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

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The effect of carbon and nitrogen sources on the production of novel aminopeptidase M inhibitors MR-387A and B by *Streptomyces* sp. SL-387 has been studied. High D-glucose and ammonia concentrations (5% and 1%, respectively) exerted a negative influence on the inhibitor formation. The suppressive effect of glucose on the inhibitor formation is probably caused by an effect of medium pH rather than that of cyclic AMP. To establish the optimum conditions for inhibitor overproduction, various nitrogen sources and ammonium ion-trapping agents were examined. The use of ammonia slow-releasing nitrogen sources such as soybean meal and fish meal, or ammonium ion-trapping agents such as kaoline, celite, and natural zeolite achieved the enhancement of inhibitor production. These results also indicate that inhibitor formation is affected by ammonium ion repression.

The bioactive peptides MR-387A and B are novel aminopeptidase M (AP-M) inhibitors isolated from the culture filtrate of *Streptomyces* sp. SL-387 (1). Our group has been involved in studies on the factors and conditions controlling the formation of these inhibitors. It was found that the biosynthesis of the inhibitors is controlled by some nutrients such as glucose, ammonia, and phosphate (2).

The negative influence exerted by the type and concentration of the carbon source on the fermentative production of secondary metabolites, has been well documented for several antibiotics (4, 6). The phenomenon of carbon catabolite repression in certain bacteria involves the binding of a complex between cyclic adenosine 3',5'-monophosphate (cyclic AMP) and a cyclic AMP receptor protein (CRP) to the promotor site of an operon. This binding stimulates the initiation of transcription by RNA polymerase. When glucose is present, its uptake inhibits adenylate cyclase, the enzyme that converts ATP to cyclic AMP, thus decreasing the concentration of cyclic AMP and inhibiting the transcription by RNA polymerase of operons subject to this control. Other forms of carbon catabolism regulation are carbon catabolite inhibition, in which metabolism of the favored carbon source inhibits the activity of a pre-existing enzyme, and carbon catabolite inactivation, in which a pre-existing enzyme is inactivated (3).

Regulatory mechanisms that control the use of nitrogen sources have also been reported in many microorganisms. Ammonia or some other readily used nitrogen source represses enzymes involved in the use of other nitrogen sources, affecting the formation of secondary metabolites (5).

In this paper, we report the effect of utilizable carbon sources on growth and AP-M inhibitor biosynthesis in this actinomycete. This paper also describes the effect of nitrogen sources and the avoidance of ammonia repression by the ammonia slow-releasing nitrogen sources or ammonia-trapping agents.

MATERIALS AND METHODS

Culture and Growth Conditions

Streptomyces sp. SL-387 was kindly supplied by the Screening Laboratory of Korea Research Institute of Bioscience and Biotechnology, KIST, Taejon, Korea. Spores of this microorganism were obtained and main-

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tained as reported previously (1). 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℃ 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 (or 3% soybean meal), 0.2% yeast extract, 0.1% beef extract, 0.01% K_2HPO_4, 0.3% NaCl, 0.0005% $CuSO_4 \cdot 5H_2O$, 0.005% $MgCl_2 \cdot 4H_2O$, and 0.0005% $ZnCl_2 \cdot 7H_2O$ 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℃ for 5 days on a rotary shaker at 170 rpm.

Fermentation by Addition of cAMP and Ammonia-trapping Agents

Cyclic AMP (10 mM) was added to 20 ml of fermentation medium containing soybean meal as nitrogen source at specified intervals (0 and 24 hours of fermentation). The cultures were returned to the shaker and the productivity of the inhibitor was determined by AP-M assays after 96 hours.

To determine the effect of ammonia-trapping agents on the total inhibitor formation, various concentrations of magnesium phosphate[$(Mg_3(PO_4)_2 \cdot 8H_2O$], kaoline (hydrated aluminum silicate), celite, and natural zeolite were added to the fermentation medium before autoclaving.

Assay for AP-M and Inhibitor Determination

AP-M activity was measured as reported previously (1). The percent inhibition was calculated by the formula $(A-B)/A \times 100$, where A is the measured 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 activity was defined as the amount of inhibitor required for 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 of original culture medium.

