Studies on the Formation of Uricase by *Streptomyces*

Part I. The Effect of Purine Bases on the Induced Formation of Uricase by the Cultured Cells*

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The induced formation of uricase by the cultured cells of *Streptomyces* sp. and the effect of purine bases on the enzyme formation were studied. The microorganism was grown in media containing urate and/or purine bases (adenine, guanine, hypoxanthine or xanthine) and the development of the uricase activity of the cells were measured at intervals. The disappearance of urate and purine bases from the media was also determined. Without the purine bases, the production of uricase was significantly low even in the presence of urate and the disappearance of urate from the medium was in a slow rate. Upon the addition of hypoxanthine or xanthine in the presence of urate, a significant increase in the uricase activity of the cells and a concomitant rapid decrease of urate in the medium were observed. The purine bases added to the media were incorporated into the cells at a relatively early period of the culture and appeared to be converted into urate within the cells. The repression of uricase formation in the cultured cells and the derepression by the addition of the purine bases were discussed.

Uricase (1.7.3.3, urate: oxygen oxidoreductase) in animal tissues has been purified from porcine liver and bovine kidney and the molecular properties and catalytic reactions have been studied.¹⁾ In microorganisms the enzyme has usually been found in the cells grown in the presence of urate.^{2~10)} Several

reports on the purification and properties of microbial uricase have been published^{11~14)} and the most of the papers have described that the enzyme is inducible. However, the studies on the induced formation of uricase have been very limited.^{15,16)}

Studies on the industrial production of microbial uricase have been undertaken in our

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16) M. F. Quetsch and W. F. Danforth, *J. Cellular Comp. Physiol.*, **64**, 115 (1964).

laboratory and one of the present authors (J. Fukumoto) has reported the extraction, purification and some properties of yeast (*Candida utilis*) uricase.¹⁷⁾ In the course of the studies, an extensive search for strong uricase producers was carried out and a strain of *Streptomyces* sp. was isolated from the soil of a poultry farm.^{18,19)} The microorganism was found to produce a large amount of uricase in the cells when it was grown in a medium consisted of peptone, meat extracts, glucose, inorganic salts and urate. The activity of the enzyme increased when the cells were incubated with urate in phosphate buffer.²⁰⁾ The enzyme extracted by sonic oscillation from the cells grown in the urate containing medium was partially purified and some of the properties of the purified enzyme have been reported.²¹⁾

The removal of meat extracts from the medium resulted in a significant reduction in the quantity of the enzyme produced by the cultured cells, but the growth of the cells was unchanged. The effect of meat extracts on the uricase formation was found to be caused, at least in part, by hypoxanthine existed in meat extracts. The present paper is concerned with the incorporation and the effect of purine bases on the induced formation of uricase by the growing cells of *Streptomyces* sp. cultured in media containing urate and/or purine bases.

MATERIALS AND METHODS

Organism. The microorganism used in the present study was a strain of *Streptomyces* sp. isolated from the soil of a poultry farm. The organism was stocked on a potato-agar slant (peptone 0.5%, glucose 1.0% and agar 1.5% were dissolved in 20% potato extracts, pH 7.0) at 4°C. Spore formation was excellent on

this medium.

Media. The basal medium (B-medium) contained peptone 2.0%, glucose 3.0%, K_2HPO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05% and NaCl 0.05%. The following media were prepared by adding to B-medium; meat extracts 1.0% (M-medium), meat extracts and urate (MU-medium), adenine (A-medium), adenine and urate (AU-medium), guanine (G-medium), guanine and urate (GU-medium), hypoxanthine (H-medium), hypoxanthine and urate (HU-medium), xanthine (X-medium), xanthine and urate (XU-medium) or urate (U-medium). Unless otherwise stated, urate and purine bases were added at the concentrations of 6×10^{-3} and 1×10^{-3} M, respectively. pH values of the media were adjusted to 7.0.

Reagents. Peptone used for the media was "Polypepton" of Daigo Eiyo Kagaku Co. and meat extracts were "Extract Bonito" of Wako Pure Chemical Industries Co. The ultraviolet absorption spectra of the solutions of "Polypepton" and "Extract Bonito" in 0.02 M phosphate buffer (pH 7.0) were shown in

FIG. 1. Ultraviolet Absorption Spectra of "Polypepton" and "Extract Bonito."

"Polypepton" and "Extract Bonito" were dissolved in 0.02 M phosphate buffer (pH 7.0) at the concentration of 0.025%. The solution of "Extract Bonito" was heated in boiling water with egg white albumin to remove turbid substances.

— "Polypepton" and ---- "Extract Bonito."

17) K. Itaya, T. Yamamoto and J. Fukumoto, *Agr. Biol. Chem.*, **31**, 1256 (1967).

