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*Escherichia coli*의 Peroxidase Isoenzyme에 미치는 溫度의 影響

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Effects of Temperature on the Isoenzymes of Peroxidase in *Escherichia coli*

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ABSTRACT

This experiment was designed to study the effects of temperature on the peroxidase isoenzymes of a mesophilic microorganism, *Escherichia coli* (grown within biokinetic zone.)

Optimum temperature for the growth of *E. coli* was 37°C. Three different temperatures, 20, 30 and 40°C, were selected. And the isoenzyme patterns of peroxidase of *E. coli*, grown respectively at each temperature, were analysed by disc electrophoresis.

The sample of 20°C showed 4 bands, that of 30°C, 5 bands and that of 40°C, 6 bands. Two dark bands (higher molecular weight, 56,000 and 54,000) and two light bands (lower molecular weight, 11,500 and 10,000) were constant at all samples. But two intermediate bands (M.W. 44,000 and 34,000) were variable; at 20°C, no banding pattern, one band (M.W. 34,000) only at 30°C, and at 40°C, two bands were appeared. And the shifts of growth temperatures between 30°C and 40°C showed the alteration of the isoenzyme patterns; the isoenzyme patterns of the sample of temperature shift from 30°C to 40°C were same as that of 40°C and *vice versa*.

INTRODUCTION

Temperature is one of the most important factors influencing the evolution and survival of microorganisms.

From ancient times, the fact that organisms live not only near 30°C, but at lower or higher temperature has been well known (Brock, 1967). Because living cells are essentially aqueous chemical systems, their existence is theoretically limited to temperature at which water exists in a liquid state.

The relationships between temperature and the activities of microorganisms were subjected to study greatly in the middle of 20th century with the physiology of thermophilism, i.e. mechanisms involved in survival (since Gaughran, 1947).

Sie *et al.* (1961) indicated that the mesophilic microorganisms could be transformed to the thermophile, when incubated with the genetic materials of thermophile.

Heibrunn (1924) and Bělehrádek (1931) observed that thermally stable organisms had lipids with higher melting points than did thermally labile organisms, and Brock (1967) joined to their proposition of the stability of membrane.

One of the first cell-free systems isolated from a thermophilic bacterium was a complex red-colored particulate fraction. This fraction showed all of the characteristics of a cytochrome system, but had a high degree of heat stability (Militzer *et al.*, 1949). And malate dehydrogenase was partially purified from the red fraction (Marsh and Militzer, 1952). After that, many of enzymes were isolated from thermophilic microorganisms and they were all thermostable (Campbell and Manning, 1961; Ohta *et al.*, 1966; Amelunxen and Lins, 1968; Hachimori *et al.*, 1970; Tanaka *et al.*, 1971; Ulrich *et al.*, 1972).

Stoll *et al.*, (1972) reported that aminopeptidase I from *Bacillus stearothermophilus* were composed of 12 subunits, two types of subunits (α, β), leading to only 3 isoenzymes ($\alpha_{10}\beta_2$, $\alpha_8\beta_4$, and $\alpha_6\beta_6$) and other isoenzymes could be formed *in vitro*, and also reported that their thermostabilities were identical, although the various isoenzymes had different substrate specificities.

Fulco (1969 and 1970) demonstrated the temperature induction of the desaturation reaction by bacilli. A mesophile, *Bacillus megaterium* desaturated palmitate to 5-hexadecencate readily at 20°C but not at 30°C. Further investigation of the temperature effect revealed that the Δ^5-desaturating enzyme of *B. megaterium* is not present at 30°C but can be induced by previously incubating a 30°C grown culture at 20°C. His experiments suggested that the shift of temperature induced the new materials, leading to the new enzyme and expression of new or masked gene.

On the base of the above some other experiments, temperature will control the expression of genes, at least in the production of isoenzymes. Authors performed some experiments to observe the influence of temperature on the induction of isoenzymes of a catabolic enzyme, peroxidase, and report some suggestions about the alteration of isoenzymes according to the shift of temperature.

