Synthesis of 5-Methyluridine by a Thermophile, *Bacillus stearothermophilus* JTS 859

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Many thermophiles, which can grow at 65°C, were examined as to their ability to produce 5-methyluridine from inosine and thymine (5-methyluracil) in the presence of phosphate and cells as enzyme sources. *Bacillus stearothermophilus* JTS 859 was selected as a strain that synthesized 5-methyluridine efficiently. The reaction is supposed to be carried out by a combination of a thermostable purine nucleoside phosphorylase and a thermostable pyrimidine nucleoside phosphorylase. Their half-lives were 7200 hr and 400 hr at 63°C, and 148 hr and 14 hr at 70°C, respectively.

Nucleosides such as inosine, adenosine, uridine and cytidine are produced by a fermentation method, but thymidine has not been produced by such a method. Thymidine is practically produced by hydrolysis and separation from deoxyribonucleic acid derived from natural sources such as milt. As an alternative to this method, we developed a method for the practical production of thymidine from non-natural sources. We tried to synthesize 5-methyluridine (5MU) from thymine (5-methyluracil) and inosine enzymatically at first by means of the ribosyl transfer reaction, as shown in the following chemical equations, and to chemically reduce the hydroxy group at the 2'-position on the ribose ring of 5MU to yield thymidine.

Inosine + Phosphate →

Ribose-1-phosphate + Hypoxanthine (1)

Ribose-1-phosphate + Thymine →

5-Methyluridine + Phosphate (2)

We selected inosine as a ribose donor because it it produced abundantly in industrial fermentations. This paper describes the synthesis of 5-methyluridine using thermostable enzymes produced by a thermophile.

The ribosyl transfer reaction has been studied for a long time.^{1~4)} This reaction is carried out by purine nucleoside phosphorylase, pyrimidine nucleoside phosphorylase, uridine phosphorylase, thymidine phosphorylase and a combination of these enzymes. The substrate specificities of these enzymes are not strict, therefore, the ribosyl transfer reaction has been applied for the synthesis of nucleoside analogues, 5-fluorouridine, 5-bromouridine and so on.^{5~7)} It is necessary that the enzymes used for practical application should be stable for a long time. Enzymes derived from mesophiles have been used for practical application. As for thermostable enzymes derived from thermophiles, only pyrimidine nucleoside phosphorylase from *Bacillus stearothermophilus* NCA10 has been reported.⁸⁾

The application of thermostable enzymes has several advantages.⁹⁾ The reaction can be carried out at high temperature for a long time and the reaction rate is also high. The higher solubility of the reactants allows operation with higher concentrations of reactants. The solubility of 5MU is 83 g/l at 25°C and above 500 g/l at 63°C. Thermostable enzyme reactors are resistant to bacterial contamination due to the high operation temperature. This is impor-

tant for practical application because usual enzyme reactors tend to be contaminated by bacteria.

As thermostable enzymes have these advantages, we screened thermophiles and finally found a thermophile, *Bacillus stearothermophilus* JTS 859, that efficiently produced thermostable enzymes for the ribosyl transfer reaction. The thermal stabilities of these enzymes were high. The half-lives of the enzymes as to the formation of 5MU at 63°C and 70°C were 400 hr and 14 hr, respectively.

Materials and Methods

Microorganisms. All strains used were isolated at 65°C from soil and sludge from hot springs and drains in Beppu, Houfu and Izu.

Chemicals. Thymine and inosine were purchased from Wako Pure Chemical Industries, Ltd. and Yamasa Shoyu Co., Ltd, respectively. All other reagents, of analytical grade, were commercially available.

Culture media. Medium A, containing (g/l of water) Bacto-Tryptone 10, Bacto-Yeast extract 5, glucose 3 and NaCl 3, was used for the cultivation of *B. stearothermophilus* JTS 859. Medium B used for screening was Medium A supplemented with pig liver infusion 25 and glycerol 18.9 (g/l). The liver infusion was prepared as follows; sliced liver (25 g) was boiled gently in water (150 ml) for 30 min, followed by filtration through gauze.

Screening. Cultivation was carried out in an Erlenmeyer flask (500 ml) containing 100 ml of medium B at 210 rpm for 4 to 5 hr at 63°C. The cells were harvested by centrifugation ($10,000 \times g$, 15 min) and 500 mg of wet cells was added to 10 ml of reaction solution A or B. Reaction solution A contained 20 mM inosine, 25 mM thymine, 60 mM KH_2PO_4 , 50 mM Tris·HCl and 11.1 mM glucose (pH 7.0). Reaction solution B contained 20 mM inosine, 20 mM thymine and 20 mM potassium phosphate (pH 7.0). Each reaction mixture was incubated for 3 hr at 65°C. After the reaction mixture had been centrifuged to remove insoluble materials, the supernatant was analyzed for 5MU.

