INJURY OF *Escherichia coli* IN PHYSIOLOGICAL PHOSPHATE-BUFFERED SALINE INDUCED BY FAR-INFRARED IRRADIATION

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Key Words: Food Chemical Engineering, Biological Engineering, Infrared Radiation, Pasteurization, Injured Cell, Antibiotic, Rifampicin, Chloramphenicol, *Escherichia coli*

Injury in *Escherichia coli* irradiated by far-infrared radiation (FIR) was examined. We used penicillin G (PCG), chloramphenicol (CP), rifampicin (RFP) and nalidixic acid (NA) as selective reagents, and tried to evaluate the injury in bacteria irradiated by FIR on the basis of the changes in the sensitivity to selective reagents. The validity of this method was examined using UV pasteurization. *E. coli* irradiated by UV became more sensitive to CP and NA, which indicated that UV irradiation damaged nucleic acid in *E. coli*. This result agrees with those demonstrated by many researchers and suggests this method is applicable to useful evaluation of the damaged part in bacteria induced by stresses. *E. coli* irradiated by FIR became more sensitive to RFP and CP, which suggests that FIR irradiation damages RNA polymerase and ribosome in *E. coli*. Though similar changes in the sensitivity were obtained in the case of thermal conductive heating, the pasteurization effect of FIR irradiation was much greater than that of conductive heating.

Introduction

Far-infrared radiation (FIR), which is the region of wavelength from about 3 to 1000 μm , is easily absorbed by water and organic materials, which are the main components of food. Therefore, application of FIR to pasteurization in food processing is desired and fundamental studies on pasteurization by FIR are needed.

In the previous study⁸⁾, the pasteurization effect of FIR irradiation was compared with that of thermal conductive heating, under the condition that the bulk temperature of bacterial suspension was elevated above the lethal temperature of bacteria. At that time, the transient behavior of the bulk temperature of bacterial suspension irradiated by FIR was the same as that heated by thermal conduction. The pasteurization effect of FIR irradiation was much greater than that of thermal conduction. It was suggested that the pasteurization effect was due to both the absorption of FIR energy within a very thin domain near the surface and the bulk temperature of the suspension.

Moreover, FIR irradiation was carried out under the condition that the bacterial suspension irradiated by FIR was cooled and the bulk temperature of the suspension was maintained below 313 K. Since the bulk temperature was below the lethal temperature of bacteria, the pasteurization effect of the bulk temperature by thermal conductive heating was negligible. Under this condition, *Escherichia coli* and *Staphylococcus aureus* in the suspension were injured and

killed by FIR irradiation¹⁰⁾. By estimating the temperature distribution in the suspension, it was suggested that bacteria were injured and killed within the very thin domain near the surface of the suspension^{9, 11)}. However, it has not yet been grasped how FIR radiation injures and kills bacteria. Study on the action mechanism of FIR pasteurization is needed for its application.

FIR radiation has a heating effect. For thermal inactivation of bacteria, several possible targets such as DNA^{33, 35)}, RNA, ribosome^{25, 28)}, cell envelope^{13, 23)}, and proteins in the cell^{20, 21, 22)}, have been proposed by many researchers. However, the mechanisms of thermal death and injury of bacterial cells have not been elucidated.

The purpose of this study is to examine the injury in bacteria induced by FIR irradiation. In this study, we supposed that sublethally injured cells induced by stress became more sensitive than intact cells to the agent which had the inhibitory action on the damaged part of the bacteria. By using the agents of which primary action were well known, we tried to evaluate the injury in *E. coli* caused by stress on the basis of the changes in the sensitivity of injured cells to the agents. At first, in order to prove the validity of this method, the injury in *E. coli* induced by ultraviolet (UV) irradiation, of which the pasteurization mechanism is well known, was examined by using this method. Then, the injury induced by FIR irradiation was examined by the same method. Moreover, since FIR is an electromagnetic wave with thermal effect, the injury of bacteria irradiated by FIR was compared with that of bacteria heated by thermal conduction.

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Fig. 1 Effect of concentration of PCG on colony-forming number. Intact cells of *E. coli* were incubated with agar medium containing various concentrations of PCG

Table 1 Maximum concentrations of selective reagents in agar medium and primary inhibitory actions

Reagent	$C_{MAX}[mg/l]$	Primary inhibitory action
PCG	14.8	synthesis of cell wall
CP	2.5	synthesis of protein
NA	1.0	synthesis of DNA
RFP	2.2	synthesis of RNA

1. Materials and Methods

1.1 Pasteurization sample

E. coli 745 was used throughout this study. *E. coli* was cultured in Brain Heart Infusion broth (Difco) at 308 K for 24 h. The culture was suspended in 0.05 M physiological phosphate buffered saline to give a final bacterial concentration of about 10^9 CFU/l. The pasteurization sample was the same as that used in previous studies^{8, 9)}.

