ENHANCEMENT OF CERULENIN PRODUCTION BY A NATURAL ZEOLITE, AN AMMONIUM ION-TRAPPING AGENT

ROKUROU MASUMA, YOSHITAKE TANAKA and SATOSHI ŌMURA*

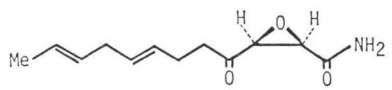
School of Pharmaceutical Sciences, Kitasato University
and The Kitasato Institute
Minato-ku, Tokyo 108, Japan

(Received for publication March 31, 1982)

Addition of a natural zeolite, a known ammonium ion-trapping agent, to a complex medium resulted in a several fold increase in cerulenin production by *Cephalosporium caerulens*. In the presence of zeolite, ammonium ion in the medium decreased, while mycelial growth increased to a small extent, and pH values remained constant. Small amounts of ammonium bicarbonate inhibited cerulenin production without affecting mycelial growth and pH values. It is thus assumed that zeolite stimulated cerulenin production by releasing the biosynthesis from the suppression by ammonium ions in *C. caerulens*.

Cerulenin, 2*R*,3*S*-epoxy-4-oxo-7,10-*trans*,*trans*-dodecadienoic acid amide (Fig. 1), is an antifungal antibiotic discovered by HATA *et al.* in 1963 in a culture of the fungus *Cephalosporium caerulens*.[1] The microorganism co-produces the sterol antibiotic helvolic acid.[2]

Fig. 1. Structure of cerulenin.



Cerulenin specifically inhibits the condensing enzymes involved in fatty acid and polyketide biosyntheses.[1] Because of its unique mode of action, the antibiotic has become an important biological agent useful in the studies on the biosyntheses of fatty acids, lipids, and polyketide-derived secondary metabolites such as leucomycin, spiramycin, tylosin[3] and nanaomycin.[4] It is also used as an aid for hybrid biosynthesis of new antibiotic derivatives[5] and for strain improvement in antibiotic fermentation.[6]

In this laboratory, cerulenin production has been carried out by the method originally reported by IWAI *et al.*[7] The maximum titer was 250 μg/ml in Sakaguchi flasks, but was lower (about 100 μg/ml or less) on larger scales. Because of the increasing demands for the supply of cerulenin and the undesirable decline of the cerulenin titer during repeated transfer of the culture, it became necessary to improve cerulenin productivity of *C. caerulens*. Relatively minor increases were brought about, however, by conventional monospore isolation techniques and/or random selection of improved mutants after mutations by ultraviolet and mutagen treatments.

Recently, ŌMURA *et al.*[8] reported that magnesium phosphate and related insoluble compounds promoted the production of the macrolide antibiotics leucomycin, spiramycin and tylosin. Magnesium phosphate acts as an ammonium ion-trapping agent in biological systems, and stimulates leucomycin production by reducing the inhibitory effect of ammonium ions.[9] Magnesium phosphate also stimulates the microbial conversion of glycine to L-serine.[10] It was of interest to examine if magnesium phosphate and other ammonium ion-trapping agents exhibit a similar stimulatory effect on cerulenin production.

* To whom all correspondence should be addressed.

The present paper describes that a natural zeolite, which is a known ammonium ion-trapping agent,[11] promotes cerulenin production by *C. caerulens* several fold. The cerulenin titer exceeded 300 μg/ml and 250 μg/ml in a Sakaguchi flask and in a 400-liter fermentor, respectively. The stimulation was assumed to be due to the release of cerulenin biosynthesis from the suppression by ammonium ions.

Materials and Methods

Microorganism

Cephalosporium caerulens KF-140 was used throughout the work.