MATERIALS AND METHODS

1. Strain and Growth

Escherichia coli 055 B5 obtained from N.I.H. was used in all these experiments. Optimum temperature for the growth of bacteria was 37°C and was subcultured on nutrient agar slants (*p*H 7.0), at the same temperature. Growth and incubation were carried out in air with shaking temperature-controlled rotary shaker (200 rpm) with nutrient broth (*p*H 7.0) at 20, 30 and 40°C. Growth phase was determined by the method of colorimetry at 530nm.

2. Preparation of Cell-free Extracts.

Bacteria at the end of logarithmic phase, grown respectively at 20, 30 and 40°C, were harvested by 10 min-centrifugation at 12,000 × g, at 4°C and washed three times with 0.05M Tris-Glycine buffer (*p*H 8.3) by the same

centrifugation.

Cells were suspended in the same buffer and mixed at equal parts with alumina. This mixtures were frozen at −25°C, ground in a cold homogenizing tube at 4°C, for 20 mins. The broken bacteria were contrifuged at 15,000 ×g for 60 mins, at 4°C and the clear supernatants were only used for disc gel electrophoresis.

3. Polyacrylamide Gel Electropherosis.

In order to analyse isoenzymes, disc gel electrophoresis was carried out by the method of Davis (Ornstein, 1964; Davis, 1964; Hong and Park, 1974) with some modifications; the concentration of the running gel was 10% instead of 7.0% acrylamide. The columns used in this experiments were 5mm in inner diameter and 80mm in length. All columns were filled with running gel from the bottom to 60mm and after polymerization, spacer gel was filled on the running gel to 10 mm.

The amount of protein of the loaded crude cell extracts on each column was within range of 200-400μg which was determined according to the method of Lowry *et al.* (1951), using human serum albumin as a standard. After 30min prerunning, electrophoresis was performed with Tris-Glycine buffer (pH8.3) at a current of 2mA/gel for 1hr, 2 hrs and 3 hrs respectively at 4°C.

4. Identification of Peroxidase by its activity.

Peroxidase activity on the gel was assayed qualitatively by the specific staining (Scandalio, 1965). Gels were taken out of the columns, washed with distilled water and incubated immediately in the staining solution, containing benzidine, H_2O_2, acetic acid for 30 min. at room temperature, 30°C and 40°C respectively.

5. Molecular Weight Determination.

Molecular weights of peroxidase isoenzymes were determined by disc gel electrophoresis of David method, using bovine serum albumin (67,000), ovalbumin (45,000), pepsin (35,000) and chymotrypsinogen (25,000) as standards.

6. Temperature shift experiments.

Incubations were performed by the same procedure, as described in strain and incubation. Two procedures were applied:

a. Cultures, grown at 30°C for 6 hrs, were grown continuously at 40°C, shifted temperature, for 18 hrs.

b. Cultures, grown at 40°C for 4 hrs, were grown continuously at 30°C, shifted temperature, for 20 hrs.

The alteration of isoenzymes of peroxidase analysed also by disc gel electrophoresis.

RESULTS

1. Growth Phase at Various Temperatures.

When growth phase was determined by colorimeter at 430, 530 and 660 nm respectively, that of 530 nm was appropriate to this condition.

As shown in Fig. 1, growth rate was decreased according to the decrease of temperature; at 40°C, cells initiate to proliferate within ca. 3 hrs and reach to stationary phase at ca. 12 hrs, at 30°C, initiate within ca. 4 hrs and reach to maximum population at ca. 15 hrs, and at 20°C, initiate within ca. 12 hrs and reach to maximum at ca. 45 hrs.

