Production of Coenzyme A by Sarcina lutea

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To develop an efficient method for the production of coenzyme A (CoA), optimal conditions for its formation from pantothenic acid, cysteine, and adenine were studied. A number of microorganisms were screened for production of CoA. Strains belonging to the genera Sarcina, Bacillus, Microbacterium, Micrococcus, and Serratia accumulated CoA. Among these, Sarcina lutea was selected as the best organism, and the culture conditions for the production of CoA were investigated with this organism. Under optimal conditions, 600 μg of CoA per ml was accumulated in the culture broth. CoA was readily isolated in high purity by the use of charcoal, diethylaminoethyl-cellulose, Sephadex G-25, and Dowex-50. Yields of isolated CoA were over 33% from culture broth.

It is well known that coenzyme A (CoA) plays an important role in many metabolic processes of living cells, such as the metabolism of carbohydrates, fats, and amino acids or the biosynthesis of ferroprotoporphyrin. Therefore, various chemical syntheses of CoA from pantothenic acid and adenosine 5'-monophosphate (7, 8, 14), and the isolation method from natural sources such as hog liver (5) and yeast (17), have been developed. However, these hitherto known methods are not satisfactory for the industrial preparation of CoA, because of the complexity of the synthetic routes or the low contents of this coenzyme in hog liver and yeast. Alternatively, the fermentative preparation of CoA, using hydrocarbon as the sole source of carbon, was reported (4). In this method, the accumulation of CoA in the fermentation broth was low. Shimizu et al. (11, 12, 15) reported production of CoA in higher yield from pantothenic acid, cysteine, and adenosine 5'-triphosphate (ATP) or adenosine 5'-monophosphate by dried cells of Brevibacterium ammoniagenes and bakers' yeast. Recently, they also reported an improved method for the fermentative production of CoA from the same substrates, using the culture broth of B. ammoniagenes (11, 13).

No report has appeared on the production of CoA from pantothenic acid, cysteine, and adenine using a microbial CoA synthesizing system.

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MATERIALS AND METHODS

Organisms. Screening tests were performed on 132 strains of bacteria and 61 strains of yeast, all from the collection in this laboratory. Sarcina lutea IAM 1099 was used for the fermentation experiments.

Reagents. Unless otherwise specified, all chemicals were Katayama (Osaka) certified reagent grade. Phosphotransacetylase from Clostridium kluyveri and acetylphosphate and luciferase were purchased from C. F. Boehringer & Soehne GmbH, Mannheim, Germany. Pigeon liver acetone powder was obtained from Sigma Chemical Co., St. Louis, Mo. Diethylaminoethyl-cellulose was obtained from Green Cross Co-Operation (Osaka).

Screening experiments. Slant cultures of bacteria were grown on an agar medium containing 0.25% peptone, 0.25% meat extract, 0.25% yeast extract, and 0.5% NaCl. Slant cultures of yeast were prepared on malt extract agar. All organisms were grown for 24 h at 30 C. The screening medium of bacteria contained 5% glucose, 0.1% yeast extract, 1% peptone, 1% meat extract, 0.5% NaCl, 1% KH2PO4, 1% K2HPO4, and 0.1% MgSO4·7H2O. The yeast cultures were grown on 5% glucose, 0.2% yeast extract, 0.5% peptone, 0.5% (NH4)2SO4, 0.2% KH2PO4, 0.2% K2HPO4, 0.1% MgSO4·7H2O, and 1% CaCO3. These media were adjusted to pH 7.0 with KOH, distributed in 5-ml amounts in test tubes, and sterilized. After inoculation from the slant cultures, shaking was carried out for 48 h at 30 C. Calcium pantothenate, cysteine-hydrochloride, and adenine, each at a concentration of 0.1%, were added to 48-h cultures, and the cultivation was continued for a further 48 h. The whole broth was boiled for 10 min and centrifuged. The supernatant fluid was employed for the determination of CoA.

