

## Formation of Cyclo-(D-ala-Me-leu) Using Partially Purified Cyclosporine Synthetase from *Aspergillus Terreus*

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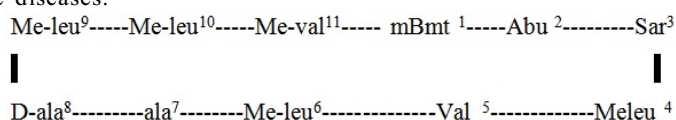
**Abstract:** An enzyme fractions which involved in the biosynthesis of cyclosporine A was partially purified. The enzyme was capable of forming covalent enzyme substrate complexes and catalyzed the ATP- pyrophosphate exchange reactions dependent on the un-methylated constituent amino acids of cyclosporine A. The cyclic dipeptide (D-ala-Me-leu) represents a partial sequence of the cyclosporine A molecule and was important in the immuno-suppressive activity. The formation of this diketopiprazine from D-alanine and L-leucine under the consumption of ATP and S-adenosyl L-methionine was investigated. The maximum out put of the cyclo ( D-ala- Me-leu) 800ug/ml was obtained by using ammonium sulphate precipitation at 60% in Tris- buffer pH 7.5, incubation time 90 min and enzyme / substrate ratio (1:2).The gel electrophoresis analysis showed that the enzyme has molecular weight about 1,689,243 dalton compared to control.

**Key words:** cyclosporine A (CyA), *Aspergillus terreus*, cyclosporine synthetase

### INTRODUCTION

Cyclosporins are produced as fungal metabolite by *Tolypocladium inflatum*. Among the know cyclosporins (Cy A) is consider to be the highly active immunosuppressive agent. Cy A exhibited strong Tcell specific immuno-suprsion specially, It is a cyclic un-decapeptide consist of eleven amino aids some of are unusual amino acid Deryfuss, *et al.*, (1976) and Borel, *et al.*, (1986).

CyA commercially know as (Sandmmiun) which play an important role in organ transplantation and the treatment of auto immune diseases.



**Fig. 1:** Structure of cyclosporine A

Bmt=(4R)-4-[E-2butenyl]-4-methyl-Lthereionine, Abu=aminobutyricacid, ala=alanine, val=valine, Meleu=N-methylleucine, Meval=N-methyl valine, Sar= Sarcosine

It has been shown to be synthesized by a large multi-enzymes system from the primary precursors, L-amino acid and D-hydroxy acid. Unlike gene-encoded peptides, the peptides synthesized in non-ribosomal system usually form families, (Kallen *et al.*, 1997).

The enzymes involved in peptides biosynthesis could be grouped into single and multi step systems. The later systems are still incompletely understood, because their restricted availability, and the kinetic analysis of enzymes with six to 29 substrate binding sites as well as the possibility of several products were difficult to follow and understand (Kleinkauf & Von Döhren, 1990).

The biosynthesis of cyclosporine A is likely to proceed by a non-ribosomal process and characterized by several unusual amino acids in this compound. Different studies so far have been made by feeding experiments with C<sup>13</sup> and C<sup>14</sup> labeled precursors. The in vitro synthesis of complete cyclosporine A molecules could not be achieved ,since all the amino acid constituent must be supplied in the un methylated form. The N-methyl group presented in a given amino acid was suggested to originate from L- methionine (Zocher *et al.*, 1986).

The present study aim to synthesis a partial sequence of the Cy A molecule(D-ala-Me-leu), using Cy A synthetase isolated from new producing strain *Aspergillus terrues* which had been recorded by Sallam *et al.*, (2003)

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## MATERIALS AND METHODS

### **Microorganism:**

The used strain *Aspergillus terreus* was obtained from the Natural and Microbial products Chemistry Research Department, National Research Centre, and maintained on standard Dox'S agar medium.

### **Chemicals:**

The authentic cyclosporine synthetase and *S*-adenosyl methionine were provided from Sigma Company .All the other chemicals used in the current study are HPLC grades.