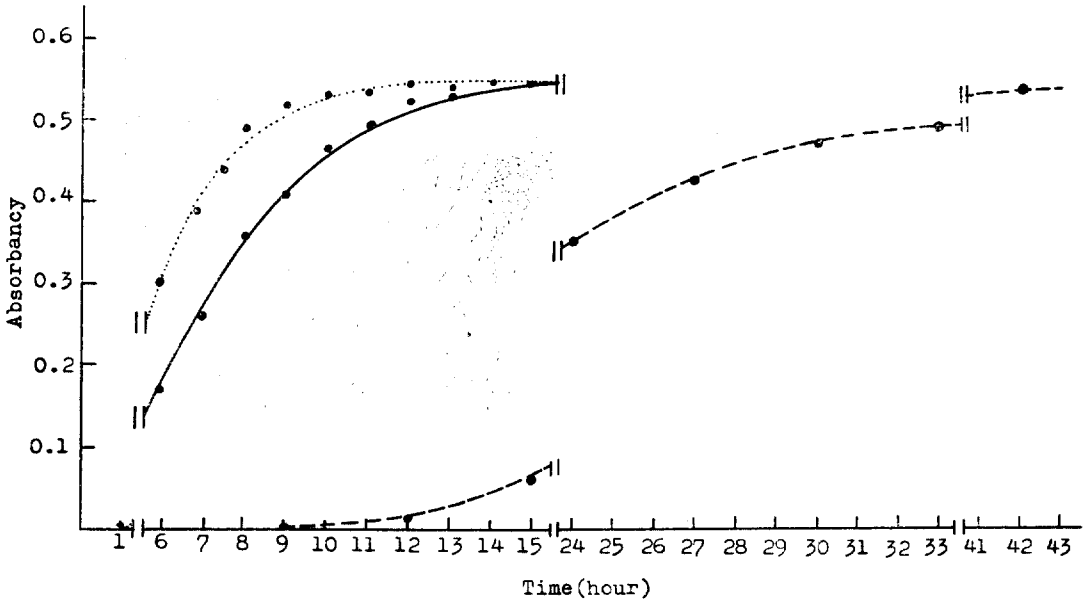

Fig. 1. Growth curves, measured by colorimeter with 530 nm, at various temperatures; 20, 30 and 40°C (·····, 20°C; ———, 30°C; ····· 40°C).

2. Isoenzymes on Electrophoretograms at Various Temperatures and their Molecular Weights.

When specific staining of peroxidase isoenzyme banded in the gels was performed at room temperature, 30 and 40°C, all temperature conditions showed the same aspect on the electrophoretograms, i.e. within selected range of incubation temperatures for staining (about 20-40°C), activities of all peroxidase isoenzymes were maintained.

All cultures at various temperatures were stopped in the end of logarithmic phase; 40°C-culture within 6 hrs, 30°C-culture within 8 hrs and 20°C-culture within 24 hrs.

When bacteria were grown for the same period under the condition of various temperatures, each culture showed the same isoenzyme banding pattern as that grown for different period at each temperature.

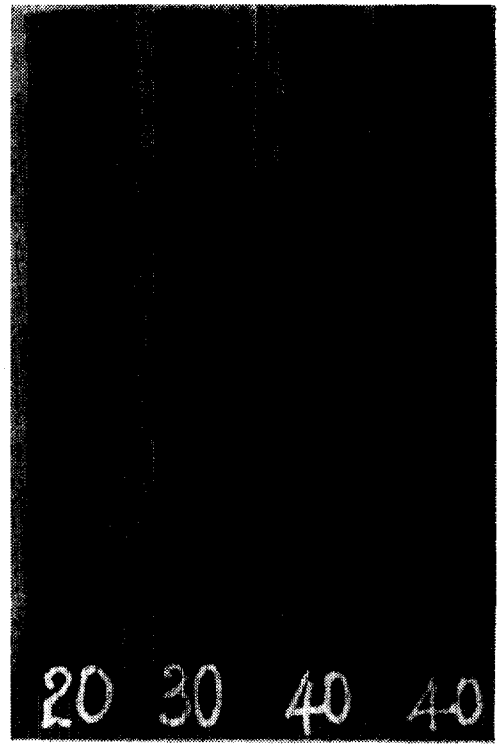

Fig. 2. Isoenzyme patterns after 2hr-running.

Fig. 2 shows electrophoretograms after 2 hr-running under the condition of various temperatures. In the extracts from 40°C-grown cells, all bands appeared; one dark broad band in the upper position and two light bands (M.W. 11, 500 and 10,000) in the lower position and two bands (M.W. 44,000 and 34,000) in the middle of the gels. In the case of 30°C, one of the two bands in the middle almost vanished; only 4 bands existed apparently in the gel and one band (M. W. 44,000) was too faint to discriminate as peroxidase activity. In the case of 20 °C, two bands in the middle all most vanished, so that it was so difficult to discriminate, as did one band in 30°C. When electrophoresis was performed for 3 hrs (Fig. 3) one dark band, a common band at various temperatures, was divided into two dark sharp bands (M. W. 56,000 and 54,000) in the upper position of the gels.