Enzyme assay. The standard reaction mixture, consisting of 20 mM inosine, 20 mM thymine, 20 mM potassium phosphate (pH 7.0) and 50 μl of the crude enzyme solution in a total volume of 1.0 ml, was incubated at 60°C for 1 hr. Fifty μl of the reaction mixture was removed and added to 950 μl of MeOH to terminate the reaction, followed by

analysis by HPLC.

Analysis. Quantitative determination of bases and nucleosides was carried out HPLC. The operating conditions were as follows: column, Chemcosorb 5-ODS-H ($\phi 4 \times 300$ mm) (Chemco Scientific Co., Ltd., Osaka); column temperature, 38°C; solvent, water-MeOH=95:5 (v/v); flow rate, 0.8 ml/min; detector, UV monitor (254 nm).

Ribose-1-phosphate was detected by HPLC. The operating conditions were as follows: column TSKgel DEAE-5PW, ($\phi 7.5 \times 75$ mm) (Tosoh Co. Tokyo); solvent, 50 mM potassium phosphate (pH 5.1); flow rate, 1 ml/min; detector, RI; retention time, 5.6 min.

Preparation of crude enzymes. *B. stearothermophilus* JTS 859 was grown in a 500 ml flask containing 100 ml of medium A at 65°C for 4.5 hr at 210 rpm. Cells were collected by centrifugation ($10,000 \times g$, 10 min, 4°C). One gram of wet cells was suspended in 10 ml of 20 mM potassium phosphate buffer (pH 7.0) and then disrupted with an ultrasonic oscillator for 10 min (20 kHz, 0~10°C). After centrifugation ($10,000 \times g$, 10 min, 4°C), the resultant supernatant, containing 15.1 mg/ml protein was used as the crude enzymes.

Preparation of acetone-treated crude enzymes. Eight ml of acetone (-20°C) was added to 10 ml of crude enzymes. After standing for 15 min below 5°C, the mixture was centrifuged ($10,000 \times g$, 10 min, 4°C). Six ml of acetone (-20°C) was added to the supernatant. After standing for 15 min below 5°C, the mixture was centrifuged ($10,000 \times g$, 10 min, 4°C). The precipitate was dissolved in 5 ml of 20 mM potassium phosphate buffer (pH 7.0). It contained 11.4 mg/ml protein and was used as the acetone-treated crude enzymes.

Results

Screening

Using 4 hr-cultured and 16 hr-cultured cells of various strains as enzyme sources, we examined the ability to form 5MU at 65°C. One hundred and seventy thermophilic strains were investigated in the survey. None of the 16 hr-cultured cells of the strains formed 5MU. As to 4 hr-cultured cells of the strains, 69 strains showed the enzymatic activity. Among them, 4 strains showed high ability as to 5MU formation (Table I). We selected JTS 859 isolated from sludge, as the most active strain per wet cell, for further investigation.

Table I. FORMATION OF 5-METHYLURIDINE BY THE SELECTED THERMOPHILES

The reaction was carried out as described in the text.

Strain	5-Methyluridine ($\mu\text{mol/hr} \cdot \text{g wet cell}$)
H-254	43
JTS 859	105
H-491	41
H-524	39

Fig. 1. Time Course of Culture.

Bacillus stearothermophilus JTS 859 was cultivated in Medium A at 65°C. Ten ml of the reaction mixture, containing 200 mg wet cells, 20 mM inosine, 20 mM thymine, 60 mM potassium phosphate and 11.1 mM glucose (pH 7.0), was incubated for 3 hr at 65°C. ○, dry cells; ●, pH; ■, 5-methyluridine (5MU).

Time course of the culture

The enzymatic activity was measured with the cultivation time (Fig. 1). Cells grew rapidly from 3 to 5.5 hr after inoculation. The enzymatic activity was high during the logarithmic phase of growth and had disappeared by 7 hr after the inoculation. Thus, the harvesting time of this thermophile was important.