Fermentation experiments. Unless otherwise noted, culture experiments for CoA production were...
carried out as follows. The respective media were distributed in 25-ml amounts in shake flasks (500-ml), sterilized, and inoculated with 1 loopful of the selected organism. The cultures were incubated at 30 C for 48 h with reciprocal shaking (140 rpm, 8-cm stroke). Additions and harvesting were carried out as described in the previous section.

Methods of analysis. (i) Determination of growth. The fermentation broth was diluted 1:100 with saline, and absorbance was measured at 660 nm with a Hitachi photoelectric photometer (model 101). Dry cell weight was estimated from a standard curve which correlated optical density to weight of lyophilized cells.

(ii) Assay of CoA synthetic activity. The reaction mixture, which contained 10 mM calcium-pantothenate, 10 mM cysteine-hydrochloride, 15 mM ATP, 10 mM MgSO4 • 7H2O, 150 mM potassium phosphate buffer (pH 6.5), and 100 mg of acetone dried cells, was incubated at 37 C for 2 h in a total volume of 1 ml. The reaction was stopped by heating for 5 min in boiling water. After centrifugation, the supernatant solution was assayed for CoA. CoA synthetic activity was defined as that activity which synthesizes 1 μg of CoA per mg of dry cells per h.

(iii) Analyses. CoA was determined with the pigeon liver method (9) in screening experiments and with phosphotransacetylase (6, 9) in fermentation experiments. Paper chromatography was carried out by the ascending technique on Toyo filter paper no. 50. The solvent system used was isobutyric acid-0.5 N ammonium hydroxide (5:3). Adenine-containing compounds were located with ultraviolet light. The spot corresponding to adenine was cut out and eluted with 0.1 N HCl. Absorbance at 260 nm was measured, and the amount of adenine was calculated by using the molecular extinction coefficient of an authentic sample. Phosphoric esters were detected with the Hanes and Isherwood spray (3) followed by ultraviolet irradiation, and sulfhydryl and disulfide compounds were detected with the Toennies and Kobl spray (19). ATP was determined with luciferase by using the Tri-Carb liquid scintillation spectrometer (model 3330, Packard Instrument Co., Inc., Downers Grove, Ill.) (18). Panthenolic acid and cysteine were determined by microbioassay using Lactobacillus plantarum ATCC 8014 (16) and Leuconostoc mesenteroides P-60 (1), respectively. Pentose was determined by the phloroglucinol reaction (2).

RESULTS

Screening experiments of organisms. CoA formation was observed with a considerable number of organisms when the reaction mixtures were incubated for 48 h at 30 C (Table 1). Marked CoA formation was found among strains of the genera Bacillus, Microbacterium, Micrococcus, Sarcina, and Serratia. Several strains of yeast accumulated CoA.

CoA-synthesizing activities were compared among the strains which produced high levels of CoA (Table 2). Of the tested organisms, Sarcina lutea IAM 1099 showed the highest activity in the production of CoA, and this strain was chosen for the following experiments.

Effect of carbon sources. The effect of carbon sources at a concentration of 5% on CoA production was investigated (Table 3). Glucose,

| Genus           | No. of strains tested | No. of CoA-forming strains |
|-----------------|-----------------------|----------------------------|
| **Achromobacter** | 15                    | 3                          |
| **Aerobacter**   | 1                     | 0                          |
| **Agrobacterium**| 2                     | 1                          |
| **Alcaligenes**  | 7                     | 0                          |
| **Arthrobacter** | 1                     | 0                          |
| **Bacillus**     | 17                    | 7                          |
| **Bacterium**    | 2                     | 1                          |
| **Brevisbacterium** | 3                  | 1                          |
| **Corynebacterium** | 3                   | 1                          |
| **Escherichia**  | 4                     | 2                          |
| **Flavobacterium** | 3                   | 1                          |
| **Klebsiella**   | 2                     | 1                          |
| **Microbacterium** | 1                    | 1                          |
| **Micrococcus**  | 1                     | 1                          |
| **Proteus**      | 16                    | 11                         |
| **Pseudomonas**  | 36                    | 3                          |
| **Sarcina**      | 12                    | 11                         |
| **Serratia**     | 3                     | 3                          |
| **Staphylococcus** | 1                    | 1                          |
| **Xanthomonas**  | 2                     | 1                          |
| **Ashbya**       | 1                     | 0                          |
| **Brettanomyces** | 1                    | 0                          |