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Fig. 1. Uric acid and purine bases were purchased from Wako Pure Chemical Industries Co. and other reagents were the highest grade commercially available.

Culture. Sixty milliliters of the media were taken in 500 ml shaking flasks, inoculated with spores and kept at 27°C with shaking. Cells were harvested by filtering with suction and washed 3 times with water. The dry matter contents of the wet cells obtained in this manner were 20% in average.

Sonic extraction. The washed cells were suspended in 10 volumes of 0.1 M borate buffer (pH 8.0) on the wet basis. The suspension was treated by sonic oscillation (10 kc, 100 W) for 15 min at 4°C to extract uricase and was centrifuged ($16,000 \times g$) for 15 min at 4°C. The supernatant thus obtained was used as a crude enzyme solution.

Uricase assay. The uricase activity was assayed by a spectrophotometric method.²²⁾ The enzyme solution 0.5 ml, H₂O 0.5 ml and the substrate solution 2.0 ml were added to a cuvette of Shimadzu Spectrophotometer QV-50. As a control, the enzyme 0.5 ml, 0.1 M KCN 0.2 ml, H₂O 0.3 ml and the substrate 2.0 ml were added to another cuvette in this order. The reaction was carried out at 30°C by adding the substrate to the test cuvette and absorbancy at 290 m μ was measured at 30 and 90 sec adjusting the absorbancy of the control to 0.500. The substrate solution was prepared by dissolving 4.0 mg of uric acid in 100 ml of 0.1 M borate buffer (pH 8.0). The enzyme solution was prepared by diluting the sonic extracts with 0.1 M borate buffer (pH 8.0) so as that the absorbancy decrease per minute is less than 0.1. The amount of urate oxidized is calculated assuming that the molar absorbancy of urate, under the conditions employed for the enzyme reaction, is 1.2×10^4 . One unit of the enzyme activity was defined as an activity which converts uric acid to allantoin at the rate of 1 μ mole per minute under the specified conditions.

Determination of urate and purine bases. Urate and purine bases in the media were determined spectrophotometrically by measuring the absorbancies at 290 m μ for urate, at 270 m μ for xanthine, at 260 m μ for adenine, at 250 m μ for hypoxanthine and at 245 m μ for guanine. The absorbancy readings were made at pH 7.0 in 0.02 M phosphate buffer. The ultraviolet

absorption spectra of the media were determined in the same buffer using Beckman spectrophotometer DB. The molar absorbancies of adenine, guanine, hypoxanthine and xanthine at the specified wavelengths were 1.3 , 1.0 , 1.1 and 1.0×10^4 , respectively.

RESULTS

The growth of the cells and the activity of uricase in the cells cultured in B-, U-, M- and MU-media are shown in Figs. 2, 3, 4 and 5, respectively. The disappearance of urate in U- and MU-media and the decrease in the absorbancies at 260 m μ of M- and MU-media are also presented. The uricase activity of the cells grown in B-medium was extremely low, although a slight increase in the activity was observed during the later part of the culture. The addition of urate to B-medium had no effect on the cell growth. A gradual increase in the uricase activity and a gradual decrease in urate were observed in the cells grown in U-medium. The supplement of meat extracts to B-medium did not bring about any change in the cell growth, but the uricase activity increased gradually in spite

FIG. 2. Growth and Uricase Activity of the Cells Cultured in B-Medium.

Cells were grown in B-medium at 27°C with shaking and harvested by filtering with suction. Uricase was extracted from the cells by ultrasonic oscillation and assayed by the spectrophotometric method.

○—○ Cells and ○---○ uricase.

22) H. M. Kalcker and M. Shafran, *J. Biol. Chem.*, **167**, 429 (1947).

FIG. 3. Growth and Uricase Activity of the Cells Cultured in U-Medium and the Disappearance of Urate in the Medium.

Urate was determined by measuring the absorbancy at 290 $m\mu$ of culture filtrates diluted with 0.02 M phosphate buffer (pH 7.0).

○—○ Cells, ●—● urate and ○---○ uricase.

FIG. 4. Growth and Uricase Activity of the Cells Cultured in M-Medium and Absorbancies at 260 $m\mu$ of the Medium.

Absorbancy readings were made at 260 $m\mu$ on culture filtrates diluted with 0.02 M phosphate buffer (pH 7.0).

○—○ Cells, ●---● absorbancy at 260 $m\mu$ and ○---○ uricase.

of the absence of urate from the medium. When urate was added to M-medium, a rapid increase in the uricase activity and a concomitant rapid disappearance of urate from the

FIG. 5. Growth and Uricase Activity of the Cells Cultured in MU-Medium, the Disappearance of Urate and Absorbancy at 260 $m\mu$ of the Medium.

