of this antibiotic is of current interest in order to increase the production of Cyclosporin A from the T. inflatum cells after fermentation in semisynthetic medium.

MATERIALS AND METHODS

Organism

The microorganism used was *Tolypocladium inflatum* (American Type Culture Collection (ATCC) 34921). The initial culture was adapted to glucose through subculturing in semisynthetic medium, SSM (Semisynthetic medium-glucose: 50 g/l, peptone: 10 g/l, KH_2PO_4: 5 g/l and KCl : 2.5 g/l. pH 5.7).

Chemicals:

All the materials used in this work except the solvents were of analytical grade, while the solvents used including water were of High Pressure Liquid Chromatography (HPLC) grade.

Determination of dry cell growth:

Cell growth was determined by taking dry weight of cells. The cells were harvested and washed twice thoroughly with distilled water and then transferred to a constant weight aluminium cup, dried at 60-70°C for 24 hrs.

Cyclosporin A analysis:

HPLC pump (Spectroflow 400) and UV detector (Spectroflow 783) were supplied from Kratos. Integrator from Shimadzu Company, Japan (C-R3A) was used for data analysis.

The 4.6 mm x 150 mm reversed phase column packed with 5 nm Spherisorb C-8 (Phase separation) was kept at 81°C by water jacket. The sample was injected via Model 7010 injection valve (Rheodyne, Cotati, CA). Mobile phase was prepared by mixing 670 ml acetonitrile and 330 ml water which contained 1 ml trifluroacetic acid (Carruthers *et al.*, 1983).

Recovery of Cyclosporin A:

At different time interval, a 10 ml portion of the culture broth was kept in a 30 ml plugged glass vial and was frozen -20°C until analyzed for Cyclosporin A. To this 10 ml culture broth which was kept frozen 10 ml of n-butyl acetate was added for extraction of Cyclosporin A. The mixed sample was incubated at 27°C in a gyratory shaker (250 rpm) for 24 hrs. After contrifugation, 5 ml of the supernatant was removed and dried at room temperature by blowing air through a multi nozzle manifold. After drying the sample, an equal amount of acetonitrile was added to redissolve the extrated Cyclosporin A, which was then filtered through a microfilter and injected to the HPLC (Dreyfuss *et al.*, 1976).

Fermentation:

Seed cultures were developed with SSM medium. The pH of the semisynthetic medium was initially adjusted to 5.7 before sterilization. Seed cultures were grown in a 250 ml flask at 200 rpm for 10 days at 27°C. The fermentation media used here are also semisynthetic media. The incubation period was for 14 days at 27°C at 200 rpm. A New Brunswick Scientific gyratory shaker (Model G76) was used for seed culture and shake flask fermentations.

In order to see the effect of amino acids on production of Cyclosporin A, 4.0 g of different amino acids per litre were added to the semisynthetic medium and fermentations were continued for 14 days. From Table 1 it can be seen that L-valine had a strong positive effect on the production of Cyclosporin A. To see the effect of time of addition of L-valine, L-valine was added at 48 hrs. time interval of fermentation up to 10 days of fermentation and in each case the fermentation period was 14 days. For determination of optimal concentration of L-valine, different concentration of L-valine such as 2 g/l, 4 g/l, 6 g/l, 8 g/l, 10 g/l, 12 g/l were added at 48 hrs of fermentation. To see whether any other amino acids can increase the production of Cyclosporin A when used with L-valine, 1 g/l of other amino acids

Effect of NaOH, Tween-80 and NaOH, Lauryl sulphate and NaOH on the extraction of Cyclosporin A from intact and homogenized cells:

Dreyfuss *et. al.* (1976) extracted Cyclosporin A from the culture broth using organic solvents like n-butyl acetate and ethyl acetate. To enhance the recovery of Cyclosporin A, treatment of the whole culture broth with a strong base like NaOH was performed here. To a 10 ml portion of the culture broth which was kept frozen at -20°C concentrated solution of NaOH was added to reach a concentration of 1N and heated at 60°C for 30 minutes. Then 10 ml of n-butylacetate was added to the NaOH treated sample and then the procedure described above was followed to analyze the Cyclosporin A.

(a) **Effect of homogenization:**

For the effect of homogenization on the recovery of Cyclosporin A, the sample was homogenized using a tissue grinder (potter-Elvehjem Company, USA) and then NaOH was added followed by heat treatment as described above.

(b) **Effect of surfactants:**

The effect of surfactants like Tween-80 or lauryl sulphate, different concentrations of Tween 80(0.2-1.0 per cent) was added to the frozen broth (the Fermentation medium was SSM with L-valine added at 48 hrs. of incubation) before treatment with n-butyl acetate. In another experiment, different concentrations of lauryl sulphate (0.05-0.6 per cent) was added to the frozen broth before treatment with n-butyl acetate. It has been shown that tween-80 at 0.5 per cent and lauryl sulphate at 0.11 aper cent could enhance the recovery of Cyclosporin A. The combined effect of tween-80 and NaOH and the combined effect of laurylsulphate and NaOH were also determined. Tween-80 was added to the frozen broth and NaOH was added as described before addition of n-butylacetate. This was repeated with homogenized broth. Lautyl sulphate was added to the frozen broth and homogenized broth prior to addition of NaOH and n-butylacetate. All the samples were analyzed for Cyclosporin A according to the procedure described above.

