Application of combined non-thermal treatments for the processing of liquid whole egg

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Abstract

The objective of this research was to investigate the applicability of non-thermal treatments for the processing of liquid whole egg (LWE). High hydrostatic pressure (HHP) was focused as the main unit operation among the non-thermal technologies, and the utilization of nisin or high-intensity ultrasound was also considered within the study. As a first step of process optimization, LWEs were subjected to a wide range of pressure (100–400 MPa), temperature (5, 25 and 45 °C), and time (0–3600 s) combinations, and the pressure-induced coagulation of LWE was determined by a rheological method. The HHP processing criteria were evaluated from the coagulation kinetics of LWE, and it was indicated that pressure treatments at lower temperatures (5 and 25 °C) had clearly better opportunities to conduct HHP treatments avoiding egg protein coagulation. The kinetic studies on the HHP inactivation of microorganisms were performed at 5 and 25 °C. The optimized HHP conditions were selected by incorporating the HHP processing criteria into the microbial inactivation kinetics, and they were 250 MPa for 886 s or 300 MPa for 200 s at the treatment temperature of 5 °C. These processing conditions ensured the minimum changes in the rheological properties of LWE, and effectively reduced the microbial loads of LWE. The combination processes of HHP with other non-thermal treatments were explored to achieve further microbial inactivation. The addition of nisin prior to pressure treatments significantly increased the lethal effects of HHP against *Listeria seeligeri*, but not against *E. coli*. The combination of ultrasound and HHP had only a marginal effect both on *Listeria* and *E. coli* within the experimental conditions. Finally, the effects of HHP and combined non-thermal processes on the functional properties of LWE were compared to those of fresh LWE. Little changes in foaming and emulsifying properties were observed by the application of HHP at optimized processing condition. The presence of nisin during HHP treatment also did not affect the foaming and emulsifying property. Therefore, LWE with superior quality could be produced by the application of appropriate (combined) non-thermal treatments.

Abstrakt

Ziel der Forschung war die Entwicklung und die Optimierung eines nicht-thermischen Verfahrens von flüssigem Vollei durch die Anwendung hoher hydrostatischer Drücke, Nisin, Leistungsultraschall oder deren Kombinationen. Als erster Schritt wurden die Koagulationsgrenzen des flüssigen Volleis während der Hochdruckbehandlung festgestellt. Die Parameter Druck (100–400 MPa), Temperatur (5, 25 und 45 °C) und Behandlungszeit (0–3600 s) wurden variiert und deren Einflüsse auf die Koagulation der Eiproteine durch ein rheologisches Meßverfahren evaluiert. Die ausgewerteten Prozeßkriterien zeigten, daß Druckbehandlungen bei niedrigen Temperaturen (5 und 25 °C) deutlich bessere Ergebnisse bei der Hochdruckbehandlung ohne Koagulation der Eiproteine. Kinetische Untersuchungen zur Abtötung der Mikroorganismen innerhalb dieser Prozeßkriterien zeigten, daß die Inaktivierung der Mikroorganismen nicht nur Behandlungsdruck und Temperatur sondern auch vom Phasenzustand des flüssigen Volleis abhängig war. Die optimierten Hochdruck Behandlungsbedingungen (250 MPa für 886 s oder 300 MPa für 200 s um 5 °C) wurden so festgelegt, daß sowohl die Erhaltung der rheologischen Eigenschaften als auch die maximale Abtötung der Mikroorganismen erreicht werden konnte. Die Kombinationsprozesse von Hochdruck mit anderen nicht-thermischen Verfahren, wie Nisin oder Leistungsultraschall, wurden erforscht um eine verbesserte Inaktivierung der Mikroorganismen zu erreichen. Der Kombinationsprozeß von Hochdruck mit Nisin zeigte ausgeprägte Synergismen gegen Gram-positive *Listerien*. Schließlich wurden die Wirkungen von Hochdruck und von Kombinationsprozessen auf die funktionellen Eigenschaften von flüssigen Volleier mit denen von frischer Volleier vergleichen. Die Ergebnisse zeigten, daß durch die Anwendung der ausgewählten nicht-thermischen-Verfahren flüssiges Vollei mit bessere Qualität produziert werden konnen.

Contents

Symbols and Abbreviations

μ_{app} : Apparent viscosity [mPa·s]

ΔV : Partial molar volume change [ml·mol^{-1}]

ΔV^* : Apparent volume of activation [m^3·mol^{-1}]

ΔW : Excess work of structure breakdown [kJ·m^3]

C_p : Specific heat [kJ·kg^{-1}·K^{-1}]

D value : Decimal reduction time [s] or [min]

D_p : Decimal reduction time by pressure treatment [s] or [min]

f : Initial population of first fraction estimated from biphasic kinetic analysis

HHP : High hydrostatic pressure

k : Rate constant [s^{-1}] or [min^{-1}]

k_1 : Rate constant of first fraction [s^{-1}]

k_2 : Rate constant of second fraction [s^{-1}]

LWE : Liquid whole egg

N : Number of viable cells [cfu/ml]

N_t^{std} : Number of viable cells in standard I agar after treatment time t [cfu/ml]

N_t^{VRBG} : Number of viable cells in VRBG agar after treatment time t [cfu/ml]

p : Pressure [MPa]

R : Universal gas constant (8.314·J mol^{-1} K^{-1})

T : Temperature [K] or [°C]

t : Time [s] or [min]

US : (High-intensity) ultrasound

VRBG : Violet red bile glucose

V : Volume [m^3] or [ml]

γ : Shear rate [s^{-1}]

τ : Shear stress [Pa]

τ_e : Equilibrium shear stress [Pa]

Chapter 1. Introduction

The egg is an important part of human diet. It is one of the most nutritious foods we consume, and it can be prepared in many different ways by itself or as a food ingredient. Egg production worldwide has been increased continuously, and 49 million tons of eggs were produced in year 1999. Today, China has about 35% of world egg production, followed by USA (10 %), Japan (5 %) and India (3.5 %) (FAO, 2000).

The majority of eggs are consumed as shell eggs. However, some amounts of them are further processed to egg products. In the UK, 12–15 % of shell eggs go into egg products, whereas the comparable figure for the USA would be 28 % (Belyavin, 1993). If these egg products are classified by processing concerns, there are three types of egg products: liquid, frozen and dried egg product. The production of liquid egg is the first step of all these egg products because frozen or dried egg products are also made of pasteurized liquid egg.

Liquid whole egg (LWE), which is the main concern of this research, is mainly used in the industrial production of bakery goods, confectioneries or ice creams. LWEs, in addition to their nutritional value, contribute the functional properties to such foods like foaming, coagulation, or emulsification. Because these properties of egg can be easily impaired by heat treatment, egg product pasteurization is conducted on a critical temperature-time regime where the egg protein coagulation may not occur. The USDA requires that LWE be heated at least to 60 °C and held for no less than 3.5 min for the average particles. LWE pasteurization requirements of other countries are 64 °C for 2.5 min in the United Kingdom; 66 to 68 °C for 3 min in Poland; 63 °C for 2.5 min in

China; 62 °C for 2.5 min in Australia; and 65 °C for 90 to 180 s in Denmark (Cunningham, 1995).

Although the heat processes used to pasteurize LWE can assure food safety by eliminating heat sensitive pathogens from egg products, some heat resistant spoilage microorganisms can survive these pasteurization requirements and spoil the LWE even under refrigerated conditions. Generally, only one or two log cycle reductions of viable cell counts are achieved by commercial thermal pasteurization, and pasteurized liquid egg products contain 10^2 or more than 10^3 microbial cells/g. The principal genera found in pasteurized egg products are *Alcaligenes*, *Bacillus*, *Proteus*, *Escherichia*, *Pseudomonas* and Gram positive cocci (Schmidt-Lorenz, 1983; Cunningham, 1995). Therefore, the shelf life of LWE is very short even under refrigerated condition. For example, pasteurized LWE and yolk are chilled to 4.4 °C with the shelf life of only 4 days (Belyavin, 1993).

Much efforts have been devoted to overcome the limitation of conventional thermal pasteurization and to extend the refrigerated shelf life of LWE. The attempted technologies for LWE processing include ultrapasteurization combined with aseptic packaging (Ball Jr. et al., 1987), ultrasonic waves (Wrigley and Llorca, 1992), irradiation (Ma et al., 1993) or thermoradiation (Schaffner et al., 1989), high electric field pulses (Ma et al., 1998), high hydrostatic pressure (Ponce et al., 1998) and the use of antibacterial agents like nisin (Knight et al., 1999) or glucose oxidase (Dobbenie et al., 1995). However, most of the studies mentioned above were concentrated on the microbiological point of view, and did not consider the changes of physico-chemical properties of LWE induced by such processes.

Non-thermal food processing technologies, such as high hydrostatic pressure, high electric field pulses, high intensity ultrasound or the utilization of antimicrobial bacteriocins, have been received great attentions by food scientists due to their potential as a new method to preserve food with minimal loss of organoleptic and nutritional quality of fresh products. The objective of this thesis was to investigate the applicability of non-thermal technologies for the LWE processing.

High hydrostatic pressure (HHP) was considered as the main unit operation among the non-thermal technologies since the instant and homogeneous transmittance of pressure throughout a food system represents a major advantage over conventional thermal proc-

essing. For the application and optimization of HHP processing in the real food system like LWE, changes of physico-chemical properties induced by HHP treatments should be considered along with microbial inactivation. Although substantial literature is available on high pressure-induced protein gelation, information regarding the effects of high pressure treatment on liquid-semiliquid-semisolid transition state which is important for the processing designing is very rare. The first objective of this research was to investigate the coagulation of LWE over a wide pressure/ temperature/ time range in order to determine the boundary conditions for high pressure treatment of LWE (Chapter 4.1). The second objective of this research was to determine the kinetic parameters of microbial inactivation by HHP treatments and to find optimized points of the HHP processing (Chapter 4.2). The combination processes of HHP with other non-thermal treatments were also attempted to achieve further microbial inactivation (Chapter 4.3). Finally, the effects of HHP treatments or combined non-thermal processes on the functional properties of LWE were investigated to verify the product quality of processed LWE (Chapter 4.4).

Chapter 2. Literature Review

2.1. Egg and Egg Products

2.1.1. Structure of shell egg

The egg can be divided into four distinct parts: (1) yolk; (2) egg white; (3) shell membrane; (4) shell. The eggs vary in relative amounts of the parts, but a general average is 10% shell, 1% shell membranes, 59% egg white and 30% yolk. The yolk is formed during the final 10 days prior to the laying of the egg, and enclosed by the vitelline membranes and anchored in the center of the egg by the mucin fibers, the chalaza. (Stadelman, 1993). The egg shell and shell membrane act as natural barriers against microbial invasions during the storage. The fibrous shell membrane is composed of outer and inner shell membrane, and together they are 0.01–0.02 mm thick. The intrinsic detail of the egg shell was observed by scanning electron microscopy (Chen et al., 1999). Moving from outer layer toward inside, the egg shell is composed of cuticle, the sponge layer and the mamillary layer.

2.1.2. Chemical property of egg

As a human food, egg can be described one of the most nutritious foods. The chemical analysis of egg is presented in Table 2.1.1. Egg provides a wide range of nutrients including proteins, minerals and vitamins. Egg proteins may be divided into egg white proteins and yolk proteins. The egg white contains as many as 40 different proteins.

The major egg white proteins are ovalbumin, ovotransferrin (conalbumin), ovomucoid, lysozyme, globulins and ovomucin. Unlike egg white proteins, lipoproteins dominate egg yolk proteins. The physical and biological characteristics of egg proteins are summarized by others (Osuga and Feeney, 1977; Li-Chen et al., 1995; Mine, 1995).

The lipids of the eggs are all contained in the yolk. Nearly all of the yolk lipids are present as lipoproteins, which are classified as high density, low density and very low density lipoproteins. A lipid component that has received much attention is cholesterol. One large egg with a 17g yolk contains about 213 mg of cholesterol (Stadelman, 1993).

Table 2.1.1. Proximate, minerals and vitamins composition of whole egg, yolk and egg white (after USDA, 1999).

	Component	Whole egg	Yolk	Egg white
Proximate (g/100g)	Water	75.33	48.81	87.81
	Protein	12.49	16.76	10.52
	Total lipid (fat)	10.02	30.87	0.00
	Carbohydrate	1.22	1.78	1.03
	Ash	0.94	1.77	0.64
Minerals (mg/100g)	Calcium, Ca	49.00	137.00	6.00
	Iron, Fe	1.44	3.53	0.030
	Magnesium, Mg	10.00	9.00	11.00
	Phosphorus, P	178.00	488.00	13.00
	Potassium, K	121.00	94.00	143.00
	Sodium, Na	126.00	43.00	164.00
	Zinc, Zn	1.10	3.11	0.01
Vitamins (mg/100g)	Ascorbic acid (C)	0.00	0.00	0.00
	Thiamin (B_1)	0.06	0.17	0.01
	Riboflavin (B_2)	0.51	0.64	0.45
	Nicotinic acid	0.07	0.02	0.10
	Pantothenic acid	1.23	3.81	0.12
	Pyridoxal (B_6)	0.14	0.39	0.00

2.1.3. Physical property of egg

When the egg is freshly laid, the pH of yolk and egg white is about 6.0 and 8.0, respectively. During the storage, the pH of yolk and egg white gradually increases to the maximum values of 6.9 and 9.7, respectively. This increase of pH is due to the loss of carbon dioxide from the egg through the pores in the shell (Li-Chan et al., 1995). The pH of liquid whole egg varies according to the condition of shell eggs, but the general average is pH 7.5.

Thermal properties of egg components are useful for the designing of heating and cooling processes. The specific heat indicates how much heat is required to change the temperature of a material. The specific heats of egg components are: 3.26 $kJ \cdot kg^{-1} \cdot K^{-1}$ for yolk; 3.93 $kJ \cdot kg^{-1} \cdot K^{-1}$ for egg white; and 3.68 $kJ \cdot kg^{-1} \cdot K^{-1}$ for liquid whole egg. The latent heats of freezing are: 188 $kJ \cdot kg^{-1}$ for yolk; 295 $kJ \cdot kg^{-1}$ for egg white; and 251 $kJ \cdot kg^{-1}$ for liquid whole egg. The density of yolk, egg white and liquid whole egg is almost the same, 1.04 $g \cdot ml^{-1}$ (Cotterill, 1995).

The viscosity of liquid whole egg behaves as a Newtonian liquid provided that the shear rates are fairly high and the egg proteins are not coagulated. Both pasteurization and freezing increase the viscosity of liquid whole egg. The viscosity of unpasteurized liquid whole egg is about 10 mPa·s (Torten and Eisenberg, 1982; Herald et al., 1989).

Eggs or egg products are extensively utilized as food ingredients because of their functional properties. The major functions are (1) coagulating; (2) foaming; (3) emulsifying; (4) nutrition; (5) flavor; (6) color. These traditional and several novel functions of egg were completely reviewed by others (Yang and Baldwin, 1995; Mine, 1995).

2.1.4. Manufacturing of egg products

The process line for egg products varies depending on the types of product. The simplest process is the production of liquid whole egg where no separation process of yolk and egg white is needed. The production of frozen and dried whole egg is a further step beyond pasteurization of liquid whole egg.

Figure 2.1.2 shows the production flow of liquid, frozen and dried whole egg. Eggs are held under refrigeration until they are broken. After the eggs are transferred to process

line, undesirable eggs are separated by visual inspection. Then the eggs are washed and sanitized by spray rinsing which contains 100 to 200 ppm of chorine. Breaking machines are used to break the eggs and, if necessary, separate them into the yolk and the egg white. Such breaking–separating system can handle 16,000–65,000 eggs per hour (Cotterill and McBee, 1995). The contents from the broken eggs are then passed through a sieve to remove any remnants of shell, chalazae and any undesirable particles.

