Kor. J. Appl. Microbiol. Biotechnol.
Vol. 19, No. 6, 588-591 (1991)

Laboratory Scale Preparation of S-Adenosyl-L-Methionine from Yeast

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효모로부터 S-Adenosyl-L-Methionine의 실험실 규모 생산

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Aabstract — S-adenosyl-L-methionine (SAM) is essential substrate for biological methylation reactions. The present work describes a reoptimized procedure of SAM preparation in laboratory scale by the method of yeast fermentation. The fermentation medium enriched with methionine and the culture conditions were reoptimized. The isolation steps consisted of 5 steps including extractions, precipitations, and chromatography. This improved procedure over original method provides relatively high yield of biologically active product within a 4 day-period.

As part of our efforts for studying some methyltransferases (1-3). we have undertaken to prepare a large amount of S-adenosyl-L-methionine (SAM). SAM is essential substrate for biological methylation reactions. The methyltransferase reactions occur in a wide variety of biomolecules such as proteins, nucleic acid, phospholipid, carbohydrate, and biogenic amines. The subject dealing with the biochemistry and biological significance of SAM has been reviewed thoroughly (4, 5). Several methods of preparation of SAM were also reported (6-10).

The present work describes a reoptimized procedure of SAM preparation in laboratory scale using simple flask-type fermentor by the cultivation of yeast originally developed by Schlenk and DePalma (6). The fermentation medium was enriched with methionine and the culture conditions were readjusted for optimum accumulation of intracellular SAM. The isolation of SAM was achieved by the method of Schlenk *et al.* with slight modification (11). Isolation steps involve perchloric acid extraction, ammonium reineckate precipitation, Dowex 50 H^+ ion-exchange chromatography, phosphotungstic acid precipitation, and extraction with a series of organic solvent systems.

Key words: S-adenosyl-L-methionine, yeast fermentation, isolation, bioassay
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Materials and Methods

Materials

Bakers' Yeast was purchased from Choheung Chemical Industrial Co (Seoul). S-adenosyl-L-methionine, S-adenosyl-L-homocysteine, ammonium reineckate, and Dowex 50 H^- ion-exchangee resin were obtained from Sigma (St. Louis) and L-methionine from Merck (Darmstadt). Phosphotungstic acid was product of Wako Pure Chemical Industries (Osaka). All other chemicals used were reagent grade commercially available.

Medium and Cultivation

A laboratory scale simple fermentor was built in a temperature controlled water-bath-type reciprocal shaker. A 2 liter erlenmeyer flask fitted with two-hole stopper was used as a cultivation chamber. The aeration was performed by a plastic tube connected to an air pump was introduced through one hole in the stopper. Into the cultivation flask 1 liter

of fermentation medium containing the following ingredients was introduced: 2g of KH_2PO_4, 1g of K_2HPO_4, 5g of $(NH_4)_2SO_4$, 1g of trisodium citrate, 0.3g of $MgCl_2$, 15g of glucose, and 0.75g of L-methionine. A portion of 12.5g of active dry bakers' yeast were pured into the medium at a temperature of 30℃. A homogeneous suspension was obtained under vigorous shaking and aeration. After 4 hours, second charge of 10g glucose was added and after 8 hours, third charge of 10g glucose was added. After 24~30 hours cultivation, the fermented yeast was harvested and washed twice with an excess of cold water.

Isolation of SAM

The washed yeast was extracted with 3 volumes of 1.5 N perchloric acid with vigorous stirring at room temperature for 2 hours. The cell debris was discarded and clear supernatant was decanted after centrifugation at 6,000×g for 20 min. Crude SAM was precipitated from the decanted supernatant by the addition of one-half volume of a cold saturated solution of reshly prepared ammonium reineckate solution. The mixture was placed at low temperature for a few hours and precipitate formed was collected by centrifugation at 14,000× for 20 min.

Dowex 50 H^+ chromatography

The precipitate was dissolved in a 10~20 fold amount of a mixture of acetone/2 N H_2SO_4 (1:1, v/v), and the resulting dark purple solution was applied to a Dowex 50 H^+ column (0.7×10 cm) which had previously been equilibrated with the same mixture. The column was washed with 1 liter of 2 N H_2SO_4 at a flow rate of 150 m*l*/hr. SAM was eluted with 6 N H_2SO_4 at a flow rate of 40 m*l*/hr. The eluting was continued until the absorbance at 256 nm was less than 0.1 (about 1 liter of the acid was required). 6 N H_2SO_4 eluate was pooled and the amount of SAM was determined by the absorbance at 256 nm ($\varepsilon = 15{,}000$).

Phosphotungstic acid precipitation

To the 6 N H_2SO_4 eluate was added 20% (w/v) phosphotungstic acid solution (10 m*l*/100 μmole of SAM) with stirring. After the mixture was stood at low temperature, precipitate formed was collected by centrifugation at 6,000×g for 20 min and washed twice with excess of cold water.