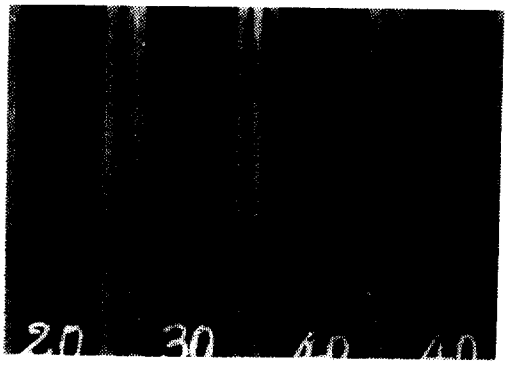

Fig. 3. Isoenzyme patterns of the upper position after 3 hr-running, appeared broadly in Fig. 2.

Therefore, the numbers of isoenzymes at various temperatures shown after 2.5 hr-running of electrophoresis were as follows (Fig. 6); 6 bands at 40°C, 5 bands at 30°C and 4 bands at 20°C. According to the decrease of temperature,

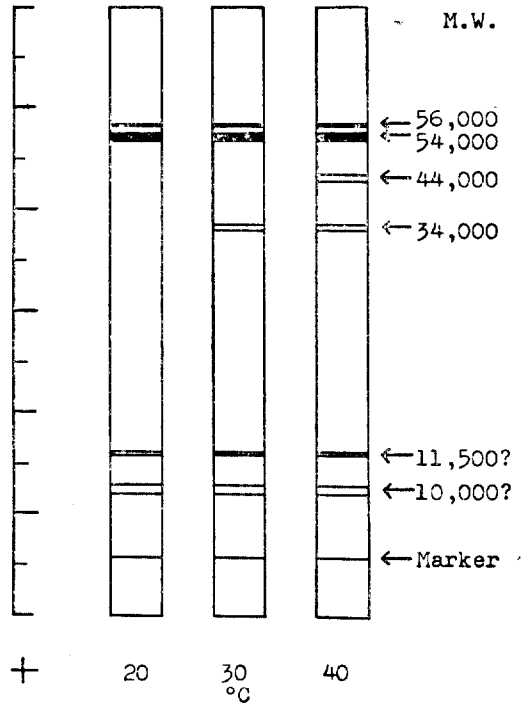

Fig. 6. Electrophoretograms after 2.5 hr-running, summarized in comparison with Figs. 2, 3 and 5.

the number of isoenzymes was decreased.

Fig. 5 shows the semi-logarithmic plotting about the relationship of molecular weights and relative mobilities. In

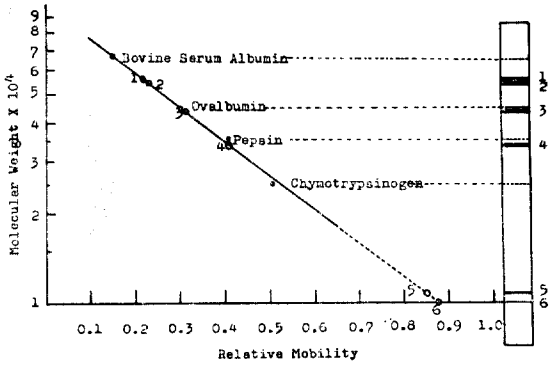

Fig. 5. Semi-logarithmic plotting of the relationship between molecular weights and relative mobilities. Bovine serum albumin (67,000), ovalbumin (45,000), pepsin (35,000) and chymotrypsinogen (25,000) were used as standards.

this figure, the expected linearity was formed and molecular weights of peroxidase isoenzymes were measured on it. Their values were already described above.

3. Induction of New Isoenzymes.

In order to investigate further above the vanished isoenzyme, temperature shift experiments for the growth of bacteria were performed.

