FERMENTATIVE PREPARATION OF S^{35}-SIOMYCIN, A SULFUR-CONTAINING PEPTIDE ANTIBIOTIC

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Siomycin is a sulfur-containing peptide antibiotic which resembles thiostrepton in structure[2] and is produced by *Streptomyces sioyaensis*[1]. The antibiotic shows high activity against various gram-positive bacteria including *Mycobacterium tuberculosis* and many clinical resistant strains of *Staphylococcus*.

YAGI *et al.*[3] recently reported that the yield of siomycin is increased three- to four-fold by the addition of elemental sulfur to the fermentation broth where the sulfur is oxidized to thiosulfate, and also that the incorporation of thiosulfate ions is one of the key steps in siomycin biosynthesis.

This work has been carried out in order to prepare S^{35}-siomycin for pharmacological use, and owes much to the above work of YAGI *et al.*[3]

Materials and Methods

1. Microorganisms Used: *Streptomyces sioyaensis* U_3-48-37 strain was used in the fermentation. The strain is one of a number of mutant strains in our stock, and has high siomycin production potency (400~700 μg/ml).

2. S^{35}-Ammonium Sulfate Solution: Five mci of carrier free S^{35}-sulfuric acid (0.25 ml of 0.05 N HCl solution in a capped vial, Daiichi Pure Chemicals Co., Ltd., Lot. 510-30) was transferred into a small flask and neutralized completely with excess 18% NH_4OH solution. The alkaline solution was mixed with 2 ml of 0.05% cold ammonium sulfate solution and dried *in vacuo*. The solid obtained was dissolved in 2 ml of water and again dried. This procedure was repeated a further three times. The resulting neutral preparation was then dissolved in water and made up to 10 ml exactly, and stored aseptically at 0°C. The radioactivity of this solution was 526 μci per ml.

3. Fermentation Process: Fresh vegetative mycelium of strain U_3-48-37 grown on BENNET's agar slant (3~7 days at 27°C) was inoculated into 10 ml of seed medium (glucose 0.5%, peptone 1.0%, beef extracts 0.5%, NaCl 0.3%, pH 7.0), and cultured on a reciprocal shaker at 29°C for 3~4 days (240 rpm). An inoculum of 5~8% of the rusulting seed broth was then used to inoculate the fermentation flasks. The composition of the fermentation medium was as follows: sucrose 7%, soybean meal 2.5%, ammonium sulfate 0.3%, pH 7.0, 25 ml of medium being used per 200 ml flask. The fermentation was carried out on a rotary shaker at 200 rpm for about 10 days at 30°C. Feeding of S^{35}-ammonium sulfate solution (see above) in 50 μci or 1 mci amounts at various stages of the fermentation was investigated. The addition of 1 mci at zero hour proved to be the most favorable.

4. Analysis of Radioactivity: A Nuclear-Chicago Liquid Scintillator (720 series) and dioxan scintillation fluid (PPO 7 g, POPOP 0.3 g, naphthalene 100 g, dioxan 1 liter) were used in this study. For TLC analysis, Aloka Radioscanner and X-ray film were also employed.

5. Isolation of S^{35}-Siomycin: The fermented broth (25 ml) was centrifuged at 3,000 rpm for 15 minutes in order to separate cellular matter from supernatant. The cellular part was extracted by stirring with 10 ml of chloroform-methanol, 1:1 mixture. The supernatant was also extracted with 20 ml of chloroform. Both solvent extracts were combined, dried with Na_2SO_4, and then dried *in vacuo*. The resulting syrup was extracted again with 4 ml of chloroform-methanol mixture, 1:1, and dried below 30°C *in vacuo*. This procedure was repeated a further three times. The final extract thus obtained was added to an authentic sample of unlabelled siomycin (8 mg). The mixture was dissolved in 0.3 ml of chloroform-methanol mixture, 1:1, and applied to Kieselgel GF plates (20×20 cm, 500 μ in

thickness, 2 plates). After developing with chloroform - methanol, 10 : 1, the siomycin zone (Rf 0.69) was removed and eluted with about 30 ml of chloroform - methanol, 4 : 1. The eluate was chromatographed again on the same Kieselgel GF plates as above. The siomycin fraction thus obtained showed 1 spot on TLC and TLC-radioautogram (Fig. 1). The purified material was recrystallized from chloroform-ether to give radioactive siomycin, which was confirmed to be identical with siomycin by recrystallization after addition of the authentic sample. In Fig. 1, the TLC-radioautographic pattern of the crude preparation is shown. The crude extract exhibited two radio active spots at Rf 0 and 0.69. The spot at Rf 0 was more radioactive than the spot at Rf 0.69 (siomycin). No other radioactive spot was detected. Therefore, it is easy to separate radioactive siomycin from other radioactive substances.

Results and Discussion

In the microbial preparation of radioactive metabolites, a cell-free enzyme system or the washed cell suspension technique is more popular than fermentation methods. In general, it is considered that the products obtained by fermentation have less radioactivity than is the case using the other techniques.

