Kinetic Study on the Thermal Resistance of E. coli Cultivated by Soybean Extract Added Medium

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The aim of this study is to investigate the thermal stress resistance properties of Escherichia coli (E. coli) to construct the optimized sterilization method for the soybean based foods. The samples of E. coli incubated on the soybean extract added LB medium (test sample) and normal LB medium (control sample) were subjected to high temperature stress of 60°C. Soybean extract which is mainly consisted of protein, lipid and water was prepared to add the LB medium. Under high-temperature condition, the survival behaviors of E. coli inoculated by the control and test sample were indicated by the first-order kinetics. And the thermal resistance of E. coli was significantly enhanced by a soybean extract. Moreover the top, middle and bottom part of soybean extract were prepared by the additional centrifugation of soybean extract. Each part is characterized by lipid, phospholipid, and protein, respectively. The thermal resistance of middle part showed the quite similar behavior to that of whole soybean extract. However, those of top and bottom parts were obviously decreased faster than that of middle part. And a significantly increase of the weight of phospholipid in a cell incubated with the middle part was observed comparing with that of control sample. It is therefore very likely that the thermal resistance of E. coli is enhanced by the effect of phospholipid in soybean extract. Furthermore, under the high pressure stress of 150 and 200 MPa, the survival curve of middle part was obviously slower decrease than control sample. Consequently, results obtained in this paper, are successfully revealed that the addition of phospholipid in soybean extract is strengthened the cell membrane or surface structure of E.coli.

Keywords: soybean extract, E. coli, high hydrostatic pressure stress, high temperature stress

1. Introduction

Temperature control is widely considered as a main form of external perturbations, triggers chemical, biochemical, and microbiological changes during food processing. Thermal treatment has been applied as the general process to inactivate microorganisms from food [1, 2]. In particular, the survival behavior of Escherichia coli, which is a cause of severe food intoxication, has been reported by previous researches [3]. Moreover, non-thermal processes such as the use of antimicrobial oxidants, high voltage electric pulses, oscillating magnetic fields, and combinations of these have gained importance in recent years as potential technologies to replace or complement the thermal processing of food [e.g. 4, 5].

Soybean has been used in many food products in Japan because its high nutrient content satisfies many human nutritional requirements [6]. However, soymilk, a soybean–based product, is also an ideal medium for microbial growth. Thus, the development of optimal sterilization methods is essential, not only to preserve product quality but also to expand the market of soybean–based food [7, 8]. Soybean is known to contain anti–nutritional components such as trypsin inhibitors. Research focusing on these inhibitors has led to progress in the practical use of soybean–based food [9]. Soybeans are used as materials of the fermented foods. In modern food industry, fermented soybean foods are distributed after being sterilized. In such cases, the microorganisms were incubated coexisting with soy components, and are sterilized in the coexistence soy components.

However, to our best knowledge, an optimal sterilization process for soybean–based food is still under development. Surprisingly, few studies have reported the survival behavior of microorganism by using systems whereby the incubation and inactivation processes are separated [10, 11]. In particular, kinetic analysis is fre-
quentely used to clarify the effects of intrinsic factors on the survival of microorganisms. Recently, Ishiwatari et al. proposed kinetics of microorganisms in food products subjected to thermal treatment by using the heat-transfer simulation model. These studies have provided sufficient insight on the kinetic behavior of microorganisms in food products [12, 13].

To optimize the sterilization method of food products, it is very important to consider that the thermal resistance of *E. coli* and other bacteria is improved by various physical and chemical factors and growth conditions, such as growth medium, growth temperature, and growth phase [14–19].

However, while a robust data set is integral for developing a model, the investigation of the survival behavior of microorganisms due to the separated system of incubation and inactivation processes has not yet been performed thoroughly. Generally, protein denaturation is a main factor causing the inactivation of microorganisms by thermal stress. And studies have reported on the effect of salts and sugar in the solvent [14, 16, 20, 21]. On the other hand, previous studies have suggested a relationship between the composition of cellular fatty acids and heat resistance exhibited by various bacteria. Specifically, changes in membrane fluidity because of fatty acids may contribute to the thermal resistance of bacteria [18]. Thus, the investigation of the effect of phospholipids is essential to clarify the mechanism of thermal resistance in *E. coli*.

