Stimulative Effect of Elemental Sulfur on Siomycin Production by Streptomyces sioyaensis

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The addition of elemental sulfur to the fermentation of Streptomyces sioyaensis in a soybean meal medium resulted in a three- to fourfold increase of siomycin. Further experiments on the effect of elemental sulfur during fermentation suggest that one of the key steps stimulating siomycin synthesis is the utilization of thiosulfate, which accumulates in the medium as the result of the oxidation of elemental sulfur.

Siomycin is a sulfur-containing, slightly water-soluble chromoprotein antibiotic isolated from the mycelium of Streptomyces sioyaensis (2). It possesses high activity against gram-positive bacteria and mycobacteria and has chemical and biological properties similar to those of thioestrept (3). In view of the high sulfur content in the mycin molecule, attention was paid to the sulfur metabolism of this organism.

In our accompanying paper (5), S. sioyaensis was found to oxidize elemental sulfur and to accumulate thiosulfate in the medium. This report shows that the addition of elemental sulfur to the fermentation medium of S. sioyaensis caused marked stimulation of siomycin production. This stimulation appears to be the result of the utilization of thiosulfate, which accumulates as an oxidation product of elemental sulfur.

MATERIALS AND METHODS

Organism and culture conditions. S. sioyaensis U4-48-37, kindly supplied by M. Mayama of our laboratory, was used. Suspensions of lyophilized spores in skim milk were inoculated on slants prepared as follows. One hundred grams of potato was autoclaved with 1 liter of distilled water at 121 C for 20 min, and the solids were removed by filtration through a cheese cloth. The filtrate was brought up to 1 liter and 2% of yeast extract (Difco), 0.05% MgSO4·7H2O, 0.05% K2HPO4, 0.05% NaCl, 0.001% FeSO4·7H2O, and 2.0% agar were added. The pH was adjusted to 7.0. Inoculated slants were cultured for 7 days at 28 C and were used within 10 days. One-half of the spores obtained from a slant were inoculated in a Sakaguchi flask (500 ml capacity) containing 100 ml of Bennett’s medium. It was incubated at 28 C for 36 to 42 hr on a reciprocal shaker at 140 strokes/min with 5-cm amplitude. The production medium was composed of 7% sucrose, 2.33% soybean meal, 0.07% elemental sulfur, and deionized water. The pH was not adjusted. The medium was sterilized for 20 min at 121 C.

Triplicate fermentations were carried out in Saka- guchi flasks (500 ml capacity) containing 100 ml of medium inoculated with 3 ml of the inoculum. The fermentation flasks were incubated at 28 C for approximately 10 days on a reciprocal shaker at 140 strokes/min with 5-cm amplitude.

Assays. Siomycin was assayed by the method of Ebata (personal communication). Twenty to 30 ml of culture of S. sioyaensis was centrifuged (1,650 × g for 10 min), and the supernatant fluid was removed. The residual mycelium, which contains all of the siomycin, was collected on filter paper and dried under vacuum. After drying, the mycelium and solids were ground in a mortar to a homogeneous powder. The ground sample was transferred to an Erlenmeyer flask (50 ml capacity). Thirty milliliters of chloroform-methanol (3:1) was poured into the flask. After extracting siomycin at 40 C, the solvent was filtered through a sintered glass filter. The extraction procedure was repeated three times. Extracts were combined and concentrated under vacuum. The concentrated extract was transferred to a volumetric flask and was diluted to a suitable volume. One-tenth volume of this sample was transferred to a Kjeldahl-type flask and concentrated under vacuum to approximately 0.2 ml. All of the concentrated sample was spotted on a thin-layer plate (20 by 20 cm, 300 to 500 μm; Kieselgel G nach Stahl, E. Merck AG, Darmstadt). The chromatogram was developed in chloroform-methanol (9:1) at room temperature. The siomycin was detected by spraying with distilled water, and this area was removed and collected in a spitz glass while the plate was still wet. The siomycin containing Kieselgel G was dried and extracted with 3 to 4 ml of chloroform-methanol (1:1), and the extract was separated from the solids by centrifugation. This procedure was repeated three times. The extract was placed in a 10-ml volumetric flask and made up to volume with extraction solvent. The absorbancy of 270 nm was determined with a Hitachi-Perkin Elmer spectrophotometer model 139. The quantity of siomycin was calculated from the standard curve for siomycin.
Thiosulfate was determined by the method of Sörbo (4), after removal of protein by Cd\(^{2+}\) ions.

