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Fermentation of MR-387A and B, Novel Aminopeptidase M Inhibitors by *Streptomyces* sp. SL-387: Phosphate Repression of Inhibitor Formation

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The effect of inorganic phosphate on the fermentative production of aminopeptidase M inhibitors MR-387A and B by *Streptomyces* sp. SL-387 has been studied. With inorganic phosphate concentrations higher than 0.78 mM, an inverse correlation was found between the maximum inhibitor production and the initial phosphate concentration added. Growth sensitivity of this actinomycete to arsenate, a phosphate analogue, and the use of magnesium carbonate, a phosphate-trapping agent, suggested that the inhibitor formation was under phosphate repression. Exogenous ATP further increased the degree of phosphate interference in both phosphate-repressed and nonrepressed culture conditions. The use of a phosphate analogue and a protein synthesis inhibitor also suggested that the phosphate itself repressed inhibitor formation.

High soluble phosphate concentrations are frequently disadvantageous for overproduction of metabolites. In a culture medium, inorganic phosphate is required for the growth of prokaryotes and eukaryotes within a range of 0.3-300 mM. However, a much lower phosphate concentration inhibits the production of many secondary metabolites. In a number of systems studied, the highest inorganic phosphate concentration which allows unimpeded production of secondary metabolites is about 1 mM; complete inhibition of production occures at about 10 mM (3).

As mentioned above, inorganic phosphate has long been known to suppress the biosynthesis of many antibiotics and other secondary metabolites (6). In these examples, orthophosphate either inhibited or repressed dephosphorylation reactions or synthetases in which orthophosphate is neither a substrate nor a product. During the biosynthesis of other antibiotics, biologically inactive phosphorylated intermediates are formed which later are enzymatically dephosphorylated to yield bioactive products (4, 7, 9). However, the phosphate regulation mechanism is not yet fully understood.

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The novel peptides MR-387A and B were isolated from the culture filtrate of *Streptomyces* sp. SL-387 in the screening of specific inhibitors against aminopeptidase M (AP-M) having an analgesic, immunopotentiating, or anti-metastatic effect (2). Our group have been involved in studies on the factors and conditions controlling the formation of these inhibitors. The biosynthesis of the inhibitors is controlled by some nutrients such as glucose, ammonia and phosphate. Particularly, production of MR-387 was repressed by phosphate concentration added to the cultures in a dose dependent manner.

This paper describes the effect of phosphate concentration on the synthesis of AP-M inhibitors by *Streptomyces* sp. SL-387. In this paper, phosphate regulation is also investigated by the use of a phosphate-trapping agent and growth sensitivity to arsenate.

MATERIALS AND METHODS

Organism and Cultivation

Streptomyces sp. SL-387 was supplied and maintained as reported previously (2). One loopful of spores was inoculated into 50 ml of a seed medium consisting of 1% glucose, 2% bacto soytone, 0.2%

yeast extract, 0.1% beef extract and 0.3% NaCl, and cultured at 28°C for 2 days on a rotary shaker (170 rpm). For inhibitor production, 0.5 ml of a seed culture was inoculated into 250 ml-Erlenmeyer flasks containing 50 ml of the following fermentation medium: 1% glucose, 2% bacto soytone, 0.2% yeast extract, 0.1% beef extract, 0.3% NaCl, 0.0005% $CuSO_4 \cdot 5H_2O$, 0.005% $MgCl_2 \cdot 4H_2O$, 0.0005% $ZnCl_2 \cdot 7H_2O$ and desired K_2HPO_4 concentrations in distilled water. After preparation, the fermentation medium was adjusted to pH 7.0 with 1 N NaOH and autoclaved at 1.3 kg/cm^2 for 15 minutes. Fermentations were carried out at 28°C for 5 days on a rotary shaker at 170 rpm.

Additions to the Fermentation

Streptomyces sp. SL-387 was streaked onto plates of Bennett's agar (1% glucose, 0.1% yeast extract, 0.2% Bacto peptone, 0.1% beef extract, 1.5% agar) containing 5 or 50 mM sodium arsenate and grown at 28°C for a week. The sensitivity to arsenate in liquid cultures was tested in the fermentation medium supplemented with 5 or 50 mM sodium arsenate.