It is well known that high pressure, which directly influences the cell membrane of microorganisms, is a useful criterion in understanding the mechanism of the stress protective function of microorganisms [2, 22–26]. A number of researchers have therefore reported on the survival behavior of *E. coli* affected by high pressure [24, 27, 28].

In this study, therefore, the thermal resistance enhancement of *E. coli* with the coexistence of soybean extract is investigated in a system where incubation and inactivation are separated. The mechanism of thermal resistance which *E. coli* obtains is discussed in terms of kinetic analysis and the morphology of *E. coli*.

2. Materials and Methods

2.1. Sample preparation

*E. coli* K12 strain was prepared for thermal and pressure treatment in this study. The cells were maintained and cultivated in slant LB medium. *E. coli* K12 was inoculated in new LB medium and distilled water. The medium used for the control sample consisted of 10 g/L Bacto Tryptone (Becton, Dickinson and Co.), 5 g/L Bacto yeast extract (Becton, Dickinson and Co.), and 10 g/L NaCl (Wako Pure Chemical Industries) at pH 7.2.

*E. coli* K12 was incubated in LB medium (control samples), and soybean extract was added to the medium (test samples). Soybean extract for the test samples was prepared from soymilk (Taishi Food Inc., Aomori, Japan). Soymilk was centrifuged at 70000 rpm at 20°C for 1 h and it clearly separated into three parts: surface, liquid, and precipitate as shown in Fig. 1, and the weight ratio of each part was 2.34, 95.87 and 1.79 %, respectively. Since soymilk is a colloidal dispersion containing oil and protein, the separation result obtained by the centrifugation was appropriate. Namely the oil body and protein were separated into the surface and precipitate phase, and the liquid part was separated as the continuous phase. These parts are referred as top, middle, and bottom parts, respectively, in this study. Flasks of inoculated medium were shaken at 110 rpm for 24 h at 37°C.

2.1.2 Content of soymilk

As contents of top, middle and bottom part of soymilk, the top part and the bottom parts mainly consisted lipid and protein, respectively. As described above, the middle part was dominated more than 95 % of total weight of soymilk, and it characterized by an abundance of phospholipids. In other words, the weights of phospholipid in top, middle and bottom part were 12.16, 561.82 and 2.66 mg per 100 g of total weight, respectively. Note that the weight of phospholipid in each part of soymilk was con-

![Fig. 1](image-url) Overview of surface, liquid, and precipitate of the centrifuged soymilk.
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2.1.3 Determination of the weight of phospholipids in a cell of *E. coli*

*E. coli* inoculated to control and test sample, respectively, and they were incubated with shaking at 37°C during 24 h. After that, their solutions were centrifuged under 4000 rpm, 4°C at 10 min. Cells of *E. coli* were collected from the precipitation of these centrifuged solutions. Cells of *E. coli* were suspected into chloroform–methanol solution, and were centrifuged (1500 rpm, 5 min, and 4°C condition). Consequently this solution was separated chloroform phase included phospholipid of *E. coli* and the other parts. Chloroform was completely evaporated, finally the remained phospholipids of *E. coli* was weighted.

2.2 Heat treatment

Heat treatment was performed in a test tube in a temperature-controlled water bath. Samples were suspended in 0.85% NaCl solution. Dispersed water was exposed to heat treatment at 60°C for up to 100 s. The test tubes were immediately transferred to ice for cooling at the end of the heating period.

2.3 Pressure treatment

Samples of control and middle part of test were packed by vacuum packing machine, respectively and each sample was settled in a stainless-steel chamber of high hydrostatic pressure machine (HR–7–BS, Hikari Kouatsu, Hirashima, Japan). Inside of the chamber is satisfied by distilled water, scilicet, a sample is soaked in water to add the hydrostatic pressure. Each sample was subjected the hydrostatic pressure of 150 and 200 MPa during 120 s at room temperature.

2.4 Cell count

Samples were diluted with distilled water, and 1 mL of the sample was inoculated on a dry sheet medium culture plate (Compact Dry EC, Nissui Pharmaceutical). Each plate is a phosphate-buffered selective medium, and it mainly consists of peptone, KNO3, NaCl, sodium pyruvate, substrates selective for gram-positive bacteria. Inoculated plates were incubated at 37°C for 1 d.