Sugar determinations were performed by the anthrone method (1).

Paper chromatography. Paper chromatography was done as previously described (4).

RESULTS

At the beginning of the investigation, the effects of various carbon and nitrogen compounds on siomycin production were studied. Sucrose and maltose were both found to be excellent nitrogen sources; soybean meal was an adequate nitrogen source. The base medium which was selected consisted of 7.0% sucrose and 2.33 soybean meal.

Stimulation of siomycin production by sulfur sources. *Streptomyces sioyaensis* grew well in this fermentation medium without the addition of a sulfur source. However, as indicated in Table 1, the addition of various sulfur sources (added concentrations to give 0.07% sulfur) markedly stimulated siomycin production.

This table shows that sulfur, supplied as \(\text{SO}_4^{2-}\), \(\text{S}_2\text{O}_8^{2-}\), methionine, and cysteine stimulated to various degrees the production of siomycin. The stimulating effect of elemental sulfur was marked with yields raised from 89 to 360 \(\mu\text{g}\)/ml.

**Effect of the amount of elemental sulfur added.**

Figure 1 shows that maximal stimulation of siomycin production occurred when 0.05 to 0.07 g of elemental sulfur was added per 100 ml of fermentation medium. With this addition, a three- to fourfold increase over yields obtained in the absence of added elemental sulfur was noted.

**Time course of the siomycin fermentation.** The time courses of siomycin fermentation in medium with or without the addition of elemental sulfur are illustrated in Fig. 2. When the pH had dropped to approximately 5.0, a marked increase in the rate of siomycin production was seen in the medium supplemented with elemental sulfur. During the next 96 hr, siomycin concentration

![Graph](https://via.placeholder.com/400)

**Fig. 1. Effect of the amount of elemental sulfur added on siomycin production.**

![Graph](https://via.placeholder.com/400)

**Fig. 2. Time course of siomycin production.** Symbols: \(\bigcirc\), the potency of siomycin in the culture with \(S^0\) (elemental sulfur, 0.07% at final concentration); \(\bullet\), the potency of siomycin in the culture without \(S^0\); \(\bigtriangleup\), the concentration of sucrose in the culture with \(S^0\); \(\bigtriangleup\), the concentration of sucrose in the culture without \(S^0\); \(\bigtriangledown\), pH changes in the culture with \(S^0\); \(\triangle\), pH changes in the culture without \(S^0\).

### Table 1. Effect of inorganic and organic sulfur compounds on siomycin production

| Sulfur compounds* | Final pH | Maximum potency (\(\mu\text{g}/\text{ml}\)) | Incubation time (hr) |
|--------------------|----------|---------------------------------|---------------------|
| \((\text{NH}_4)_2\text{SO}_4\) | 4.7 | 264 | 240 |
| \(\text{Na}_2\text{SO}_4\) | 5.5 | 150 | 240 |
| \((\text{NH}_4)_2\text{SO}_4\) | 5.2 | 0 | |
| \(\text{Na}_2\text{SO}_4\) | 5.0 | 0 | |
| \((\text{NH}_4)_2\text{S}_2\text{O}_8\) | 5.3 | 162 | 168 |
| \(\text{Na}_2\text{S}_2\text{O}_8\) | 5.2 | 123 | 192 |
| \(S^0\) (elemental sulfur) | 5.3 | 360 | 216 |
| L-Cysteine | 5.0 | 120 | 240 |
| L-Methionine | 5.0 | 218 | 216 |
| D,L-Methionine | 5.0 | 180 | 240 |
| Taurine | 7.3 | 0 | |
| L-Cysteic acid | 4.3 | 0 | |
| None | 6.0 | 89 | 216 |

* Sulfur compounds were added to the basal medium to give a final concentration of 0.07% sulfur.
appreciable decrease of thiosulfate was observed after 144 hr of cultivation. The timing of the thiosulfate decrease coincided with the time of active antibiotic production (Fig. 2). Polythionates were barely detectable throughout the fermentation, either spectrophotometrically or paper chromatographically.

Timing of addition of elemental sulfur. Elemental sulfur was added to fermentation flasks at 0, 48, 96, 144, and 196 hr after inoculation, and the fermentation was continued until 240 hr. Figure 4 shows that each addition of elemental sulfur resulted in an increase in the rate of siomycin production. The highest yield was obtained when the sulfur was added at 48 hr.