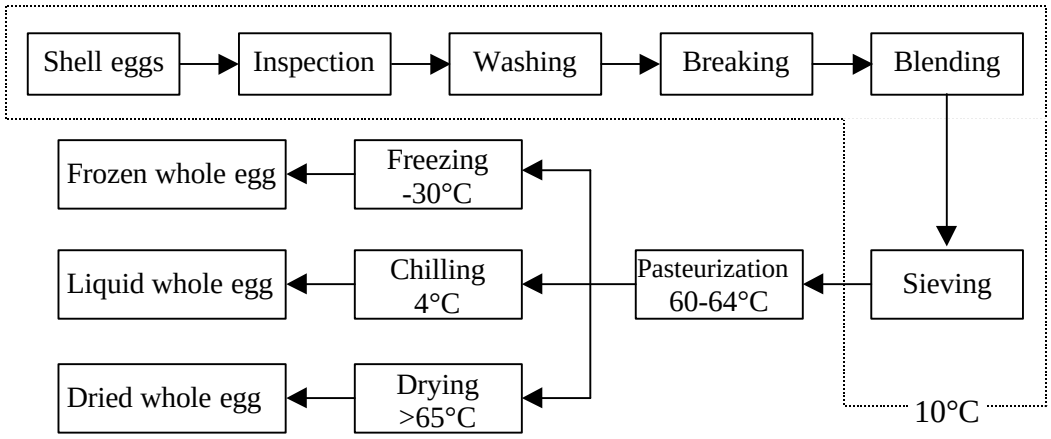

Figure 2.1.1. Processing flow of whole egg products

The pasteurization is the most important processing point of egg product. The objective in pasteurization is to reduce the hazard of potential pathogenic microorganisms and still retain the physical and functional properties of the raw liquid egg. Some countries including Germany have taken the position that liquid eggs must be pasteurized by a method which renders the egg products *Salmonella* negative, while other countries specified the conditions under which liquid eggs must be pasteurized. For example, pasteurization requirements for the egg products are described in the regulations in the United States. The holding times and temperatures listed in Table 2.1.2 are the minimum. Although the heat processes used to pasteurize liquid whole egg assure food safety by eliminating *Salmonella*, some heat resistant spoilage microorganisms can survive these pasteurization requirements and spoil the liquid whole egg even under refrig-

erated conditions. Therefore, all these pasteurized egg products had to be frozen rapidly in a blast-air freezer or dried by spray drier and held in this form until use. However, more recently there has been a tendency in large scale plants to chill the products and transport them in refrigerated vehicles to their point of use (Belyavin, 1993).

Heat pasteurization and frozen storage cause higher viscosity of liquid whole egg. Samples which had received 64.4 °C treatment showed viscosity values which were approximately double to the value of untreated one (Torten and Eisenberg, 1982). An apparent viscosity at a shear rate of 30 s^{-1} of a freshly prepared LWE was approximately 10 mPa·s, but it increased to 80 mPa·s when LWE was heated to 70 °C for 3.5 min (Hamid-Samimi et al., 1984). The increase in apparent viscosity with heat treatments was attributed to the denaturation, insolubilization and aggregation of proteins via intermolecular bonds. The apparent viscosity of LWE pasteurized at 68 °C increased approximately four fold after 24 hr of frozen storage (Herald et al., 1989).

Table 2.1.2. Pasteurization requirements for various type of liquid egg product (after Cunningham, 1995).

Liquid egg product	Minimum holding	
	Temperature (°C)	Time (min)
Egg white (without chemicals)	55.6	3.10
Whole egg	60.0	3.50
Whole egg blends (2 % ingredient)	60.0	3.10
Salted whole egg (2 % or more salt)	62.2	3.10
Sugared whole egg (2 to 12 % sugar)	60.0	3.10
Yolk	60.0	3.10
Salted yolk (2 to 12 % salt)	62.2	3.10
Sugared yolk (2 % or more sugar)	62.2	3.10

2.2. High Hydrostatic Pressure

2.2.1. General introduction

In high pressure processing of foods, foods are subjected to high hydrostatic pressure (HHP), generally in the range of 100–1000 MPa. The processing temperature during pressure treatments can be adjusted from below 0 °C to above 100 °C. The most unique property of HHP is it's ability to be transferred instantly and uniformly throughout food system. Thus the application of HHP is independent of sample size and geometry. This property of hydrostatic pressure allows not only the flexibility of product package but also the compliance of process scale-up. If the temperature gradient within the pressure vessel is not significant, the optimum process condition acquired from laboratory scale experiments can be directly translated to the pilot or plant scale processes, because pressure itself does not make any energy gradient within the pressure vessel.

Further key advantages of HHP to food processing include (1) inactivation of microorganisms; (2) modification of biopolymers; (3) quality retention, such as color and flavor; (4) changes in product functionality (Knorr, 1993). This chapter will cover the physical, chemical and biological aspects of HHP in the viewpoint of food technology.

2.2.2. Physical and chemical aspects of high hydrostatic pressure

Dimension and nomenclature

Some units of pressure are listed in Table 2.2.1. The SI unit of pressure is the pascal [Pa]. However, pressure units from *cgs* or *fps* system are also encountered in older articles.

Table 2.2.1. Units of pressure

atm	Pa	bar	kg/cm^2	psi
1.000	$1.013 \cdot 10^5$	1.013	1.034	1.470·10

Because the pressure level of HHP in food processing is much higher than several pascal, the standard prefix mega- (10^6) is often multiplied to the pascal and yields the unit of mega-pascal [MPa]. Thus, 100 MPa is about 987 atm.

Generation of high hydrostatic pressure

In nature, hydrostatic pressure increases by one atmospheric pressure for every ten meter depth of water. Thus the pressure at the bottom of Mariana Trench, one of the deepest sea in our planet, reaches up to 116 MPa (Yayanos, 1998). As mentioned above, HHP in food processing uses the pressure range of 100–1000 MPa, which is almost the same level or even ten times higher than the pressure of the deepest sea. Special equipment is needed to generate and endure such high pressure.

A typical HHP system consists of a high pressure vessel and its closure, a pressure-generation system, a temperature control device (see Figure 3.1). The heart of the HHP system is obviously the pressure vessel, which is in many cases a forged monolithic, cylindrical vessel constructed in low alloy steel of high tensile strength. The wall thickness of the monobloc vessel determines the maximum working pressure. Depending on the internal diameter of the vessel, the use of monobloc vessels is typically limited to maximum working pressure of 400–600 MPa. In case higher pressures are required, pre-stressed vessel designs like multilayer vessels or wire-wound vessels are used (Mertens, 1995). Safety codes of ASME require the vessel to crack to allow leakage to relieve pressure ("leak before break") and thus avoid catastrophic failure of the pressure vessel (USDA, 2000).

HHP can be generated either by direct compression and indirect compression. In the case of direct, piston-type compression, the pressure medium in the high pressure vessel is directly pressurized by a piston, driven at its larger diameter end by a low pressure pump. The indirect compression method uses a high pressure intensifier which pumps the pressure medium from the reservoir into the closed and de-aerated high pressure vessel, until the desired pressure is reached. Most of industrial cold, warm and hot isostatic pressing systems use the indirect pressurization method (Mertens, 1995).

The required work to increase the pressure to the desired pressure can be estimated if we know the compressibility of pressure medium. Assuming the pressure [P] increases

following the equilibrium path, the element of work [*W*] to increase the pressure (or decrease the volume [*V*]) of a system can be written:

$$dW = -p \cdot dV \tag{2.2.1}$$

The variation of V with P at constant T defines the compressibility b of a substance:

$$b = -\frac{1}{V} \cdot \left(\frac{\partial V}{\partial P} \right)_T \tag{2.2.2}$$

Introducing Equation 2.2.2 to Equation 2.2.1 yields:

$$W = \int pVb \; dp \tag{2.2.3}$$

The temperature within the pressure vessel does not remain constant during pressure build-up. The temperature within the pressure system will increase as the pressure increases. Equation 2.2.4 can be used to predict this adiabatic heat generation during pressure build-up (Pfister et al., 2001).

$$\left(\frac{\partial T}{\partial P} \right)_{adiabat} = \frac{b \cdot T}{r \cdot c_p} \tag{2.2.4}$$

where, ρ is the density, c_p is the specific thermal capacity of the substance.

Effects of high hydrostatic pressure on chemical bonds

The most basic concept to interpret the effects of pressure on chemical reactions is the Principle of Le Chatelier. That is, the pressure enhances chemical processes that result in volume decreases and diminishes those that result in volume increases. Therefore, we can roughly estimate how pressure will affect any process if we know the net change in volume that accompanies the process (Table 2.2.2.).

For example, pressure tends to dissociate electrostatic interactions because ionization in aqueous system is accompanied by a decrease in volume. Pressure also tends to disrupt hydrophobic interactions because the disrupt of hydrophobic interaction results in vol-

ume contraction. On the contrary, the breakage of hydrogen bond and covalent bond is restricted by pressure because that should result in the volume expansion.

Table 2.2.2. Volume changes associated with biochemically important bond breakage at 25°C (after Marquis, 1975; Gross and Jaenicke, 1994).

Bond type	Example	ΔV (ml/mole)	Effect of pressure
Ionic	$H_2O \rightarrow H^+ + OH^-$	-21	Disrupts electrostatic interactions
Hydrophobic	CH_4 in hexane → CH_4 in water	-23	Disrupts hydrophobic interactions
Covalent	C–C	+12	Inhibits bond breakage
Hydrogen	$–OH^{\cdots}O= \rightarrow –OH + O=$	+ 4	Enhances hydrogen bonding
Protein (denaturation)	Myoglobin (pH 5.0) (Native → Denatured)	-98	Enhances denaturation
Protein (dissociation)	Lactate dehydrogenase ($M_4 \rightarrow 4M$)	-500	Enhances dissociation

2.2.3. Effects of high hydrostatic pressure on protein systems

Stability of proteins

The protein is a polymer of amino acids which are linked one another by a peptide bond. This sequence of amino acids is called the primary structure of the protein, and determine the most important properties of a protein. Secondary structure is based on the formation of regular structural elements, such as α helix and β sheet. The tertiary structure refers to the totally folded, three dimensional structure of protein. Now, the protein has more complex interactions between elements of secondary structures and their side chains. Fully folded native polypeptide chains frequently interact with other polypeptide chains and yield the quaternary structure of protein (Zubay, 1988).

However, the native conformation of a protein is only marginally stable. The difference in free energy (ΔG) between the folded and unfolded states in typical proteins under

physiological conditions is in the range of -20 to -65 kJ/mol. The narrow margin of protein stability can be depicted if we compare the ΔG of protein denaturation with the ΔG of the covalent bond formation which is in the range of -200 to -460 kJ/mol. The chemical interactions that stabilize the native conformation of protein include disulfide bonds, and the weak non-covalent interactions such as hydrogen bonds, hydrophobic interactions, ionic interactions, and van der Waals interactions (Lehninger et al., 1993). Because of this lower stability the native structure of protein is easily affected by the environmental conditions such as solvent composition, temperature and pressure. The term 'denaturation of protein' indicates the phenomena in which the higher structure of protein is ruptured by environmental changes, while the primary structure is kept without damage (Masson, 1992).

Mechanism of high pressure induced protein denaturation

The denaturation of a protein by HHP was first observed by Bridgman (1914). However, more systematical studies on the pressure induced denaturation of protein have been conducted after 50 years later using ovalbumin (Suzuki et al., 1963), RNase A (Brandts et al., 1970), chymotrypsinogen (Hawley, 1971), or metmyoglobin (Zipp and Kauzmann 1973). These quantitative evaluation of pressure-induced protein denaturation proposed p/T diagrams which revealed elliptic contours indicating that proteins can yield heat and cold denaturation.

A fundamental difference between pressure and temperature induced protein denaturation is that no change in covalent bonding has been observed in the pressure induced protein denaturation (Masson, 1992). Further, the structure of pressure denatured proteins differs significantly from that of heat denatured proteins. The pressure denatured proteins are relatively compact and retain elements of secondary structure while the heat denatured proteins have the extended, nearly random coil configurations (Ghosh et al., 2001).

As mentioned in previous section, pressure enhances the processes that result in volume decreases. Thus the main targets of pressure on proteins are the electrostatic and hydrophobic interactions which maintain the higher structure of protein and are accompanied by negative ΔV on disruption. Further, the structural transitions by disrupting electro-

static and hydrophobic interactions are accompanied by large hydration changes which may result in further volume decreases. Pressure-induced ionization, increased solvent exposure to amino acid side chains and peptide bonds, or the diffusion of water into the cavities located in the hydrophobic core should be the main resources of such further volume decreases (Masson, 1992). The resulting net volume decreases accompanying by protein denaturation were calculated -30 to -300 mL/mol (Richards, 1977; Heremans, 1982; Weber and Drickamer, 1983).

Because hydrophobic interactions which play an important role in the stabilization of the quaternary structure of proteins can be easily disrupted by pressure treatments, oligomeric proteins tend to dissociated into their subunits at low pressure level (150–200 MPa) and then reassociate under higher pressure levels (Ohmiya et al., 1989).

The high molecular weight aggregates are formed after pressure induced unfolding of proteins and stabilized by intermolecular disulfide bonds. These disulfide bonds are considered to be formed either by SH/S-S interchange reactions or by SH oxidation reactions (Funtenberger et al., 1995; Van Camp et al., 1995; Messens et al., 1997).

Frequently, researchers tried to explain the stability of a protein against high pressure treatments in terms of adiabatic compressibility of a protein because it comprises the hydration and cavity effects besides changes of partial molar volume. Although this parameter could give new insights the properties of protein, it is still unknown whether the highly compressible proteins are more labile than lowly compressible proteins under high pressure (Gekko and Hasegawa, 1986; Gekko and Yamagami, 1991).

Effects of high hydrostatic pressure on food proteins

HHP leads to protein denaturation. However, the denaturation processes strongly depend on protein system (e.g. type of proteins, pH, ionic strength), the applied pressure, treatment time and treatment temperature (Messens et al., 1997). Therefore, it should be kept in mind that the phenomena taking place in the model system with one sort of protein and a well-defined buffer do not necessarily predict the behavior of proteins in foods, since the presence of other proteins, salts, sugars and other things will modify the observed mechanisms and kinetics of protein denaturation. For example, protein concentration affects the denaturation process by HHP: At low protein concentrations

(0.05–0.2 %), pressure-induced denaturation is often partly or completely reversible. On the contrary, intermolecular interactions and irreversible aggregation are favored at higher protein concentration (10 %) (Wong and Heremans, 1988). Thus each food system should be investigated independently.

Since the finding of Bridgman (1914) that egg white coagulates with pressure, many studies have been devoted to the denaturation and gelation of egg proteins. Ovalbumin was denatured under high pressure, as confirmed by the decrease in its α-helical content and DSC endothermic enthalpies (Hayakawa et al., 1992). The results indicated that only conformational changes are induced by pressure treatments because no change in PAGE pattern was observed. HHP can make gels, but HHP can also melt gels. A heat induced ovalbumin gel melted completely with pressure over 600 MPa, whereas gels of galycinin, gelatin and agarose did not melt up to 700 MPa (Doi et al., 1991). Because ovalbumin gel is known to be stabilized by hydrophobic interactions and other gels to be stabilized by hydrogen bonds, these results verify that high pressure tends to disrupt hydrophobic interactions, but not hydrogen bonds. Pressure treatments up to 800 MPa increased the viscosity and the protein digestibility of egg white as a consequence of protein unfolding, whereas the forming and heat gelling properties were retained (Iametti et al., 1999). SDS-PAGE of high pressure treated egg white was similar to that of fresh egg white, indicating that pressure processing did not form the protein aggregates stabilized by disulfide interactions. High pressure induced egg white and yolk gels were more elastic and softer than heat-induced gels although the gels tended to increase in hardness and to decrease in adhesiveness with an increase of pressures. Taste and flavor of pressure-induced gels were free of cooked flavor and there was no destruction in vitamins and amino acids (Okamoto et al., 1990; Hayashi et al., 1989). The rate of dissociation is assumed to be much larger than that of gelation in pressure-induced gelation process which is contrarily to the heat-induced gelation process (Kanaya et al., 1996). The synergistic effects of high pressure with high temperature on protein gelation was described (Van Camp et al., 1996). For the temperatures of -5 to 50 °C, hardness of ovalbumin gel was a minimum at 25 °C at all pressures applied. This was consitent with the usual elliptical P-T diagram of protein denaturation which has a minimum around room temperature. However, pressure cannot induce any gela-

tion of egg yolk below 10 °C. Therefore, the usual elliptical P-T diagram of protein denaturation was not reflected by gel hardness, indicating the P-T dependency of protein denaturation strongly depends on the protein system (Dumoulin et al., 1998). Egg white concentrates, which easily form heat set gels, are only capable of forming a weak coagulum or gel network structure under high pressure (400 MPa, 30 min) at an initial temperature of 50 °C (Van camp et al., 1996).