2.5 The theory of kinetic analysis [2]

The resistance of cells in certain bacterial populations is described by first-order kinetics as follows:

\[ \frac{N}{N_0} = e^{-kt} \]  

\[ \ln \left( \frac{N}{N_0} \right) = -kt \]

where \( N_0 \) is the initial number of cells (CFU/mL), \( N \) is the number of survivors (CFU/mL), \( t \) is the exposure time (s), and \( k \) is the first-order inactivation rate constant (s\(^{-1}\)).

3. Results

3.1 Survival curve of *E. coli* under temperature stress

The survival curve of control and test samples under temperature stress is shown in Fig. 2. A linear decrease in survival ratio was observed in both control and test samples. Consequently, the entire behavior of the survival curve for the control and test samples can be described well by a first-order kinetic equation.

Figure 3 shows the survival curve of the top, middle, and bottom parts, which were separated by centrifuging the test sample, test, and control sample (shown in Fig. 2), respectively. A linear decrease in survival rate was confirmed with high accuracy for all samples. Moreover, the survival ratio of the test and middle parts of the test sample were similar and were obviously slower than those of the control sample, top, and bottom parts of the test sample. These results therefore successfully show that the high temperature resistance of *E. coli* was obviously enhanced with the addition of soybean extract, and components of the middle part contributed to the enhancement in resistance.

![Fig. 2 Survival curves of *E. coli* of soybean extract added LB medium (test sample) and LB medium (control sample) at 60°C during 0–100 seconds.](image)
Moreover, Fig. 4 depicts the complementary experimental result of the survival ratio of *E. coli* by using phosphate buffer (pH 6.86, Wako Pure Chemical Industries Ltd.) for incubation instead of 0.85% NaCl solution. The survival ratio of control sample was faster than that of test, and this behavior was similar with that by using 0.85% NaCl solution for incubation (i.e., Fig. 2). These results therefore suggested that the effect of salt in solution for the improvement of thermal resistance of *E. coli* may be slight in the case of this study.

**3.2 Behavior of inactivation rate constant**

Table 1 summarizes the inactivation rate constant obtained in this study, compared with some similar studies. In previous studies, the inactivation rate constant of *Bacillus cereus*, which was cultivated in LB medium and suspended in phosphate buffer or milk, was reported to be around 0.001 and 0.21 [30]. The constant of *E. coli* in culture solution cultivated in LB medium was around 0.0085 [30]. However, the result obtained from the middle part of the soybean extract in this study was 0.016.

**3.3 Effect of phospholipids on the resistance improvement of *E. coli***

As we described above, soybean, especially the middle part obtained by centrifugation of soymilk, contains abundant phospholipids. Therefore, the influence of phospholipids, which coexist with *E. coli* during incubation, was investigated.

Changes in phospholipids in a cell of control sample and middle parts of the test sample after incubation is shown in Table 2. The weight of phospholipids in the test sample was obviously heavier than that of control sample, respectively. Thus, when *E. coli* is cultivated with the contents of the middle part, the phospholipids in the cells increased obviously.

**3.4 Survival curve of *E. coli* under high hydrostatic pressure stress**

Figure 5 shows the survival curve of *E. coli* in the middle part of the test and control sample under high hydro-

![Image of survival curve](image-url)
static pressure stress. The effect of sterilization decreased because of the addition of soybean extract. This behavior corresponds to that observed in the case of thermal stress.

4. Discussion

The thermal resistance of *E. coli* incubated with soybean extract added to the LB medium (test) was improved noticeably. Moreover, this behavior was only confirmed in the middle part of the test sample separated by centrifugation. The middle part contains phospholipids abundantly, and the weight of phospholipids in an *E. coli* cell in the test sample is approximately three times higher than that of the control sample. Therefore, it is important to note that phospholipids in the soybean extract may contribute to improvements in the thermal resistance of *E. coli*. In other words, the contribution of the triacylglycerol and protein which characterizes top and bottom part of soymilk may be smaller than that of phospholipids to gain the thermal resistance.

The results supported the insight reported by Annous *et al.* [18]. Namely, cell membrane fluidity may decrease because of the abundance of phospholipids. However, the details of this mechanism were not described. The phospholipids in soymilk may have been taken into the membrane, and crystalline structures may have been formed.