Identification

The JTS 859 strain was identified as *Bacillus stearothermophilus* based on its taxonomical properties, referring to Bergey's Manual of Systematic Bacteriology (Table II).¹⁰⁾

Isolation and identification of 5MU from the reaction mixture

Four ml of the crude enzyme solution was added to 36 ml of 20 mM potassium phosphate buffer (pH 7.0) containing 804 mg (3 mmol) inosine and 378 mg (3 mmol) thymine. The

Table II. TAXONOMICAL PROPERTIES OF STRAIN JTS-859

Morphology
Rod-shaped (0.7~0.9) μm X (5.4~6.5) μm . Endospores (1.1~1.2) μm X (2.0~2.3) μm . Gram positive. Motile.
Cultural characteristics
Nutrient agar colonies: moderate growth, circular slightly irregular, smooth, raised, opaque and white slightly yellow
Nutrient slant agar: moderate growth, smooth, raised, opaque and white slightly yellow
Liquefaction of gelatin: positive
Litmus milk: coagulation
Anaerobic growth: negative
Physiological Characteristics
Catalase: positive
Oxidase: positive
Urease: negative
Arginine dihydrolase: positive
Anaerobic growth: negative
V-P test: negative
MR test: negative
O/F test: fermentative
Acid from D-glucose: positive
Acid from L-arabinose: negative
Acid from D-xylose: negative
Acid from D-mannitol: positive
Gas from glucose: negative
Hydrolysis of casein: positive
Hydrolysis of gelatin: positive
Hydrolysis of starch: positive
Utilization of citrate: negative
Degradation of tyrosine: negative
Deamination of phenylalanine: negative
Egg-yolk lecithinase: no growth
Nitrate reduced to nitrite: positive
Formation of indole: negative
Formation of dihydroxyacetone: negative
Growth pH range: pH 5.7~8.5, optimum pH 6.0~7.0
Temperature: 40~71°C, optimum 62~66°C
Growth with lysozyme present: negative
Growth with azide present: negative
GC content: 47.3%, T_m = 88.7°C

reaction was carried out for 16 hr at 65°C. The reaction mixture was applied on a column of Dowex 50WX (H^+ form, 100 ml) and then elution was carried out with water (300 ml). The eluate containing 5MU and thymine was obtained. 5MU was isolated from the eluate by HPLC (Licrosorb RP-18 $\phi 8 \times 250$ (Merck, Darmstadt), water-MeOH = 97.5:2.5 (v/v), 4 ml/min), 177.1 mg of 5MU being obtained; NMR δ_{C} (CD_3OD): 152.5 (s, C-2), 166.2 (s, C-

Fig. 2. Effect of Temperature.

Reaction mixtures containing 20 mM inosine, 20 mM thymine, 20 mM potassium phosphate and 50 μ l of crude enzymes in a total volume of 1 ml, were incubated at various temperatures. $\circ$, 40°C; $\bullet$, 50°C; $\square$, 60°C; $\blacksquare$, 70°C; $\triangle$, 80°C.

4), 111.4 (s, C-5), 138.3 (d, C-6), 12.4 (g, -Me), 90.4 (d, C-1'), 75.4 (d, C-2'), 71.3 (d, C-3'), 86.2 (d, C-4'), 62.3 (t, C-5'); NMR δ_H (CD₃OD): 7.82 (1H, d, J =1.3 Hz, C6H), 5.89 (1H, D, J =4.6 Hz, C1'H), 4.19 (1H, d, J =4.6 Hz, C2'H), 4.15 (1H, d, J =4.7 Hz, C3'H), 4.00 (1H, m, C4'H), 3.81 (1H, d, J =3.1 Hz, C5'H), 3.77 (1H, d, J =3.1 Hz, C5'H), 1.88 (3H, d, J =1.3 Hz, C5-Me).

The effect of temperature on the reaction rate

The reaction was carried out at 40~80°C (Fig. 2). Up to 70°C, 5MU and hypoxanthine were synthesized with time, but heat denaturation was observed at 80°C. This showed that the enzymes were heat stable.

The effect of the phosphate concentration

As shown in Fig. 3, the formation of hypoxanthine increased with the concentration of phosphate, but the formation of 5MU remained almost the same. Without potassium phosphate, the reaction did not proceed. 5MU was also formed in sodium phosphate buffer, in place of potassium phosphate buffer (data not shown). Thus, phosphate is necessary for this reaction.

Fig. 3. Effect of the Phosphate Concentration.

Reaction mixtures containing 20 mM inosine, 20 mM thymine, 50 μ l of crude enzymes and various concentrations of potassium phosphate (pH 7.0), in a total volume of 1 ml, were incubated for 1 hr at 65°C. $\circ$, hypoxanthine; $\bullet$, 5-methyluridine.