### **Inoculum Preparation:**

According to the method described by Agathos *et al.*, (1986) and Sallam *et al.*, (2003),where pure cultures from Dox' S agar slants was introduced into Erlenmeyer flask 250 ml containing 50ml of the following composition malt extract 2 %; yeast extract 0.4% (MY) medium, pH 5.3 .incubated at 200 rpm, 27 C<sup>0</sup> for 72 hr,

### **Cultivation Process:**

5ml from the above prepared inoculum was used to inoculated, Erlenmeyer flask (250ml) containing sterile 50 ml of the following composition g/L (glucose 50, peptone 10, KH<sub>2</sub>PO<sub>4</sub> 5, KCL 2.5) at pH 5.3. The fermentation continued at 200 rpm, 27 C<sup>0</sup> for 10 days (Agathos *et al.*, 1986, Zocher, *et al.*, 1986).

Enzyme extraction and purification 25gm of freeze dried mycelium was homogenized with in a mortar and extracted with Tris- buffer pH 7, stirring gently for 20 min, the homogenate was centrifuged at 4000 for 20 min using cooling centrifuge (4C<sup>0</sup> ), then saturated ammonium sulfate solution was gradually used (30-35%). 10 ml of the suspended (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> material were loaded on to a fractogel HW.55(F) column 4x63 cm and eluted with buffer consists of (0.1 M Tris-HCL, pH 7.8, 4 mM EDTA, and 15% (w/v) glycerol,.The crude extract precipitated using polyethylene all these were achieved according to the method described by Lawen and Zocher, (1990), active fraction were pooled and undergo analysis.

### **Amino Acids Activation:**

The amino acids constituents of cyclosporine A molecule must to be activated firstly. Cyclosporine synthetase activates the amino acids to amino acyl adenylates and binds them covalently via thioester linkages at prosthetic phosphopantetheine groups. Seven of the substrate amino acids become *N*-methylated by *S*-adenosylmethionine via respective methyl transferase activities of cyclosporine synthetase. This activation process is required because these amino acids shown to be covalently bonded as ester to the enzyme. The activation process involved using labeled C<sup>14</sup> *S*-adenosyl methionine Therefore, it was possible to labeled cyclosporine synthetase Zocher ,*et al.*, (1986), Billich and Zocher, (1987)

### **Assay of Yclo (D-ala-Meieu):**

The assay mixture contained active enzyme fraction 10 ul ATP (7.3 mM), MgCl<sub>2</sub> (7.3 mM, D-alaine 7.3 mM Me-leu 1-C<sup>14</sup> in total volume 250 ml after 30 min incubation at 25°C, 2ml of H<sub>2</sub>O and ethyl acetate was added to the assay mixture. However, the rate of cyclosporine A formation decreases to about 50% compared to the reaction with Mg<sup>+2</sup>.

The amount of diketopiprazine(cyclo D-ala-Meieu) formed was calculated by measuring the peak areas of scanning curves obtained with Berthold thin layer scanner system BF210. A calibration curve was made with C<sup>14</sup> L-leu as internal standard (Zocher *et al.*,1986, Lawen and Zocher, (1990).

## RESULTS AND DISCUSSION

### **Separation Of Cyclosporine A Synthetase:**

The separation of CyA synthetase achieved according to the methods described by (Lawen and Zocher, (1990). The separation of cyclosporine synthetase was achieved by different techniques (ammonium sulphate precipitation, polyethylene precipitation and fractogel column).

**Table 1:** Different techniques for the purification of cyclosporine synthetase

Technique	Volume (ml)	Total protein (mg)	Total activity(u)	Sp. Activity u/mg protien
1- Crude extract	500	2.226	470	211
2-NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> precipitation	90	299	988	16.746
3- Fractogel HW55 column	103	59	505	1.689
4- polyethylene precipitation	87	45	420	0.91

The results presented in table (1) showed that the total activity affected by the applied method for precipitation, the best activity 988 u was obtained by using the ammonium sulphate precipitation., compared to polyethylene precipitation and fractogel column analysis. These results correlated with that stated by Lawen and Zoicher, (1990) and Zoicher, *et al.*, (1986) on using Cy A synthetase from *T. inflaum*.

#### Effect Of Different Ammonium Sulphate Concentration:

This study interested in using different  $(\text{NH}_4)_2\text{SO}_4$  concentrations for the precipitation of Cy A synthetase, where different conc. (0-90%) was investigated. The results in table (2) suggested that the best purification was obtained at 60% since, the maximum specific activity(60.9 u/ml) was achieved. At the higher and lower conc. reduced activities were noticed (Zoicher, *et al.*, 1986). The formation of the diketopiperazine cydo-(D-ala-Me-leu) strongly resembles that of cyclo (D-phe-pro) in the gramicidin S system (Srinivasa, *et al.*, 2007), when only D-phe-andL-pro are present as the substrate amino acids of gramicidin S synthetase (Otani, *et al.*, 1988).