After 24 hours fermentation, phosphate, arsenate, and chloramphenicol were added to the cultures growing in the fermentation medium described above with low phosphate concentration (approximately 0.78 mM). The cultures were returned to the shaker and at desired times 1 ml of sample was withdrawn for further analysis.

To determine the effect of phosphate trapping agents on total inhibitor formation, varous concentrations of magnesium carbonate [$(MgCO_3)_4 \cdot Mg(OH)_2 \cdot 5H_2O$] were initially added to the fermentation medium supplemented with 10 mM phosphate (phosphate-repressed culture).

In order to characterize the exogenous ATP effect on phosphate-repressed cultures, 5 and 10 mM ATP were added to phosphate-repressed cultures at the beginning of fermentation.

Assay for AP-M and Inhibitor Determination

AP-M activity was measured as reported previously (2). The percent inhibition was calculated by the formula (A-B)/A × 100, where A is the measured value by the enzymatic reaction in the system without an inhibitor and B is that with an inhibitor. At specified intervals, the production of inhibitor was monitored by AP-M inhibitory activities. One unit of inhibitor formation was defined as the amount of inhibitor required for the 50% inhibition of 1 mU of AP-M. Specific inhibitor production was expressed as units per mg dry cell weight. Total (volumetric) productivities refer to units per ml original culture medium.

Cell Growth and Phosphate Determination

Cell growth was measured by dry cell weight (DCW) determination. Samples of mycelia (2 ml) were harvested by centrifugation, and washed twice with equal volume of distilled water. After centrifugation, the pellet was dried in an oven at 105°C for 3 hours and then weighed.

The inorganic phosphate was determined from the absorbance at 595 nm by the modification of the microcolorimetric method of Taussky and Shorr (11). 0.2 ml of ferrous sulfate-molybdate reagent was added to samples (0.01 ml) in a 96 well microplate and the intensity of the color was read at 595 nm in a microplate reader (Biorad Model 3550).

The experiments reported here were repeated at least twice (two independant experiments) and the results reported are the mean value. The observed variations were consistently less than 10%.

RESULTS AND DISCUSSION

Effect of Inorganic Phosphate Concentration

Streptomyces sp. SL-387 was able to grow but not to produce AP-M inhibitors in a synthetic medium. Therefore, a complex medium containing Bacto soytone as a nitrogen source was used for fermentation studies. Fig. 1 shows maximum growth and specific inhibitor formation of this microorganism with inorganic phosphate concentrations ranging from 0.78 to 28.4 mM. As shown in the figure, with the use of inorganic phosphate concentrations added to the cultures higher than 0.78 mM an inverse correlation was found between the maximum inhibitor production and the initial phosphate concentration added. The inorganic phosphate concentration necessary for the growth of *Streptomyces* sp. SL-387 and inhibitor pro-

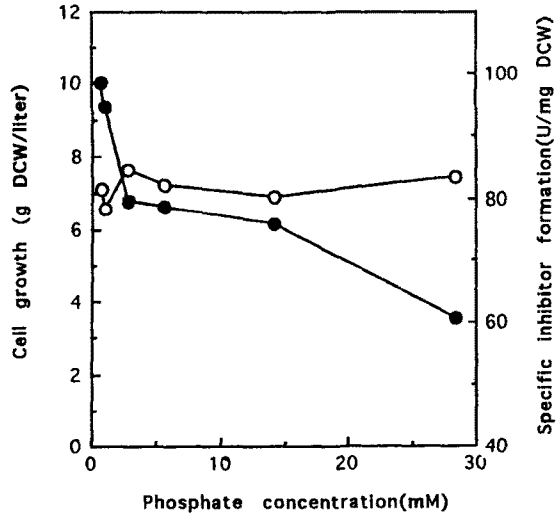

Fig. 1. Effect of phosphate concentrations on the growth and specific inhibitor formation.
○ : Cell growth, ● : Specific inhibitor formation.

duction was 0.78 mM contained in the complex nutrients such as Bacto soytone, yeast extract and beef extract. Furthermore, additions of phosphate to the cultures did not inhibit the cell growth but specifically prevented inhibitor formation.