Cell Growth Determination

Cell growth was measured by dry cell weight (DCW) or packed cell volume (PCV) determination. For DCW determination, samples of mycelia (2 ml) were harvested by centrifugation, and washed twice with equal volumes of distilled water. After centrifugation, the pellet was dried in an oven at 105℃ for 3 hours and then weighed. For PCV determination, 10 ml of culture broth was centrifuged at 3000 rpm for 10 minutes. PCV (%, v/v) was expressed as a partition of packed cell volume per total broth volume.

Reproducibility of Results

The experiments reported 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 D-Glucose Concentration on Fermentations

Streptomyces sp. SL-387 was able to utilize D-glucose as a carbon source for growth and AP-M inhibitor formation (1). Fig. 1 shows maximum growth and total inhibitor production of this actinomycete in fermentation with glucose concentrations ranging from 0.05 to 5%. As shown in Fig. 1, growth of culture increases in proportion to the glucose concentration added to the fermentation medium (up to 2%). In regard to total productivity of the inhibitor, an inverse correlation was found between the maximum inhibitor produced and the initial carbohydrate concentration added to the fermentation medium, with a maximum effect shown at 5%. A close correlation between the medium pH and inhibitor production was observed at all sugar concentrations tested, indicating this possibly, as the cause of inhibitor repression. With fermentations in flasks, pH is controlled by adding mineral acids, $CaCO_3$, phosphate salts, ammonia, and Na_2CO_3 to the fermentation media. Addition of $CaCO_3$ to the fermentation media containing 1% glucose resulted in an increase of inhibitor productivity, even though it is a slight increase (Table 1).

Effect of cyclic AMP Additions on Inhibitor Formation

The effect of cyclic AMP on inhibitor production was

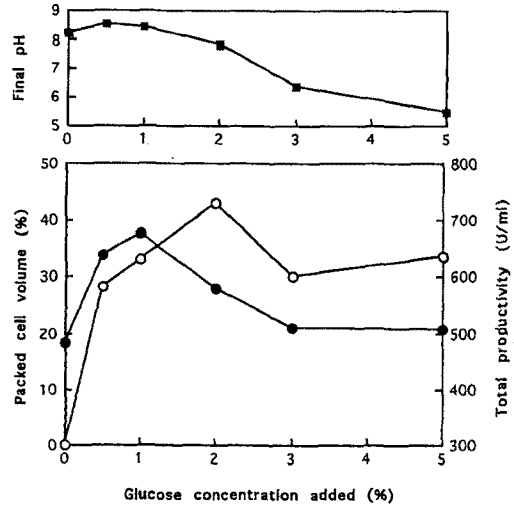

Fig. 1. Effect of different D-glucose concentrations on the maximum growth(○), total productivity of the inhibitor(●), and final pH of the medium(■).

evaluated. The cell growth was slightly increased but the specific inhibitor formation was slightly reduced when cyclic AMP was added to the cultures at specified intervals (0 and 24 hours of fermentation, Table 2). The slight reduction of inhibitor production by addition of cyclic AMP shows that cyclic AMP is not a major regulatory influence on the inhibitor biosynthesis in *Streptomyces* sp. SL-387. Therefore, the inhibitory effect of glucose on AP-M inhibitor formation from the results of medium pH and cyclic AMP additions is probably caused by an effect of medium pH rather than that of cyclic AMP.

The biochemical mechanisms of pH effects are not known. It is assumed that growth at a suitable pH is favorable for the synthesis of a relevant enzyme, or that the medium pH coincides with the optimal pH of a key biosynthesis enzyme. The inhibitory effect of glucose on bacitracin production resulted from a pH effect (7). Addition of short-chain fatty acids often resulted in a decrease in both mycelial growth and antibiotic production. Tanaka *et al* (7) proposed that this is caused by an effect on intracellular pH.

Fig. 2 shows the effect of initial pH on inhibitor production. As shown, inhibitor productivity of below 7.0 initial pH was lower than that of above 7.0. In general, actinomycetes are among alkalophilic microorganisms (7). They produce extracellular enzymes with optimal pH values in the alkaline range. Although initial pH values are independent of medium final pH, the above results indicate that alkaline conditions are more effective than acidic conditions for inhibitor formation.

Table 1. Effect of $CaCO_3$ concentrations on the inhibitor production.

$CaCO_3$ added(%)	Final pH	Total productivity(U/ml)
0	8.2	631.7
0.1	8.6	711.7
0.2	8.6	719.4
0.3	8.4	736.4
0.4	8.5	696.4
1.0	8.5	688.2

$CaCO_3$ was added to the fermentation media containing soybean mealas nitrogen source before autoclaving. Fermentation was done for 4 days at 28℃ on a rotary shaker (170 rpm).