RESULTS

Fermentation

From the results in Table 1 it has been shown that L-valine showed the highest positive effect on the production, while the rest of the amino acids except L-leucine had a negligible effect. For all the tested amino acids, there was no significant effect on dry cell wt. of T. inflatum. The optimal time of addition of L-valine was second day of the fermentation (Table 2). The concentration response of externally added L-valine on Cyclosporin A synthesis has shown that the optimum concentration was 3 g/l (Table 3). All other constituent amino acids diminished the positive effect of L-valine in SSM medium (Table 4).

Effect of NaOH, T-80 and SDS on recovery of Cyclosporin A:

It has been shown that the recovery of Cyclosporin A can be enhanced by treatment of the whole culture broth with a strong base like NaOH (Table 5). Cyclosporin A recovery was better with homogenized cell when the Standard procedure (cells were treated with only n-butyl acetate) but the recovery was better with the intact cells when treated with NaOH and n-butyl acetate. Also, a surfactant like tween-80 or lauryl sulphate could enhance the extraction yield (Table 5). However, the best results were obtained with NaOH treatment. It produced a 70 per cent improvement over the standard procedure of extraction with butylacetate alone (Table 5).

Table 1: Effect of amino acids on Cyclosporin A production by Tolypocladium inflatum in semisynthe medium.

Amino acids	Dry cell weight (g/l)	Cyclosporin A (mg/l)
L-leucine	10.2	78.0
D, L-alanine	7.8	61.0
D-valine	10.5	76.5
L-valine	8.2	118.0
D,L-valine	11.8	97.2
Control	8.0	50.0

Table 2: Effect of adding time of L-valine on Cyclosporin A production by T. inflatum

Time of addition (days)	Dry cell weight (g/l)	Cyclosporin A (mg/l)
0	9.0	84.0
2	9.6	105.0
4	8.8	82.0
6	7.7	62.0
8	8.0	60.0
10	8.2	63.2
Control	8.2	41.0

Table 3: Determination of optimum concentration of L-valine for production of Cyclosporin A by T. inflatum in SSM.

Concentration of L-valine (g/l)	Cyclosporin A (mg/l)
2.0	71.0
4.0	110.0
6.0	116.0
8.0	98.0
10.0	100.0
12.0	96.0
Control	55.0

Table 4: Effect of L-Leucine and D,L-alanine on Cyclosporin A production in semisynthetic medium.

Amino acids	Dry cell weight (g/l)	Cyclosporin A (mg/l)
L-leucine + L-valine	8.4	82.0
D, L-alanine + L-valine	8.0	64.0
L-valine	7.8	112.0
Control	7.6	61.0

Table 5: Effect of homogenization on recovery of Cyclosporin A

Surfactants	Cyclosporin A (mg/l)			
	Intact sample		Homogenized sample	
	W/NaOH	W/O NaOH	W/NaOH	W/o NaOH
Control	165	105 164	131	
T-80	174	130 175	150	
SDS	170	125 168	143	

Notes:

1. Potter-Elvehjem tissue grinder was used for homogenizing.
2. NaOH conc. = 1N
3. Tween-80 = 0.5 per cent
4. SDS = 0.1 per cent
5. Heat treatment at 60°C for 30 mins. after adding NaOH (1N).
6. Time of extraction = 24 hrs.
7. Control - sample with equal volume of butyl acetate (without any surfactant).

Table 6: Effect of Surfactants (tween-80, SDS and NaOH/ heating treatment) on the recovery of Cyclosporin A from whole cultured broth.

Treatment Procedure	Cyclosporin A (mg/l)
Control	90.0
Control/NaOH/heat	170.0
T-80	151.1
T-80/NaOH/heat	155.0
SDS	146.3
SDS/NaOH/heat	152.5

Notes:

1. NaOH conc. = 1N
2. tween-80 = 0.5 per cent
3. SDS = 0.1 per cent
4. Heat treatment at 60°C for 30 mins. after adding NaOH (1N).
5. Time of extraction = 24 hrs.
6. Control = sample with equal volume of butyl acetate (without any surfactant).

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DISCUSSION

Several constituent amino acids of the cyclosporin A molecule were tested to increase drug productivity. L-valine showed significant enhancement of Cyclosporin A synthesis. According to Kobel and Traber (1982), the constituent amino acids except L-alanine can enhance the synthesis of Cyclosporin A molecule. This present finding may suggest that L-valine may have a regulatory effect onsynthesis of this drug, preferably as a precursor. Demain and Matteo (1976) found that in the case of Gramacidin S fermentation L-phenylalanine has a stimulatory effect on the production, which may be due to limitation of L-phenylalanine in the intracellular amino acid pool. A similar stimulatory effect was found in the case of the bacitracin fermentation by L-leucine (Haavik and Froyshowe, 1982). The stimulatory effect of L-valine at the second day of fermentation mg mean that L-valine is being used preferentially for cell growth when it is added at the beginning of the fermentation.

Cyclosporin A is a hydrophobic substance and the major portion of the drug produced remain cell-bound. Therefore, this drug is recovered from the total broth with organic solvents. The increment in the recovery of this drug by treatment with NaOH may be due to the tenderization of the fungal cell wall.

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