Method of Cultivation

Spores and mycelia of *C. caerulens* KF-140 were inoculated into a test tube (20 cm×2 cm) containing 10 ml of medium S[11] (2% glucose, 0.5% meat extract, 0.5% peptone, 0.3% dried yeast cells and 0.5% NaCl, pH 7.0 before autoclaving), and incubated at 27°C for 2 days with reciprocal shaking (280 strokes/minute). The seed culture (1 ml) thus obtained was transferred into a 500-ml Sakaguchi flask containing medium P[7] (3% glycerol, 1% glucose, 0.5% peptone, 0.2% NaCl and 0 or 1% zeolite, pH 7.0 prior to sterilization), and incubated for 1 to 4 days with reciprocal shaking (130 strokes/minute). A natural zeolite preparation heated at 180°C for 3 hours was mainly used, as will be described in the text. A 30-liter jar fermentor containing 15 liters of medium P was run at 27°C for 4 days with aeration (7.5 liters/minute) and with agitation (200 rpm). A 400-liter fermentor containing 200 liters of medium P was carried out in a similar manner.

Antibiotics

Pure cerulenin and helvolic acid were prepared by the methods previously described.[7]

Zeolites

The natural zeolite (40 mesh or smaller) was a product of Fusseki Kako Co. According to the supplier's manual, it contains 70% of zeolites consisting of mordenite $[Na_2(AlSi_5O_{12})_n \cdot 6H_2O]$ and clinoptiolite $[Na_3(Al_3Si_{15}O_{36})_n \cdot 12H_2O]$, and 30% of other types of zeolites and clays. The synthetic zeolite (type A-3) (200 mesh or smaller) was purchased from Wako Chemical Co.

Analysis

The cultured broth (10 ml) was centrifuged (2,500 rpm, 5 minutes) and the packed mycelial volume of the sediment was measured and taken as the amount of mycelial growth. It was expressed as ml of packed mycelial volume/100 ml of culture broth. The zeolite fraction in the sediment was subtracted, when necessary. The supernatant fluid was used for the assay of the antibiotic titer. The amount of cerulenin was determined by paper disk assay with *Candida albicans* KF-1 as the test organism (glucose-potato agar, pH 6, 37°C, overnight). The helvolic acid titer was estimated with *Corynebacterium paurometabolum* KB-121 as the test organism (nutrient agar, 37°C, 48 hours).[7] The amount of ammonium ion was determined colorimetrically by the indophenol method,[12] glucose by the *o*-toluidine method,[13] and glycerol by the periodate method as described by IWAI *et al.*[14]

Results

Effect of a Natural Zeolite on Cerulenin Production

In the previous papers[8,9,15] from our laboratories, it was reported that leucomycin production by *Streptoverticillium kitasatoensis** and spiramycin production by *Streptomyces ambofaciens* were significantly promoted when an ammonium ion-trapping agent such as magnesium phosphate, calcium phosphate or sodium phosphotungstate was added to the fermentation medium. It was of interest

* The former name *Streptomyces kitasatoensis*[16] is revised hereafter according to the recent proposal.[17]

Table 1. Effect of ammonium ion-trapping agents on cerulenin production by *Cephalosporium caerulens* KF-140.

NH_4^+-trapping agent	Amount added (%)	Growth	pH	Cerulenin produced (μg/ml)
None	—	++	4.5	40.5
Magnesium phosphate	0.5	++	7.3	28.5
	1.0	++	7.4	15.5
Calcium phosphate	0.5	+	5.9	37.5
	1.0	+	6.0	40.0
	2.0	+	6.1	32.5
Sodium phosphotungstate	0.5	++	5.0	<10
Natural zeolite	0.5	+++	6.7	61.0
	1.0	+++	5.2	69.5
	2.0	+++	5.5	76.0

The fermentation was carried out at 27°C for 3 days in test tubes (20 cm×2 cm) containing 10 ml of medium P.

Table 2. Effect of a natural zeolite on antibiotic production by *C. caerulens* KF-140.

Zeolite added (%)	Packed mycelial volume (ml/100 ml)	pH	Antibiotics produced (μg/ml)	
			Cerulenin	Helvolic acid
None	5.5	5.0	100	21
0.5	6.0	5.2	180	8
1.0	7.0	5.0	215	8
2.0	7.0	5.2	200	3

The fermentation was carried out in Sakaguchi flasks at 27°C for 2 days.

if these compounds and the known ammonium ion-trapping agent, zeolite, would also promote cerulenin production.