However, S^{35}-siomycin of relatively high specific activity can be prepared by the following fermentation method. Techniques for both the fermentation and the isolation are very simple to perform and the incorporation ratio is relatively good.

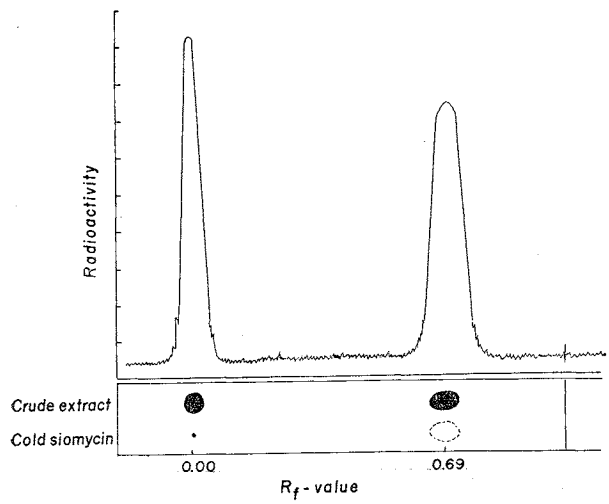
Experiment 1: In this lot, 1.0 mci of S^{35}-ammonium sulfate (2.0 ml of stock solution) was added to 25 ml of fermentation medium at the zero hour stage. All the fermentation and isolation steps were carried out as described above. The fermentation was continued for 10 days, the pH of the fermentation broth being 5.0 at 6 days and 4.6 at 10 days. By adding 8 mg of cold siomycin to the hot siomycin fraction, 15 mg of hot siomycin was obtained. The specific activity was 3.3×10^6 dpm per mg, and the incorporation ratio was 2.3 %.

Experiment 2: In this run, feeding of S^{35}-ammonium sulfate (1 mci per 2 ml) was carried out at 6 days. Otherwise the procedures were as in experiment 1. A final yield of 14.6 mg of purified S^{35}-siomycin was obtained. The specific activity was 1.0×1.0^6 dpm per mg, and the incorporation ratio was 0.6 %.

From above two experiments, it it clear that the incorporation ratio in experiment 2 was considerably lower than that in experiment 1. The sole difference between experiments 1 and 2 was the feeding stage of hot ammonium sulfate. Therefore, it would appear that the early feeding at 0 hour is more effective than the late feeding at 6 days.

In order to increase the incorporation ratio, several trials were carried out involving modification of the fermentation medium including omission of the ammonium sulfate. All the trials were not successful however. A marked decrease in incorporation ratio (to about one tenth) was observed when the ammonium sulfate was omitted. Therefore, the presence of cold ammonium sulfate is important in this technique even

Fig. 1. TLC-autoradiogram and TLC-radioactinogram
Silicagel Kieselgel GF (Merck).
Solvent system : $CHCl_3$-MeOH, 10 : 1



though S^{35}-sulfate is thereby diluted to 2.7 $\times 10^{-3}$. However, the specific activity of S^{35}-siomycin may be increased by reducing the amount of cold ammonium sulfate in the fermentation medium to some extent.

As shown in experiment 1, 15 mg of S^{35}-siomycin preparation (5×10^7 dpm in total) was isolated from 25 ml of broth which contained 1.0 mci of S^{35}-ammonium sulfate. The incorporation ratio is therefore 2.3 %. In assuming (1) the purified hot siomycin contains 8.31%* of sulfur, and (2) the net yield of hot siomycin is about 7 mg (the remaining 8 mg is cold authentic sample), it can be concluded that 69 % of the sulfur atoms in the hot siomycin produced (*ca.* 7 mg) are derived from inorganic sulfate. This conclusion confirms strongly the report of YAGI *et al.*[3] that the sulfur atoms of siomycin are derived to a large extent from inorganic thiosulfate and that incorporation of sulfate ions is one of the key steps in siomycin biosynthesis.

Summary

S^{35}-Labelled siomycin showing significant radioactivity was prepared by a fermentation method using *Streptomyces sioyaensis* U_3-48-37. In a typical lot, hot siomycin (5×10^7 dpm in total) was obtained from a fermented broth which contained 1.0 mci of S^{35}-ammonium sulfate, with an incorporation of 2.3 %. The fermentation was carried out at 29°C for about 10 days on a rotary shaker. The feeding of hot ammonium sulfate at zero hour stage was more effective with regard to the radioactive yield than was feeding at 6 days. Both the fermentation and isolation techniques of this method are very simple and easy to perform. The incorporation of sulfur from inorganic sulfate into siomycin is highly efficient, approximately 70 % of the sulfur atoms in the hot siomycin preparation being derived from sulfate ions in the fermentation medium. The results confirm the conclusions of YAGI *et al.* that the utilization of inorganic sulfate is one of the key steps in siomycin biosynthesis.

References

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* From elemental analysis of cold authentic siomycin.