**Table 1. Screening of microorganisms for CoA formation**

**Table 2. CoA formation by yeast strains**

**Table 3. Effect of carbon sources on CoA formation**

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TABLE 2. Accumulation of CoA by selected bacteria

| Microorganism                  | CoA (µg/ml) |
|-------------------------------|-------------|
| Achromobacter liquidum        | 10.2        |
| Bacillus aneurinolyticus      | 36.7        |
| B. cereus                     | 41.7        |
| B. subtilis                   | 30.0        |
| Flavobacterium arborescens    | 31.3        |
| Microbacterium flauum         | 36.8        |
| Micrococcus rubens            | 40.5        |
| Pseudomonas myxogenes         | 23.1        |
| Sarcina aurantiaca            | 69.8        |
| S. lutea                      | 90.0        |
| S. variabilis                 | 18.0        |
| Serratia marcescens           | 49.3        |
| Xanthomonas pruni             | 18.3        |

TABLE 3. Effect of carbon sources on CoA formation by S. lutea

| Carbon source | Growth (mg/ml) | CoA (µg/ml) |
|---------------|----------------|-------------|
| Ribose        | 6.0            | 46.2        |
| Xylose        | 6.5            | 17.0        |
| Fructose      | 17.0           | 60.0        |
| Galactose     | 8.0            | 22.2        |
| Glucose       | 18.6           | 100.4       |
| Mannose       | 16.0           | 60.7        |
| Lactose       | 7.2            | 10.0        |
| Maltose       | 21.0           | 150.0       |
| Sucrose       | 23.0           | 104.6       |
| Raffinose     | 7.0            | 21.5        |
| Dextrin       | 11.2           | 38.5        |
| Sorbitol      | 6.3            | 9.2         |
| Acetate       | 0.7            | 6.2         |
| Fumarate      | 6.0            | 7.7         |
| Succinate     | 10.0           | 31.4        |
| Citrate       | 2.2            | 16.2        |

* In addition to the above carbon sources, all media contained 0.1% yeast extract, 1% peptone, 1% meat extract, 1%KH,PO4, 1% K2HPO4, and 0.1% MgSO4·7H2O (pH 7.0). The cultivation was carried out under the conditions described in Materials and Methods.

Maltose, and sucrose were found to be favorable for both growth and production of CoA. Glucose, the most inexpensive carbon source, was used in the following experiments. Throughout the culture period, CoA was found in the cells but seldom in the broth filtrate.

Effect of nitrogen sources. During preliminary experiments, it was found that the organism could not utilize inorganic nitrogen. To choose the most favorable nitrogen source, several organic nutrients, each at a concentration of 0.1% nitrogen, were examined for their effects on the production of CoA. As shown in Table 4, corn steep liquor was effective for both growth and production of CoA. The combination of corn steep liquor and peptone was even better. Part of this stimulation could be due to the higher pH observed with these additives.

Effect of glucose and organic nutrients on CoA formation. The above experiments revealed the advantage of glucose as a carbon source and the combination of corn steep liquor and peptone as organic nutrients. The ratio of glucose and these nutrients was also studied. Five percent glucose, 2.2% corn steep liquor, and 1.35% peptone proved to be most favorable. The amounts of CoA accumulated in the culture broth reached 220 µg per ml in 24 h.

Effect of inorganic phosphate and magnesium ion. ATP is an essential intermediate in the biosynthesis of CoA from pantothenic acid, cysteine, and adenine, and the production of ATP is influenced by inorganic phosphate. The effect of inorganic phosphate on CoA formation was investigated. As shown in Fig. 1, maximum production of CoA, ATP, and pentose (which is an essential for formation of ATP) was observed in the presence of 0.8% inorganic phosphate. The effect of magnesium ion on CoA formation was studied by varying the concentration in the medium. The optimal level of magnesium sulfate was about 0.1%.