Table 1 shows that the former three compounds inhibited cerulenin production, while a natural zeolite stimulated it to a small extent. One percent of zeolite was optimal for cerulenin production as shown in Table 2. The increase in the cerulenin titer was accompanied by an increase in the mycelial growth and a decrease in the helvolic acid titer. A zeolite preparation with particle size smaller than 200 mesh was more effective than the one with a larger particle size (40 mesh) (data not shown).

Effect of the Time of Zeolite Addition on Cerulenin Production

A culture of *C. caerulens* was started without zeolite, followed by its addition at varying times of cultivation, and continuation of the culture for a total of 48 hours. Table 3 indicates that the cerulenin production was the highest when zeolite was added initially to the medium, but was lower when added at later periods.

Effect of Pretreatments of a Natural Zeolite on Cerulenin Production

Zeolites have high affinity to ammonium ion and several cations. The species of the cation with the highest affinity depends on the zeolite.[11] The natural zeolite employed in the present study shows the highest affinity to ammonium ion (according to the supplier's manual). The zeolite might have trapped various cations during its reserve under natural weather conditions before the harvest, since considerable amounts of ammonium ion were released from the zeolite, as detected by NESSLER's

Table 3. Effect of the time of zeolite addition on cerulenin production.

Addition time (hour)	Packed mycelial volume (ml/100 ml)[1)]	pH[1)]	Antibiotic production (μg/ml)	
			Cerulenin	Helvolic acid
0	7.5	4.8	320	6.4
6	7.5	4.8	200	7.8
12	8.5	4.8	140	9.0
24	8.0	5.6	140	4.5
(No addition)	7.0	5.0	80	10.7

The fermentation was carried out in Sakaguchi flasks for a total of 48 hours. One percent zeolite was added.

1) Values after 48 hours.

Table 4. Effect of pretreatments of natural and synthetic zeolites and Amberlite IRC-50 (H^+ type) on cerulenin production.

	Pretreatment	Packed mycelial volume (ml/100 ml)	pH		Cerulenin produced (μg/ml)
			Just after inoculation	After 48 hours	
(Natural zeolite)	Washing with water	6.5	6.6	4.6	250
	10% NaCl	7.0	6.2	4.8	170
	1 N HCl	6.5	6.2	2.0	120
	1 M $(NH_4)_2SO_4$	5.5	6.6	2.0	70
	Medium P	4.5	6.8	4.6	140
	Heating at 180°C, 3 hours	6.5	6.8	4.6	200
	Not treated	9.0	7.6	4.8	170
(Synthetic zeolite (A-3))	Washing with Medium P	1.0	7.2	6.0	10
	Not treated	1.0	7.0	5.8	<3
(Amberlite IRC-50 (H^+))	Washing with Medium P	2.5	6.4	5.0	9
	Not treated	2.0	6.6	2.0	<3
	(No addition)	7.0	6.6	5.0	80

A slurry (1 g) of the indicated ammonium ion-trapping agent was suspended in 10 ml of the indicated solution and incubated overnight with shaking, washed with water, then added to a Sakaguchi flask containing 100 ml of medium P. Another portion (1 g) was heated and added. Cerulenin production after 48 hours is shown.

reagent, when the zeolite was suspended in a sodium chloride solution at room temperature. Accordingly, it was expected that the extent of stimulation of cerulenin production might be affected by various pretreatments of the zeolite, if the stimulation was related to the capacity of the zeolite to trap ammonium ion in the cerulenin production medium.

A slurry of the natural zeolite was shaken (280 strokes/minute) at 27°C overnight in water containing various compounds, washed thoroughly with water, and then added to the cerulenin production medium. Heated zeolite was also tested. A synthetic zeolite (type A-3) and the cation-exchange resin Amberlite IRC-50 (H^+ type) were treated in the same manner, and the effect on cerulenin production was studied. As summarized in Table 4, natural zeolite preparations obtained by washing with water or heating promoted cerulenin production somewhat more than non-treated zeolite did. Washing with sodium chloride did not virtually influence the stimulatory effect. On the other hand, washing the zeolite with ammonium sulfate eliminated the stimulating ability. It is likely that the ammonium

ion-trapping capacity increased in the water-washed or heated zeolite, or that some contaminating inhibitory substances were removed.

