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論　文　名	「Conditional generation and its mechanism of oxidative injury in heated and irradiated *Escherichia coli* cells （大腸菌の加熱および照射細胞における酸化損傷の条件的発生とそのメカニズム）」
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論文要旨

Target number three, the goal No.12 of Sustainable development goals (SDGs – United Nations), stated that “by 2030, halve per capita global food waste at the retail and consumer level and reduce food losses along production and supply chains, including post-harvest losses”. The food industry is very demanding regarding food safety, and one of the biggest problems that cause the loss of food production in supply chains is microbial contamination. It relates to the whole supply chain: harvesting, transport, storage, and processing. Various chemical and physical treatments have been applied to reduce and control the risk of foodborne pathogens. Thermal treatment is one of the oldest techniques still in daily use. Depending on the target organisms, heat treatments can be classified into pasteurization and sterilization treatment. Heat treatment has multitargets action on membranes, DNA, RNA and protein in a microbial cell. However, on the need for sterilization or disinfection in heat-sensitive materials or food production, radiation

treatment has been promoted as an alternative treatment with a large number of applications in many countries except Japan. Irradiation treatment uses energy transmitted through space in a wave pattern (including ionizing and non-ionizing radiation) to kill or inhibit bacteria. Radiation treatment can easily penetrate cell membranes, alters the molecular structure, damages cell components, and can kill/inhibit contaminating microorganisms, and react with water molecules to form oxygen radicals. Both methods have their advantages and disadvantages, but the main goal of any preservation method is to maximize the quality of the product while achieving the highest safety level.

After the preservation treatments, bacterial cells are divided into three groups as lethal population (dead), resistant population (survived with less severe damage) and sublethal population (injured). Bacterial injury arising from food preservation techniques has received much attentions over the past 50 years. Injured populations show a prolonged lag period before re-growing and re-gaining their pathogenicity under favorable conditions and these cells pose a potential threat to food safety. The degree of injury can be influenced by many factors, such as the stress level, processing and storage conditions, bacterial species, and so on. The generation of oxidative stress as a secondary damage to bacterial cells has been observed in various stresses such as heat, antibiotic, acidity, and radiation treatments. The relevance and mechanisms of cell repair in considering the effect of oxidative stress in injured cells is necessary to understand bacterial injury.

Identifying factors that can affect the bacterial inactivation, using *E. coli* as a model microorganism, with the conditional effects of oxidative injury was a critical objective in this study. In this study, the semi-synthetic and complex media for cell growth and recovery were employed to evaluate the effects of heat and irradiation treatments. Furthermore, the survival statistics of heated and irradiated populations by adding antioxidant compounds to the recovery process was also aimed to investigate the effects of oxidative damage on bacterial cells in those control treatments. It is necessary to explore the relationship between oxidative injury and the resuscitation of microbial injure cells in the preservation treatments in order to develop an efficient strategy to control foodborne pathogens. The dissertation content contains 6 chapters as summarized as follows:

Chapter 1 provides general information on some of the commonly used disinfection

treatments in the food industry. This chapter also introduces the definition of bacterial injury, characteristics of the injured cells and current knowledges regarding repair mechanisms. Besides induced primary stress, the impact of oxidative secondary injury on bacterial cell survival and detection methods for injured cells are introduces.

Chapter 2 presents an overview and evidence on the involvement of oxidative stress during and after thermal treatment. The thermal treatment has been used as a method of preventing microbial spoilage since ancient ages. After treatment, injured cells may recover to a normal physiological state under favorable conditions which may pose a potential threat to food safety. The presence of oxidative stress after heat treatment may contribute to the inactivation of spoilage bacteria. In this study, we investigated the heat sensitivity of *Escherichia coli* cells and the oxidative stress injury by assessing the rescue effects of several reducing and reactive oxygen species (ROS) scavengers in two different conditions. Heat resistance in *E. coli* cells grown in the enriched minimal M9 (EM9) medium was higher than those cells grown in Luria broth (LB) medium. In addition, the survival rate of heat-treated cells was increased with the addition of ROS scavengers in liquid and agar media evaluated by plate counting and the growth delay analysis as well as the most probable number methods. The heat sensitivity of the cells and the effects of ROS scavengers on that depended on the growth conditions were demonstrated. Furthermore, the enumeration of the secondary injury, the resuscitation mechanisms of injured cells and the potential of the rescuing antioxidants for bacterium were also discussed.

The repair mechanisms of heat-injured *E. coli* cells were discussed in the **chapter 3**. In this chapter, the author investigated the effect of heat treatment on bacterial cell physiology in terms of oxidative stress and the repair mechanisms in injured cells. The effects of heat treatment on the activities of two antioxidative enzymes, involving in the intracellular reactive oxygen species and redox potential were examined. The activities of catalase and superoxide dismutase increased slightly during heating period and gradually decreased to the baseline level within 4 hr of the post-heating incubation. The generation of superoxide radicals and alteration of redox potential during heat treatment seemed to be heterogeneous in *E. coli* cells grown on both minimal and rich media. In addition, a complex nutritional system may be involved in the oxidative damages in heat-treated cells grown in the rich medium, while the repair of the membrane damage suggested to be

crucial for cells grown and recovered in the minimal medium. The addition of antioxidants to agar media also rescued many injured cells even in the presence of some of the metabolic inhibitors. In both treatments, the electron transport system seemed to critically affect (both positively and negatively) to the generation of and revival from the oxidative injury in heated cells. The results obtained in this study emphasize once again the potential application of different antioxidants to rescues heat-damaged cells depending on the recovery medium. This finding supports the current knowledges of the inactivation action of heat, oxidative secondary injury and the repair mechanisms in heat-treated bacterial cells.