As shown in Figs. 4 and 7, temperature shift from 30°C to 40°C induced isoenzyme and this isoenzyme vanished again in 30°C-grown culture by temperature decrease from 40°C to 30°C; banding pattern of temperature shift from 30°C to 40°C was same as that of 40°C-grown culture and *vice versa*.

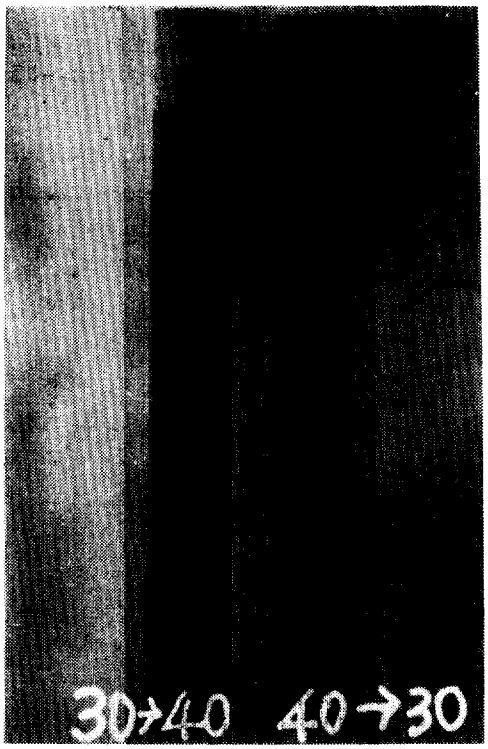

Fig. 4. Isoenzyme patterns after 2.5 hr-running according to the temperature shift experiment.

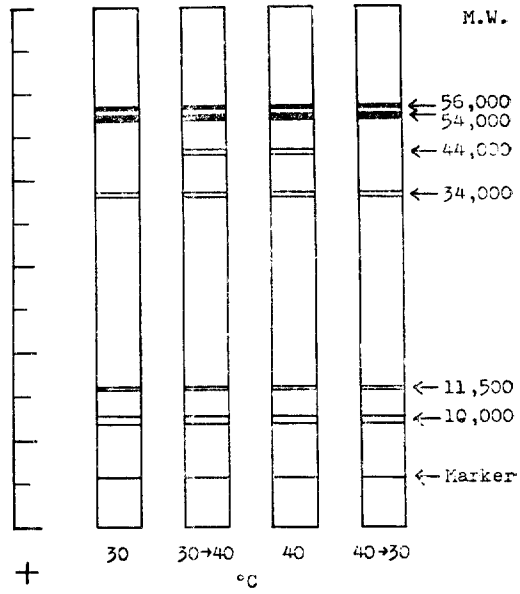

Fig. 7. Electrophoretograms summarzied in comparison with Figs. 4 and 5.

In the temperature shift experiments, when the post-incubations were stopped in the end of the maximum growth (in 30°C-40°C, for 12 hrs, in 40°C--30°C, for 10 hrs), isoenzyme banding patterns were similar to each other.

DISCUSSION

The results of this study show that peroxidase isoenzymes in cells of *E. coli*, mesophile, are able to be altered by the changes of temperature.

Hagen *et al.* (1964), Haight and Morita (1968) and Nash and Sinclair (1968) reported thermally induced leakage from psychrophilic bacteria and yeasts. Crisan (1973) also reported that bands of intracellular soluble protein, on electrophoretograms in fungi were decreased according to the increase of temperature, which was higher than that of biokinetic zone, that is, the increase of temperature induces the leakage of the intracellular

soluble protein of microorganisms, leading to collapse of the integrity of membrane, as proposed early by Brock (1968). But these reports were all about the range, in which microorganisms cannot express their life phenomena. In practice, it is very dangerous to discuss the data under the abnormal conditions without the data under the normal conditions.

Fulco (1969 and 1970) suggested also the alteration of enzymes by temperature shift within biokinetic zone, as previously described in Introduction. However, the idea of enzymes in this sort of experiment is very rough, because there are many isoenzymes in a single enzyme. As reported by Stoll *et al.* (1972), in a given condition, only some kinds of many isoenzymes, exist in living cells and other kinds can be formed *in vitro*. It may suggest that the protein had a high degree of hydrophobic nature, based on the amino acid analysis.