A synthetic zeolite and Amberlite IRC-50 (H^+ type) did not stimulate cerulenin production, but severely inhibited it as well as mycelial growth. Even when the two materials were washed with the production medium (medium P), the inhibition was not reversed. This is different from the results reported by TONE *et al.*[18], who showed an anion-exchange resin preparation washed with production medium promoted salicylic acid production from naphthalene.

In the following experiments, a heated preparation of the natural zeolite (40 mesh or smaller) was used for convenience.

Effect of Seed Medium and Seed Culture Age on Cerulenin Production

In the course of the studies on cerulenin production using jar fermentors in the presence of zeolite, considerable fluctuation of the cerulenin titer was noted. In an attempt to reduce this fluctuation for efficient cerulenin production, the effects of seed medium and seed culture age were examined using Sakaguchi flasks. The first seed culture was obtained by the standard method, and transferred to two kinds of second seed medium, media S and P (Materials and Methods). At varying times of the second seed cultures, aliquots were withdrawn and transferred to a fermentation medium (medium P). All the flasks were incubated at 27°C for two days with reciprocal shaking. The results are summarized in Table 5.

Table 5. Effect of seed medium and seed culture age on cerulenin production.

2nd Seed		Zeolite added to fermentation medium (%)	Packed mycelial volume (ml/100 ml)	pH	Cerulenin produced (μg/ml)
Medium	Culture age (hours)				
Medium S	11	0	3.5	5.0	22
	24		2.5	5.0	15
	35		3.5	5.0	23
	49		3.0	5.4	30
	11	1	11.0	4.8	300
	24		5.5	4.8	220
	35		6.0	4.8	200
	49		5.5	5.0	150
Medium P	11	0	2.0	5.0	—
	24		1.5	5.2	trace
	35		2.0	5.2	trace
	49		2.0	5.2	trace
	11	1	11.0	4.8	260
	24		7.0	5.0	260
	35		11.0	5.0	230
	49		8.0	5.0	250

C. caerulens KF-140 was grown for 38 hours in medium S. For the 2nd seed culture, an aliquot (1 ml) was transferred into 100 ml of medium S or medium P, as indicated, in a Sakaguchi flask. At the intervals indicated, a 1 ml portion of the 2nd seed culture was transferred to a Sakaguchi flask containing 100 ml of medium P supplemented with or without zeolite. Cerulenin titers after 48 hours of the cultivation are shown.

Zeolite markedly stimulated cerulenin production irrespective of the composition of the second seed medium. With the second seed culture carried out in medium S, the cerulenin titer at 48 hours of cultivation was the highest (300 μg/ml) when the second seed culture was transferred at 11 hours, but became lower when transferred later. On the other hand, when the second seed culture was carried out in medium P, the cerulenin titer at 48 hours was constant at about 250 μg/ml independent of the seed culture age. In all the cases, the pH values were around 5.0 in the presence of zeolite.

The mycelia were pulpy in the presence of zeolite, but were in pellets in its absence. The apparent increases in packed mycelial volume in the presence of zeolite were due partly to this difference in mycelial form. Dry mycelial weight was also 1.5~2 times as high in the presence of zeolite as that in its absence (data not shown). This indicates that a net increase of cerulenin production occurred in the presence of zeolite.

A larger inoculum size favored higher production of cerulenin (Table 6). The fluctuation of the cerulenin titer was also reduced (not shown).