Thus, *E. coli* may have been surrounded by a thin biofilm similar to a liposome, which mainly consists of phospholipids.

Furthermore, improvement in the high-pressure resistance of *E. coli* was revealed by improvements in the membrane of *E. coli*. Cheftel and Chlioli have reported that high-pressure treatment affected the membrane structure and permeability of the cell membrane of microorganisms resulting from the crystallization of phospholipids. Therefore, it is very likely that the membrane of the incubated *E. coli* was influenced by the coexistence of phospholipids [31].

Interestingly, the resistance of *E. coli* could not be obtained by addition of other parts of the soybean extract. Biofilms, especially the cell membrane, mainly consist of hydrophobic vesicles. The phospholipids in soybean extract form micelles. Therefore, the mixing of micelles and vesicle phospholipids may arise with the addition of soybean extract. Takajyo *et al.* reported that extra structures were formed from mixing micelles and vesicles [32]. Moreover, properties of the new structures depended on the ratio between existent micelles and vesicles. In particular, the vesicle structure may stabilize on addition of appropriate short-chain phospholipids that form micelles. In our study, the protective effect of phospholipids originating from soybean extract may be optimized by components in the middle part.

5. Conclusion

To develop an optimal sterilization method for soybean–based food, this study demonstrated the fundamental behavior of *E. coli* in LB medium with added soybean extract. The survival curve of *E. coli* in medium with added soybean extract (test sample) was slower than that in normal medium (control sample) under high temperature conditions of 60°C. This trend was confirmed only in the middle part of the soybean extract as separated by centrifugation. The weight of phospholipids in the middle part was three times higher than that in the control sample. Moreover, under high hydrostatic pressure of 150 and 200 MPa, the survival curve of the middle part was slower than that of the control sample.

It is suggested that the phospholipids in soybean extract contributed to enhancements in the membrane or surface structure of *E. coli*. These results successfully demonstrated that soybean–based food might enhance the stress resistance of *E. coli*, consequently highlighting the need of special sterilization methods for soybean–based food.
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大豆抽出物添加培地で培養した大腸菌の熱死滅挙動に関する速度論的研究

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食の安全安心の観点から、食中毒の原因となる微生物の殺菌は重要である。しかし、過度の殺菌処理が施されると、品質が劣化するためプロセスの最適化が必要である。これまで微生物を標準的な培地で増殖させた後、被験食品に添加することで殺菌条件の検討が行われてきただが、食品中で増殖した微生物の死滅挙動については十分には解明されていない。そこで、本研究では、大豆系食品に対する殺菌プロセスの最適化的ため、豆乳中で培養した大腸菌についてその熱死滅挙動の解析を行った。指標微生物として大腸菌（K-12株）を、培養用試料として、LB培地と豆乳（水分：89.6%、脂質：2.7%、タンパク質：5.0%）、豆乳を遠心（70000 rpm, 25℃, 3時間）により分画して得られた上相（top part）、中間相（middle part）と下相（bottom part）にLB培地粉末を溶解させた培地を準備した。各培地に大腸菌を植菌し、37℃、24時間振盪培養した。培養液を0.85% NaCl溶液に懸濁し、60℃で加熱処理を施した。高温条件下の大腸菌の死滅曲線は大豆抽出物添加培地培養（Test sample）、LB培地培養（control sample）とも一次反応式で記述され、大腸菌は、大豆抽出添加培地で培養することにより耐熱性が増すことが示された。さらに、中間相の死滅速度は上相と下相より遅いことが確かめられた。中間相はリン脂質が豊富に含まれていることから、大腸菌のリン脂質含量を定量したところ、豆乳中で培養した大腸菌のリン脂質含量は、コントロールに比べて増加していた。リン酸緩衝液中で死滅させた場合においても、中間相の死滅が抑制される結果となった。したがって、大豆抽出物中のリン脂質の効果により、大腸菌の耐熱性が向上する可能性が示唆された。また、細胞膜に作用すると考えられる高圧試験を実施した結果、150および200 MPaの高圧処理においても、中間相の死滅速度は明らかにコントロールより遅くなった。以上の結果から、大豆抽出物中のリン脂質によって強化される可能性が示唆された。すなわち、大豆系食品では、微生物の熱耐性向上を考慮して殺菌プロセスを構築することが重要であると考えられた。

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