After 96 hours of fermentation, inorganic phosphate was totally consumed when added to the medium at about 0.39 mM. Nevertheless, an inverse correlation between the phosphate consumed and inhibitor formed was observed (Fig. 2). It is therefore appropriate that inorganic phosphates usually are not further added to complex media, because they are included in complex nutrients and because high concentrations of them do not favor inhibitor biosynthesis.

Growth Sensitivity to Arsenate

Growth sensitivity of *Streptomyces* sp. SL-387 to arsenate was tested. On the solid medium Bennett's agar, the strain showed poor growth with the addition of 5 mM sodium arsenate, and did not grow under the condition of addition of 50 mM sodium arsenate. In the liquid medium, the same results were also obtained (Table 1). These results indicate that AP-M inhibitor formation by *Streptomyces* sp. SL-387 is under phosphate regulation.

All sensitive strains to phosphate analogue arsenate were also sensitive to phosphate regulation (4). Therefore, selection of *Streptomyces* mutants resistant to the toxic phosphate analogue arsenate is an efficient way of overcoming the sensitivity of many antibiotic biosynthetic pathways to repression by inorganic phosphate in the medium (8). Most of these arsenate-resistant mutants show an uptake of phosphate identical to that of the wild type. However, changes have been found in energy metabolism, phospholipid synthesis and alkaline phosphatase regulation, and a decreased repression of enzymes of antibiotic synthesis by inorganic phosphate has been observed. The mechanism of arsenical resistance in streptomycetes is still unknown. Anyway, development of arsenate-resistant mutant by mutagenesis or genetic manipulations may be an efficient way of overcoming phosphate regulation for overproduction of these inhibitors.

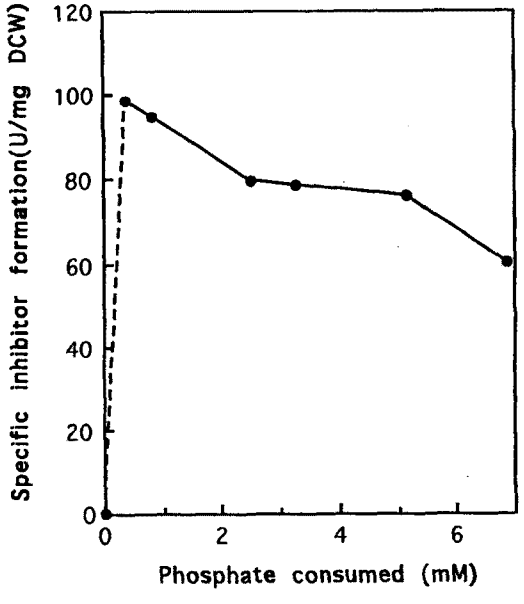

Fig. 2. Relationship between phosphate consumed and the specific inhibitor formation at 96 hours of fermentation.
Cultures were grown in the fermentation medium with phosphate ranging from 0.78 to 28.4 mM.

Effect of Exogenous ATP on Phosphate-Repressed Cultures

To investigate the effect of exogenous ATP on the fermentative production of AP-M inhibitors, ATP was added to the non-phosphate repressed (0.78 mM phosphate) and phosphate repressed (17.4 mM phosphate) cultures at the beginning of fermentation. In both culture conditions, ATP further increases the degree of phosphate interference (Table 2). ATP does not transport into the cell. If the peptide inhibitor is extracellulary synthesized by a peptide synthetase whose activity is dependent on ATP (1), specific inhibitor formation will be increased in a dose-dependent manner. The degree of phosphate repression when ATP was added to the medium was stronger in a dose-related fashion than that of phosphate repression with no addition of ATP. The inhibitor synthesis therefore may be carried out by an intracellular enzyme. Practically, the inhibitor synthesis by a cell-free system was dependent on ATP, precursor amino acids, reaction time, and active enzyme (data not shown).

Effect of Phosphate Trapping Agent on Inhibitor Formation

Table 1. Growth sensitivity of *Streptomyces* sp. SL-387 to arsenate and the specific inhibitor formation in the medium containing arsenate.