Time Course of Cerulenin Production in Jar Fermentors in the Presence of Zeolite

With medium P for the second seed culture, cerulenin production was carried out in a 30-liter jar fermentor. Fig. 2-A indicates the time course of cerulenin production in the presence and absence of zeolite. In the presence of zeolite, the cerulenin titer reached a maximum of 230 μg/ml at 76 hours of cultivation. The titer was eight times as high as in its absence (30 μg/ml). The helvolic acid titer reached 10 μg/ml at 19 hours, and then decreased in the presence of zeolite. The titer was lower than in its absence (14 μg/ml at 76 hours). Mycelial growth was about two times higher in the presence of zeolite. pH values were about 5.0~5.4 in the presence of zeolites, when active cerulenin production continued, while they gradually decreased to 4.7 in the absence of zeolite. Thus, the results obtained in flasks were substantially reproduced using a 30-liter jar fermentor.

In a 400-liter fermentor, cerulenin production reached 250 μg/ml at 43 hours of cultivation and remained at this level during later periods (data not shown). The cultured broth (180 liters) was centrifuged and cerulenin was purified from the supernatant fluid by the procedures previously described[7] with minor modification as follows. The supernatant fluid was extracted with benzene.

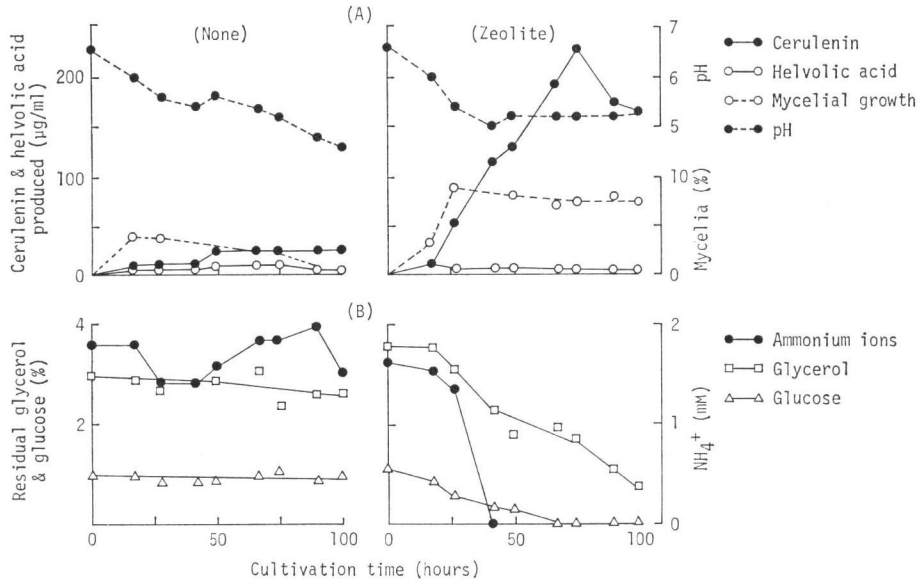
Table 6. Effect of inoculum size on cerulenin production.

2nd Seed medium	Zeolite added to fermentation medium (%)	Inoculum size (%)	Packed mycelial (ml/100 ml)	pH	Cerulenin produced (μg/ml)
Medium S	0	1	2.5	5.0	15
		3	3.5	5.0	32
	1	1	5.5	4.8	220
		3	5.5	5.0	250
Medium P	0	1	1.5	5.2	trace
		3	1.0	5.4	trace
	1	1	7.0	5.0	260
		3	11.0	5.0	300

The 2nd seed culture was carried out with medium P for 24 hours. The other conditions are the same as described for Table 5.

Fig. 2. Time course of cerulenin production and ammonium ion concentration in a 30-liter jar fermentor in the presence or absence of zeolite.

C. caerulens KF-140 was grown at 27°C for 40 hours in a Sakaguchi flask containing 100 ml of medium S. A 5 ml portion of this culture was transferred into five Sakaguchi flasks containing 100 ml of medium P, and incubated at 27°C for 24 hours with reciprocal shaking. The culture (500 ml) thus obtained was transferred into a 30-liter jar fermentor containing 15 liters of medium P supplemented with or without 1% heated zeolite. The jar fermentors were run at 27°C for 4 days with aeration and agitation. At the times indicated an aliquot (30 ml) was withdrawn from each vessel, and used for the assay.