O'Brien *et al.* (1973) isolated and purified methylen tetrahydrofolate dehydrogenase from *Bacillus stearothermophilus*. Two bands were always seen upon disc gel electrophoresis. When the purified bands were subjected to electrophoresis a second time, two bands were again appeared on the electrophoretogram. This observation might suggest an equilibrium relationship for the two forms. Sedimentation centrifugation demonstrated a single molecule of 60,000 daltons. Thus two electrophoretic bands are not products of an association equilibrium, but are probably products of a conformational equilibrium.

In comparison with the above described reports, Figs. 2, 3, 4, 6 and 7 give us some suggestions; changes of temperatures within biokinetic zone are able to alter the isoenzymes in living bacteria. This alteration occurs gradually, parallel to temperature decrease. The results from temperature shift experiment (Figs. 4 and 7) may strongly suggest that the expression of genes is able to be altered by changes of temperature, as suggested by Fulco (1969 and 1970), with respect to the expression of different genes from that expressing fixed or constant bands.

However, if isoenzyme bands could appear by separation and/or recombination of its subunits, as described in aminopeptidase I(Stoll *et al.*, 1972) and methylenetetrahydrofolate dehydrogenase (O'Brien *et al.*, 1973), the flexible bands may not be products expressed by new or masked genes, but a secondary products formed non-enzymatically. Such possibility can be assumed in comparison of molecular weights of isoenzyme bands with each other (Fig. 5). If two bands of lower molecular weights (11,500 and 10,000) are equivalent to two types of isoenzymes (A, 11,500 and B, 10,000), then the band of M.W. 56,000 may be composed of A_4B_1, that of M.W. 54,000, A_3B_2, that of M.W. 44,000, A_3B_1, that of M.W. 34,000, A_2B_1 or A_3B_0. Thus, two flexible intermediate bands (44,000 and 34,000) may be formed by the aggregation of isoenzyme A and/or B.

In order to solve these problems, it must be demonstrated that the bands of higher molecular weights (M.W. 56,000 and 54,000) and the intermediate bands (M.W. 44,000 and 34,000) can be dissociated by treating of those isoenzymes with high salt or temperature, to form

the bands of lower molecular weights (11,500 and 10,000), and also immunological assay may be essentially required to elucidate the origin or vanished band, because if this band was derived from other bands or formed by re-assembly of the subunits, the antibodies against this band cross-react with other bands, and if it was produced by the expression of new or masked genes, switched on or off by temperature, then the antibodies against it must not reveal cross-reactivity.

At all events, on assumption that each isoenzyme reacts in the different site of metabolisms, the alteration of banding patterns of isoenzymes of peroxidase suggest the changes of metabolism due to only one environmental factor, temperature itself within biokinetic zone.

摘 要

Mesophile인 *Escherichia coli*의 peroxidase isoenzyme의 温度에 依한 變化를 實驗, 觀察하였다.

*E. coli*의 生長 最適温度는 37°C이며 20°C, 30°C, 40°C가 本實驗에서 採擇되었다. Isoenzyme은 disc 電氣泳動法으로 分析하여 그 banding pattern을 相互 比較하였다.

細胞의 生長率은 温度의 降下에 따라 減少하였으며 peroxidase isoenzyme의 數도 温度의 降下에 따라 減少하여 40°C에서는 6個, 30°C에서는 5個, 20°C에서는 4個이었다. 分子量이 56,000인 것과 54,000인 두 個의 짙은 bands와 分子量이 11,500, 10,000인 弱한 두 個의 bands는 세 温度條件下에서 모두 같이 나타났으나, 分子量이 44,000인 것과 34,000인 것은 變化를 보여 40°C에서는 모두 나타났으며, 30°C에서는 分子量 44,000인 것만이 나타났고, 20°C에서는 모두 사라졌다.

温度에 따라 變化를 보이고 있는 이 두 個의 bands에 對해 温度 變化를 培養 途中에 加한 結果, isoenzyme의 banding pattern은 變化를 보여, 30°C에서 培養하던 것을 40°C로 옮겼을 때는 40°C의 banding pattern과 같았고 40°C에서 30°C로 옮긴 것을 30°C와 같은 banding pattern을 보였다.

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