Production of Nucleic Acid Related Substances by Fermentative Processes

Part VI. Accumulation of 5'-Xanthylic Acid by Guanine-Requiring Mutants of *Micrococcus glutamicus*

I. Isolation and Characterization of 5'-Xanthylic Acid

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It was found that a guanine and adenine-requiring mutant of *Micrococcus glutamicus* accumulated the ultraviolet-absorbing substance in the culture fluid. A KY9978 strain, which accumulated the largest amount of the substance, we selected from the guanine auxotrophs derived from the guanine-adenine doubleless mutant. The substance was isolated in a crystalline form from the culture fluid by the use of ion exchange resins, Diaion SA 21A and SK No. 1, and identified chemically and enzymatically as 5'-xanthylic acid, an intermediate from 5'-inosinic acid to 5'-guanylic acid on the purine nucleotide biosynthesis.

INTRODUCTION

5'-Xanthylic acid (abbreviated as XMP) is an intermediate on purine nucleotide biosynthesis from 5'-inosinic acid leading to 5'-guanylic acid, and its sodium salt has been known a flavorenhancing agent¹⁾ Magasanik et al.^{2,3)} reported the accumulation of xanthosine in culture fluids of guanine-requiring mutants of *Aerobacter aerogenes* and *Salmonella typhimurium*, respectively. Nakayama et al.⁴⁾ developed a complex medium producing a large amount of xanthosine in culture medium by the Magasanik's strain. The culture, however, was found not to accumulate XMP, because of its high level of phosphatase activity.⁵⁾

In attempt to search for microorganisms capable of accumulating 5'-purine nucleotide, it was found that guanine and adenine doubleless or guanineless-mutants of *Micrococcus glutamicus* (a glutamate accumulating strain) produced a copious amount of XMP in their culture liquid. The work reported here was undertaken to select KY9978 strain most suitable for XMP production, to isolate pure XMP from its culture liquid, and to definitely characterize the compound.

EXPERIMENTAL

Materials and Methods

Strains. A guanine- and adenine-requiring strain, KY 9938 of *Micrococcus glutamicus* and a guanine-requiring strain, KY9978 derived from KY9938 strain were mostly used.

Culture methods. Twenty ml of seed culture medium containing 2% of glucose, 1% of yeast extract, 1% of peptone and 0.25% of NaCl were poured into a 250 ml-Erlenmeyer flask and the inoculated medium were

1) A. Kuninaka, *J. Agr. Chem. Soc. Japan*, **34**, 489 (1960).

2) B. Magasanik and M.S. Brooke, *J. Biol. Chem.*, **206**, 83 (1954).

3) B. Magasanik, H.S. Moyed and L.B. Gehring, *ibid.*, **226**, 339 (1957).

4) K. Nakayama, T. Suzuki, Z. Sato and S. Kinoshita, *Amino Acid and Nucleic Acid* (in Japan), **8**, 88 (1963).

5) M. Misawa, T. Nara and K. Nakayama, *J. Agr. Chem. Soc. Japan*, **38**, 167 (1964).

shaken at 30°C for 24 hours. Two ml of the grown seed was inoculated to 20 ml of the fermentation medium in a 250 ml-Erlenmeyer flask. All fermentations were run at 30°C for 5 to 6 days on a rotary shaker, which operated at 220 rpm. and described 5 cm in circle. The composition of the fermentation medium was shown in tables and figures.

Analytical methods. XMP and xanthine in culture filtrates were analyzed by paperchromatography. The filter paper (No 51A type made by Tōyō Rōshi Co.) was developed with a solvent system, containing 10 parts of isobutyric acid and 6 parts of 0.5 N-NH₄OH.⁶⁾

The R_F values of XMP, xanthosine and xanthine are approximately 0.22, 0.47 and 0.51, respectively. After drying, XMP and xanthine were detected with the ultraviolet absorbing detector, and their spots on paper were cut off and eluted with water at 100°C for 30 minutes. The absorbance of the eluates was determined by a Beckman spectrophotometer at 258 m μ for xanthyllic acid and at 266 m μ for xanthine.