Effects of pressure on milk proteins have been extensively investigated. Pressure treatment resulted in no effect on the emulsifying property of casein (Denda and Hayashi, 1992), while that of whey protein was reduced by HHP due to the aggregation of whey proteins (Galazka et al., 1995). HHP could regulate the curd formation (Ohmiya et al., 1988), or selective digestion of β-lactoglobulin in milk (Hayashi et al., 1987). The application of pressure treatments to cheese processing was investigated in order to reduce the ripening period (Messens et al., 2000; Johnston and Darcy, 2000). The rheological parameters and meltability of immature Mozzarella cheese were converged to those of ripened cheese by pressure treatment of 200 MPa due to casein dissociation and internal moisture redistribution. The hardness of cheese increased again above 200 MPa due to the protein denaturation and aggregation.

The nature of muscle proteins under high pressure can provide basic information for the application of high pressure to meat and fish products. Myosin molecules were associated through head-to-head interactions to form oligomers with increasing pressure up to 210 MPa (Yamamoto et al., 1992). The helical structure in myosin tail is considered to remain intact at such treatment condition. The metmyoglobin was denatured and formed dimer by the pressure of 7.5 kbar (Defaye et al., 1995; Defaye and Ledward, 1995). The dimer was presumably stabilized by hydrophobic and/or hydrogen bonds since it was dissociated in SDS. The rheological properties of muscle proteins were investigated with the HHP treated sausage (Huang et al., 1999), or with HHP treated fish samples (Ashie et al., 1999). Interestingly, muscle proteins of tropical fish species showed greater stability against pressure than those of cold acclimatized fish species, although the structural basis for species related differences in stability is not clearly understood.

2.2.4. Effects of high hydrostatic pressure on microorganisms

Inactivation mechanism

High hydrostatic pressure brings about a number of changes in the morphology, cell membrane or biochemical reactions of microorganisms, and all these processes are related to the inactivation of microorganisms. Especially, the cell membrane is considered to be the major target for the pressure-induced inactivation of microorganism, and it is generally accepted that the leakage of intracellular constituents through the permeabilized cell membrane is the most direct reason of cell death by high pressure treatment. However, if applied pressure was not severe enough to induce a total permeabilization of cell, the permeabilization took place only outer membrane in the case of Gram negative bacteria, and permeabilized membrane was rapidly restored after pressure release (Hauben et al., 1996). This result was verified again with the membrane proteins of *Salmonella* (Ritz et al, 2000). They observed that most of the outer membrane proteins were disappeared after HHP, whereas the cytoplasmic membrane bounded proteins were still retained after HHP. The fluidity of cell membrane plays an important role in the susceptibility of microorganisms to pressure treatments. Microorganisms with less fluid membranes were more sensitive to HHP (Macdonald, 1992). Conversely, an increased membrane fluidity protected against pressure inactivation (Steeg et al., 1999).

The denaturation of key enzymes in microorganism by pressure has been regarded another important reason of cell death. Because intracelluar enzymes seemed to not a determining factor of pressure resistance (Simpson and Gilmour, 1997), membrane bounded ATPase was considered to such a key enzyme (Wouters et al., 1998). Indeed, the observations on acid efflux and ATP pool suggest that ATPase is affected by HHP earlier than the intracellular enzyme system related to glycolysis.

Bacterial spores are known to be pressure resistant, and the inactivation mechanism is different from that of other vegetative microorganisms. It was assumed that pressure caused inactivation of spores by first initiating germination and then inactivating germinated forms (Sale et al., 1970). Biochemically, binding of germinant to it's receptor is believed to promote the germination process followed by the efflux of Ca^{2+} and other ions and the influx of water into the spore, which result in the activation of spore-specific cortex lytic enzyme (Wuytack, E. Y. et al., 2000). This germination process

can be enhanced by pressure treatments, because the volume of system decreases during germination as a result of increased solvation of spore's components (Clouston and Wills, 1969; Heinz and Knorr, 1998). It was also suggested that pressure-induced germination involves activation of physiological pathway and is therefore not merely a physico-chemical process in which water is forced into the spore protoplast, because inhibitors of nutrient-induced germination also inhibit pressure-induced germination (Wuytack, E. Y. et al., 2000). However, when high pressure is applied to spores with elevated temperature, the coretex lytic enzymes in spores could be directly inactivated and the inactivation of spores took place without the germination step (Heinz and Knorr, 2001).

Critical parameters for microbial inactivation by high hydrostatic pressure

Besides pressure level and treatment time, the critical parameters for high pressure induced microbial inactivation are pH, water activity (a_w) and the treatment temperature. Various combinations of these parameters have been investigated and the general rules are as follows: (1) Microorganisms become more susceptible to pressure at lower pH. Further, sublethally injured microorganisms induced by HHP can be reactivated in an nutrition-rich environments, but fail to repair at acidic conditions (Linton, 1999); (2) A reduction of water activity exerts protective effect for microorganisms against pressure treatments (Oxen and Knorr, 1993; Palou et al., 1997); (3) The treatment temperatures above or below room temperature tend to increase the inactivation rate of microorganisms (Knorr and Heinz, 1999).

Inactivation kinetics

There are several ways in which the speed of microbial inactivation can be reported. When a suspension of microorganisms is heated at constant temperature, the decrease in the number of viable organisms is described by a first order reaction:

$$-\frac{dN}{dt} = k \cdot N \tag{2.2.3}$$

where N is number of viable organisms and k is the first order rate constant for microbial inactivation. Integrating equation 2.2.3 using the initial condition, $N=N_0$ at t=0:

$$\ln(\frac{N}{N_0}) = -k \cdot t \tag{2.2.4}$$

Equation 2.2.4 suggests a linear plot of *N* against t in a semilogarithmic scale. Equation 2.2.4 can be expressed in common logarithm:

$$\ln(\frac{N}{N_0}) = 2.303 \cdot \log(\frac{N}{N_0}) \tag{2.2.5}$$

And the decimal reduction time (D value), the time required to reduce the viable organisms by a factor of 10, is defined as:

$$\log(\frac{N}{N_0}) = -\frac{t}{D} \tag{2.2.6}$$

The first order inactivation rate (*k*) and the decimal reduction time (D value) is the most frequently used concept to explain the thermal inactivation of microorganisms. By analogy with D value for thermal inactivation, decimal reduction times for pressure treatment (D_p value) can be defined. Indeed, much of works have been conducted to calculate the D_p values of various microorganisms (Table 2.2.3).

However, much of the inactivation curves follow non-linear inactivation pattern in a semilogarithmic scale. Actually, some of inactivation data presented in Table 2.2.3 should not be handled with simple D value concept, because their inactivation curves deviated from first order reactions, i.e., the inactivation curves contained shoulder or tail, or both of them. Mathematical models based on Gompertz equation (Heinz and Knorr, 1996; Patterson and Kilpatrick, 1998) or quadratic polynomial expression (Reyns et al., 2000) were devised to predict the inactivation of microorganisms with non-linear characteristics. Such models could predict the inactivation of microorganisms at various pressure-temperature combinations and provide flexibility in selecting optimum processing conditions without compromising microbiological safety.

If a set of kinetic parameters was collected, the pressure sensitivities of the kinetic parameter can be evaluated. The inactivation kinetics of *Listeria monocytogenes* by high pressure was analyzed either by Arrhenius type model and pressure death time model

(Mussa et al., 1999). The volume change of activation ($\Delta V^{\neq}$) and pressure Z value (Z_p) of *L. monocytogenes* was calculated to $-4.17 \cdot 10^{-5}$ m^3mol^{-1} and 134 MPa, respectively. Reyns et al. (2000) calculated Z_p values of various microorganisms either from their own experiments or from other publications. The Z_p values were: 66.5 MPa for *Zygosaccharomyces bailii*; 68 MPa for *Saccharomyces cerevisiae*; 130–200 MPa for *Listeria species*; 155 MPa for *Salmonella typhimurium*; 134 MPa for *Salmonella seftenberg*; 75 MPa for *E. coli*; 100 MPa for *Lactobacillus casei*.

Inactivation of microorganisms in foods

The first HHP processed commercial food product was fruit jams (Horie et al., 1991). Strawberry jams subjected to 294 MPa for 20 min inactivated yeast with natural color and flavor. The application of HHP to fruit products has been considered to be the most realistic, because the inherent low pH of fruits can inhibit the growth of most spoilage bacteria. Further, the yeast and mold which survive such low pH range are relative susceptible to HHP (Aleman et al., 1996; Raso et al., 1998; Garcia-Graells et al., 1998; Linton et al., 1999; Zook et al., 1999; Prestamo et al., 1999).

The spoilage bacteria in vegetables come from soil and the varieties of them are extremely wide. Application of 300 MPa was able to reduce indigenous microflora in lettuce and tomato, but the treatment did cause changes in appearance and structure (Arroyo et al., 1997). Other examples of HHP applications on vegetable products are the increased shelf life of vegetable juices by HHP (Lee et al., 1996) or fermentation control without the quality loss of fermented vegetable (Sohn and Lee, 1998). However, the limiting parameter of vegetable processing is often the presence of browning enzymes which need higher pressure levels to inactivate (Eshtiaghi and Knorr, 1993; Seyderhelm et al., 1996).

High pressure treatments of protein rich foods such as egg, meat, or fish are limited because HHP induces protein denaturation. Further, these food materials have fairy high fat content which is known as a common baroprotective component for microbial inactivation (Styles et al., 1991).

Applications of high pressure to liquid egg products are very rare. *Listeria* and *E. coli* were inoculated in liquid whole egg and pressurized up to 450 MPa (Ponce et al., 1998).

Table 2.2.3. Pressure induced decimal reduction times (D_p values) of various microorganisms

Microorganisms	Medium	Temp [°C]	Press [MPa]	D value [min]	Reference
Salmonella seftenberg	Chicken medium	23	340	7.1	Metrick et al., 1989.
	Phosphate buffer (pH 7.0)	23	340	4.2	
Salmonella typhimurium	Chicken medium	23	340	7.6	
	Phosphate buffer (pH 7.0)	23	340	7.4	
Pseudomonas flourescens	Minced beef muscle	20	150	24.0	Calrez et al., 1993.
Listeria innocua		20	330	6.5	
Citrobacter freundii		20	230	15.0	
Listeria monocytogenes	UHT milk	45	375	17.0	Simpson and Gilmour, 1997.
	Minced raw chicken	45	375	5.0	
	PBS buffer (pH 7.0)	45	373	8.9	
Zygosaccharomyces bailli	Broth (pH 3.5; a_w 0.98)	21	310	2.0	Paulo et al., 1997.
		21	431	2.1	
Lactobacillus sake	0.1% peptone solution	25	345	10.1	Kalchyanand et al., 1998.
Leuconostoc mesenter.		25	345	5.6	
Listeria monocytogenes		25	345	4.0	
E. coli O157:H7		25	345	3.0	
Staphylococcus aureus		25	345	1.7	
Pseudomonas flourescens		25	345	0.6	
Salmonella typhimurium		25	345	0.6	
Serritia liquefaciens		25	345	0.6	
Saccharomyces cerevisiae	Orange juice	Ns*	350	0.6	Parish, 1998
		Ns	500	0.1	
Listeria monocytogenes	Fresh pork loin	25	414	2.2	Ananth et al., 1998.
Salmonella typhimurium		20	414	1.5	
Saccharomyces cerevisiae (in ascopore)	Orange juice (pH 3.9)	37	350	2.8	Zook et al., 1999.
	Apple juice (pH 3.9)	37	350	2.5	
Pseudomonas flourescens	Ewe's milk (pH 6.7; fat 7.6%)	2	250	3.9	Gervilla et al., 1999.
		25	250	4.6	
		50	250	2.8	
E. coli	Ewe's milk (pH 6.7; fat 7.6%)	2	300	5.4	
		25	300	5.2	
		50	300	2.5	
Zygosaccharomyces bailii	Tris-HCl buffer (pH 6.5)	-1	180	79.1	Reyns et al., 2000.
		20	180	237.0	
		40	180	55.5	
		-1	260	3.7	
		20	260	12.8	
		40	260	1.8	

*Ns: Not stated.

Although substantial levels of microbial inactivation were accomplished by this pressure treatment, changes in physical properties of liquid whole egg during pressure treatments were not considered.

Fresh minced meat is a highly perishable product, whose shelf life is limited by the growth of different strains of spoilage bacteria contaminated during different steps of processing such as mincing, mixing or packaging. High pressure treatments inactivated *Citrobacter*, *Pseudomonas* and *Listeria* in minced meats (Carlez et al., 1993). *Listeria* was the most resistant ($D_{330\ MPa}$=6.5 min) among the three species. Higher (50 °C) or lower (4 °C) temperature enhanced the effects of pressure treatments. However, partial discoloration of minced beef was observed above 150 MPa. Processed meat product such as spreadable sausage may be more adequate to pressure treatment than fresh meat. Inactivation kinetics of *E. coli* and *Listeria innocua* inoculated in spreadable sausage were investigated and pressure-time contours for 5 log cycle reductions of microorganisms were established (Zenker et al., 2000).

The higher content in free amino acids and nitrogenous materials make fresh fish quite susceptible to spoilage microorganisms. A number of studies have demonstrated that HHP can extend the shelf life of fish products such as cod (Ohshima et al., 1993), minced mackerel (Fujii et al., 1994), prawns (Lopez-Caballero et al., 2000), smoked salmon cream (Capri et al., 1995)or surimi (Miyao et al., 1993).

Milk was found to provide the microorganisms protection against HHP. Only 2 log cycles of inactivation at 340 MPa was observed when *Listeria* was treated in milk whereas almost 7 log cycles of inactivation was observed when the same microorganisms were treated in buffer solution (Styles et al., 1991). The protective effect of milk against HHP were observed again with other species of microorganisms (Patterson et al., 1998). However, there were no significant difference between skim and whole milk. HHP of 400 MPa at 7 °C reduced aerobic plate counts of whole milk and skim milk equally by 1 log cycle. The pressure treatment did not inactivate the plasmin in milk, since considerable beta- and alpha-casein hydrolysis took place during refrigerated storage after HHP (Garcia-Risco et al., 1998). All these results indicated that the medium in which the microorganisms are treated is an important determinant factor of the level of inactivation by HHP.

2.3. Nisin

2.3.1. Structure and physical properties of nisin

Nisin is a positively charged small peptide which is produced by *Lactococcus lactis*. Nisin shows the antimicrobial activity against a broad spectrum of Gram-positive bacteria and has been used in the food industry as a natural preservative. Actually, nisin is the only bacteriocin which has been approved by WHO to be used as a food preservative (Delves-Broughton, 1990). The complete structure of nisin was given in Figure 2.3.1.

Nisin is composed of 34 amino acids of which 13 are post-translationally modified. These modifications include the dehydration of serine and threonine, resulting in three dehydroalanine and five dehydrobutyrine residues. Five of these dehydro residues are subsequently linked and result in the characteristic (β-methyl) lanthionine rings (Breu-

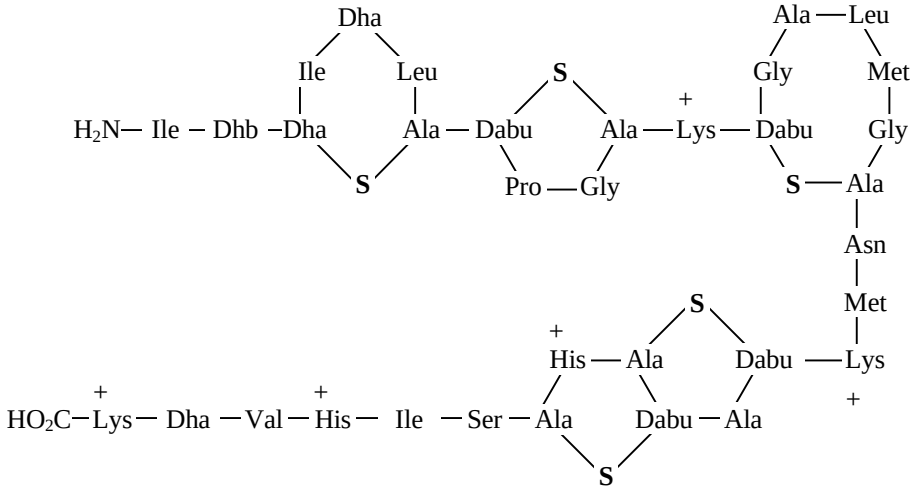

Figure 2.3.1. Primary structure of nisin (after Gross and Morell, 1971). Dha = dehydroalanine, Dhb=dehydrobutyrine, Dabu=2-aminobutyric acid moiety of 3-methyllanthionine, Dha-S-Ala=lanthionine ring, Dabu-S-Ala=β-methyllanthionine ring.

kink et al., 1998).