1.2 Pasteurization by far-infrared irradiation

The FIR irradiation apparatus and the FIR irradiation method were the same as those used in previous studies^{8, 9, 10, 11)}. The radiative power irradiated on the bacterial suspension surface was $3.22 \text{ kW} \cdot \text{m}^{-2}$, which was measured according to the method reported in the previous study¹²⁾. The suspension temperature was measured by CA-thermocouples sheathed by a stainless steel tube (0.25 mm ϕ). Measurement was carried out according to previous studies^{10, 11)}. Since the evaporation loss from the surface of the suspension during FIR irradiation was less than a few percent of the weight, the variance of the depth of suspension was less than a few percent.

1.3 Pasteurization by conductive heating

The conductive heating apparatus and the conductive heating method were the same as those used in the previous study⁸⁾. By varying electric power supplied to a submerged heater, the transient behavior of the bulk temperature of the bacterial suspension in the test tube was controlled to be the same as that irradiated by FIR. The suspension temperature was measured by CA-thermocouples sheathed by a stainless steel tube. The measurement was carried out according to the previous study⁸⁾.

1.4 Pasteurization by UV irradiation

The bacterial suspension of 0.02 l was dispensed in

Fig. 2 Transient behavior of temperature of bacterial suspension

Fig. 3 Effect of FIR irradiation and conductive heating on surviving fraction of *E. coli*

a petri dish made of stainless steel (14.5 $\times$ 88.5 mm ϕ). The dish was placed under a UV lamp (Mitsubishi GKL-15), and UV irradiation was carried out at 298 K (room temperature). The wavelength of the lamp was 254 nm. Irradiation distance was 0.28 m.

1.5 Microbial counts

After pasteurization, the suspension was diluted with physiological saline. The diluent was pour-plated with agar medium. Sensitivity Disk Agar-N (Nissui) was used as the non-selective medium. Sensitivity Disk Agar-N containing the selective reagent was used as the selective medium. Penicillin G (PCG, Sigma), chloramphenicol (CP, Sigma), nalidixic acid (NA, Daiichiseiyaku), and rifampicin (RFP, Daiichiseiyaku) were used as selective reagents in order to specify the character of injury in *E. coli*. Their primary inhibitory actions are shown in Table 1. The colonies were

Fig. 4 Changes in sensitivity of *E. coli* irradiated by FIR to selective reagents: (A) 5 min, (B) 6 min, (C) 7 min, (D) 8 min

enumerated after incubation at 310 K for 48 h.

2. Results

2.1 Determination of concentration of selective reagent in agar medium

For uninjured cells, the number of colonies on the selective medium is the same as that on the non-selective medium. However some of the injured cells form colonies on the non-selective medium but not on the selective medium. The difference in both colony counts with and without the selective reagent can be taken as a measure of injury. The effects of various concentrations of the selective reagents on the recovery of *E. coli* in the selective medium were examined to find the maximum concentrations where the selective reagent had no effect on colony formation of intact cells. Figure 1 shows the effect of PCG on the surviving fraction (N_1/N_{10}) of intact cells of *E. coli*. The surviving fraction of *E. coli* was unity at PCG concentration lower than 14.8 mg/l and decreased rapidly above that concentration. The results of four selective reagents were summarized in Table 1.

2.2 Pasteurization by FIR irradiation

As shown in Fig. 2, FIR pasteurization was carried out under the condition that the transient behavior of bulk temperature of bacterial suspension irradiated by FIR irradiation was the same as that heated by thermal conduction. In FIR pasteurization, the heat transfer to the bacterial suspension depends on only radiation. It is assumed that the optical and thermal properties of the bacterial suspension are approximately equal to those of water. The external reflectivity of water to FIR is 0.04. Therefore, 96 % of irradiation is absorbed by the bacterial suspension and causes a rise in the temperature of the suspension. Figure 3 shows the pasteurization effects by FIR irradiation and thermal conductive heating. For the same heating time, the pasteurization effect by FIR irradiation is much greater than that by thermal conduction.