Effect of shaking and temperature. In preliminary experiments, shaking cultures possessed much higher enzyme activity than stationary cultures. The effect of aeration was studied by varying the amount of media in the flasks. Maximal CoA formation was attained with 20 to 25 ml of medium in a 500-ml flask (Fig. 2). The effect of temperature was also studied. The best temperature was 30 C for both enzyme and CoA formation (Table 5).

Effect of cultural period on CoA synthetic activity. The data for a typical fermentation under optimal conditions are given in Fig. 3. A 500-ml flask containing 25 ml of medium composed of 5% glucose, 2.2% corn steep liquor, 1.35% peptone, 0.5% KH,PO4, 0.5% K2HPO4, and 0.1% MgSO4·7H2O (pH 7.0) was inoculated with 1 loopful of the organism, and a shaking culture was carried out at 30 C. CoA synthetic activity increased parallel with growth, and reached the maximal level at about 36 h, the point at which the culture entered the stationary phase. On the other hand, the maximal level of pentose was attained at 48 h. The addition of calcium-pantothenate, cysteine-hydrochloride, and adenine at this point is considered to be best for accumulation of CoA.

Effect of pantothenic acid, cysteine, and adenine. In preliminary experiments, single additions of pantothenic acid, cysteine, and
Table 4. Effect of complex nutrients on CoA formation

| Complex nutrient | pH | Growth (mg/ml) | CoA (µg/ml) |
|------------------|----|---------------|-------------|
| Corn steep liquor (2.2%) | -  | -  | -  |
| Yeast extract (0.9%) | -  | -  | -  |
| Peptone (0.9%) | -  | -  | -  |
| Meat extract (7.0%) | -  | -  | -  |
| Malt extract (1.0%) | -  | -  | -  |
| pH | 5.0 | 4.5 | 8.0 |
| Growth (mg/ml) | 4.8 | 23.0 | 186.9 |
| CoA (µg/ml) | 0.9 | 0.9 | 7.0 |

*In addition to the above nitrogen sources, all media contained 5% glucose, 1% KH2PO4, 1% K2HPO4, 0.1% MgSO4·7H2O, and 1.1% casein hydrolysate (pH 7.0). The cultivation was carried out under the conditions described in Materials and Methods.

Adenine only scarcely stimulated CoA production as compared with simultaneous addition. It is thus essential that pantothenic acid, cysteine, and adenine be simultaneously added to the culture medium. To investigate the most effective concentration of substrates, the concentration of one precursor was varied whereas the other two were held at a concentration of 0.1% (Fig. 4). The effect of pantothenic acid and cysteine was remarkable as compared with that of adenine. The optimal concentrations were 0.2% for pantothenic acid and cysteine and 0.1% for adenine.

**Table 5. Effect of temperature on CoA formation**

| Temp (C) | Cultivation | pH | Growth (mg/ml) | CoA (µg/ml) |
|----------|-------------|----|---------------|-------------|
| 30       | 30          | 6.0 | 35.0          | 340.0       |
| 30       | 37          | 6.0 | 30.9          | 110.0       |
| 37       | 30          | 6.0 | 22.5          | 80.0        |
| 37       | 37          | 6.0 | 21.0          | 72.0        |

*The medium contained 5% glucose, 2.2% corn steep liquor, 1.35% peptone, 0.5% KH2PO4, 0.5% K2HPO4, and 0.1% MgSO4·7H2O (pH 7.0). The cultivation was carried out under the conditions described in Materials and Methods. Analysis was made after 72 h.
was immersed for 10 min in boiling water, and cells were centrifuged off after adjusting pH to 3.0 with diluted HCl. The supernatant solution was placed on an activated charcoal column (4 by 40 cm). The column was washed with water, and the substances adsorbed were eluted with 40% acetone containing 0.28% ammonia. The eluate was concentrated to about 560 ml under conditions) with 0.2% pantothenic acid, 0.2% cysteine, and 0.1% adenine. Formation of ATP from adenine and consumption of pantothenic acid and cysteine occurred at the same time; then CoA increased linearly with the consumption of these substrates. During the reaction, the pH was maintained at around 6, which was optimal for the reaction. The amount of CoA accumulated in a medium reached 600 µg/ml in 20 h.