$Na_2HAsO_4 \cdot 7H_2O$ added (mM)	Growth on Benett agar	Growth in liquid medium[1)]	
		DCW(g/l)	Specific inhibitor formation (U/mg DCW)
0	++	6.1	98.3
5	(+)	0.8	14.6
50	-	0.2	9.7

++, good growth, (+), poor growth, -, no growth.
[1)] Liquid medium was the fermentation medium described in Materials and Methods. *Streptomyces* sp. SL-387 inoculated in the medium was cultured at 28°C for 5 days in a shaker (170 rpm).

Table 2. Effect of exogenous ATP on the phosphate-repressed cultures.

ATP added (mM)	Dry cell weight (g/l)	Specific inhibitor formation (U/mg DCW)
Non-repressed cultures;		
0	5.6	98.3
5	7.9	62.1
10	9.1	38.5
Repressed cultures;		
0	5.9	69.9
5	7.8	49.5
10	8.0	37.4

Additions of ATP were done at 24 hours cultures grown in the fermentation medium with low concentration of phosphate. Non-repressed cultures were done in the presence of 0.78 mM phosphate. Phosphate (17.4 mM) was added to cultures for phosphate repression at the beginning of fermentation.

In order to confirm phosphate repression and to overcome the repression, magnesium carbonate, a phosphate trapping agent, was added to phosphate-repressed media before autoclaving. Total inhibitor formation was maximum in the medium supplemented with 0.05% magnesium carbonate indicating the concentration at which derepression of the phosphate supression occurs (Fig. 3). Tanaka and Omura (10) developed a new fermentation technique using phosphate-trapping agents such as allophane (a noncrystalline clay of aluminosilicate) and magnesium carbonate to achieve 2- to 10-fold increases in the production of many types of antibiotics. To overproduce the AP-M inhibitor MR-387A and B, the "phosphate ion-depressed fermentation" technique can be applied.

Addition of Inorganic Phosphate, Arsenate and Chloramphenicol During the Fermentation

In order to characterize the phosphate effect, a high ion concentration was added to the cultures growing in low phosphate at 24 hours of fermentation. At this time, the growth was in the stationary phase and once the production of inhibitors had started. As seen in Fig. 4, in contrast to the control without additional phosphate, suplementation with 50 mM phosphate reduced the rate of inhibitor biosynthesis. The addition of chloramphenicol (0.15 mM), a protein synthesis inhibitor, to low phosphate fermentations also exerted a negative effect on inhibitor production, similar to that observed at a high phosphate concentration. These results suggest that phosphate regulation is a repressive rather than an inhibitory action on the inhibitor formation.

To establish whether or not phosphate *per se* was responsible for this effect sodium arsenate (50 mM), a non-metabolizable phosphate analogue, was supplied to cultures at stationary phase with low phosphate. As revealed in Fig. 4, in contrast to a control without further addition, arsenate significantly decreased inhibitor production. The magnitude of this effect was similar to that obtained at a high phosphate concentration. Therefore, the use of a phos-

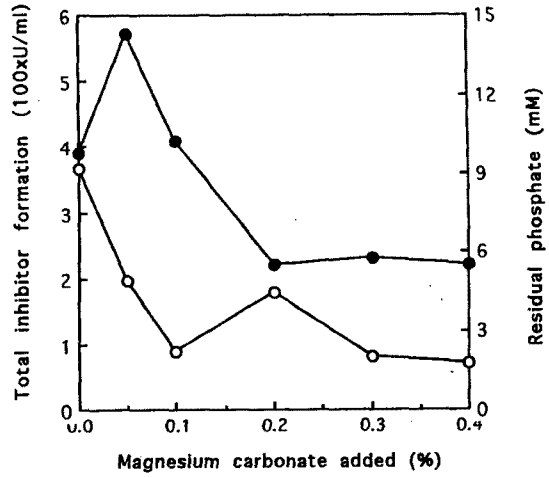

Fig. 3. Effect of phosphate trapping agent (magnesium carbonate) on the phosphate-repressed cultures.
Magnesium carbonate was added to fermentation medium (17.4 mM phosphate) ranging from 0.05 to 0.4% before autoclaving. ○: residual phosphate (soluble phosphate), ●: total productivity.