○—○ Cells, ●—● urate, ●---● absorbancy at 260 $m\mu$ and ○---○ uricase.

medium were observed. The absorbancy at 260 $m\mu$ of the MU-medium decreased in an earlier stage of the culture. The second decrease in the absorbancy noticed in MU-medium seemed to be attributable to the decrease of urate which has absorption maxima at about 235 and 290 $m\mu$. The concentration of purine bases in M-medium was estimated as 1×10^{-3} M from the absorbancy decrease of

FIG. 6. Comparison of the Cell Growth in the Media Containing Various Purine Bases.

○—○ Basal, guanine, hypoxanthine, xanthine and urate media and ●—● adenine medium.

about 10 measured at $260\text{ m}\mu$ during the culture and assuming an average molar absorptivity of purine bases at this wave-length as 10×10^3 .

The growth curves of the microorganism in the media prepared by adding urate or various purine bases to the basal medium at the concentration of $1 \times 10^{-3}\text{ M}$ were compared in Fig. 6. There was no significant difference between the growth of the cells in the basal medium and the purine base containing media except for that in A-medium, where a slight inhibition of the growth was observed. The growth inhibition of adenine has been reported for other microorganisms.²³⁾

The decrease of the purine bases in A-, G-, H-, X- and U-media during the culture is

FIG. 7. Decrease of Purine Bases in Culture Filtrates of the Media Containing Purine Bases.

Purine bases and urate were added to B-medium at the concentration of $1 \times 10^{-3}\text{ M}$ and were determined by measuring absorbancies of culture filtrates diluted with 0.02 M phosphate buffer (pH 7.0) at the wavelengths corresponding to their absorption maxima.

●---● Adenine, ○---○ guanine, ○---○ hypoxanthine, ●---● xanthine and ○---○ urate.

shown in Fig. 7. The purine bases examined, except for urate, disappeared from the media in a relatively early period of the culture, in the order of hypoxanthine, guanine and xanthine, and adenine. In contrast to these purine bases, urate decreased in a significantly slow rate. The ultraviolet absorption spectra of the culture filtrates of these media were taken at intervals during the culture and are shown in Fig. 8. No significant shift was noticed in the spectra of the purine bases added to these media.

The uricase activity of the cells grown in the media containing one of the purine bases is shown in Fig. 9. For a comparison, the activity of the cells grown in M-medium is also given. Except for the cells cultured in A-medium, the activity of uricase in the cells reached to a maximum value at 60 to 72 hr of the culture. The highest activities in the cells grown in H-, X- and M-media were all found at 60 hr of the culture and were almost comparable to each other, although the time courses of the uricase formation in these cells were not identical.

Figures 10 and 11 indicate the activity of uricase in the cells cultured in the media containing urate and one of the purine bases, and the decrease in the amount of urate in these media during the culture, respectively. The results obtained by culturing the cells in MU-medium are also given for a comparison. The decrease in the absorbancies of the media at the wavelengths corresponding to the ultraviolet absorption maximum of each purine base was shown in Fig. 12.

A rapid increase in the uricase activity in the cells and a rapid decrease of urate in the medium were also observed in HU- and XU-media as found in MU-medium. The effect of guanine on the uricase formation and the urate disappearance seemed to be somewhat weaker than those of hypoxanthine and xanthine, and that of adenine was almost negligible. The decrease in the absorbancies at $245\text{ m}\mu$ of GU-, $250\text{ m}\mu$ of HU-, $260\text{ m}\mu$ of

23) C. N. Remy and S. H. Love, *J. Bacteriol.*, **96**, 76 (1968).

FIG. 8-a. Ultraviolet Absorption Spectra of Culture Filtrates of A-Medium.

Culture filtrates were diluted 15 times with 0.02 M phosphate buffer (pH 7.0) and ultraviolet absorption spectra were taken against culture filtrates of the basal medium diluted in the same manner. — 0 hr and 24 hr.

FIG. 8-b. Ultraviolet Absorption Spectra of Culture Filtrates of G-Medium.

FIG. 9. Increase in Uricase Activity of the Cells Cultured in the Media Containing Purine Bases.

○—○ B-, ●—● A-, ○—○ G-, ○—○ H-, ●—● X-, ○—○ U- and ●—● M-media.

— 0 hr, 18 hr and —·— 24 hr.

FIG. 8-c. Ultraviolet Absorption Spectra of Culture Filtrates of H-Medium.

— 0 hr, 15 hr and —·— 18 hr.

FIG. 8-d. Ultraviolet Absorption Spectra of Culture Filtrates of X-Medium.