The benzene extracts were evaporated *in vacuo*, and the residue was subjected to a silica gel column chromatography, which was developed with chloroform - ethyl acetate (10: 1). Active fractions were collected and evaporated. Cerulenin was crystallized from benzene - petroleum ether, and recrystallized to afford 9.7 g of pure cerulenin (23% of recovery), which showed identical physicochemical properties as authentic cerulenin. The yield was about 2 to 3 times higher than obtained previously without zeolite.

Effect of Zeolite on Ammonium Ion Concentration in the Medium

Preliminary results showed that the level of ammonium ion in the zeolite-supplemented culture was lower than in the non-supplemented medium. A typical time course of the ammonium ion level in the presence or absence of zeolite is shown in Fig. 2-B. In the presence of zeolite, ammonium ion decreased promptly to an almost undetectable level (<0.1 mM) while active cerulenin producion continued. On the other hand, ammonium ion retained a level of around 2 mM throughout the fermentation in the absence of zeolite. It increased slightly at a later period of fermentation. Glycerol and glucose were consumed more promptly in the presence of zeolite than in its absence, coinciding with the higher mycelial growth in the presence of zeolite.

These results strongly suggest that cerulenin biosynthesis is suppressed by high concentrations of ammonium ion, but is stimulated in the presence of zeolite because of the trapping of ammonium ion. Cerulenin production continued in the presence of high amounts of residual glycerol, if only

glucose remained, but ceased when glucose was exhausted. It thus appears that glucose is necessary for cerulenin production, as is the case of candicidin biosynthesis by *Streptomyces griseus*.[19]

Effect of Ammonium Salts and Methylamine on Cerulenin Production

The above time course study suggested a possible correlation between cerulenin biosynthesis and ammonium level in the medium. To verify this, ammonium chloride and methylamine, an unmetabolizable ammonium analog, were added to the medium at zero time, and their effect on cerulenin production was examined. Table 7 shows that the two compounds inhibited cerulenin production. However, the mycelial growth was also affected to small extents, and the pH values decreased considerably at the same time: thus the effect of ammonium ions on cerulenin biosynthesis remained unclear in this experiment.

In another experiment, ammonium bicarbonate was added. Again, the ammonium salt at 6.3 mM or higher severely inhibited cerulenin production (Fig. 3). In this case, neither the mycelial growth (not shown) nor the pH values were affected. After 50 hours of cultivation, cerulenin production was restored. Probably the ammonium ion added initially to the medium were consumed to give a low level not inhibitory to cerulenin biosynthesis.

Fig. 3. Effect of ammonium bicarbonate on cerulenin production.

C. caerulens KF-140 was grown in Sakaguchi flasks as described. To the flasks, a portion of ammonium bicarbonate solution was added in a final concentration of 0 (●), 6.3 (△) or 12.5 (○) mM. At the intervals indicated, a 2-ml portion was withdrawn from each culture, and used for the assay of the cerulenin titer (——) and pH (------).

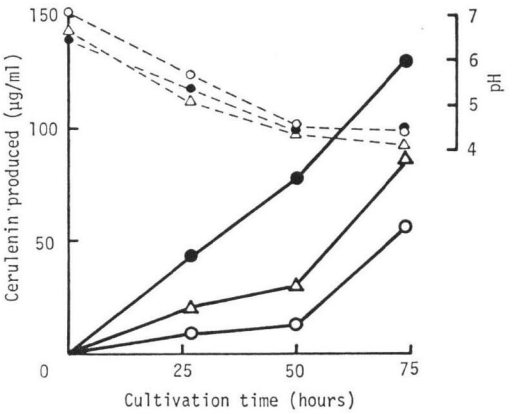


Table 7. Effect of ammonium chloride and methylamine on cerulenin production.

