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Calcium inhibition of efrotomycin production by *Nocardia lactamdurans*

William H. Cover, A.C. Kirpekar, Hugh George and R.W. Stieber

MCMD, Merck and Co., Inc., Elkton, VA, U.S.A.

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SUMMARY

Efrotomycin is a modified polyketide antibiotic of the elfamycin family that has use in the area of pig husbandry. Optimization of the fermentation medium for production of efrotomycin by *Nocardia lactamdurans* revealed that the fermentation is sensitive to hard water and certain lots of cottonseed flour used to prepare a complex fermentation medium. A limited metal ion analysis of the hard water indicated that calcium ions are present at concentrations found to be inhibitory by the addition of calcium chloride to medium prepared with distilled water. Similarly, a correlation between lots of cottonseed flour that poorly supported the fermentation and high calcium levels is presented. Further experimentation revealed that by altering the sterilization conditions of the cottonseed flour, the inhibitory effect of poor lots could be prevented.

INTRODUCTION

Improvements in fermentation processes are traditionally achieved by the selection of strains that produce increased titers of product and by optimizing the composition of the fermentation medium for product formation and stability [5]. Economic constraints for industrial scale fermentations normally require that the process be carried out in a complex medium consisting of raw materials available in bulk quantities, usually of animal or vegetative origin [6]. Thus, the concentration of many substances in a typical complex medium fluctuate dependent upon the amount present in each lot a raw material. When the concentration of a component critical to the fermentation fluctuates in one raw material, either a stimulatory or inhibitory effect on product formation will be observed. For example, the potassium content of blackstrap molasses medium is critical for optimal fermentations of itaconic acid with *Aspergillus terreus* K26 [10,11] and for the fermentation of citric acid the levels of iron and manganese must be below certain critical levels [10]. Sterilization methods and conditions also can significantly effect fermentation titers, presumably by altering the chemical nature or bioavailability of nutrients or inhibitory substances in the medium [15]. For these reasons, procedures for selecting consistent raw materials and optimum sterilization conditions are necessary to minimize variability in a fermentation process.

In testing lots of cottonseed flour as the main nitrogen source for the fermentation of efrotomycin by *Nocardia lactamdurans*, we observed variation in titers of flask fermentations dependent upon the lot of cottonseed flour used in the medium. The fermentation was also found to be sensitive to the source of water used in preparing the medium. Based on these observations it was thought that the fermentation was sensitive to a metal ion present in variable amounts in the cottonseed flour and the water. Herein we report experiments testing this hypothesis and present data showing an inverse correlation of titers to calcium levels in the fermentation medium originating from either the cottonseed flour or the water. Data also is presented demonstrating that the fermentation is sensitive to the sterilization conditions as previously reported [16]. The sensitivity of the fermentation to calcium and possible other metal ions in relation to the sterilization conditions is discussed.

MATERIALS AND METHODS

A mutant of the original soil isolate of *Nocardia lactamdurans* was used for all experiments. Originally this culture was deposited with the Northern Regional Research Laboratories (NRRL3802) as *Streptomyces lactamdurans* [7]. The culture for each fermentation was prepared by inoculating the seed medium with frozen vegetative mycelia as described previously [16]. Seed and fermentation media

Correspondence: W.H. Cover, MCMD, Merck and Co., Inc., P.O. Box 7, Elkton, VA 22827, U.S.A.

preparations were made as described previously [7,16]. For routine flask fermentations, the medium was sterilized by autoclaving for 20 min. To alter the soluble protein of the medium, a continuous high temperature short term (HTST) sterilization of cottonseed flour slurry was carried out prior to mixing it with the remainder of the medium sterilized in an autoclave. The continuous HTST sterilization process involved preparation of cottonseed flour slurry in cold industrial grade hard water, pH adjustment with NaOH, and sending the slurry through the sterilizer at a fixed flow rate and temperature. The sterilization time is an inverse function of the flow rate and can be determined from the sterilizer volume and the medium flow rate. By changing the amount of NaOH added to the cottonseed flour slurry, the sterilization time and the temperature of sterilization, the soluble protein concentration in the sterile medium was altered.

Assays for efrotomycin were performed essentially as described previously [16]. Protein was determined by the BCA method (Pierce Chem.) using bovine serum albumin as a standard [8]. Elemental analyses were performed by Free-Col Laboratories, Meadville, PA.

RESULTS

Effect of the source of water and cottonseed flour lot on flask fermentations.