Effect of Ammonium nitrate Additions on Inhibitor Formation

In order to characterize the ammonia effect, ammonium nitrate was added to cultures (soybean meal medium) in different concentrations ranging from 0.3% to 1%. As shown in Fig. 3, in contrast to the control without additional ammonia, a closely inverse correlation was found between inhibitor production and the initial ammonia concentration added to the fermentation medium. Only slight differences were observed in the medium pH at all ammonia concentrations, excluding this possibility as the cause of ammonia repression of inhibitor production.

Table 3 shows the effect of various nitrogen sources on inhibitor production in a medium with glucose, galactose, and mannose as a carbon source. Among them, the complex nitrogen sources such as soybean meal, fish meal, and soytone were effective on inhibitor production at all sugar tested. Enzymatic or acid-hydrolysates of casein such as tryptone or casamino acid were less effective than the complex nitrogen sources. Otherwise, inorganic nitrogen source (such as ammonium nitrate)

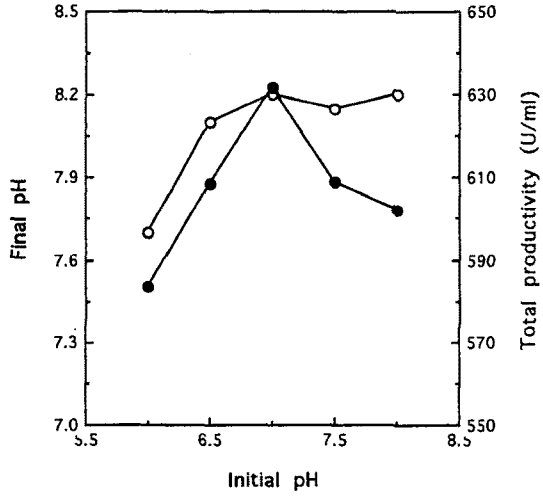

Fig. 2. Effect of initial pH on the inhibitor production.
Initial pH of medium containing 3% soybean meal in the fermentation medium was adjusted with 1 N NaOH or 1 N HCl. Fermentation was done for 4 days at 28℃ on a rotary shaker (170 rpm). ●: total productivity of the inhibitor ○: final pH in the medium.

Table 2. Effect of cyclic AMP on the specific inhibitor formation.

Addition of cAMP	Dry cell weight (g/l)	Total productivity (U/ml)	Specific inhibitor formation (U/mg DCW)
No addition	5.94	719.4	121.1
Addition at 0 hour	7.02	699.8	99.7
Addition at 24 hours	6.69	705.2	105.4

10 mM of cAMP was added to the cultures at 0 or 24 hours of fermentaion. After 96 hours of fermentation, the productivity of AP-Minhibitors was determined in the fermentation broths.

or some amino acids gave no growth or no inhibitor production. High productivity of the inhibitor by soybean meal may be due to avoidance of nitrogen metabolite repression of the inhibitor biosynthesis via the slowness of its breakdown into repressive amino acids and ammonia (3).

These results suggest a possibility that the production of AP-M inhibitor MR-387A and B by *Streptomyces* sp. SL-387 are under ammonia repression.

Overcoming Ammonia Repression

To overcome ammonia repression of inhibitor overproduction, the use of an insoluble ammonia slow-releasing nitrogen source such as soybean meal was found suitable, as mentioned above. Another method is the use of ammonia trapping agents (7). Treatment with most ammonium ion-trapping agents such as magnesium phosphate, kaoline, celite or natural zeolite achieved the enhancement of total productivity of the inhibitors except magnesium phosphate as shown in Table 4. It is also well known that zeolite, kaoline, celite, and other natural minerals interact with microbial cells to suppress pellet formation (8). This can lead to enhancement of antibiotic production if increased uptake of oxygen and nutrients, which occurs with suppressed pellet formation, favors secondary metabolite biosynthesis. In this experiment, the results indicate that kaoline, celite, or natural zeolite functions as a pellet formation-suppressing agent as well as as an ammonia ion-trapping agent for enhancement of inhibitor production.