Nisin is more soluble at lower pH. It exhibits also greatest stability under acidic conditions. For example, nisin was stable by autoclaving (115.6 °C) at pH 2.0, but 40 % of activity was lost at pH 5.0, and more than 90 % was lost at pH 6.8. Loss of nisin activity also occurs during storage. Losses of nisin activity during storage were more pronounced at higher pH and higher temperatures. However, nisin was stable over 30 weeks in pasteurized cheese spreads (pH 5.8) at the storage temperature of 20 °C (Delves-Broughton, 1990).

2.3.2. Antimicrobial mechanism

Nisin shows the antimicrobial activity against a wide range of Gram-positive bacteria. The antimicrobial mechanism of nisin is based on the pore formation in the cytoplasmic membrane of the target microorganisms which leads to a loss of small intracellular molecules and ions and a collapse of the proton motive force (Driessen et al., 1995). Gram negative bacteria are generally resistant to nisin since the outer membrane of Gram negative bacteria does not allow the entry of nisin (Helander et al., 1998).

Nisin should carry on a multi-step process in order to exert the antimicrobial activity. The first step in the antimicrobial mechanism of nisin is to pass through the cell wall of Gram positive bacteria. In general, it is assumed that nisin passes the cell wall by diffusion. However, the cell wall can act as a molecular sieve against to nisin according to it's composition, thickness or hydrophobicity (Crandall and Montville, 1998). The removal of the cell wall from nisin-resistant *Listeria* resulted in the loss of nisin resistance, suggesting that the cell wall plays a role in the differences in susceptibility toward nisin (Davis and Adams, 1994).

The next step of the antimicrobial process of nisin is to associate with the cytoplasmic membrane of target microorganism. It has been regarded that nisin interacts electrostatically with the negatively charged phosphate groups of phospholipid at the surface of the membrane (Henning et al., 1986). Indeed, the removal of positive charge either from the N- or C- terminal region of nisin reduced the association of nisin with membrane (Giffard et al., 1997). However, it was also suggested that Lipid II, the pentapeptide of the membrane anchored cell wall precursor, would be the sole binding tar-

get of nisin (Breukink et al., 1999). In the absence of Lipid II, nisin induced permeabilization of model membrane at the concentration of 1 μM, whereas only 1 nM of nisin was enough for the permeabilization in the presence of Lipid II. They also explained why *Escherichia coli* is more resistant to nisin than *Micrococcus flavus* in terms of Lipid II content. Lipid II content in cytoplasmic membrane of nisin resistant *E. coli* was about 2000 molecules per cell, whereas nisin sensitive *M. flavus* contains about 10^5 Lipid II molecules per cell.

Models have been proposed to explain the insertion and the pore formation of nisin. The surface-bound nisin molecules may flip into the membrane and disorder the bilayer structure of membrane in the presence of a trans-membrane electrical potential (Driessen et al., 1995). This model assumes the locally disturbed bilayer may form an integral part of nonspecific, water-filled nisin dependent pore. Quenching methods were adopted to reveal the real orientation of nisin within the cell membranes (Martin et al., 1996; Breukink, 1998). Martin et al. (1996) suggested that the C-terminal region of nisin inserted more deeply into the hydrophobic core of the membrane. The composition of phospholipid was found to be important, because the negatively charged lipid pull the nisin more deeply in the membrane. However, more detailed experiments presented completely different observations (Breukink et al., 1998). The results showed an overall parallel average orientation of nisin in the membrane, with respect to the membrane surface, with N-terminal region slight deeply inserted into the center of membrane than the C-terminal region. They suggested that the C-terminal part of the nisin might be further re-oriented to causing membrane permeabilization.

Although the mechanism of permeabilization by nisin is not yet fully understood, the result from this permeabilization process is obvious. The loss of soluble low-molecular mass compounds (amino acids, ions and ATP) from the cytoplasm and the consequential breakdown in membrane potential and cessation of biosynthetic activity (Garcera et al., 1993; Martin et al., 1996).

2.3.3. Application of nisin in food

In 1969 FAO/WHO expert committee on food additives reviewed the toxicological data of nisin and recommended its acceptance for food use. In 1990, nisin was allowed as a food additive in forty-seven countries including U.K. and U.S.A. (Delves-Broughton, 1990).

Nisin has been proved to be a effective preservative in pasteurized processed cheese and cheese spread. Nisin at levels of 500 to 10,000 IU/g prevented the growth of *Clostridium botulinum*. For milk processing, 30 to 50 IU/ml of nisin doubled the shelf life of pasteurized milk, where 1 mg of purified nisin is correspond to 40,000 IU (Luek and Jager, 1995). The nisin can be introduced during the fermentation process to prevent the growth of lactic acid bacteria in beer and wine. It was indicated that the spoilage lactic acid bacteria were sensitive to nisin, although the yeast was completely unaffected by nisin (Delves-Broughton, 1990). Application of nisin to liquid egg products was also reported. Addition of nisin at a level of 5 mg/l resulted in a significant increase in the shelf life of pasteurized liquid whole egg (Delves-Broughton et al., 1992). Nisin increased the lethal effects of heat treatment, especially at lower temperature (< 58 °C), indicated by the decreased D-value of *Listeria* (Knight et al., 1999). However, nisin showed no additional effects when the treatment temperature was increased to 60 or 63 °C.

2.4. High Intensity Ultrasound

2.4.1. Physical property of ultrasound

High- and low-intensity ultrasound

Humans can hear the sound which has the frequencies in the range of 20 Hz–20 kHz. Ultrasound is the sound whose frequency is too high for humans to hear, i.e., the frequency of ultrasound is above 20 kHz.

The application of ultrasound in food processing can be classified into two categories. The low-intensity ultrasound and the high-intensity ultrasound. The low-intensity ultrasound uses very small power levels, typically less than 1 $W \cdot cm^{-2}$, with the frequency range of 5–10 MHz (McClements, 1995; Masson, 1998). Because of the small power levels the low-intensity ultrasound causes no physical and chemical alterations in the properties of the material through which the wave passes, and it can be used to measure the texture, composition, viscosity or concentration of food.

In contrast, the high-intensity ultrasound uses much higher power levels, typically in the range of 10–1000 $W \cdot cm^{-2}$, with the frequency of 20–100 kHz (McClements, 1995; Mason, 1998). The high-intensity ultrasound causes physical disruption of the material to which it is applied and promote certain chemical reactions. The current uses of high-intensity ultrasound are cleaning, liquid degassing, homogenization and welding. The possible effects of high-intensity ultrasound in food processing include the disruption of microorganisms and enzymes, generation of dispersion and emulsion or promotion of certain chemical reactions, and thereby high-intensity ultrasound is considered to be a potential unit operation of nonthermal processing of food.

Amplitude, frequency, wave length and attenuation coefficient of sound wave

A sound wave is characterized by its amplitude [A] and frequency [f], which can be chosen by investigator, and wavelength [λ] and attenuation coefficient [α], which are fundamental characteristics of a material (Figure 2.4.1). At a fixed position within a material, the displacement varies sinusoidally with time. At any instant in time, the ampli-

tude [*A*] decreases with increasing distance because of attenuation by the sample where the attenuation coefficient [α] is a measure of the decrease in amplitude of an ultrasonic wave as it travels through a material. The attenuation coefficient of a material can be defined by following equations:

$$A = A_0 \cdot e^{-ax} \tag{2.4.1}$$

where, A_0 is the initial amplitude of the sound wave, and x is the distance traveled.
The major causes of attenuation are adsorption and scattering. Adsorption is caused by physical mechanism which converts the ultrasound energy to heat. Scattering occurs in heterogeneous materials, such as emulsions, suspensions and foams. Unlike the adsorption, the scattered energy is still stored as ultrasound energy, but it is not detected because it's propagation direction and phase have been altered (McClements, 1995).

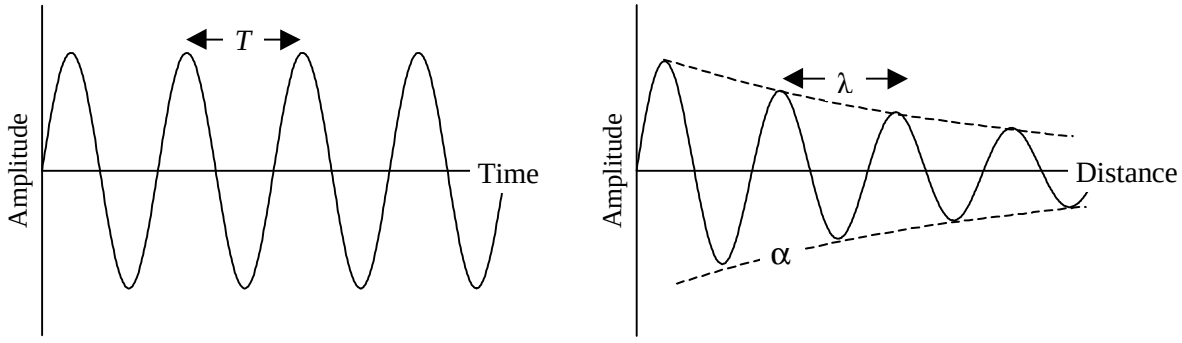

Figure 2.4.1. Changes of amplitude of ultrasounds at a fixed position within a material (left), and at an instant in time (right). *T* is the distance between the successive maxima, λ is the wave length and α is the attenuation coefficient (after McClements, 1995).

2.4.2. Generation of ultrasound

The ultrasonic transducers convert electrical or mechanical energy to sound energy. There are three types of ultrasonic transducers in common usage including liquid-driven transducer, magnetostrictive transducer and piezoelectric transducer (Mason, 1998). Piezoelectric transducers are the most common devices employed for the generation of ultrasound. Piezoelectric material such as such as barium titanate or lead metaniobate expands and contracts in alternating electrical field and thereby generates ultrasonic waves. The piezoelectric elements commonly used in ultrasonic transducer are potentially brittle and so it is normal practice to clamp them between the metal blocks (the front and back drivers) (Figure 2.4.2). Liquid-driven transducers and magnetostrictive transducers are also used to generate ultrasounds. These two systems have very robust construction although the frequency range is restricted.

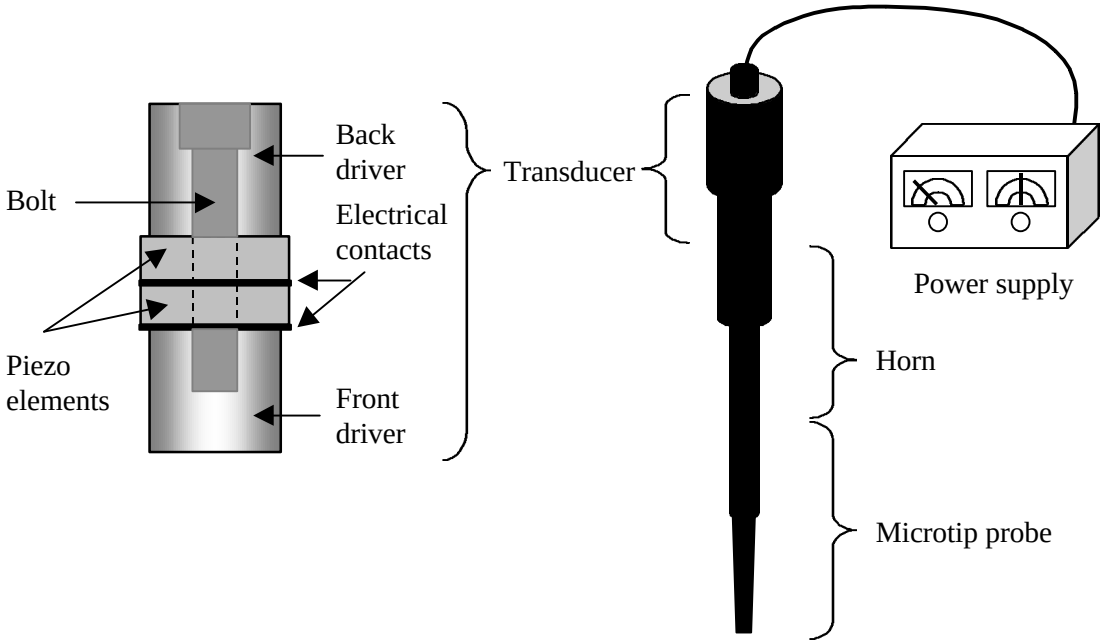

Figure 2.4.2. A probe type high-intensity ultrasound system with a piezoelectric sandwich transducer.

2.4.3. Effects of high-intensity ultrasound on food system

Cavitation

The cavitation designates a series of process which includes the formation, growth and violent collapse of small bubbles in liquid as a result of acoustic pressure fluctuation. Driven by sound fields, the bubbles can undergo either low energy oscillation called 'stable cavitation', or high energy oscillation called 'transient cavitation' (Leighton, 1998). It is generally regarded that cavitation generates high temperature and shock waves in the boundaries of the gas bubbles and the surrounding liquid. Therefore, cavitation is considered to be in charge of various physical, chemical or biological reactions by ultrasound such as accelerating chemical reactions, increasing diffusion rates, dispersing aggregates or breakdown susceptible particles such as enzyme and microorganism. The occurrence of cavitation depends on dissolved gas, temperature and hydrostatic pressure of liquid medium. However, the frequency of ultrasound is the determining factor of cavitation. At very high frequencies , i.e., above 1MHz., cavitation is more difficult and above 2.5 MHz cavitation does not occur (Sala et al., 1995).

Heating

Ultrasound may cause a localized temperature increase within a food system as a result of specific absorption of acoustic energy by biomaterials such as biological membranes. Some investigators claim that this localized temperature can increase up to 5,000 °C (Floros and Liang, 1994). However, this localized temperature increase by ultrasound treatment is difficult to measure and an overall increase of temperature is observed during treatment, which depends on the nature of the medium and the total energy input of ultrasound treatment.

Effects on Viscosity

When fluids are placed under high-intensity ultrasound, the produced dynamic agitation and shear stresses affect their structural properties, particularly their viscosity. Usually, Newtonian fluids maintain their Newtonian characteristics, but dilatant and thixotropic fluids tend to either stiffen or become less viscous, respectively (Floros and Liang, 1994). High-intensity ultrasound in low-viscosity liquids produces violent agitation

which can be utilized to disperse particles. At liquid/solid or gas/solid interfaces, acoustic waves cause extreme turbulence known as “acoustic streaming” or “micro-streaming”. This reduces the diffusion boundary layer, increases the convective mass transfer, and considerably accelerates diffusion which can lead to reduce drying or re-hydration times (McClements, 1995).

Inactivation of Enzyme

Prolonged exposure periods were required to inactivate the enzymes by high-intensity ultrasound. The inactivation of enzyme is probably due to the intense pressure, temperature and formation of free radicals generated by cavitation. The influence of the dissolved gas on the enzyme inactivation can also be explained by the formation of free radicals by cavitation. Some enzymes, such as catalase, Yeast invertase, or pepsin is very resistant against ultrasound (Sala et al., 1995). Hardly any effect on milk enzymes was observed when ultrasound was applied without thermal treatment, but the inactivation of enzyme was increased when ultrasound was applied above 61 °C (Villamiel and de Jong, 2000). Ultrasound can assist the thermal processing by reducing the thermal resistance peroxidase (De Gennaro et al., 1999). The resistance of enzyme was also dependent on the power of ultrasound, the geometry of sonotrode and the volume of suspensions submitted to the treatment.