The data presented in Table 1 show that the fermentation of efrotomycin in flasks is sensitive to the type of water used to prepare the complex medium. Titers of 50.7 and 52.0 units of activity were achieved when distilled water was used compared to only 37.0 and 39.2 when industrial grade hard water was used, the water normally used for large scale fermentations. Of the different water sources tested, only steam condensate of the industrial grade hard water produced titers comparable to laboratory distilled water. Both potable and well water inhibited the fermentation. A limited elemental analysis of the industrial grade hard water of samples taken at 24-h intervals for 5 days shows that the industrial grade hard water contains considerably more calcium and magnesium ions than distilled water (Table 2).

TABLE 1

Effect of the source of water on the production of efrotomycin in flask fermentations

Water grade	Efrotomycin (Units/l)	
	Flask 1	Flask 2
Potable[a] (from tap)	19.3	9.5
Distilled	50.7	52.0
Well water[b]	39.9	41.7
Industrial grade (hard water)	37.0	39.2
Steam condensate of industrial grade hard water	49.3	46.4

[a] Rahway, N.J.
[b] Elkton, VA.

TABLE 2

Selected elemental analysis of industrial grade hard water (IG) and distilled water

Sample (day)	Element (mg/l)			
	Potassium	Magnesium	Calcium	Sodium
Distilled water	ND	0.05	ND	ND
IG (1)	1.9	10.6	22.9	3.0
IG (2)	1.7	10.2	20.5	2.8
IG (3)	1.8	10.3	21.7	3.1
IG (4)	1.9	11.5	23.0	3.3
IG (5)	1.8	10.7	22.4	3.1

ND, not detected; IG, industrial grade hard water.

A second ingredient of the complex medium expected to fluctuate in metal ion concentrations is the cottonseed flour, which serves as the main nitrogen source for the fermentation. A summary of a series of flask fermentations in medium prepared with distilled water and different lots of the cottonseed flour is presented in Table 3. Lots designated A, G and H supported the fermentation of titers to less than 50% of that achieved with the designated control lot D or B, C, E and F. Lots A, G, and H contain considerably higher amounts of calcium than the lots that supported the fermentation of higher titers of efrotomycin. No apparent correlation was observed between efrotomycin and the absolute amount of

TABLE 3

Concentration of calcium and amount of efrotomycin produced from cottonseed flour lots

Cottonseed lot	Calcium mg/kg	Efrotomycin[a] (% of control)
A	4710	48
B	2044	93
C	1780	113
D (Control)	1429	100
E	1639	107
F	1124	102
G	3280	37
H	4000	38

[a] Productivity is expressed as a percent of control lot D.

magnesium, potassium or sodium in the cottonseed flour (data not shown).

Inhibition of efrotomycin production by calcium chloride

To determine if an increase in the calcium concentration could inhibit the production of efrotomycin, calcium chloride was added at various concentrations to medium prepared with distilled water and the control lot D of the cottonseed flour. As shown in Fig. 1, concentrations of calcium chloride greater than 0.05 g/l inhibited the fermentation of efrotomycin. The addition of 0.1 g/l calcium chloride resulted in titers comparable to that observed when the medium is prepared with the industrial grade hard water (see Fig. 1 and Table 1). A concentration of 0.1 g/l calcium chloride increased the total calcium in the medium by approximately 27 mg/l which is comparable to the amount of calcium found in the industrial grade hard water (Table 2). The addition of magnesium chloride, sodium chloride and potassium chloride did not inhibit the fermentation in contrast to the inhibition observed by the addition of calcium chloride (data not shown).

Effect of sterilization conditions on flask fermentations

Since it has been previously reported that the fermentation of efrotomycin is sensitive to the sterilization conditions of the cottonseed flour [16], a low production lot of the cottonseed flour (lot A) was sterilized under varying conditions and tested for the ability to support the fermentation of efrotomycin. Increased amount of NaOH, sterilization time and temperature of sterilization, all separately or in combination, resulted in higher soluble protein. As shown in Fig. 2, sterilization conditions that resulted in higher soluble protein concentrations supported the production of higher titers of efrotomycin.

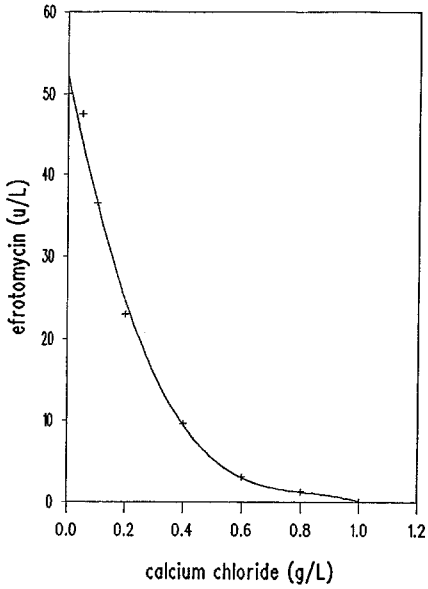

Fig. 1. Effect of calcium chloride on efrotomycin production.