Extraction of SAM with organic solvent system

The precipitate was dissolved in 4~6 volumes of acetone/water mixture (1:1, w/v). The acetone/water mixture was extracted with 4 volumes of a mixture of isoamyl alcohol and ether (1:1, v/v). The upper organic solvent phase was discarded. The extraction was repeated four times further with 2 volumes of the isoamyl alcohol/ether mixture. Residual acetone and isoamyl alcohol were removed by five extractions, each with several volumes of ether. To this solution, a stream of nitrogen gas was blown through to remove ether. The concentration of this preparation was determined by absorbance at 256 nm. The solution was diluted to 12 mM with distilled water and stored as 3 m*l* protions at −20℃.

Analysis of prepared SAM

In order to identify the prepared SAM and determine homogeneity of the product, paper chromatogrphy was carried out. Appropriate amount of the product was chromatographed on Whatman No. 1 paper with ethanol/water/acetic acid (65:34:1) solution. Commercial SAM and methionine were chromatographed in parallel. After chromatography was finished, ninhydrin solution was sprayed on the paper.

The biological activity of prepared SAM was determined by assaying the activity of catechol-o-methyltransferase. The enzyme was extracted from rat liver and the activity was assayed spectrophotometrically using p-nitrocatechol as substrated (1). The reaction mixture contained 0.1 M sodium phosphate buffer (pH 7.8), 2 mM $MgCl_2$, 0.4 mM SAM, 0.04 mM p-nitrocatechol, and the enzyme solution (protein; 0.02~0.03 mg) in a final volume of 1.0 m*l*. One unit of the enzyme activity is defined as 1 nmole of the product formed in 1 min at 37℃ under the standard assay condition.

Results and Discussion

As summarized in Table 1, the effects of various

Table 1. Fermentation condition of bakers' yeast on the yield of SAM

#	T (℃)	pH	degree of aeraction	degree of foaming	glucose adding (g/*l*) 0 hr	4 hr	6 hr	8 hr	yield of SAM (% of control[3])
1	30	6.41	below average	below average	15			10	85
2	30	6.26	above average	extensive[2]	15		10		104
3	30	5.45	average[1]	below average	15		20		65
4	30	5.00	above average	extensive	15	5		5	163
5	30	4.83	minimal	minimal	30	20			69
6	30	—	minimal	average	15	10	10		70
7	30	4.98	average	extensive	30		10		70
8	35	5.26	below average	minimal	15		10		79
9	35	5.16	minimal	below average	15		10		48
10	37	5.69	average	average	15		20		57

[1] Average aeration refers to 3 *l*/min air indroduction to the cultivation flask. Above average was more than 5 *l*/min. At this aeration condition the dissolved oxygen was kwown to be enough to saturate the medium.
[2] The extensive foaming indicates usually continuous overflowing of foaming medium from the flask.
[3] Control refers to the yield of 14 μmoles/1 g of yeast. The control yield was obtained from a preliminary culture condition.

fermentation conditions such as pH, aeration, and glucose addition on the yield of SAM were cross-checked. The best result was achieved by the condition No. 4. Addition of glucose by several portions gave better result than addition in one single portion. When the extent of aeration and shaking was maintained as vigorously as possible, the yield was improved considerably. Under the standard condition of pH 5.0 and 30℃ cultivation, an optimal yield of SAM was 24 μmoles/1g of yeast. This yield is about 20% better than the original procedure developed for bakers' yeast (6). However using other strain of yeast *S. sake* K-6, it is achieved much higher yield of SAM (9). After SAM was extracted with perchloric acid solution, SAM was precipitated by the addition of ammonium reineckate solution. The precipitate formed was dissolved and applied to Dowex 50 H^+ column. The chromatography was performed with 6 N H_2SO_4 solution, and the result was shown in Fig. 1. By the extensive washing with 2 N H_2SO_4, the contaminant sulfonium compounds such as S-ribosyl-L-methionine and S-adensoyl-L-homocysteine were removed. The purity of SAM in each step of isolation was performed by paper chromatography.

In order to identify the prepared SAM, UV-spectrum ranged from 210 nm to 290 nm was obtained.