The concentration of XMP was calculated from that of xanthosine by the Nakayama's method,⁴⁾ and the quantity of xanthine was estimated spectrophotometrically by comparing with that of the authentic xanthine. The growth of cells shown as D.C.W. in the tables, was estimated from turbidity at 660 m μ of cell suspension which was prepared by centrifugation of cultured broth. Glucose was analyzed by the method of Somogyi.⁷⁾

RESULTS

Selection of Strains.

A preliminary observation showed the presence of a strongly ultraviolet-absorbing substance in the culture filtrate of KY9938 strain. The ultraviolet-absorbing substance was later identified as 5'-XMP; however, probably because of its double requirement, it showed poor growth and was found to accumulate 5'-inosinic acid together with XMP.⁴⁾

Therefore, to obtain a strain which accumulates the large amounts of XMP, some guanine-less mutants of *M. glutamicus* derived from KY 9938 strain were incubated and their productivities examined.

The results were tabulated in Table I. Although all of the auxotrophs tested were

TABLE I. XMP ACCUMULATION BY AUXOTROPHS OF *Micrococcus glutamicus*

Strain No.	Marker	6-Day incubation			
		pH	DCW mg/ml	XMP mg/ml	X* mg/ml
9938	ad ⁻ , gu ⁻	4.8	10.5	2.00	0.25
9969	gu ⁻	4.8	21.5	1.37	0.30
9973	"	4.8	19.0	1.95	0.50
9974	"	5.0	17.9	2.00	0.48
9978	"	5.0	15.0	2.57	trace
9979	"	4.8	14.3	2.40	trace
9980	"	4.8	16.1	2.56	0.33
9982	"	4.8	13.7	2.05	0.43
9983	"	4.8	12.0	2.28	0.43
9984	"	4.8	12.6	2.28	0.40

Seed medium: Glucose 2.0%, Yeast extract 1.0% Peptone 1.0%, NaCl 0.25%, pH 7.2 20 ml/250 ml flasks.

Fermentation medium: Glucose 10.0%, (NH₄)₂SO₄ 2.0%, Yeast extract 1.0%, KH₂PO₄ 0.2%, K₂HPO₄ 0.2%, MgSO₄·7H₂O 0.4% and CaCO₃ 2.0%. pH 7.8 20 ml/250 ml flask.

* Xanthine.

FIG. 1. Purification Procedure for Obtaining Crystalline XMP.

able to produce XMP significantly, KY 9978 strain was selected as the most suitable strain

6) B. Magasanik, E. Vischer, R. Doniger, D. Elson and E. Char-gaff, *J. Biol. Chem.*, **186**, 37 (1950).

7) M. Somogyi, *ibid.*, **160**, 61 (1945).

from view-point of XMP productivity.

Hence, in all of the subsequent experiments, KY9978 strain was exclusively used.

Isolation of XMP.

Isolation procedures are diagrammed in Fig. 1. Cultured broth, 1.4l, was centrifuged for removal of bacterial cells and the pH of the supernatant was adjusted to 8.5 with 5 N-NaOH. It was then passed through a column of about 600 ml of Diaion SA-21A (OH form) ion exchange resin (strongly alkaline anion exchanger). The column was washed with water. The effluent and the washed water were free of both XMP and xanthine.

Four tenths 1 N-HCl solution, 1.5l, was then poured on the column and the eluates were collected; 600 ml of rich cut fractions of the eluates was passed through a column packed with about 600 ml of Diaion SK-No. 1 (H form) ion exchange resin (strongly acidic cation exchanger), and 850 ml of rich cut fractions of effluents were adjusted to pH 7.5 with 5 N-NaOH, concentrated in vacuo to approximately 10 ml, and eventually chilled overnight. The gummy material which separated on cooling was removed by centrifugation. When the moisture of the material was eliminated by its drying in vacuo, a yellow crystalline material was obtained.