Figure 4 shows the changes in the sensitivity of *E. coli* to the selective reagents at FIR irradiation times of 5, 6, 7 and 8 min. The abscissa C/C_{MAX} represents the ratio of a specified concentration to the maximum concentration

Fig. 5 Changes in sensitivity of *E. coli* to selective reagents by conductive heating for 8 minutes

of selective reagent shown in Table 1. After an irradiation time of 5 min, there was no decrease in surviving fraction and no changes in the sensitivity to the selective reagents (Fig. 4 (A)). When the suspension temperature became higher than the lethal temperature of test bacteria (approximately 330 K), the sensitivities to RFP, CP, PCG increased at 6 min, and injury in *E. coli* was observed (Fig. 4 (B)). After an irradiation time of 7 min, the changes in sensitivity to RFP and CP were higher than those of the others (Fig. 4 (C)). An increase in FIR irradiation time markedly enhanced the sensitivity to CP (Fig. 4 (D)).

2.3 Pasteurization by conductive heating

Figure 5 shows changes in the sensitivity of *E. coli* heated by thermal conduction for 8 min under the conditions shown in Fig. 2. *E. coli* heated by conduction showed similar changes in sensitivity to four selective reagents in the case of FIR irradiation; at first, the sensitivity to RFP increased, and next, the sensitivity to CP increased. Changes in the sensitivity of *E. coli* irradiated

Fig. 6 Effect of UV irradiation on surviving fraction of *E. coli*

by FIR were larger than those heated by thermal conduction (Figs. 4 (D) and 5).

2.4 Pasteurization by UV irradiation

Figure 6 shows the results of UV pasteurization. UV irradiation caused a reduction of surviving fraction to 3.0×10^{-1} after 20 s. Changes in the sensitivity of *E. coli* to four selective reagents after 20 s are shown in Figure 7. The surviving fraction of *E. coli* decreased with an increase in $C/C_{\max}$ of NA, CP and PCG, which indicated that the sensitivities to NA, CP and PCG increased.

3. Discussion

3.1 Validity of this method

The pasteurization effect of UV is due to the absorption of UV by the nucleic acid of bacteria, and its pasteurization mechanism is understood well³². The most damaging range, around 260 nm, is consistent with the wavelength range absorbed by nucleic acids such as DNA and RNA in cell. UV irradiation causes single- and double-stand breaks in the DNA and leads to bacterial death. As shown in Fig.7, UV irradiation increased the sensitivities to NA and CP. The modes of action of NA and CP are DNA-synthesizing systems^{5, 6, 17, 24}) and protein-synthesizing systems depending on reaction with the 50S subunit of ribosome, respectively^{4, 16, 18, 19}). The 50S subunit contains two kinds of RNA and about 30 kinds of proteins. These results suggest that UV irradiation induces injury of nucleic acid such as DNA and RNA, and are consistent with those obtained by many researchers³²). It was also reported that ionizing irradiation damages cell membranes³). The mode of action of PCG is synthesis of cell wall^{27, 31}), and the increase in the sensitivity to PCG

Fig. 7 Changes in sensitivity of *E. coli* to selective reagents by UV irradiation for 20s

indicates damage to the cell envelope, including the cell wall and membrane. Therefore, this method could be applied as a simple method to finding the damaged part in *E. coli*.

In this method, changes in the permeability of the cell membrane by heat treatment is one problem. It was reported that heat treatment caused increases in the cell surface hydrophobicity of *E. coli* and in the sensitivity to hydrophobic antibiotics¹⁴). The order of magnitude of hydrophobicity of the antibiotics used in this study is CP, RFP, NA and PCG. However, the increases in permeability of CP and PCG were not observed, even if the surface hydrophobicity increased^{15, 29}). The concentrations of the antibiotics used as selective reagents in this experiment ($C_{\max}$) are also markedly lower than those in the literature^{29, 30}) or minimal inhibitory concentrations (MIC). Moreover, in FIR irradiation and conductive heating, the sensitivity of *E. coli* to NA did not increase. Therefore, in this method, the increase in sensitivity due to change in permeability of cell membrane does not seem to be a serious problem.