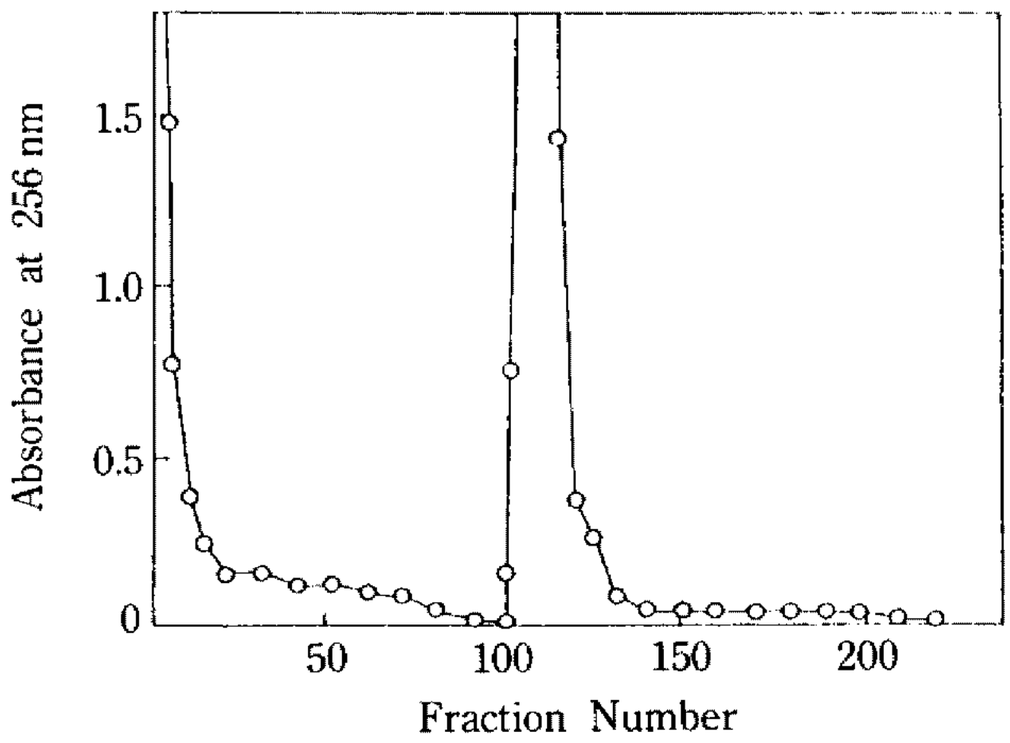

Fig. 1. Dowex 50 H^+ Chromatography of SAM. Column was washed with 2 N H_2SO_4 and SAM was eluted with 6 N H_2SO_4.

As shown in Fig. 2, the peak at 256 nm of prepared SAM was similar to that of authentic SAM. Paper chromatography was also performed to identify the product and determine the purity. For comparison, commercial SAM (SAM iodide), S-adenosyl-L-homocysteine, and methionine were chromatographed in paralled. The R_f value for prepared SAM was 0.4 and was identical to that of commercial SAM. For all instances, the prepared SAM revealed only one spot. We assayed biological activity of the prepared SAM by assaying the activity of catechol-o-methyltransferase (1). For comparison, commercial