Inactivation of Microorganisms

High-intensity ultrasound has been used to facilitate the microbial decontamination of various types of food, probably because the high pressure, shear forces and temperature generated in the material disrupt the integrity of the microorganisms. Ultrasound seems to be particularly effective when used in combination with other decontamination techniques, such as heating, extremes of pH or chlorination (McClements, 1995). However, the resistance to ultrasound varies widely depending on the microbial species. Generally microorganisms of bigger size are more sensitive to ultrasound, Gram-positive more than Gram-negative and aerobic more than anaerobic species (Sala et al., 1995).

Chapter 3. Materials and Methods

3.1. Rheological study

3.1.1. Preparation of liquid whole egg

Fresh shell eggs, no older than 10 days after laying, were purchased from a local super-market and were held overnight under refrigeration. The eggs were broken by hand and blended in a mixer at the lowest speed for 60 s (K1000, Braun, Germany). The pH of LWE was determined by a laboratory pH meter (HI9318, Hanna Instruments, Italy) for each new batch as an indication of freshness. The pH of LWE used in this study ranged from 7.4 to 7.8. Twenty five ml of LWE were transferred into polyethylene bags and heat sealed for the high pressure treatments.

3.1.2. High hydrostatic pressure treatment

High pressure treatments for the rheological study were performed in a laboratory scale high pressure system (National Forge Europe, Belgium). The HHP system consists of a high pressure vessel with a filling volume of 0.6 L, a closure for the vessel, a pressure-generation system, a temperature control device, and a control panel (Figure 3.1). The reachable pressure increase rate was approximately 11 MPa per second and the pressure release occurred within a few seconds (< 5 s). Pressure treatment time reported in this study did not include the pressure build-up and releasing time.

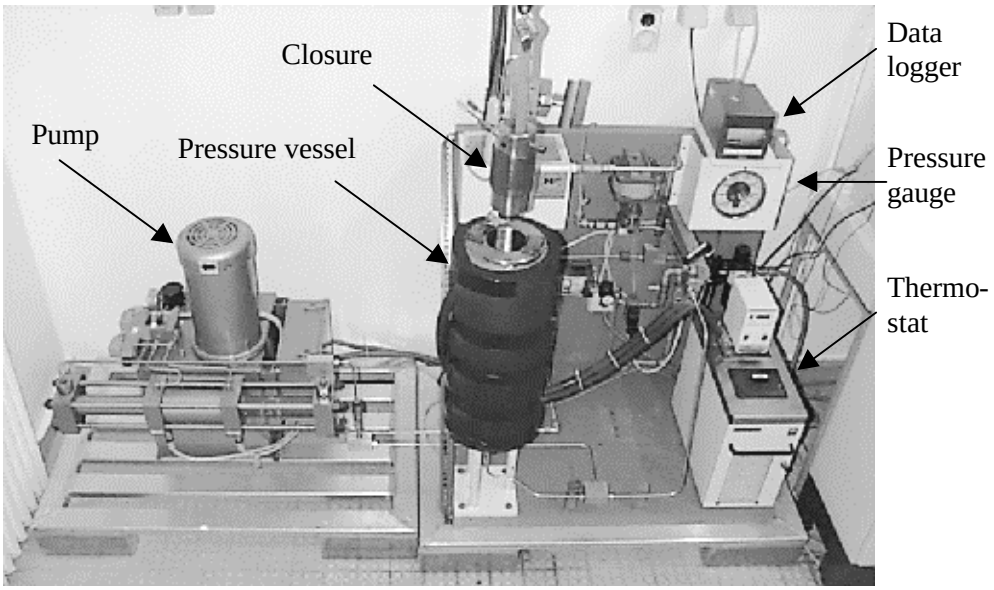

Figure 3.1. A lab-scale high hydrostatic pressure system. The maximum pressure of the system is 600 MPa with a filling volume of 0.6 L (National Forge Europe, Belgium).

High pressure treatments for the rheological study were carried out at 5, 25 and 45 °C in the pressure range of 100 to 400 MPa. Before pressure treatment the samples were submerged in the pressure vessel for 10 min to allow temperature equilibration of the LWE.

3.1.3. Rheological measurement

Non treated control and high pressure treated LWE were allowed to equilibrate to 20 °C in a temperature controlled water bath prior to the rheological measurements. Rheological measurements were made with the Haake Rotovisco RV-12 equipped with NV measuring head (Haake Inc., Germany), which was controlled to 20 °C by an external thermostat (KT33, Haake Inc., Germany). The samples were sheared for 5 min at a fixed rotational speed of 55 rpm (corresponding to a Newtonian shear rate of 300 s^{-1}). During the shear, torque was recorded every second by a data acquisition interface board (Quin-Curtis computer boards Inc., USA) which was connected to a personal

computer. Great care must be taken to the handling and loading the sample onto the viscometer, because the structure breakdown of partially coagulated LWE is an irreversible process.

3.1.4. Analysis of rheological data

Structure breakdown is a time-dependent process which could occur in emulsions, suspensions with aggregated particles and sols (Figoni and Shoemaker, 1983). The classical approach to characterize structure breakdown is the measurement of the hysterisis loop (Barbosa-Canovas and Peleg, 1983; Lewis, 1987). Another approach to characterize structure breakdown is the measurement of stress decay at a constant shear rate. The major advantage of this method is that the increasing and decreasing shear rates which play an important role in the hysterisis loop method need not to be considered. Thus, calculation and comparison of structure breakdown become more easier. Not coagulated LWE, including LWE control, when placed under steady flow at a constant shear rate, showed a changing shear stress over time until an equilibrium value was reached (Figure 3.2.A). The retarded response of LWE to shear results from a combined viscous and elastic nature and can be characterized by the Maxwell model (Figoni and Shoemaker, 1981).

An example of shear stress curve as a function of time with structure breakdown is shown in Figure 3.2.B. The stress overshoot is characteristic of a system in which structure breakdown occurs. In the steady shear analysis, the total strain to which the sample is subjected is quite large compared to that of dynamic shear measurement (Elliott and Ganz, 1975). As a consequence of this large strain, the coagulated structures which could be broken with mechanical force were broken down during the measurement, and the equilibrium was obtained within a few minutes depending on the degree of coagulation. The excess work of structure breakdown (ΔW) is directly proportional to the shaded area under the stress overshoot peak and can be calculated from it as follows (Elliott and Ganz, 1977):

$$\Delta W = \dot{g} \cdot \int (t - t_e) dt \qquad (3.1)$$

where $\dot{g}$ is shear rate, τ is the shear stress, τ_e is the equilibrium shear stress. The integral part of equation was solved numerically by Plotit software package (Scientific Programming Enterprises, USA). The apparent viscosity, η_{app}, can be acquired at the equilibrium state.

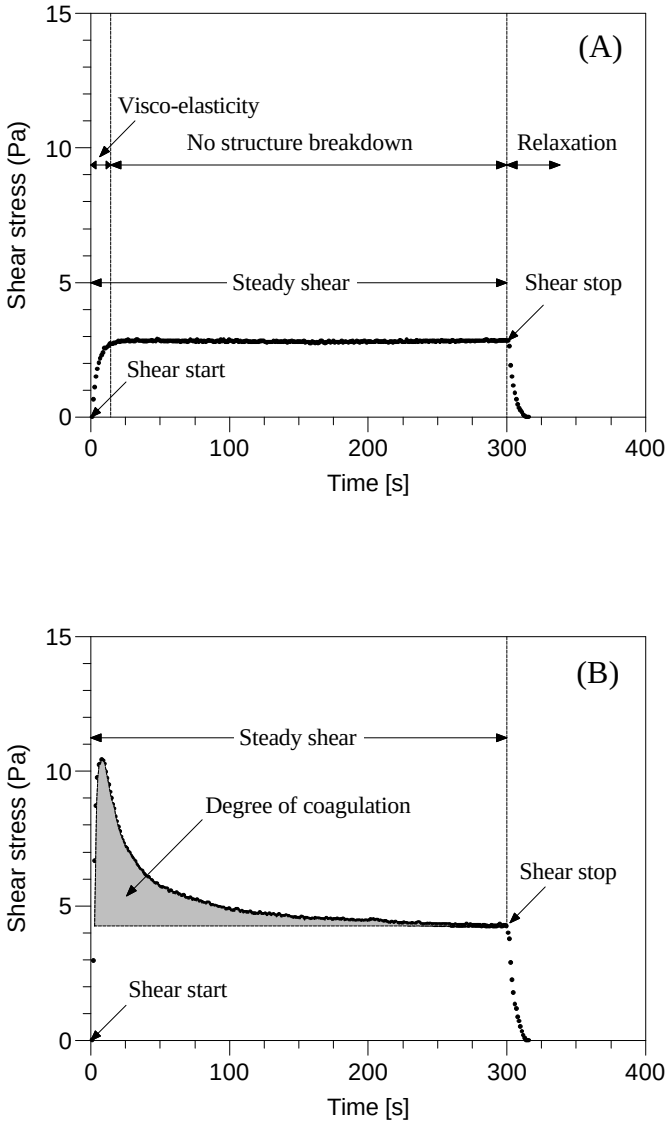

Figure 3.2. (A) Flow behavior of a fresh liquid whole egg during steady shear motion and after its cessation. (B) Flow behavior of a partially coagulated liquid whole egg undergoing structure breakdown during steady shear.

3.2. Microbiological study

3.2.1. Microorganisms

Escherichia coli K12 DH 5α was obtained from Hygiene Institute Hamburg (Hamburg, Germany). The subcultured *E. coli* was transferred to 20 mL of standard I broth (Merck, Darmstadt, Germany) and grown in a shaking incubator at 30 °C. After 24 h of incubation, 50 µl of the suspension were transferred to 20 mL of fresh broth and grown again for 24 h.

Three psychrophiles, *Pseudomonas fluorescens* DSM 50090, *Paenibacillus polymyxa* DSM 36 and *Listeria seeligeri* NCTC 11289 were obtained from DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) and NCTC (National Collections of Type Cultures and Pathogenic Fungi, Colindale, U.K.), and cultured as described previously. These strains were used to verify the inactivation effects of HHP at selected critical pressure conditions.

3.2.2. Preparation of liquid whole egg and inoculation of microorganism

Fresh shell eggs were washed with tap water, soaked in 70 % ethanol for 10 min and allowed to air dry. These sanitized eggs were aseptically broken, and the contents were transferred into a sterile stomacher bag and homogenized for 1 min in a stomacher laboratory blender 400 (Seward Medical, UK). Then 1 ml of microorganism suspension was inoculated to 100 ml of LWE. The initial concentration of *E. coli* was approximately 10^8 CFU/ml. The initial concentration of the other psychrophiles was approximately 10^7 CFU/ml.

3.2.3. High hydrostatic pressure treatment

A five-vessel high hydrostatic pressure unit U111(UNIPRESS, Poland) is shown in Figure 3.3. This equipment was adequate for the study of microbial inactivation kinetics because five samples of different treatment times could be treated at a time with

identical pressure condition. Furthermore the five vessels could be immersed in a single bath of a thermostat (Huber CC245, Offenburg, Germany), thereby the pressure vessels had a quick response to temperature overshoots caused by adiabatic heating and was more nearby isothermal conditions (Arabas et al., 1998). However, only microbial tests could be conducted with this equipment due to the small volume of pressure vessel (inner volume of 2 ml). Each pressure vessel was made of high strength beryllium copper alloy and equipped with individual K type thermocouple and manganin pressure gauge. High pressure up to 700 MPa could be generated by the pressure intensifier. Silicon oil was used as a pressure transmitting medium.

Approximately 1.5 ml of inoculated LWE was filled into a cryogenic vial (Nunc, Denmark) and put into the pressure vessel. Pressure treatments were conducted at 5 and 25 °C in the pressure range of 250 to 400 MPa with the pressure build-up rate of 10 MPa/s. The pressure release occurred within a few seconds. Pressure treatment time reported in

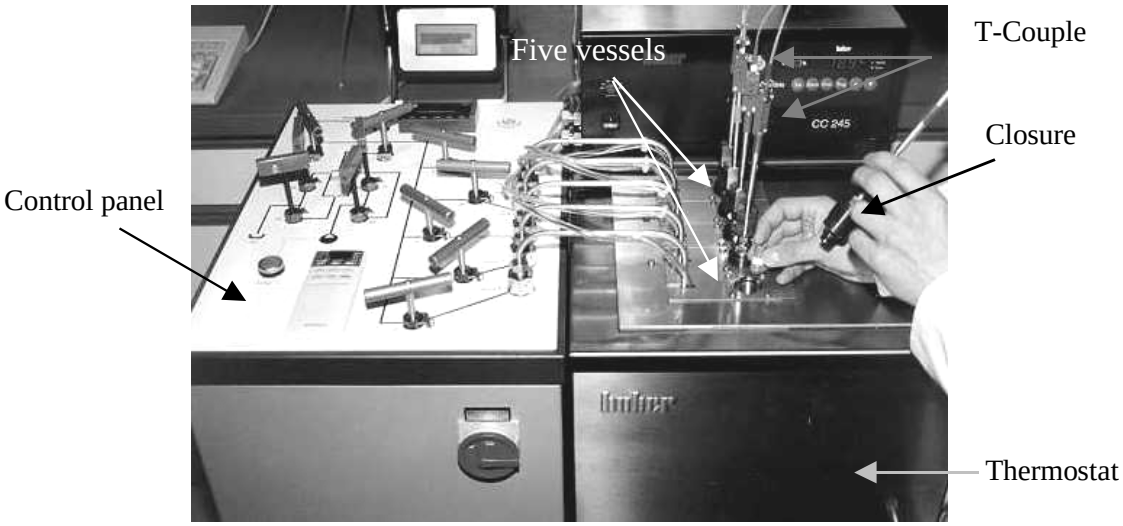

Figure 3.3. A five-vessel high hydrostatic pressure unit U111(UNIPRESS, Poland). The five vessels are immersed in a thermostat (Huber CC245, Germany). The maximum pressure of the system is 700 MPa with a filling volume of approximately 2.0 ml.

this study did not include the pressure build-up and release time.

3.2.4. Determination of viable cell counts and sublethal injury

For *E. coli* experiments, 1 ml of untreated and HHP treated LWE were serially diluted in 9 ml of sterile Ringer solution (Merck, Darmstadt, Germany). Duplicates of 50 µl from an appropriate dilution were plated onto either standard I agar (Merck, Darmstadt, Germany) and VRBG agar (Oxoid, Basingstoke, U.K.) to determine viable cell counts and sublethal injury. Both uninjured and injured cells grow on standard I agar, whereas only uninjured cells grow on VRBG agar because of the presence of bile salts in VRBG (Ray, 1979). Plates were incubated at 37 °C for 24 hr and survivors (CFU/ml) were enumerated. The microbial inactivation was determined by the logarithmic reduction on standard I agar, i.e., $\log(N_0^{Std}/N_t^{Std})$, whereas the sublethal injury in the surviving cell population was calculated as the logarithmic ratio of the cell counts in standard I agar and VRBG, i.e., $\log(N_t^{Std}/N_t^{VRBG})$ where N_0^{Std} is the initial number of cells in standard I agar, N_t^{Std} and N_t^{VRBG} are the number of cells after HHP treatment in standard I agar and VRBG agar, respectively (Hauben et al., 1996).

For *Pseudomonas*, *Paenibacillus* and *Listeria*, only viable cell counts were enumerated. Duplicates of 50 µl from an appropriate dilution of HHP treated and untreated control samples were plated on standard I agar and incubated at 30 °C for 48 hr.

3.2.5. Kinetic analysis

Inactivation data of *E. coli* were analyzed by a first order biphasic model. This model consists of two fractions which follow independently first order kinetics (Cerf, 1977; Xiong et al., 1999). For the application of the model, the first inactivation fraction was designated as fraction I with a rate constant of k_1, and the second inactivation fraction was designated as fraction II with a rate constant of k_2 (Figure 3.4).