**Table 2:** Investigation of different ammonium sulphate concentrations

Fraction %	Total protein (mg)	Total activity (U)	Specific enzyme activity(u/mg)	Production %
Filtrate	2600	7821.8	30.08	100
0-60	556.5	3723	60.9	47.6
60- 90	137	425.9	60.27	10.5

#### pH relations:

The present investigation involved estimation of the effect of different pH value of the used buffer. The pH was ranged from (6.5, 7.5 and 8.5), the assay procedure was achieved at different incubation time.

The results presented in Table ( 3) showed that the maximum out put (750 u/ml) of Cylco(D-ala- Me-leu) was achieved at pH 7.5 and optimum time. The chain-like complexes could be observed dependent of the age and specific activity of the preparations and the pH value of the surrounding medium. This accounts for a certain structural instability of the whole complex as a results of pH changes, (Billich and Zoicher, 1990, Michael, *et al.*, 2001)

**Table 3:** Effect of different pH value of the used buffer and the incubation, time on the formation of cyclo (D ala-Me leu).

Buffer pH	Time (min)	Yield ug/ml	En/sub
6.5	1020304050	5075140210230	0.0423611111
7.5	1020304050	315427750730680	0.0423611111
8.5	1020304050	70120175240250	0.0423611111

**Table 4:** Effect of different enzyme substrate ratio on the formation of cyclo (D ala-Me leu).

Enzyme/substrate	Time (min)	Yield (u/ml)	Ph
1:1	10	315	7.5
	20	427	
	30	750	
	40	730	
	50	680	
1:2	10	350	7.5
	20	475	
	30	800	
	40	780	
	50	790	
1:3	10	530	7.5
	20	575	
	30	760	
	40	780	
	50	740	

#### Enzyme Substrate Ratio:

The present study extended to investigate the effect of enzyme substrate ratio on the rate of diketpeprazine formation. Where, the formation rate of cyclo(D-ala-Me-leu) under different enzyme substrate ratio(1:1, 1:2, 1:3) at the selected optimum pH value 7.5 .The results given in table (4) showed that the best yield (800ug/ml) was obtained at ratio (1:2) and time 30 min.

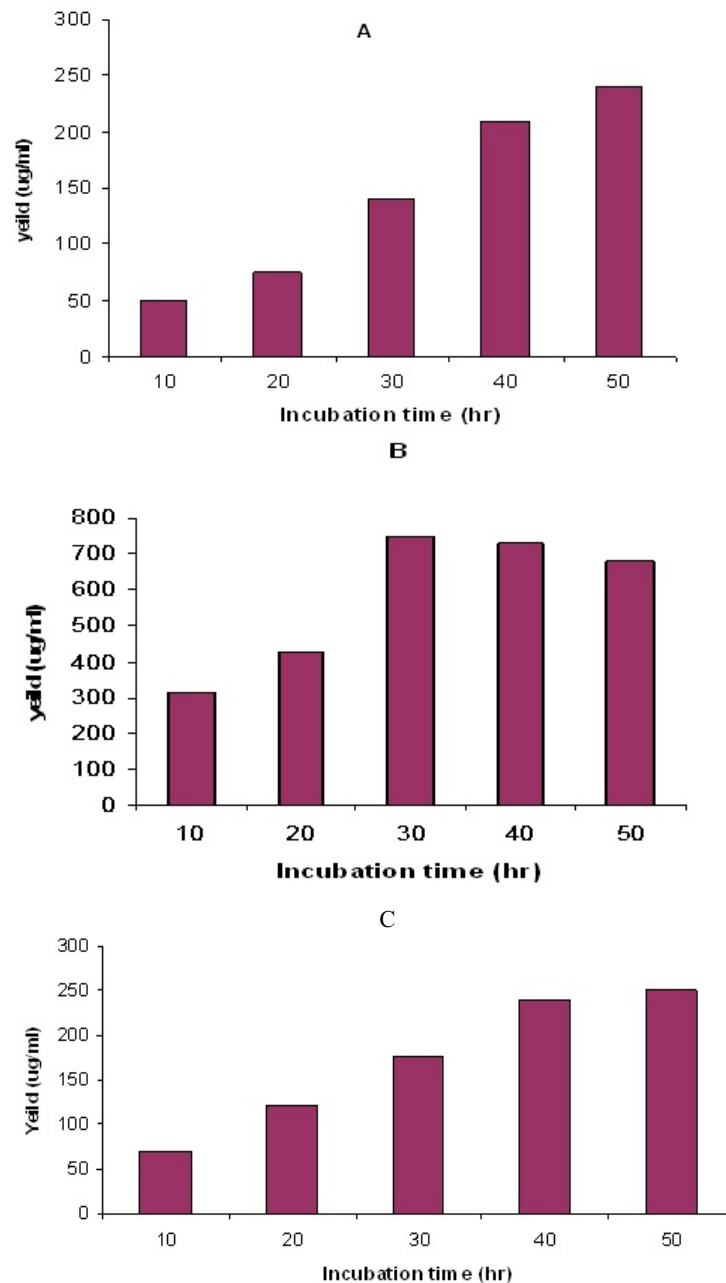
On the other hand, at the ratio (1:1and 1:3) a considerable out puts (750 and 780 ug/ml) was obtained respectively, (Kleinkauf and VonDohren 1994, 1996)