For the further purification, the crude crystal was dissolved in 20 ml of water, its pH adjusted to 4.5 with HCl, and 10 g of active carbon thereto added. The carbon was separated by filtration and XMP adsorbed by carbon was eluted with 3% ammonium hydroxide in 50% ethanol solution. After the eluate was evaporated to dryness in vacuo, crystalline XMP was obtained. Yield 134 mg.

Xanthine was eluted with 3.5% ammonium hydroxide solution from a column of Diaion SK No. 1, but crystallization of xanthine by the concentration of the eluate was unsuccessful.

Identification of XMP.

The ultraviolet spectrum of the isolated material was shown in Fig. 2, and it was almost identical with that of a commercial preparation of xanthosine (made by Nutritional Biochemical

Corp.) instead of a standard XMP, for it is not commercially available at the present time.

The sample was hydrolyzed enzymatically by 5'-nucleotidase of the snake venom. The reaction mixture used consisted of 6.64 mg of the material (its final concentration was 3.64 μ moles as XMP per ml of reaction mixture), 1 mg of the enzyme and M/5 tris (hydroxymethyl) aminomethane buffer, pH 8.0 in a total volume of 5 ml. It was incubated at 30°C for 4 hours. The liberated products were identified as xanthosine, both by the R_F value of paperchromatogram with a solvent system described in the experimental and by the ultraviolet absorption spectrum, and as phosphate by Allen's method⁸⁾, respectively.

The hydrolysate contained 3.24 μ moles per ml of xanthosine and 3.15 μ moles per ml of phosphate.

To 1 ml of the solution containing 1.66 mg of the isolated material per ml of water (final concentration was 2.28 μ moles as XMP per ml of reaction mixture), 1 ml of 2 N-HCl was added, and the mixture was placed in a test tube and heated in a boiling water bath for 1 hour. The products of its hydrolysis by acid were identical with xanthine and ribose-5-phosphate, based on their R_F values of paperchromatograms and the ultraviolet absorption spectrum for the former.

FIG. 2. U.V. Absorption Curve of XMP Obtained from Fermentation Beer of KY 9978 Strain.

- (1) The spot of XMP on the paperchromatogram was cut off and eluted with water.
- (2) One drop of 2 N-HCl solution was added to the eluate (1).
- (3) One drop of 5 N-HCl solution was added to the eluate (1).

8) R.J.L. Allen, *Biochem. J.*, **34**, 858 (1940).

The solvent system for ribose 5-phosphate consisted of 20 ml of *tert*-butanol, 1 g of picric acid and 5 ml of water.⁹⁾ The R_F value of liberated ribose 5-phosphate was found to be 0.44, in good accord with that of the authentic ribose-5-phosphate. The concentration of xanthine was 2.22 μ moles per ml of the hydrolysate, and the molar % ratio of xanthine formed to XMP initially present was 97.5%. The quantity of ribose in the isolated material which was estimated colorimetrically with orcinol reagent,¹⁰⁾ was 0.21 μ moles per ml in 0.228 μ moles per ml of the material.

From these results, the molar ratio of xanthine,

ribose and phosphate was calculated to be 97.5% : 92% : 97%. Therefore, when the concentration of xanthine was assumed to be 1.0, the composition (xanthine : ribose : phosphate) of the isolated material was shown to be 1 : 0.92 : 0.95.

The sodium salt of the isolated material was shown to have very agreeable taste, as previously reported by Kuninaka.¹⁾

From the results above described, the product was proved to be identical with 5'-XMP, and its purity was approximately 97% as disodium salt.

All of the data reported so far led to a conclusion that the UV absorbing substance accumulated by *M. glutamicus* KY 9978 (guanineless) is identical with 5'-XMP.

9) C.S. Hanes and F.A. Isherwood, *Nature*, **164**, 1107 (1949).

10) M.L. Morse and C.E. Carter. *J. Bacteriol*, **58**, 317 (1949).