A new subculture method for evaluation and quantitative analysis of growth delay time has proposed and presented in **chapter 4.** The microcolony formation at 30°C on EM9 agar plates by individual *Escherichia coli* cells heated at 50°C was monitored using a time-lapse shadow image analysis system, MicroBio μ3D^{TM} AutoScanner. The distribution of microcolony detected every half an hour for the unheated cells appeared to be consistent with the normal distribution. Meanwhile, the heated population demonstrated the growth delay probably resulting from cell injury and distributed in a deformed pattern with a tailing. Cumulative patterns of microcolonies appearing during the post-heating cultivation period could be expressed with three different mathematical models. The presence of antioxidants also affects the formation time of microcolony in heated cells. This approach can be proposed as a predictable rapid culture method to enumerate viable and repairable injured cells in practical use.

In **chapter 5,** the effects of gamma and UV irradiations on the inactivation of *E. coli* cells and the oxidative secondary injuries were evaluated. The direct interaction with cell components of and/or indirect modification by free radicals resulting from water radiolysis is known as the inactivation mechanism in the radiation treatment. In relation to the effect of oxidative secondary injury presented in the previous chapter, the experiments concentrated on the conditional generation of oxidative injury population induced by ionizing and non-ionizing radiation treatment. *E. coli* cells grown in two different media irradiated with UV and gamma-ray treatments, then recovered in liquid and solid medium were used to evaluate the effects of growth and recovery media to the radio-sensitization and oxidative damage. The sensitivities to UV and γ-rays of *E. coli* cells depended on both

media, although the recovery medium was more important. Also, oxidative damage was also produced in irradiated cells and was dependent on the recovery medium. This study suggested a different approach for the analysis of the irradiation injury mechanism and also contributes to the understanding of the effects of oxidative stress in the application of radiation to control pathogenic microorganisms.

Firstly, we confirmed the presence of oxidative stress as secondary injury in heated or irradiated cells by using the antioxidant agents. The rescue effect of these agents was examined in both liquid and solid media, which liquid media was less effective than in solid media in heat-induced cells. Reversely, secondary injury caused by UV and γ-rays irradiation was observed at lower rates than heat treatment and the effectiveness of ROS scavengers in irradiated cell is altered depending on the recovery medium. Oxidative status, ROS generation, growth/recovery condition and antioxidant function influence oxidative secondary damage caused by heat and radiation treatment. In particular, membrane damage repair is critically involved in the resuscitation of heat-injured cells, especially those grown in semi-synthetic media. In both medium, the inhibition of the electron transport chain attenuated the viability of heat-injured cell as confirmed in the results obtained. Moreover, heat-induced oxidative secondary stress is also associated with bacterial cell inactivation. Finally, we have introduced and proposed a novel method to enumerate the growth delay in microcolony formation time by using the time-lapse shadow image analysis. Interestingly, the addition of antioxidants in agar not only rescued oxidative damage but also participates to the colony formation in heat-injured cells.

In conclusion, this study should contribute to the understanding of the injurious actions of heat and radiation treatments on microbial cells, as well as the significance of oxidative stress in relation to the oxidative secondary injury and resuscitation processes in injured bacterial cells.

審査結果の要旨

食品の損耗を防ぎ、安全で食味豊かな食材を持続的に提供することは地球規模の課題となっている。特に食品の安全性を脅かす食中毒菌の防除については食味など食品の品質を最大限に確保しながら、最小限の処理条件で行うなど、殺菌に求められる条件が厳しさを増している。特に殺菌後の食品に残存し、保存中に回復する恐れのある損傷菌の発生メカニズムを知り、適切な殺菌処理法を開発することが喫緊の課題である。本論文においては損傷菌発生の原因として酸化ストレスに注目し、代表的な物理的殺菌法である加熱殺菌と放射線殺菌に的を絞り、微生物モデルとして大腸菌を材料として以下の成果を得ている。

(1) 栄養培地及び無機合成培地を用いた場合の大腸菌の加熱、紫外線、^{60}Co γ線感受性の違いを明らかにし、培養中に種々の抗酸化剤、活性酸素消去剤を添加した場合の感受性から微生物細胞の二次傷害としての酸化ストレスの存在を確認した。

(2) 熱処理が細菌の細胞生理に及ぼす影響を、酸化ストレスと損傷菌細胞の修復機構の観点から検討し、細胞内の代表的な抗酸化酵素、カタラーゼとスーパーオキシドディスムターゼの活性は、加熱中にわずかに上昇し、加熱後 4 時間以内にベースラインレベルまで徐々に低下することを見出した。紫外線およびγ線に対する感受性は両培地に依存し、抗酸化剤添加による回復促進効果も軽微であった。以上を含めて種々の要因について検討した結果、損傷を受けた細胞の二次損傷回復過程に細胞膜や呼吸系の酸化的二次損傷と培地組成との関連性が重要であることを考察した。

(3) タイムラプスシャドウ画像解析法を用いて、目視できない寒天培地上のマイクロコロニー生長の動態解析を可能とし、殺菌処理後の回復過程の細胞増殖動態を簡便に測定する新方法を導入・提案した。

以上の研究結果は、損傷菌の回復過程における基礎的知見の蓄積に加えて、更に高度な測定、解析法の提案により、当該分野の研究の加速を期待させるものであり、食品業界が食品の微生物学的安全性を保証するための衛生管理システムを構築するためにも有用な示唆を与えるものとして今後の大きな発展を期待させるものである。これらの成果は申請者が自立して研究活動を行うに必要な能力と学識を有することを証したものである。