#### Gel Electrophoresis:

The active fractions containing active cyclosporine A synthetase were subjected to separation using gel electrophoresis technique in comparison with standard for molecular weight determination. The procedure of electrophoresis was completed according to the method described by (Srinivasa, *et al.*, 2007).

The results presented in fig.(2) showed that the fraction no 2 which obtained by using ammonium sulphate precipitation produced highly purified fraction, since it appears nearly to that of standard. Fractions 3 and 4 for polyethylene and fractigel column respectively, showed overlapped and condensed bands. Cyclosporine synthetase is one of the largest enzymes in nature. Its structure is of special interest, because the enzyme molecule is composed of one polypeptide chain organized in (at least functionally) separate modules instead of separate polypeptides (i.e. subunits) Weber *et al.*, (1994).

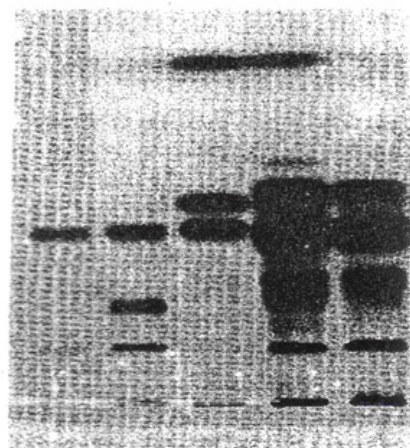
The corresponding coding region of cyclosporin synthetase contains an 45.8 kb that encodes a peptide with a calculated molecular mass of 1,689,243 Da. The predicted gene product contains 11 amino-acid-activating modules that are very similar to one another and to the domains of other peptide synthetases (Weber *et al.*, 1994) each module is responsible for the recognition, activation and modification of a substrate amino acid. Seven of these modules harbor *N*-methyltransferase functions localized on methyltransferase domains (Husi *et al.*, 1997). Some of these features are reflected in the overall structure of the cyclosporine synthetase molecules.



**Fig. 2:** Effect of different pH values on the production process, A at pH 6.5, B at pH 7.5 and C at pH 8.5.

**Conclusion:**

The trial for the biosynthesis of cyclosporine A started by separation of the cyclosporine A synthetase and purification. Each module of cyclosporin synthetase essentially consists of a central adenylation domain (A-domain; recognition, activation), thiolation domain (T-domain; covalent binding of adenylated amino acid on phosphopantetheine) and condensation domain (C-domain; elongation step). Seven modules harbor an additional methyltransferase domain (M-domain; N-methylation). Presently it is not clear whether the domains form a structurally compact module or if they act as somehow independent beads on a chain. Nevertheless, based on the fact that the amino acid is bound on the phosphopantetheine, all the domains of one module have to be in close vicinity to each other.



Control 1 2 3 4

**Fig. 3:** Purification of cyclosporine A synthetase using gel electrophoresis technique.

The synthesis of the whole CyA molecule has not been completed until now. Synthesis of cyclosporine in vitro revealed that the cyclosporine synthetase needs an appropriate amino acids in unmethylated form (including D-alanine or a homologue D amino acid (Lowen, *et al.*, 1989) the nonchiral amino acid glycine can also substitute D-alanine, ATP, Mg<sup>2+</sup>).

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