— 0 hr, 18 hr and —·— 24 hr.

FIG. 8-e. Ultraviolet Absorption Spectra of Culture Filtrates of U-Medium.

— 0 hr, 36 hr and —·— 60 hr.

FIG. 10. Increase in Uricase Activity of the Cells Cultured in the Media Containing Urate and Purine Bases.

○—○ U-, ●—● AU-, ○—● GU-, ○—● HU-, ●—● XU- and ●—● MU-media.

AU- and 270 $m\mu$ of XU-media appeared to occur in two steps. The first corresponded to the decrease of the purine bases and the second to that of urate in the media, as was seen in the absorbancy decrease at 260 $m\mu$ of MU-medium shown in Fig. 5.

DISCUSSION

The microorganism used in this study showed

FIG. 11. Decrease of Urate in Culture Filtrates of the Media Containing Urate and Purine Bases.

○—○ U-, ●—● AU-, ○—● GU-, ○—● HU-, ●—● XU- and ●—● MU-media.

a strong uricase activity when cells are grown in MU-medium (Fig. 5). When meat extracts are omitted from the medium, however, a significant reduction in the uricase formation and urate consumption was found, while the cell growth was unaffected (Fig. 3). As shown in Fig. 1, meat extracts contained ultraviolet absorbing substance(s) (O.D._{max}, 250 $m\mu$) which disappeared from the media during the early period of growth (Figs. 4 and 5). These results suggest that purine bases, especially hypoxan-

FIG. 12. Decrease in Ultraviolet Absorbancies of Culture Filtrates of the Media Containing Urate and Purine Bases at the Wavelengths Corresponding to Absorption Maxima of Purine Bases Added to the Media.

●—● AU-, ○---○ GU-, ○---○ HU-, ●—● XU-, and ○—○ MU-media.

thine, might have some effects on the induced formation of uricase by this microorganism.

The purine bases added to the basal medium were incorporated into the cells in an earlier stage of the culture (Fig. 7). The formation

of a small amount of uricase in the cells grown in the basal medium supplemented with purine bases, especially hypoxanthine and xanthine, might be based on the intracellular production of urate, the endogenous inducer, from the purine bases. The formation of uricase in the cells cultured in M-medium may similarly be accounted to the purine bases in meat extracts (Fig. 9). The involvement of the sequential induction in the purine base metabolism of yeast has been discussed.²⁴⁾ Most of the incorporated purine bases seemed to be used for the synthesis of nucleosides and nucleotides²⁵⁾ during the early phase of growth, since the ultraviolet absorption maxima of the culture filtrates did not change substantially. On the contrary, when the washed cells of the microorganism were incubated in phosphate buffer with hypoxanthine, for example, the absorption maximum of the filtrate shifted to the longer wavelength, suggesting the oxidation of hypoxanthine to xanthine, then to urate.²⁶⁾ The synthesis of nucleosides and nucleotides from the purine bases during the early phase of growth might also be suggested by the lag existed in the times of the uptake of the purine bases and of the uricase formation (Figs. 7 and 9). On the contrary, when the uricase formation was induced by urate added to the medium, the formation of the enzyme took place simultaneously with the decrease of urate from the medium (Figs. 10 and 11).

The formation of uricase seemed to be metabolically repressed in the cells grown in the B- and U-media. The enzyme activity of the cells grown in U-medium was much lower than that of the cells fully induced under the most favorable conditions as in HU-, XU- and MU-media for 60 hr. It seemed that the supplement of some of purine bases, especially

24) A. H. Roush, L. M. Anestiaux and A. J. Domnas, *J. Cellular Comp. Physiol.*, **54**, 275 (1959).

25) M. Friedkin and H. Kalckar, "The Enzymes," 2nd ed., Vol. V, ed. by P. D. Boyer, H. Lardy and K. Myrback, Academic Press, Inc., New York, 1961, p. 237.

26) Y. Watanabe and J. Fukumoto, in preparation.

hypoxanthine and xanthine, in addition to urate to the basal medium would be indispensable for the complete induction of uricase formation by this microorganism. The existence of urate did not seem to interfere with the uptake of the purine bases, while the urate uptake was competitively inhibited by xanthine.^{27, 28)} The production of the endoge-

nous inducer from these purine bases would not fully account for their effects on the uricase formation, since the uricase activity in the cells grown in HU-medium, for example, was in large excess of the sum of those grown in H- and U-media. Further investigations are necessary to determine the actual effects of these purine bases which would cause the stimulation or derepression of the uricase formation in this microorganism.

27) M. F. Quetsch and W. F. Danforth, *J. Cellular Comp. Physiol.*, **64**, 123 (1964).

28) Y. Watanabe and J. Fukumoto, in preparation.