Table III. REACTION OF RIBOSE-1-PHOSPHATE AND BASES

Reaction mixtures, containing 20 mM ribose-1-phosphate, 20 mM base, 20 mM potassium phosphate and 25 μ l of acetone-treated enzymes, in a total volume of 250 μ l, were incubated at 60°C.

Added base	Nucleoside formed (inosine* or 5-methyluridine**)	
	15 min	30 min
Hypoxanthine	1.86 mM*	3.40 mM*
Thymine	2.47**	4.48**

Reaction of ribose-1-phosphate and bases

Ribose-1-phosphate and thymine or hypoxanthine were incubated with the crude enzymes, respectively. The corresponding nucleosides were formed (Table III).

A. solution containing 20 mM inosine and 20 mM potassium phosphate was incubated with the crude enzymes for 6 hr at 65°C. Ribose-1-phosphate was detected in this solution by HPLC.

Heat stability of the enzymes

The heat stabilities of the enzymes were investigated at 63°C and 70°C (Fig. 4). The half-lives of the enzymes synthesizing 5MU were 400 hr and 14 hr, respectively. On the

Fig. 4. Heat Stabilities of the Enzymes.

The crude enzymes were kept with 1 mM inosine, 1 mM thymine and 20 mM potassium phosphate (pH 7.0) at 63°C and 70°C, respectively. The remaining activity was assayed by a standard method. ○, PUNP activity; ●, PYNP activity.

other hand, the half-lives of the enzyme for hypoxanthine were 7200 hr and 150 hr at 63°C and 70°C, respectively. The half-lives of the enzymes synthesizing 5MU and hypoxanthine were different, so the ribosyl transfer reaction was considered to be carried out by two kinds of enzymes.

Discussion

As the result of screening of many thermophiles, we found that *B. stearothermophilus* JTS 859 produced thermostable enzymes that synthesize 5MU from inosine and thymine in the presence of phosphate. *B. stearothermophilus* JTS 859 grew rapidly and its enzymatic activity also changed rapidly, as shown in Fig. 1. It was considered that these are characteristics of a thermophile. Due to the rapid growth of many thermophiles, only 4 hr-cultured cells might show the enzymatic activity on screening.

It was considered that this ribosyl transfer reaction was carried out by a combination of a purine nucleoside phosphorylase (PUNP) (EC 2.4.2.1) and a pyrimidine nucleoside phosphorylase (PYNP) (EC 2.4.2.2), due to the following facts. First, phosphate was necessary for the reaction. Second, the reaction of ribose-1-phosphate and bases with the crude enzymes resulted in the formation of the corresponding nucleosides. The incubation of inosine and

potassium phosphate with the crude enzymes yielded ribose-1-phosphate. Third, the heat stability of the enzyme synthesizing hypoxanthine was different from that of the enzyme synthesizing 5MU. Therefore, it is considered that this reaction consists of the two ribosyl transfer reactions shown above, i.e., Eq. (1) and (2).

The optimal temperature for the reaction was 70°C. Even at 80°C, 5MU and hypoxanthine were synthesized within 1 hr, but the enzymes were apparently denatured at this temperature. Regarding the PUNP, it was stable at 70°C, i.e., above the optimal growth temperature (62~66°C), although the phenomenon of that a thermophile enzyme is rapidly inactivated at a slightly higher temperature than the optimal growth temperature has been shown to be characteristic of several thermophile enzymes.¹¹⁾ The half-lives of PYNP and PUNP were 400 hr and 7200 hr at 63°C, respectively. It is interesting that their heat stabilities were very different, although they were derived from the same strain and their functions are similar. As for a purine nucleoside phosphorylase and a uridine phosphorylase derived from a mesophile, *Enterobacter aerogenes*, the half-lives at 60°C were about 170 hr.⁷⁾ The enzymes from the present thermophile are extremely stable compared to the above mentioned ones from a mesophile. As for PYNP derived from a thermophile, *B. stearothermophilus* NCA10, it was stable at 60°C for 1 hr but was denatured at 70°C for 1 hr.⁸⁾ The PYNP from *B. stearothermophilus* JTS 859 is more stable than the PYNP from *B. stearothermophilus* NCA10. The PUNP produced by *B. stearothermophilus* JTS 859 is the most thermostable nucleoside phosphorylase, as far as we know. Due to their heat stabilities, PUNP and PYNP produced by *B. stearothermophilus* JTS 859 are useful for practical application, especially as immobilized enzymes.

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