Addition		Packed mycelial volume (ml/100 ml)	pH	Cerulenin produced (μg/ml)
Compound	Amount (mM)			
None	—	5.5	4.1	140
NH_4Cl	9.4	3.5	2.1	92
	18.7	3.5	2.0	40
$CH_3NH_2 \cdot HCl$	7.2	6.0	2.4	95
	14.4	ND	2.1	52
	28.8	5.5	2.0	17

C. caerulens KF-140 was grown in Sakaguchi flasks containing medium P supplemented with the indicated compound. Cerulenin titers after 48 hours are shown. ND, not determined.

Discussion

The addition of a natural zeolite to a complex medium resulted in a several fold increase in cerulenin production by *C. caerulens*. Since zeolites are ammonium ion-trapping agents, it was speculated that the stimulation of cerulenin production by zeolite was related to the ammonium ion level in the medium.

The biosynthesis of many antibiotics is susceptibe to carbon, nitrogen or phosphate regulation.[20] Nitrogen regulation was reported in the biosynthesis of cephamycin, novobiocin, candihexin and fusidic acid.[20,21] The present authors reported the suppression of leucomycin production by ammonium ion.[15] A decline of the cerulenin titer was also observed when a zeolite preparation washed with an ammonium sulfate solution was added to the medium (Table 4). Ammonium bicarbonate inhibited cerulenin production without affecting mycelial growth and pH value (Fig. 3). These findings are in agreement with our previous observation[7] that ammonium sulfate and ammonium chloride were not suitable nitrogen sources in a chemically defined medium for cerulenin production. All of these results strongly suggest that ammonium ion suppresses cerulenin biosynthesis in *C. caerulens*.

In the presence of zeolite, cerulenin production increased, while ammonium ion in the medium decreased to a level of less than 0.1 mM, which was far lower than that (2 mM) in the absence of zeolite (Fig. 2-B). The period of low ammonium ion level coincided with the period of active cerulenin production. Accordingly, it is reasonable to assume that the natural zeolite stimulated cerulenin production by releasing cerulenin biosynthesis from the suppression by ammonium ion in *C. caerulens*. There is a gap, however, between the ammonium ion level (2 mM) in the control medium (Fig. 2-B) and the level (6.3 mM) of ammonium bicarbonate exhibiting sufficient inhibition of cerulenin production when added to the control medium (Fig. 3). For studying this point in more detail, further experiments with a defined medium will be required.

Magnesium phosphate, a proposed ammonium ion-trapping agent and a stimulator of the production of macrolide antibiotics,[8,9] did not promote cerulenin production, but rather inhibited it (Table 1). The precise mechanism of the inhibition is not known. It is speculated that inorganic phosphate released from magnesium phosphate in an acidic condition inhibited cerulenin production, or that the neutral pH values (7.0~7.5) in the presence of magnesium phosphate did not favor efficient production of cerulenin. Neither of the two possibilities is excluded at present, because the suppression of cerulenin production by inorganic phosphate was previously suggested,[7] and acidic pH values (5.0~5.5) were more favorable for cerulenin production according to our preliminary results (data not shown).

A variety of synthetic and natural zeolites are available today. To date, they have been utilized as catalysts in chemical industry[11,22] and as soil-modifying agents in agricultural and engineering works.[11] The present results suggest a new use of zeolites in fermentation technology. Although the present study was concerned with one kind of natural zeolite, it would be of interest to examine other kinds of natural and synthetic zeolites for efficient production of cerulenin.

Acknowledgement

The authors are grateful to Mr. H. MAMADA and Mr. H. KOBAYASHI for their helpful technical assistance.