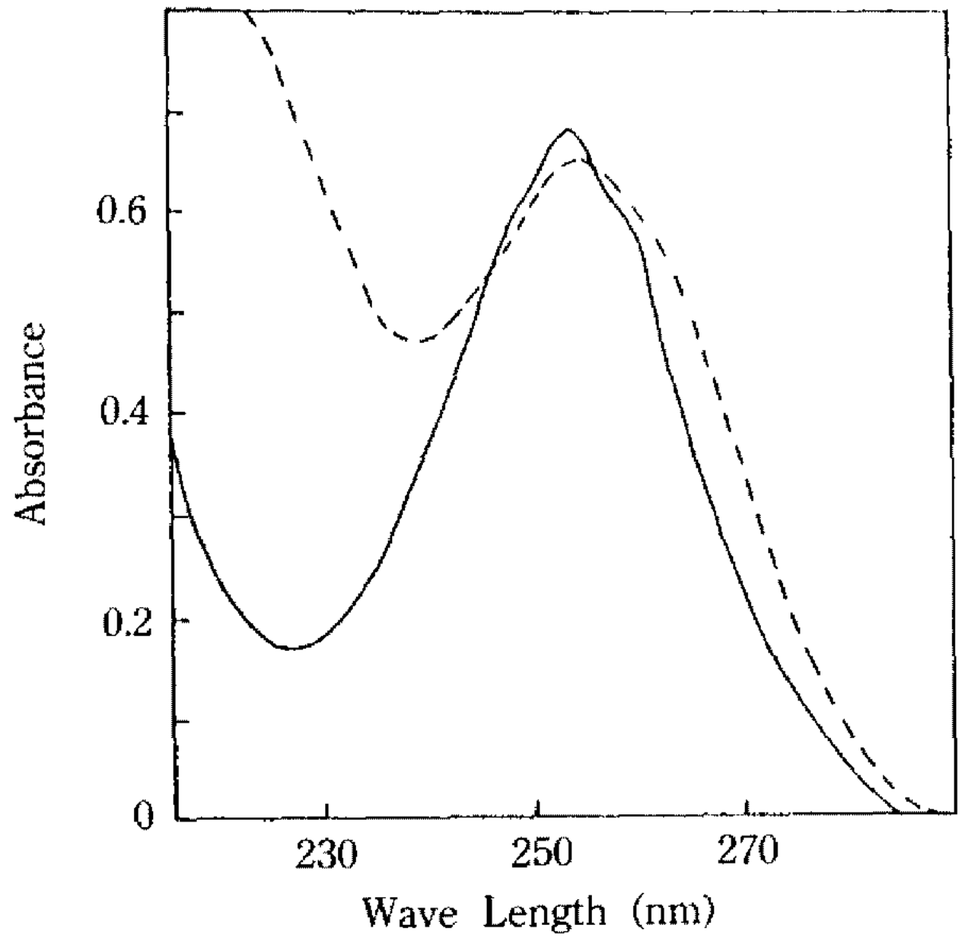

Fig. 2. UV-spectra of prepared SAM (—) and commercial (---) SAM.

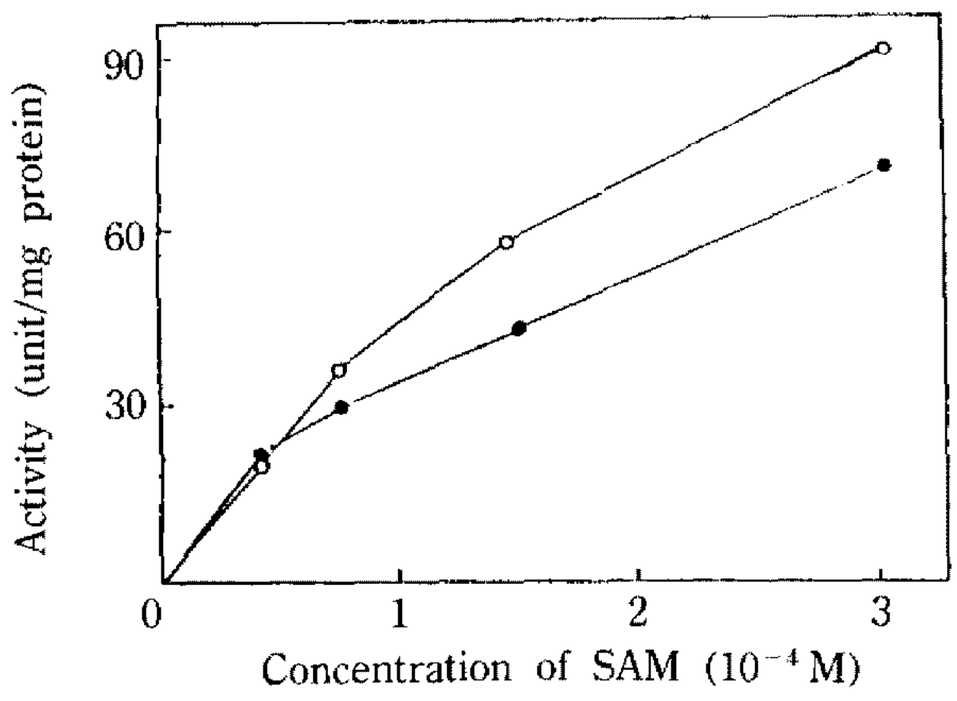

Fig. 3. Bilological activity of prepared SAM (○−○) and commercial SAM (●−●).
Catechol-o-methyl-transferase was employed for the assay of biological activity of SAM.

SAM was assayed in paralled. From the result in Fig. 3, we could confirm that the prepared SAM is more biologically active than commercial SAM. For obtaining the biologically active SAM extensive extraction with organic solvent (isoamyl alcohol/ether) was essential. Since the trace amount of acetone seemed to have the inhibitory effect to SAM activity.

In summary, the reoptimized procedure for biologically active SAM by the method of bakers' yeast fermentation in a methionine enriched medium has been achieved by altering cultivation conditions including glucose addition. A major advantage over the original method was in respect to its high yield of biologically active product. In an one-liter bench scale, about 300 μmoles (ca. 150 mg) of SAM was obtained within a 4 day-period.

요 약

S-adenosyl-L-methionine(SAM)은 생체 메칠화 반응에 긴요한 기질이다. 이 논문은 효모 발효에 의한 SAM의 실험실 규모 생산의 최적조건을 다시 검토한 것이다. 발효 배지는 메치오닌을 첨가했으며 배양조건들을 재조절하였다. 분리과정은 추출, 앙금 및 크로마토그래피를 포함한 다섯단계로 이루어졌다. 이 향상된 과정은 원래 방법보다 비교적 높은 생산 수득률로 생활성있는 SAM을 4일 이내에 제공해준다.

Acknowledgement

This study was supported in part by the Basic Science Research Institute Program, Ministry of Education, 1989, project No. ED89-37.

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(Received July 24, 1991)