3.2 Comparison of pasteurization by FIR irradiation and conductive heating

The bulk temperature rise of the suspension heated by FIR irradiation was the same as that heated by thermal conductive heating. The lethal temperature of test bacteria, *E. coli* 745, was approximately 330 K. For both FIR irradiation and conductive heating, increases in the sensitivity of *E. coli* were observed at near the lethal temperature. As shown in Figs 4 and 5, the changes in sensitivity to the selective reagents by FIR irradiation showed similar tendencies to conductive heating. At first, the sensitivity to RFP increased, and next, the sensitivity

to CP increased. However, the pasteurization effect by FIR irradiation was much higher than that by conductive heating. FIR irradiation caused death of *E. coli* when the bacterial suspension irradiated by FIR was cooled and the bulk temperature was maintained below the lethal temperature. In this case, since the influence of the bulk temperature on bacteria is negligible, the pasteurization effect is due to the absorption of infrared radiation energy by suspension in a very thin domain near the surface. Injury in *E. coli* within the domain is under investigation by this method. However, by estimating the temperature distribution in the suspension, the temperature of the very thin domain near the surface is much higher than the bulk temperature measured by the thermocouples^{8, 10}. Therefore, the difference in the pasteurization effect between FIR irradiation and conductive heating, and the pasteurization effect of FIR below the lethal temperature depend on the heating of bacteria within this domain. It seems that the pasteurization effect by FIR irradiation is due to the heating effect.

3.3 Injury in *E. coli* heated by FIR irradiation and thermal conduction

When *E. coli* in physiological phosphate buffered saline was irradiated by FIR, the sensitivity to RFP increased (Fig. 4). RFP acts on RNA polymerase and inhibits initiation of DNA-dependent RNA synthesis^{7, 26, 36}. RNA polymerase consists of α , β , β' and σ , subunits. In gene expression in *E. coli*, σ factor binds to core RNA polymerase and transcription starts from promoter on gene. Recently, the mechanism underlying induction of heat shock proteins (HSP) is a central issue in the heat-shock response and studied intensively in *E. coli*³⁴. When cells are shifted rapidly from low to high temperature, synthesis of HSP is induced quickly. At that time, the exchange in σ factor binding to RNA polymerase occurs ($\sigma^{70} \rightarrow \sigma^{32}$), and the synthesized HSPs play an important part in protection of cells against stress. Therefore, the function of RNA polymerase in cells is one of the most sensitive parts to temperature change. In the early stage of FIR irradiation and conductive heating, the increase in the sensitivity to RFP suggests injury of the function of RNA polymerase in *E. coli* and does not contradict with the understandings of heat response.

The surviving fraction decreased markedly with increasing irradiation time and the sensitivity to CP increased rapidly. CP, a well-known inhibitor of protein synthesis, binds to large ribosomal subunits (50S), and inhibits peptidyltransferase reactions^{4, 16, 18, 19}. The increase in the sensitivity to CP indicates damage to ribosome and rRNA in ribosome. It was reported that ribosome and rRNA in heated cells was destroyed^{25, 28}. Ribosome separates to 30S and 50S ribosomal subunits and decomposes to smaller particles. On the other hand, the sensitivity of PCG was almost the same. PCG inhibits the synthesis of cell walls in bacteria^{1, 2}. These results suggest that FIR irradiation and thermal conduction damage RNA polymerase and ribosome before DNA and cell membranes and lead to bacteria death.

4. Concluding Remarks

In order to examine the injury in bacteria irradiated by FIR, we tried to evaluate the injury in *E. coli* induced by stress on the basis of the changes in sensitivity to four selective reagents (PCG, CP, RFP and NA). UV pasteurization was carried out to prove the validity of this method. *E. coli* irradiated by UV became more sensitive to CP and NA, which indicated that UV irradiation damaged nucleic acid in *E. coli*. This result agrees with those demonstrated by many researchers. We considered that this method could provide an evaluation of the damaged part in *E. coli* induced by stresses, and evaluated the injury in *E. coli* irradiated by FIR. The following results and conclusions were obtained.

1) Changes in the sensitivity to selective reagents by FIR irradiation showed similar tendency to those by conductive heating. It is considered that the pasteurization effect by FIR irradiation is due to a heating effect. However, the pasteurization effect of FIR irradiation was much greater than that of conductive heating.

2) When *E. coli* was heated by FIR irradiation and thermal conduction, at first, the sensitivity to RFP increased, and next, the sensitivity to CP increased. These results suggest that FIR irradiation and conductive heating damage to RNA polymerase and ribosome in *E. coli* and lead to death.

Acknowledgment

The authors wish to thank Daiichiseiyaku Co.Ltd. for a generous gift of antibiotic.

Nomenclature

C	= concentration of selective reagent	[mg·l ⁻¹]
N_1	= concentration of viable cells in bacterial suspension	[CFU·l ⁻¹]
T_1	= bulk temperature of the bacterial suspension	[K]
t_h	= heating time	[s]
t_i	= irradiation time of UV	[s]

<Subscripts>

MAX	= maximum
0	= initial state

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(Presented in part at the 27th Autumn Meeting of the Society of Chemical Engineers, Japan at Kasugai, September 1994)