Then each fractions are expressed as:

$$\frac{dN_1}{dt} = -k_1 \cdot N_1(t), \qquad N_1(0) = N_{01} \tag{3.2}$$

$$\frac{dN_2}{dt} = -k_2 \cdot N_2(t), \qquad N_2(0) = N_{02} \tag{3.3}$$

where N_1 and N_2 are the number of cells in first and second fraction, t is the treatment time, k_1 and k_2 are the rate constant. The surviving cells at time t are the sum of individual fractions:

$$N(t) = N_1(t) + N_2(t) \tag{3.4}$$

The analytical solution of the above equation is:

$$N(t) = N_0(f \cdot e^{-k_1 \cdot t} + (1 - f) \cdot e^{-k_2 \cdot t}) \tag{3.5}$$

where N_0 is the initial number of cells and f is the initial proportion of the first fraction (N_{01}/N_0). Equation 3.5 can be written as follows for microbiological data:

$$\log\left(\frac{N(t)}{N_0}\right) = \log(f \cdot e^{-k_1 \cdot t} + (1 - f) \cdot e^{-k_2 \cdot t}) \tag{3.6}$$

The initial proportion of the first fraction f and the rate constants k_1 and k_2 were esti-

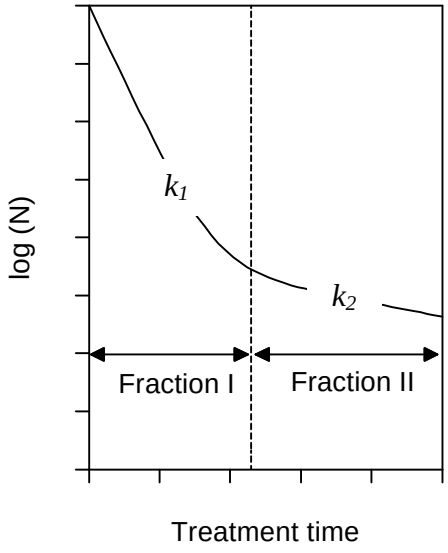

Figure 3.4. A typical biphasic inactivation curve. k_1 is the rate constant of fraction I and k_2 is the rate constant of fraction II.

mated by fitting *E. coli* inactivation data to equation 3.6 using a nonlinear regression procedure with a curve fitting program (Table Curve ver.4.07, SPSS Inc., U.S.A.).

3.2.6. Pressure dependence of rate constants

The pressure dependence of the rate constants was analyzed by an Arrhenius-type model. The pressure dependence of rate constant is given in equation 3.7:

$$\left(\frac{\partial \ln k}{\partial P}\right)_T = -\Delta V^* / RT \tag{3.7}$$

where k is first-order rate constant in s^{-1}, P is pressure in MPa, ΔV^* is the apparent volume of activation in $m^3 \cdot mol^{-1}$, R is the gas constant of $8.314 \cdot 10^{-6}$ $m^3 \cdot MPa \cdot mol^{-1} \cdot K^{-1}$, and T is the temperature in K. Equation 3.7 indicates that a plot of ln(k) versus pressure at a constant temperature should yield a straight line with a slope of $-\Delta V^*/RT$. Equation 3.7 can be applied to any sort of process for which rate data can be obtained, including complex ones such as microbial growth or death (Marquis, 1976).

3.3. Combined non-thermal processes

3.3.1. Preparation of LWE and inoculation of microorganism

The preparation of sanitized LWE was conducted as described previously (Section 3.2.2). Then 1 ml culture suspension of *E. coli* or *Listeria seeligeri* was inoculated to 100 ml of LWE. The initial concentrations of *E. coli* and *Listeria seeligeri* were approximately 10^8 CFU/ml and 10^7 CFU/ml, respectively.

3.3.2. Nisin treatments

The commercial preparation of nisin (Sigma Chemical, St. Louis, USA) contains 25 mg nisin/g. A stock solution of 1 mg nisin/ml was prepared by suspending 0.4 g of nisin preparation in 10 ml of deionized water. The stock solution was then sterilized through a 0.2 μm-pore-size filter and stored at 4 °C until use. An aliquot of stock solution or appropriate dilution of stock solution was added to LWE before pressure treatments, yielding final concentrations of 0.5 to 20 mg nisin/l. The concentrations of nisin were selected considering the commercial dosage of nisin carried out in a UK egg processing factory where 5 mg/l of nisin was added to the raw liquid egg prior to heat pasteurization (Delves-Broughton et al., 1992).

3.3.3. High-intensity ultrasound treatments

High-intensity ultrasound treatments were carried out using a lab-scale ultrasound equipment. The equipment was composed of an electric generator (HD 70, Bandelin electronic, Germany), a transducer (UW70, Bandelin electronic, Germany) and an amplifying horn with a radiating tip (SH70 and KE76, Bandelin electronic, Germany). The power supply converted the standard 50 Hz AC power to the frequency of 20 kHz with a maximum power of 60W. This high frequency electrical energy was fed to a transducer where the electrical energy is transformed to the ultrasound energy through the

mechanical vibration of the same frequency. The generated ultrasound was amplified in the amplifying horn and emitted through the radiating tip.

For the high-intensity ultrasound treatments, approximately 10 ml of inoculated LWE was filled into a glass test tube which is cooled in an iced water bath. Then the ultrasound tip was immersed into the LWE, and the ultrasound was radiated with the powers of 24.6, 34.6, or 42.0 W up to 300 s. The temperature changes during ultrasound treatments were monitored with a K-type thermocouple connected with a data logger.

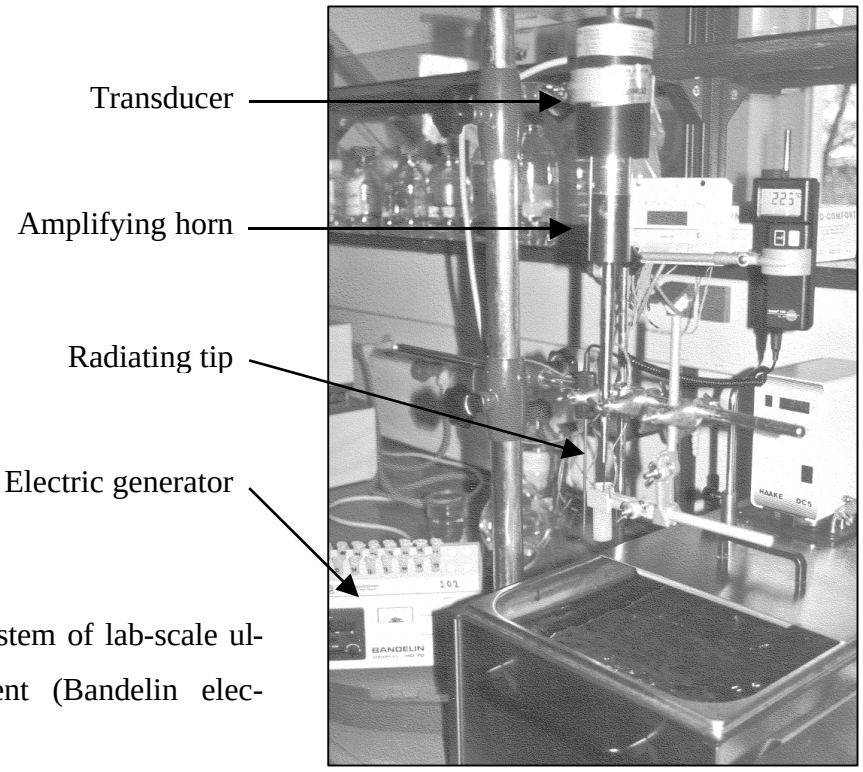

Figure 3.5. A system of lab-scale ultrasound equipment (Bandelin electronic, Germany).

3.3.4. Combined treatments of nisin and high-intensity ultrasound with high hydrostatic pressure

The consecutive combinations of nisin with high hydrostatic pressure (Nisin-HHP) and ultrasound with high hydrostatic pressure (US-HHP) were explored to achieve enhanced microbial inactivation in liquid whole egg processing (Figure 3.6).

High pressure treatments were conducted using a five-vessel high hydrostatic pressure unit as described in section 3.2.3. The HHP processing conditions were fixed to either 250 MPa for 886 s or 300 MPa for 200s, which have been determined as the optimized HHP processing conditions considering egg protein coagulation and microbial inactivation kinetics. The *E. coli* and *Listeria* were counted as described in chapter 3.2.4.

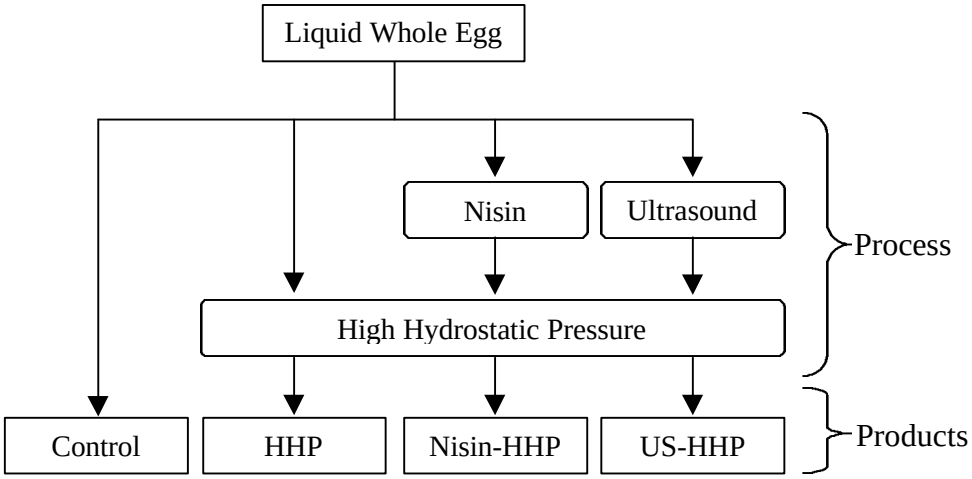

Figure 3.6. Process flow for the combined non-thermal treatments of liquid whole egg.

3.4. Functional properties

3.3.1. Preparation of LWE and combined non-thermal treatments

The preparation of LWE was conducted as described previously in chapter 3.1.1. The following combined processes were employed to determine the changes occurring in the functional properties of LWE. (i) Untreated control; (ii) HHP; (iii) Nisin in combination with HHP; (iv) Ultrasound followed by HHP. The addition of nisin (10 mg/l), the radiation of ultrasound (34.6 W for 30 s) and high pressure treatment (300 MPa for 200 s at 5 °C) were conducted as described in chapter 3.3.

3.4.2. Color measurement

The Hunter L-, a-, and b-values were measured using a Minolta Colorimeter (CR-300, Minolta, Japan). The L value denotes lightness, a value denotes redness (or greenness), and the b value denotes yellowness (or blueness). The colorimeter was calibrated by a white ceramic plate provided by the manufacturer. Each measurement was repeated more than three times and average values were reported.

3.4.3. Foaming Property

The foaming property is expressed by foaming power [%Overrun] and foam stability [%Stability]. The foams of LWE were prepared by beating LWE using a cook mixer equipped with a double whipping beater (K1000, Braun, Germany). 100 ml of LWEs were added into the bowl of the mixer and beaten for 5 min at the speed of '5' which was specified as an 'egg whipping speed'. Then the produced foams were gently filled into the weighing boats (25 ml) avoiding the formation of air pockets, and the excess foams were scraped off the top of the weighing boat using a spatula. The efficiency of foam production was expressed in terms of %Overrun (Phillips et al., 1990):

$$\%\,\text{Overrun} = \frac{(\text{wt 25 ml LWE}) - (\text{wt 25 ml foam})}{(\text{wt 25 ml foam})} \times 100 \tag{3.7}$$

Foam stability was determined by monitoring the drainage of foam at ambient temperature. The foams were filled into a 5 ml pipette with a tip diameter of 5.0 mm, and the weight of drained foams was measured. The foam stability was calculated by the ratio of the remained foam after 20 min of drainage time:

$$\%\,\text{Stability} = \frac{(\text{wt of foam}) - (\text{wt of drained foam})}{(\text{wt of foam})} \times 100 \tag{3.8}$$

Each measurement for the calculation of %Overrun and %Stability was repeated more than three times and average values were reported.

3.4.4. Emulsifying Property

The emulsifying property of LWE was examined by preparing an oil-in-water emulsion. Initially, 1 ml of LWE and 5 ml of commercial sunflower oil were blended at 8000 rpm for 20 s using a laboratory homogenizer (T25, IKA, Germany). Then 5 ml of deionized water were added and homogenized at 800 rpm for 1 min. This two-step preparation of emulsion prevented the foaming of LWE during homogenization. Immediately after preparation, the oil droplets of emulsions were observed with 250 magnifications under a phase contrast microscope (Orthoplan, Leitz, Germany) connected with a color video camera head (TK 1070E, JVC, Japan).

3.4.5. Statistical Analysis

The whole experiments were performed in triplicate and the resulting data were analyzed using a statistical analysis program (SPSS, USA). The differences between means were compared using Duncan's multiple range test with the significance of $p<0.05$.

Chapter 4. Results and Discussion

4.1. Evaluation of processing criteria for the high pressure treatment of liquid whole egg: Rheological study

4.1.1. Introduction

Liquid whole egg (LWE) or blends of this product with salt, sugar and other ingredients are used in many food products. LWEs, in addition to their nutritional value, contribute physico-chemical properties to foods such as coagulating, foaming and emulsifying (Yang and Balwin, 1995). Because these properties of egg can be easily impaired by heat treatment, egg product pasteurization is conducted on a critical temperature-time condition where the egg protein coagulation may not occur.

If an emerging technology such as high hydrostatic pressure is applied to LWE processing, effects of treatment on the rheological properties of LWE must be considered as well as the inactivation of microorganisms. Although substantial literature is available on high pressure induced protein gelation, information regarding the effects of high pressure treatment on liquid-semiliquid-semisolid transition states which are important for designing the processing limit is very rare. Therefore the phase changing process of LWE from liquid to semisolid state over a wide pressure/ temperature/ time range was investigated to determine the boundary conditions for high pressure treatment of LWE.

4.1.2. The excess work of structure breakdown according to pressure treatments.

The degree of egg protein coagulation as a result of pressure treatment is presented in Figure 4.1.1, 4.1.2, and 4.1.3 in terms of the excess work of structure breakdown (ΔW). The values of ΔW were calculated by the steady shear analysis, which measures the force needed to breakdown the aggregated particles in LWE (see Chapter 3.1.4). Each point in figures represents the average of more than two experiments.

In Figure 4.1.1, the excess work of structure breakdown according to pressure and treatment time at 5 °C is shown. No coagulation was found at 100 and 150 MPa until 3600 s. Furthermore the apparent viscosity remained almost constant as that of the untreated control, i.e. the apparent viscosity of high pressure treated LWE ranged from 9.70 to 10.3 mPa·s, while the apparent viscosity of untreated control was about 10.0 mPa·s (data not shown). Although the apparent viscosity of partially coagulated LWE can be acquired at the equilibrium state, the rheological properties are quite different from its rest state because the structure in the system is already broken down.

Coagulation occurred at pressures higher than 200 MPa. Important information that could be obtained from this graph was the starting point of coagulation (lag phase) and the rate of coagulation after lag phase. As shown in Figure 4.1.1, the lag phase was shortened and the rate was increased, with increasing pressure. The curve fitting was conducted by least square analysis and the results are summarized in Table 4.1.1.

In Figure 4.1.2, the excess work of structure breakdown according to pressure and treatment time at 25 °C is shown. No coagulation was found at 100 and 150 MPa until 3600 s. At 400 MPa, coagulation occurred almost instantaneously. Some data points above 100 kJ/m^3 were excluded in curve fitting, since the measurement was not reliable at that region. Compared to Figure 4.1.1, the lag phase was shortened and the rate was increased at the corresponding pressure. Only one exception occurred at 200 MPa. It could be explained by the change of coagulation mechanism if different combinations of pressure and temperature are used (Wong and Heremans, 1988; Messens et al., 1997). Another reason could be that some proteins whose coagulation effects are masked by other dominant proteins at higher pressures coagulate exclusively at this pressure and may show increased coagulation at 5 °C.