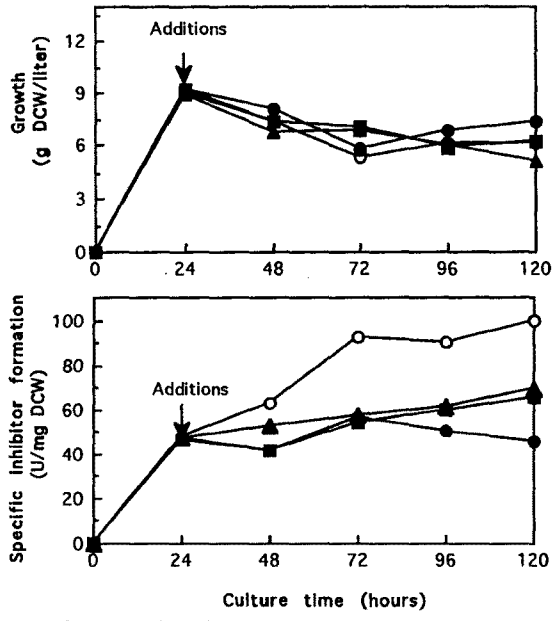

Fig. 4. Effect of phosphate, arsenate, and chloramphenicol addition on the course of growth and specific inhibitor formation.
Additions were done in 24 hours cultures grown in the fermentation medium with 1.07 mM phosphate. ○: control without further addition, ●: phosphate (50 mM), ■: sodium arsenate (50 mM), ▲: chloramphenicol (0.15 mM).

phate analogue and a protein synthesis inhibitor suggested that the phosphate itself repressed inhibitor formation.

REFERENCES

1. Banko, G., S. Wolfe, and A. L. Demain. 1985. Cell-free synthesis of δ-(L-α-aminoadipyl)-L-Cysteine, the first intermediate of penicillin and cephalosporin biosynthesis. *Biochem. Biophys. Res. Comm.* **137**: 528-535.
2. Chung, M. C., H. K. Chun, H. J. Lee, and Y. H. Kho. 1994. Taxonomic characteristics of strain producing MR-387A and B, new inhibitors of aminopeptidase M, and their production. *Kor. J. Appl. Microbiol. Biotechnol.* **22**: 447-452.
3. Crueger, W., A. Crueger, and A. G. Bayer. 1984. *Biotechnology-A textbook of industrial microbiology*, p. 9-48. Sinouer Associate, Sunderland.
4. Hanel, F., H. Krugel, and G. Fiedleir. 1989. Arsenical resistance of growth and phosphate control of antibiotic biosynthesis in *Streptomyces. J. Gen. Microbiol.* **135**: 583-591.
5. Majumdar, M. K. and S. K. Majumdar. 1970. Isolation and characterization of three phosphoamido-neomycins and their conversion into neomycin by *Streptomyces fradiae. Biochem. J.* **120**: 271-278.
6. Martin, J. F. 1989. Molecular mechanisms for the control by phosphate of the biosynthesis of antibiotics and other secondary metabolites, p. 213-237. *In* S. Shapiro (ed.), *Regulation of secondary metabolism in actinomycetes*, CRC press, Boca Raton.
7. Miller, A. L. and J. B. Walker. 1970. Accumulation of streptomycin phosphate in cultures of streptomycin producers grown on a high-phosphate medium. *J. Bacteriol.* **104**: 8-12.
8. Naharro, G., I. A. Gil, I. R. Villaneva, and J. F. Martin. 1981. Study of the molecular mechanism of phosphate control of candicidin biosynthesis using phosphate-deregulated mutants, p. 135-140. *In* M. Moo-Young, C. Vezina and K. Sigh (ed.), *Advances in Biotechnology*, vol. III, Pergamon press, Toronto.
9. Shirafugi, H., I. Nogami, M. Kida, and H. Yoneda. 1982. Two alkaline phosphatases from a butirosin A producer Bacillus vitellinus. *Agric. Biol. Chem.* **46**: 2465-2476.
10. Tanaka, T. and S. Omura. 1988. Regulation of biosynthesis of polyketide antibiotics, p. 418-423. *In* Y. Okami, T. Beppu, and H. Ogawara (ed.), *Biology of Actinomycetes '88*, Japan Science Society Press, Tokyo.
11. Taussky, H. H. and E. Shorr. 1953. A microcolorimetric method for the determination of inorganic phosphorus. *J. Biol. Chem.* **202**: 675-685.

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