References

1) ŌMURA, S.: Cerulenin. Methods in Enzymology. 72: 520~532, *Ed.*, J. M. LOWENSTEIN, Academic Press, New York, London, 1981
2) OKUDA, S.; S. IWASAKI, K. TSUDA, Y. SANO, T. HATA, S. UDAGAWA, U. NAKAYAMA & H. YAMAGUCHI: The structure of helvolic acid. Chem. Pharm. Bull. 12: 121~124, 1964
3) ŌMURA, S. & C. KITAO: Biosynthesis of macrolide antibiotics. Ferment. Ind. 37: 749~764, 1979
4) KITAO, C.; H. TANAKA, S. MINAMI & S. ŌMURA: Bioconversion and biosynthesis of nanaomycins using cerulenin, a specific inhibitor of fatty acid and polyketide biosyntheses. J. Antibiotics 33: 711~716, 1980
5) SADAKANE, N.; Y. TANAKA & S. ŌMURA: Hybrid biosynthesis of derivatives of protylonolide and M-4365 by macrolide-producing microorganisms. J. Antibiotics 35: 680~687, 1982
6) MCGUIRE, J. C.; B. K. HAMILTON & R. J. WHITE: Approaches to development of the daunorubicin fermentation. Proc. Biochem. 14: 2~5, 1979
7) IWAI, Y.; J. AWAYA, T. KESADO, H. YAMADA, S. ŌMURA & T. HATA: Selective production of cerulenin by *Cephalosporium caerulens* KF-140. J. Ferment. Technol. 51: 575~581, 1973
8) ŌMURA, S.; Y. TANAKA, H. TANAKA, Y. TAKAHASHI & Y. IWAI: Stimulation of the production of macrolide antibiotics by magnesium phosphate and related insoluble materials. J. Antibiotics 33: 1568~1569, 1980

9) TANAKA, Y.; Y. TAKAHASHI, R. MASUMA, Y. IWAI, H. TANAKA & S. ŌMURA: Enhancement and cultural characteristics of leucomycin production by *Streptomyces kitasatoensis* in the presence of magnesium phosphate. Agric. Biol. Chem. 45: 2475~2481, 1981
10) TANAKA, Y.; H. TANAKA, S. ŌMURA, K. ARAKI & K. NAKAYAMA: Magnesium phosphates stimulate microbial conversion of glycine to L-serine by release from regulation by ammonium ions. J. Ferment. Technol. 59: 447~455, 1981
11) HARA, N. & H. TAKAHASHI: Zeolites. Kōdan-sha Co., Tokyo, 1978
12) RUSSEL, J. A.: The colorimetric estimation of small amounts of ammonia by the phenol-hypochlorite reaction. J. Biol. Chem. 156: 457~461, 1944
13) EX, J. & E. HULTMAN: Determination of glucose and levulose in body-fluid. Nature 181: 780~781, 1958
14) IWAI, Y.; S. ŌMURA & T. HATA: Studies on the determination of glycerol in fermentation broth of antibiotic production by *Streptomyces* sp. J. Ferment. Technol. 49: 842~846, 1971
15) ŌMURA, S.; Y. TANAKA, C. KITAO, H. TANAKA & Y. IWAI: Stimulation of leucomycin production by magnesium phosphate and its relevance to nitrogen catabolite regulation. Antimicrob. Agents Chemother. 18: 691~695, 1980
16) HATA, T.; F. KOGA & H. KANAMORI: Studies on leucomycin. II. Bacteriological properties of *Streptomyces kitasatoensis*. J. Antibiotics, Ser. A 6: 109~112, 1953
17) BALDACCI, E. & R. LOCCI: Genus. II. *Streptoverticillium*. BERGEY's Manual of Determinative Bacteriology, 8th Edition, p. 829~842, *Ed.* R. E. BUCHANAN, The Williams & Wilkins Co., Baltimore, 1974
18) TONE, H.; A. KITAI & A. OZAKI: A new method for removal of inhibitory fermentation products. Biotechnol. Bioeng. 10: 689~692, 1968
19) LIU, C.-M.; L. E. MCDANIEL & C. P. SCHAFFNER: Factors affecting the production of candicidin. Antimicrob. Agents Chemother. 7: 196~202, 1975
20) MARTIN, J. F. & A. L. DEMAIN: Control of antibiotic biosynthesis. Microbiol. Rev. 44: 230~251, 1980
21) AHARONOWITZ, Y.: Nitrogen metabolite regulation of antibiotic biosynthesis. Ann. Rev. Microbiol. 34: 209~233, 1980
22) RABO, J. A.: Zeolite, Chemistry and Catalysis. ACS monograph 171, Am. Chem. Soc., 1976