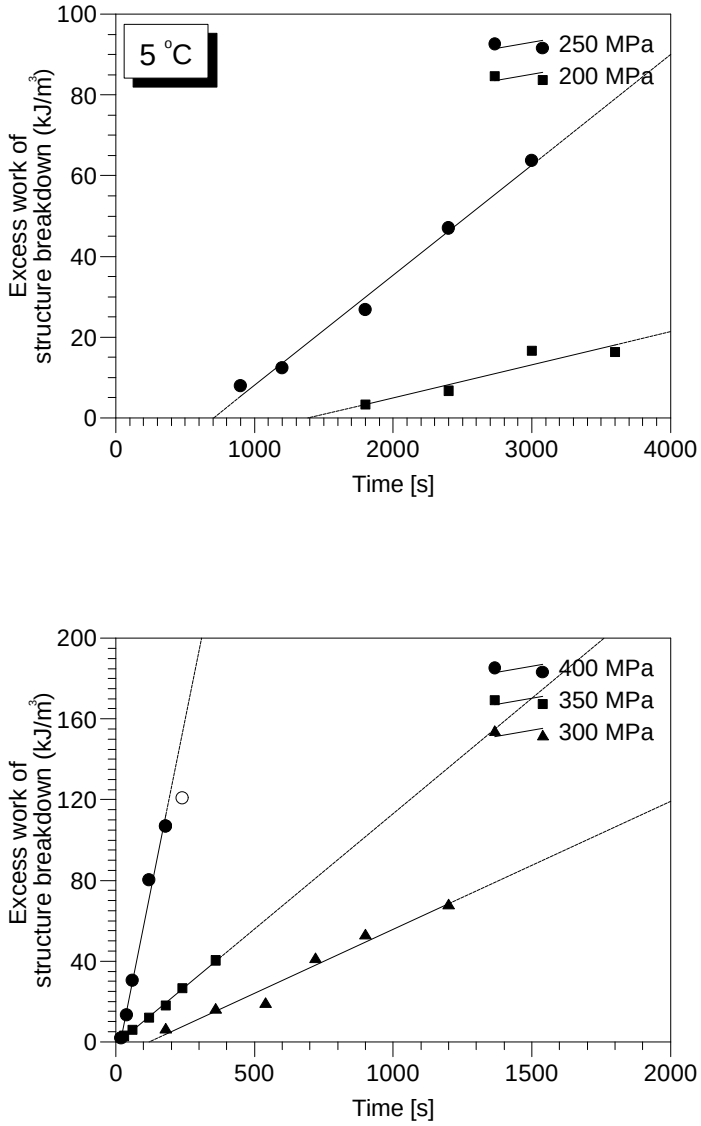

Figure 4.1.1. Pressure- and treatment time-dependent coagulation of liquid whole egg. Each treatment was performed at 5 °C. Opened symbol refers to data point whose value is above 100 kJ/m^3 and is excluded in curve fitting.

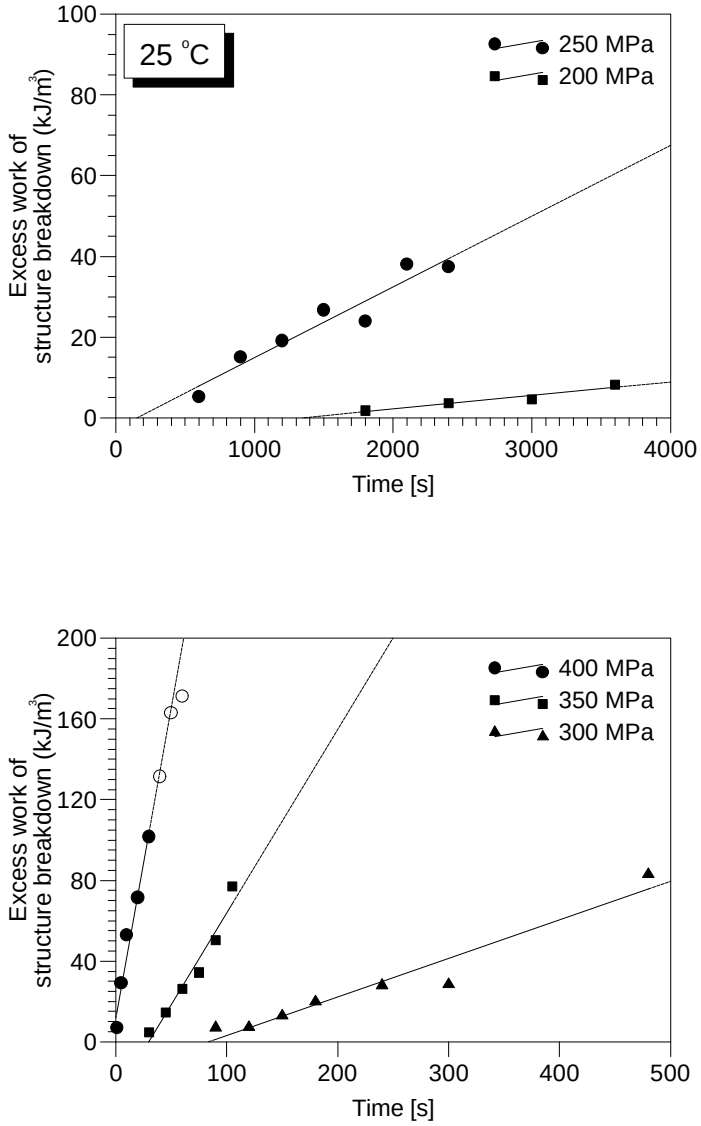

Figure 4.1.2. Pressure- and treatment time-dependent coagulation of liquid whole egg. Each treatment was performed at 25 °C. Opened symbol refers to data point whose value is above 100 kJ/m^3 and is excluded in curve fitting.

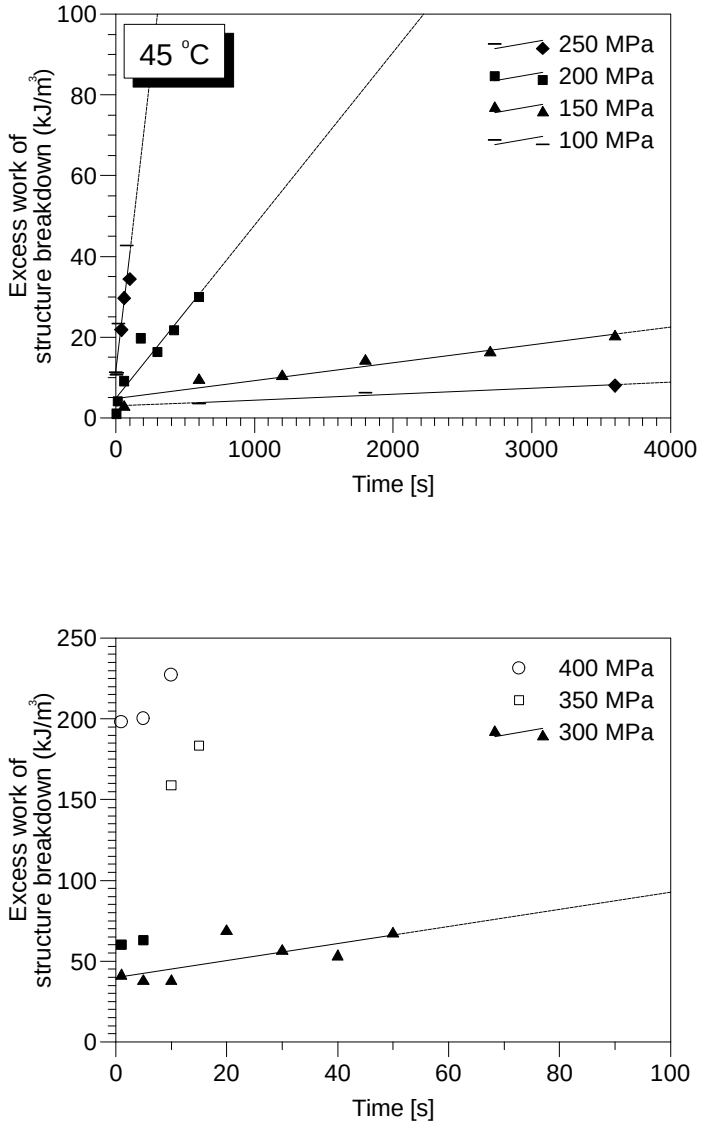

Figure 4.1.3. Pressure- and treatment time-dependent coagulation of liquid whole egg. Each treatment was performed at 45 °C. Opened symbol refers to data point whose value is above 100 kJ/m^3 and is excluded in curve fitting.

Table 4.1.1. Fitted equations and critical time.

Temp.	Pressure	Fitted equation	R^2	Critical Time[1)]
5 °C	100 MPa	N.C.[2)]		
	150 MPa	N.C.		
	200 MPa	$\Delta W=-11.3+0.00815 \cdot t$	0.873	2000
	250 MPa	$\Delta W=-19.2+0.0273 \cdot t$	0.991	886
	300 MPa	$\Delta W=-7.66+0.0634 \cdot t$	0.969	200
	350 MPa	$\Delta W=-1.23+0.114 \cdot t$	0.996	55
	400 MPa	$\Delta W=-10.9+0.684 \cdot t$	0.959	23
25 °C	100 MPa	N.C.		
	150 MPa	N.C.		
	200 MPa	$\Delta W=-4.54+0.00337 \cdot t$	0.936	2830
	250 MPa	$\Delta W=-2.66+0.0175 \cdot t$	0.923	438
	300 MPa	$\Delta W=-15.8+0.191 \cdot t$	0.937	109
	350 MPa	$\Delta W=-26.9+0.909 \cdot t$	0.952	35
	400 MPa	$\Delta W=12.0+3.07 \cdot t$	0.966	I.C. [3)]
45 °C	100 MPa	$\Delta W=2.98+0.00146 \cdot t$	0.948	1384
	150 MPa	$\Delta W=4.75+0.00444 \cdot t$	0.938	56
	200 MPa	$\Delta W=4.86+0.0429 \cdot t$	0.881	3
	250 MPa	$\Delta W=11.4+0.296 \cdot t$	0.846	I.C.
	300 MPa	$\Delta W=39.8+0.528 \cdot t$	0.545	I.C.
	350 MPa	N.D.[4)]		I.C.
	400 MPa	N.D.		I.C.

[1)] Time in sec where the excess work of structure breakdown (ΔW) reaches 5.0 kJ/m^3

[2)] N.C. No coagulation was found at this condition until 3600 s of pressure treatment.

[3)] I.C. Instant coagulation occurred at this processing condition.

[4)] N.D. Not determined.

In Figure 4.1.3, the excess work of structure breakdown according to pressure and treatment time at 45 °C is shown. Unlike others, it was also possible to detect coagulation at 100 and 150 MPa. At pressures higher than 250 MPa, coagulation occurred even at 1 s. of treatment, i.e. no lag phase existed. High protein concentration of LWE (approx. 13 %) can explain the accelerated response at higher temperature, because at high protein concentrations, intermolecular interactions and irreversible aggregation are favored over reversible denaturation (Wong and Heremans, 1988; Dumay et al., 1994).

4.1.3. The constant coagulation levels in LWE during pressure treatments

Figure 4.1.4 shows constant coagulation levels in LWE during high pressure treatment at different temperatures. The equivalent lines are drawn in the pressure-time plane. Below the line of ΔW=5.0 kJ/m^3, rheological properties of processed LWE were very similar to that of fresh LWE and it is desirable to conduct high pressure treatment under this criterion. Between ΔW=5.0 kJ/m^3 and ΔW=20.0 kJ/m^3, the LWE may be in transition state, from liquid to semi-liquid state. Above ΔW=100 kJ/m^3 the LWE showed rather semi-solid characteristics than semi-liquid characteristics. At low temperature clearly better chances exist to conduct high pressure treatment without egg protein coagulation. Pressure treatment at higher temperature caused an increased coagulation and allowed only a limited possibility to perform high pressure treatment. Since many processes undergo simultaneously in a complex system like LWE under pressure, kinetic data need to be accumulated for the purpose of processing designing. Steady shear measurements provided information on the critical points where the rheological properties of LWE changes from liquid to semi-liquid state. Further, the results of more processing possibilities of HHP at lower temperature can give some advantages to LWE processing since low temperature processing can help to retain nutritional quality and functionality of the raw LWE and could allow maintenance of consistently low temperatures during postharvest treatment, processing, storage, transportation and distribution periods of the life cycle of LWE. However, these results must be combined with microbial inactivation kinetics and product performance data in order to determine a complete process optimization.

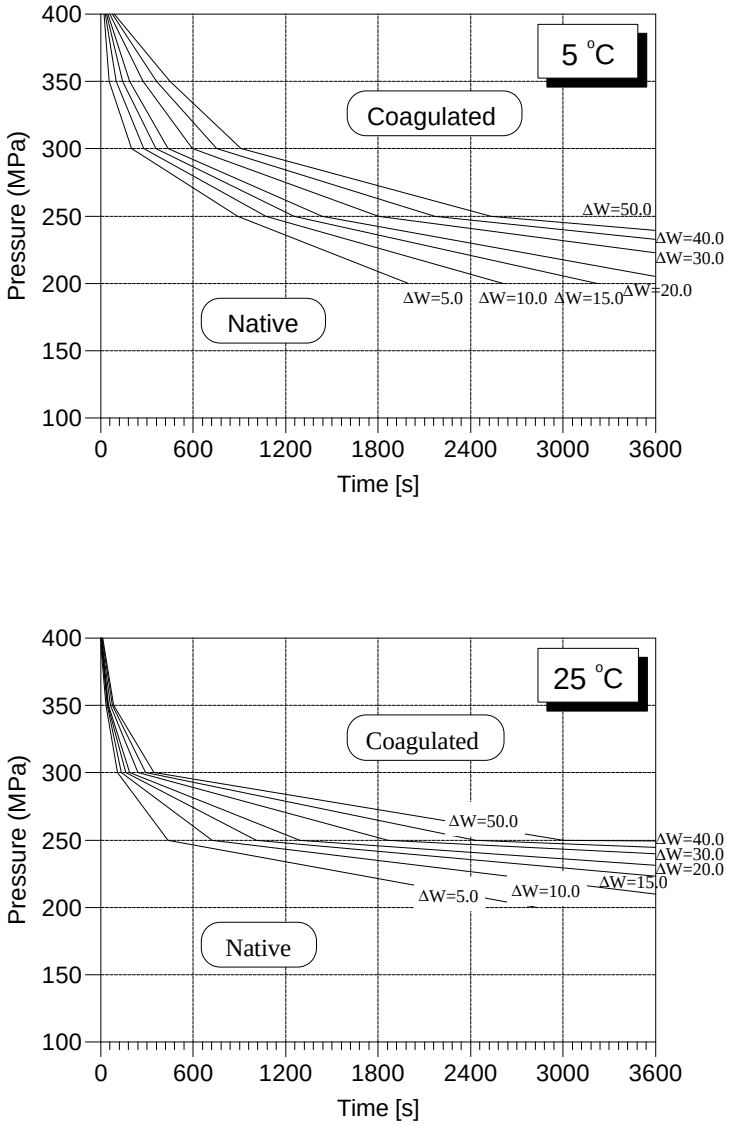

Figure 4.1.4. Constant coagulation levels in liquid whole egg during high pressure treatment. Pressure treatments were performed at 5 and 25 °C

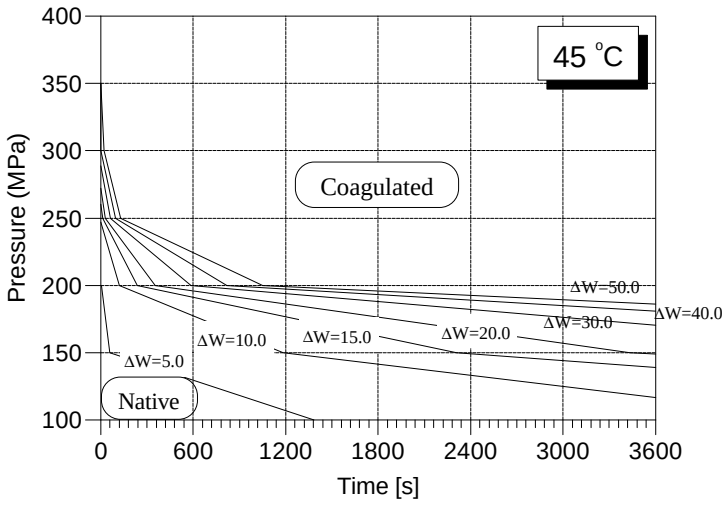

Figure 4.1.4. (Continued) Constant coagulation levels in liquid whole egg during high pressure treatment. Pressure treatments were performed at 45 °C

4.2. Biphasic inactivation kinetics of *Escherichia coli* in liquid whole egg by high hydrostatic pressure treatments

4.2.1. Introduction

Shell eggs are efficiently protected against bacterial spoilage. The protection of shell egg includes the blocking of microbial entry by shell and membrane or the antibacterial factors in egg white. Unfortunately, liquid whole egg (LWE) loses these protections during breaking and mixing, and becomes highly perishable. For example, the antimicrobial activity of ovotransferin and lysozyme ceases when the egg white is mixed with the yolk (Elliott and Hobbs, 1980). Further, the heat sensitivity of LWE precludes pasteurization at higher temperatures. Although the heat processes used to pasteurize LWE, e.g., 60 °C for 3.5 min in U.S.A. or 64 °C for 2.5 min in U.K., assure food safety by eliminating *Salmonella*, some heat resistant spoilage microorganisms can survive these pasteurization requirements and spoil the LWE even under refrigerated conditions. Generally only one or two log cycle reductions of viable cell counts are achieved by commercial thermal pasteurization, and pasteurized liquid egg products often contain 10^2 or more than 10^3 microbial cells/g. The principal genera found in pasteurized egg products are *Alcaligenes*, *Bacillus*, *Proteus*, *Escherichia*, *Pseudomonas* and Gram positive cocci (Schmidt-Lorenz, 1983; Cunningham, 1995).