Isolation of CoA. Purification suitable for industrial application was carried out as diagrammed in Fig. 6. Culture broth (1.8 liters)
reduced pressure below 40°C. After adjusting pH to 7.0 with lithium hydroxide, 140 ml of 2-mercaptoethanol was added to the concentrate, and the mixture was allowed to stand for 2 h at room temperature. The mixture was applied to a column of diethylaminoethyl-cellulose (Cl-, 4 by 30 cm). The column was washed with 0.02 M LiCl in 0.003 N HCl and was eluted by using a linear gradient technique. Figure 7 shows the elution pattern obtained. The fraction containing peak I was lyophilized. The residue was dissolved in a small volume of water, and the lithium salt of CoA was precipitated by the addition of an acetone-methanol mixture (10:1). The lithium salt was collected and washed repeatedly with acetone-methanol mixture. To improve the purity, lithium salt was dissolved in a small volume of water; subsequent gel filtration of Sephadex G-25 (1.5 by 85 cm) yielded a pure CoA fraction (CoA, 362 mg). After lyophilization of the CoA fraction, 420 mg of CoAlI₂-6H₂O was obtained. Analysis: calculated for C₁₃H₁₈O₁₄N₇P₃S₂Li₃·6H₂O: C, 28.22; H, 5.08; N, 10.97; P, 10.40; found: C, 27.96; H, 4.51; N, 10.92; P, 10.20. The purity was 98% by means of the phosphotransacetylase method (6). To obtain the free acid form, the lithium salt was dissolved in a small volume of water and was applied to Dowex 50 (H⁺, 1.5 by 17 cm). The eluate was lyophilized. After this process, 381 mg of CoA-2½H₂O was obtained (Table 6). Analysis: calculated for C₁₃H₁₆O₁₄N₇P₃S₂·2½H₂O: C, 31.03; H, 5.09; N, 12.06; P, 11.44; found: C, 31.31; H, 4.91; N, 12.04; P, 11.81. The purity of CoA preparation was 96% (6). The retardation factor was 0.56 (isobutyric acid-0.5 N NH₄OH[5:3]).

**Table 6: Isolation of CoA**

| Step            | A₅₆₀  | CoA (mg) | Purity (%) | Yield (%) |
|-----------------|-------|----------|------------|-----------|
| Culture extract | 642,000 | 1,060 | 100 |         |
| Charcoal        | 377,000 | 923  | 5   | 87       |
| DEAE-cellulose  | 11,500  | 540   | 90  | 51       |
| Sephadex G-25   | 7,373   | 362a  | 98  | 34       |
| Dowex 50        | 7,335   | 360a  | 96  | 33       |

*a A₅₆₀ absorbance at 260 nm; DEAE, diethylaminoethyl.

*b Calculated as the dehydrated form.

**DISCUSSION**

In recent years, increasing attention has been paid to CoA because of its widespread involvement in many metabolic pathways. This interest has spread throughout the fields of biochemistry, nutrition, and physiology. In 1970, Ogata et al. (10) reported a new preparative method of CoA from pantothenic acid, cysteine, and ATP using dry cells of *B. ammoniagenes*. This method is rapid, although it requires preparation of dry cells as the enzyme source. The method described here (T. Kakimoto, N. Nishimura, T. Shibatani, and I. Chibata, Proc. Annu. Meet. Jap. Agr. Chem. Soc., p. 128, 1971) gave a higher yield of CoA from pantothenic acid, cysteine, and adenosine. Shimizu et al. recently (11, 12) reported an improved method for the fermentative production of CoA from pantothenic acid, cysteine, and adenosine 5'-monophosphate, and the yield of CoA in the cultured broth reached more than 3 mg/ml. Although the formation of CoA with *S. lutea* is only 0.6 mg/ml, the present procedure has the advantage of employing the inexpensive purine, adenosine, rather than its expensive derivative, ATP. Moreover, our method results in the isolation of the highly purified CoA in the free acid form.

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