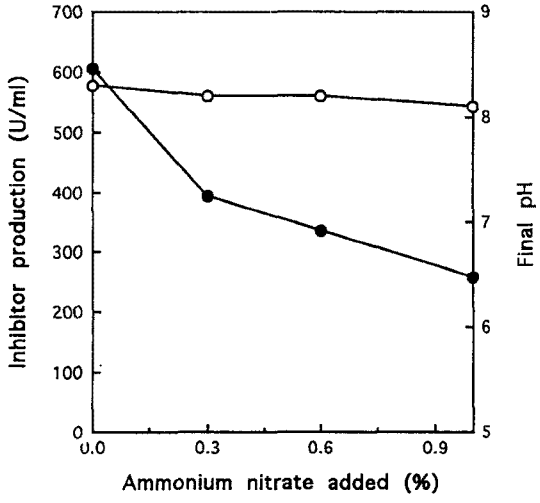

Fig. 3. Effect of ammonium nitrate added on the inhibitor production.
●: total productivity of the inhibitor, ○: final pH in the medium.

Table 4. Enhancement of the inhibitor production by ammonium ion-trapping agents.

Trapping agents	Amount added (%)	Total productivity (U/ml)
Mg-P	0.5	578.0
	1.0	520.8
kaoline	0.5	736.4
	1.0	659.2
celite	0.5	674.8
	1.0	648.1
Natural zeolite	0.5	676.9
	1.0	654.3
No addition	-	631.7

Mg-P : magnesium phosphate, $Mg_3(PO_4)_2 \cdot 8H_2O$, kaoline : hydrated aluminium silicate(particle size : 0.1~4 μm). Ammonia-trapping agents were added to the fermentation medium containing 3% soybean meal as a nitrogen source before autoclaving. Fermentations were done for 4 days at 28℃ on a rotary shaker (170 rpm).

Table 3. Effect of several nitrogen and carbon sources on the inhibitor production by *Streptomyces* sp. SL-387 (KCTC 0102BP).

Nitogen sources (1%)	Carbon sources (2%)								
	Glucose			Galactose			Mannose		
	Growth	pH	%I[1)]	Growth	pH	%I	Growth	pH	%I
Polypeptone	++	8.5	40	++	8.3	37	++	8.4	33
Casamino acid	+	7.0	42	++	7.0	40	++	7.5	48
Soybean meal	++	7.5	75	++	5.7	72	++	6.1	81
Fish meal	++	5.8	53	++	5.6	67	++	6.2	78
Soytone	++	7.5	72	++	8.2	82	++	7.3	85
Tryptone	++	8.2	63	+	8.3	39	++	8.3	60
Bactopeptone	++	7.9	60	+	8.5	40	++	8.5	23
Beef extract	++	7.7	63	+	8.4	46	++	6.6	62
Malt extract	±	5.5	15	±	6.0	0	±	6.2	6
Yeast extract	++	7.9	40	+	8.5	32	++	7.1	40
L-Phenylalanine	−	5.6	24	−	5.9	23	−	5.8	19
L-Valine	−	5.6	22	−	5.6	21	−	5.9	16
L-Proline	−	5.5	19	−	5.6	18	±	6.2	28
NH_4NO_3	−	5.4	15	−	5.5	14	−	5.5	14
None	−	5.7	14	−	5.8	12	−	6.3	25

[1)]Inhibition percent of 5 μl broth against AP-M (reaction vol.=0.2 ml), Degree of growth : ++; abundant growth, + ; moderate growth, ± ; poor growth, − ; no growth Spores (100 μl, ~10^7/ml) of *Streptomyces* sp. SL-387 were inoculated into test tube containing 10ml of media consisted of 0.2% NaCl, 0.025% K_2HPO_4, carbon and nitrogen sources, and then incubated in shaking incubator at 28℃ for 4 days.

Finally, this paper describes effect of D-glucose on the growth and MR-387 biosynthesis in *Streptomyces* sp. SL-387. The inhibitory effect of glucose on inhibitor formation is probably caused by an effect of the medium pH rather than that of cyclic AMP. Growth under glucose limitation or pH controlled conditions are necessary for the avoidance of glucose repression. The results of this experiment suggest also that inhibitor formation is under ammonium ion repression. Therefore, to overcome the ammonium ion repression for inhibitor overproduction, the use of an ammonia slow-releasing nitrogen source or ammonium ion-trapping agent (pellet formation suppressing agents) are suitable.

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