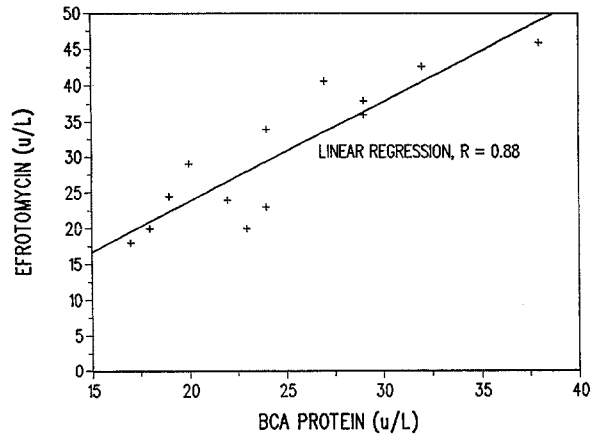

Fig. 2. Correlation between total soluble protein in the complex medium after sterilization and efrotomycin production in flasks.

DISCUSSION

The sensitivity of the efrotomycin fermentation to different lots of cottonseed flour and water obtained from different sources reflects the critical nature of secondary metabolism to minerals and trace elements. The correlation of high levels of calcium in particular lots of cottonseed flour to poor productivity indicates that lots should be tested for calcium content prior to use in industrial scale fermentations. It is also apparent that for large scale fermentations, water containing calcium should be avoided unless strains can be selected that produce efrotomycin in the presence of calcium.

The improved fermentation performance with lot A, a lot with a high calcium content, by adjusting the sterilization conditions is not fully understood. However, the sterilization conditions that resulted in increased soluble protein in the medium produced higher titers of efrotomycin. Since the soluble protein assay is based upon the ability of proteins to chelate copper [18], the increased soluble protein conceivably reduces the availability of copper and other metals to the organism during the fermentation. One would also expect a portion of these proteins to chelate calcium and thus regulate the amount of calcium available to the organism. Measuring the binding capacity of the medium to calcium and any additional inhibitory ions in media prepared under different sterilization conditions would determine if this were true. Currently it is not known if calcium is the only inhibitory substance in the cottonseed flour. Additional minerals or trace elements could possible be involved in effecting

efrotomycin production that fluctuate in concentrations in the various lots parallel to calcium.

Calcium has been identified as an inhibitory substance to the biosynthesis of cephamycin C in resting cells of *N. lactamdurans* [4]. Therefore, calcium probably does not directly interfere with the biosynthetic enzymes for either cephamycin C or efrotomycin. Inhibition more likely is caused by calcium interfering with an enzyme or cellular process more general to secondary metabolism. If a transport system exists to regulate the cytoplasmic calcium concentration similar to the ATPase transport systems described by Silver et al. [14] to regulate toxic metal concentrations, in the presence of high calcium concentrations, the cell would expend a large amount of energy removing calcium from the cytoplasm. This might deplete the ATP supply to a level that could not support antibiotic synthesis. A second possibility is that the calcium ions activate an enzyme such as a protease whose activity results in a change in the regulatory functions of the cell in favor of a different secondary metabolite. Calcium has been reported to stabilize proteinase K [1] and either stimulate or decrease protease activity in streptomyces isolated from the rhizosphere of soybean plants [9]. Calcium ions are also involved in the regulation of aerial mycelium formation in a number of actinomycetes [12].

In vitro protein synthesis experiments with cell free extracts from mutants of *E. coli* indicate that a correlation exists between a requirement for increased levels of magnesium for optimal in vitro protein synthesis and resistance to the inhibition of in vitro protein synthesis by the addition of kirromycin, an antibiotic similar to efrotomycin [13]. If calcium competes for binding sites or interferes with the availability of magnesium for protein synthesis in *N. lactamdurans*, then mutants that overproduce efrotomycin because of increased resistance to the antibiotic could similarly require more magnesium. Calcium would be expected to interfere with the fermentation of efrotomycin in certain overproducing strains.

In this paper we have shown that calcium ions inhibit the fermentation of efrotomycin. The mechanism of calcium inhibition has not yet been elucidated. Similar inhibitory effects for the production of secondary metabolites have been reported. Calcium inhibits the fermentation of beromycin [3], cephamycin C [4] and a *Bacillus subtilus* production of iturin A [2]. Whether or not a common mechanism for calcium inhibition exists among these secondary metabolites is unknown.

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