Effect of thiosulfate on siomycin production. To demonstrate further the role of elemental sulfur in antibiotic synthesis, thiosulfate was added to the fermentation at various times. Figure 5 shows that the medium supplemented with thiosulfate gave results similar to those obtained with medium supplemented with elemental sulfur except that the highest siomycin production was observed when thiosulfate was added at the start of fermentation rather than at 48 hr, as was the case with elemental sulfur. Figure 6 shows the rate of disappearance of thiosulfate.

Changes in the thiosulfate concentration of the medium with and without the addition of elemental sulfur are shown in Fig. 3. A slow but constantly increased. At 240 hr, siomycin synthesis ceased in spite of the fact that about one-third of the initial sucrose remained. Elemental sulfur inhibited sugar utilization to some extent (Fig. 2).

Fig. 3. Changes of concentration of thiosulfate during production of siomycin. Symbols: O, culture supplemented with S⁰; ●, culture without added sulfur sources except that contained in soybean meal.

Fig. 4. Effect of the addition of elemental sulfur at various times during the fermentation on siomycin. S⁰ (elemental sulfur; 0.07% at final concentration), was added at the times indicated by the arrows. Curves A, B, C, D, and E show the potency of siomycin in the cultures added S⁰ at various times. Curve F shows the potency of siomycin in the culture without added S⁰.
from these media. The decrease in thiosulfate was accompanied by a concomitant increase in siomycin production. From these results it may be concluded that siomycin production occurred at the expense of thiosulfate.

**DISCUSSION**

The results of the study reported here show that a significant stimulation of siomycin synthesis occurred when elemental sulfur was added to the medium. This probably means that either the content of sulfur in the medium is not sufficient for the maximal production of siomycin, which contains sulfur in its molecule, or that the sulfur in the medium is in a form not readily utilizable as a building block for siomycin. This investigation also shows that the timing of increased siomycin synthesis coincided with that of a decrease in thiosulfate, which is the oxidation product of elemental sulfur. This observation can be compared to those presented in our accompanying paper (5), where a large amount of the oxidation products of elementary sulfur was thiosulfate. The production of siomycin in the medium used in this accompanying paper was less than that in present medium by at least one order of magnitude. This suggests that the elemental sulfur, which was oxidized to thiosulfate, was utilized as a sulfur source for siomycin synthesis.

A marked stimulation in antibiotic synthesis also occurred with the addition of ammonium thiosulfate (Fig. 5 and 6) or ammonium sulfate (unpublished data). In medium supplemented with ammonium sulfate, thiosulfate at first accumulated and then decreased as siomycin production increased which implies that one of the limiting steps in the stimulation of siomycin synthesis is the utilization of thiosulfate. Although the manner in which S. sioyaensis metabolizes thiosulfate is unknown, further study may provide information concerning the role of sulfur compounds in the biosynthesis and regulation of siomycin synthesis.

This study is believed to be the first to show that elemental sulfur, the cheapest of sulfur compounds, can be used as a sulfur source for the fermentation medium of a heterotrophic microorganism. Since, in our accompanying paper (5), other streptomycetes have been shown to oxidize sulfur, its addition may be beneficial in the production of other antibiotics.

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**LITERATURE CITED**

1. Morris, D. L. 1948. Quantitative determination of carbohydrates with Dreywood's anthrone reagent. Science 107:254-255.
2. Nishimura, H., S. Okamoto, M. Mayama, H. Ohtsuka, K. Nakajima, K. Tawara, M. Shimohira, and N. Shimaoka. 1961. Siomycin, a new thioestrepton-like antibiotic. I. Antibiot. Ser. A 14:255-263.
3. Pagano, J. F., M. J. Weinstein, H. A. Stout, and R. Donovick. 1955/56. Thioestrepton, a new antibiotic. I. In vitro studies. Antibiot. Annu., p. 554-559.
4. Sörbo, B. 1937. A colorimetric method for the determination of thiosulfate. Biochim. Biophys. Acta 23:412-416.
5. Yagi, S., S. Kitai, and T. Kimura. 1971. Oxidation of elemental sulfur to thiosulfate by Streptomyces. Appl. Microbiol. 22:157-159.