For optimization of HHP processing in a real food system like LWE, changes of physical properties induced by HHP treatments must be considered along with microbial inactivation. The objectives of this chapter were to determine the kinetic parameters of microbial inactivation by HHP treatments and to identify optimized points of the processing of LWE by incorporating previous rheological studies.

4.2.2. Inactivation of *E. coli* inoculated in liquid whole egg at 5 °C

The inactivation curves of *E. coli* in LWE by pressure treatments at 5 °C are presented in Figure 4.2.1. The inactivation curves of *E. coli* in the pressure range of 250 to 400 MPa were not linear on a semi-logarithmic scale. Rather, it proceeded according to a

biphasic pattern. That is, after a rapid inactivation phase, a slow inactivation phase, i.e., tailing, was followed. The traditional first order kinetics model could not adequately describe these inactivation curves due to the larger deviation from linearity.

Therefore, a first order biphasic model (Equation 3.6) was adapted to depict these characteristic inactivation curves. The experimental data were fitted to the biphasic model, and the rate constants of both fraction (k_1 and k_2) and the initial proportion of the first fraction (f) were estimated (Table 4.1). The solid lines in Figure 4.2.1 were drawn with these estimated parameters, and exhibited good agreements ($r^2>0.95$) with the experimental data. As thermal treatments of LWE, HHP treatments could induce the coagulation of egg protein. Thus, HHP treatments should be conducted on critical pressure-time conditions where egg protein coagulation may not occur. The effects of high pressure on the physical properties of LWE in a wide pressure/ temperature/ time range were already investigated, and the degrees of coagulation (ΔW) at the corresponding pressure treatments were incorporated into the inactivation curves as coagulating lines (see legend in Figure 4.2.1).

After incorporating coagulating lines into *E. coli* inactivation curves, it was noticed that the fractional points of the inactivation curves agree with the starting points of LWE coagulation. As compared by the degree of coagulation (ΔW), the first rapid inactivation fraction (fraction I) coincided with the liquid phase of LWE (i.e., $\Delta W<5.0$), whereas the relative slow inactivation fraction (fraction II) was observed in the partially coagulated phase of LWE (i.e., $5.0<\Delta W<100$).

The biphasic inactivation curves of microorganisms could be explained by the existence of more resistant sub-population, de novo protein synthesis of heat shock proteins during heat treatment (Humpheson et al., 1998) or protection of varying environmental factors such as accumulation of dead cells, products of cell destruction or localized sites with low water activity (Cerf, 1977). In the case of LWE processing by HHP treatment, LWE acts as a substantial environmental factor of target microorganism. However, the physical properties of LWE do not remain constant, but change from liquid to partially coagulated phase as the pressure treatments increase. Therefore, it seems reasonable that the inactivation curve deviates from the traditional first order kinetics which is developed under the assumption of constant environmental factors.

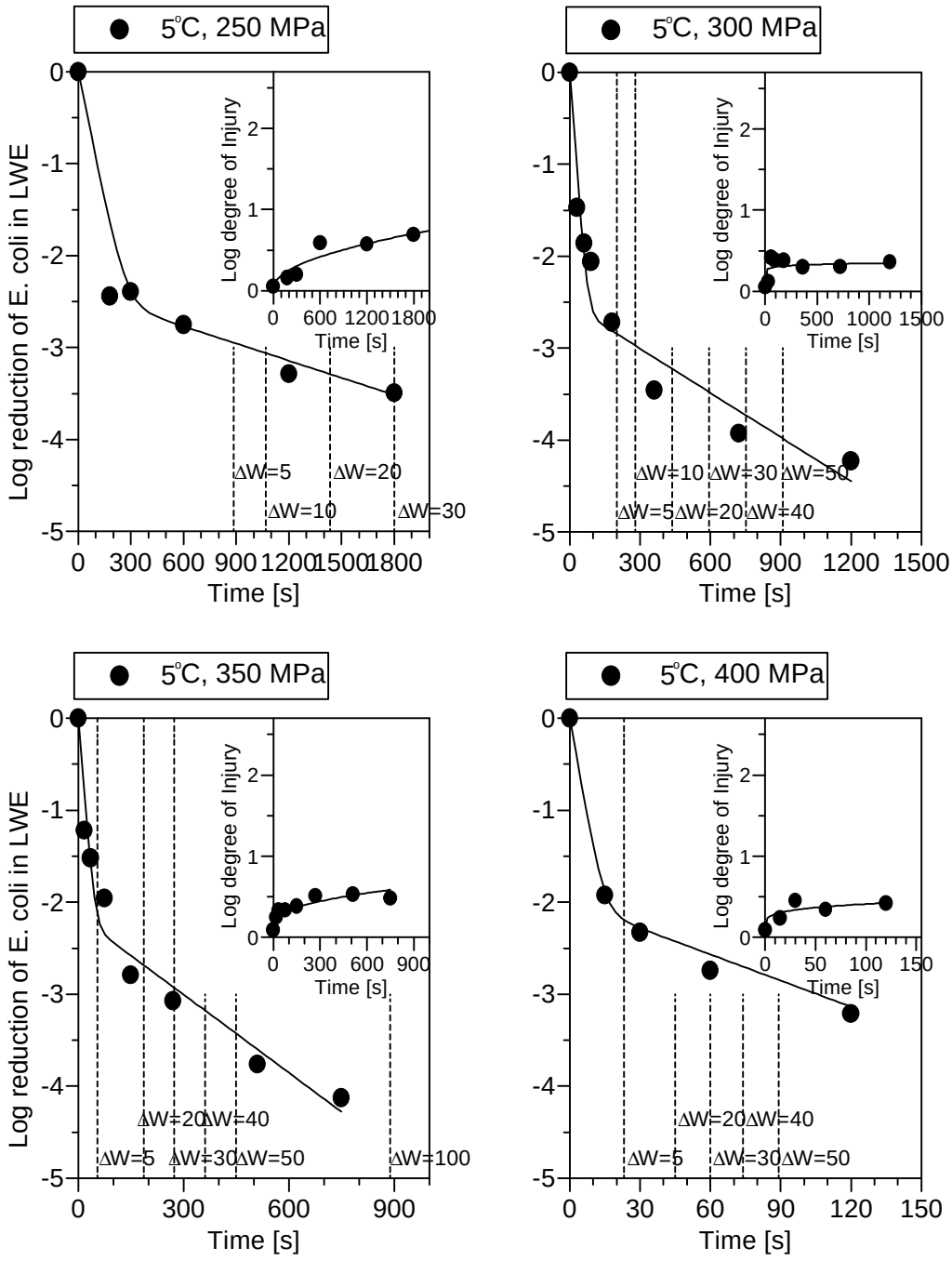

Figure 4.2.1. High pressure inactivation of *E. coli* in LWE at 5 °C. The solid curve represents the regression line calculated by a first order biphasic model. The dashed lines which are parallel to Y axis indicate the degree of egg protein coagulation (ΔW) at the corresponding to HHP treatment. Within the line of ΔW=5.0 kJ/m^3, LWE is in liquid phase. Between ΔW=5.0 and 100 kJ/m^3, LWE is in a partially coagulated phase. The insets show the degree of sublethal injury in surviving cells by a log scale.

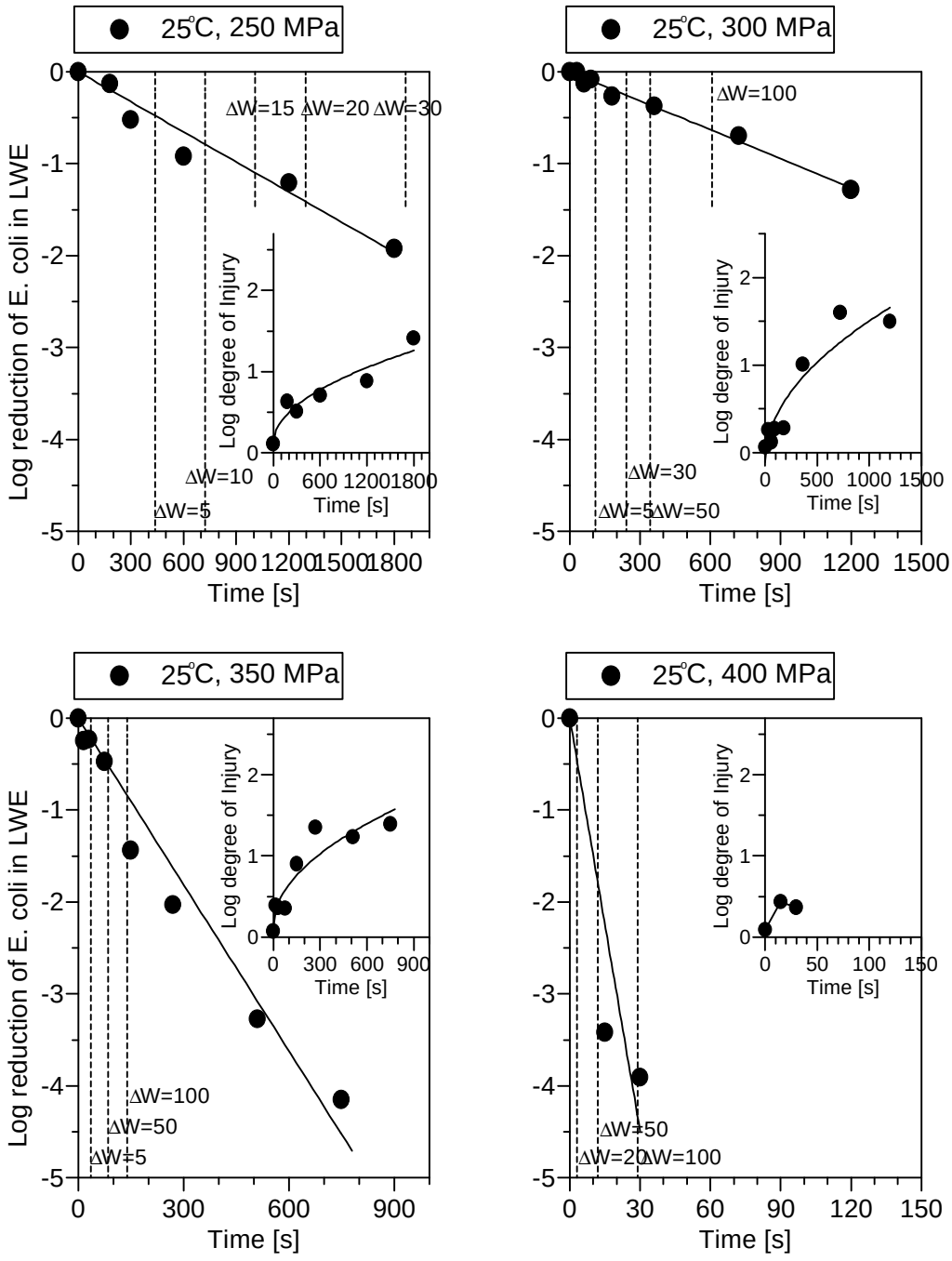

Figure 4.2.2. High pressure inactivation of *E. coli* in LWE at 25 °C. See the caption in Figure 4.2.1 for the detailed explanation.

Surviving populations of HHP treated *E. coli* were evaluated in more detail by the degree of sublethal injury (Insets in Figure 4.2.1). Both structural and metabolic injury are considered to take place by HHP treatment, although the exact mechanism of injury are not known (Hauben et al., 1996). The proportion of sublethal injury in the surviving population is of significance for the process development or the design of combination processes because further microbial inactivation can be readily achieved by addition of other stressing factors. In some instances of high acid foods such as fruit juices, only subsequent storage after pressure treatment enables such a further microbial inactivation (Garcia-Graells et al., 1998; Linton et al., 1999).

4.2.3. Inactivation of *E. coli* inoculated in liquid whole egg at 25 °C

Figure 4.2.1 shows the HHP inactivation of *E. coli* in LWE at 25 °C. Unlike 5 °C, inactivation curves at 25 °C were linear on a semi-logarithmic scale. However, the situation of HHP treatments at 25 °C was quite different from that of 5 °C. As shown by the rapid appearances of coagulation lines in comparison with those of at 5 °C, pressure treatments at 25 °C caused an increased coagulation of LWE and allowed only a limited area of treatments. For example, pressure treatments of 300 MPa at 5 °C initiated the phase transition of LWE approximately at 200 s, but it occurred only after 110 s if the treatment temperature increases to 25 °C. Further, *E. coli* showed greater resistance against HHP treatments at 25 °C than 5 °C as reported by others (Ludwig et al., 1992; Ter Steeg et al., 1999). Thus, *E. coli* inactivations within the liquid phase of LWE were very limited, and lacked any significance in kinetic analysis. Therefore, the fraction I was ignored during fitting the inactivation data to biphasic model by fixing *f* value to zero, and the overall inactivation kinetics were converged to a simple first order kinetics of fraction II (Table 4.2.1).

At 250 and 300 MPa, less than two log reductions of *E. coli* were obtained within the experimental conditions and almost no reduction of viable counts were achieved within the liquid phase of LWE. At 350 MPa, only 30 s of pressure treatment initiated coagulation and instantaneous coagulation occurred at 400 MPa. Although more than 4 log cycle reductions in viable counts could be achieved under these treatment conditions, no viable count reduction was observed within the liquid phase of LWE.

The degree of sublethal injury by HHP treatments at 25 °C is presented in insets in Figure 4.2.2. In contrast to the viable count reduction, an increase in the degree of sublethal injury was observed at 250, 300 and 350 MPa.

Table 4.2.1. Pressure, temperature and inactivation kinetic parameters for *E. coli* in LWE. The initial population of first fraction (*f*), inactivation rate constants at first fraction (k_1) and second fraction (k_2) are estimated by the first order biphasic kinetic analysis.

Temp. [°C]	Press. [MPa]	*f*	k_1 [s^{-1}]	k_2 [s^{-1}]	Coef. Det. r^2
5	250	0.996	0.0218	0.00143	0.997
	300	0.997	0.0800	0.00372	0.953
	350	0.994	0.1120	0.00503	0.979
	400	0.990	0.3400	0.02180	0.999
25	250	0	-	0.00251	0.953
	300	0	-	0.00242	0.989
	350	0	-	0.01390	0.962
	400	0	-	0.34500	0.810

4.2.4. The effect of pressure on the *E. coli* inactivation rate constants

The effect of pressure on the rate constants (*k*) can be expressed in terms of the apparent activation volume (ΔV^*). The plot of ln(*k*) versus pressure yielded a straight line and the ΔV^* value for each inactivation fraction was calculated from the slope (Figure 4.2.3). At a treatment temperature of 5 °C, the slopes for k_1 and k_2 were 0.0171 and 0.0170 and the apparent activation volume was calculated to $-3.95 \cdot 10^{-5}$ and $-3.93 \cdot 10^{-5}$ $m^3\ mol^{-1}$, respectively. These ΔV^* values do not indicate the microbial resistance against pressure

but the pressure sensitivity of rate constants. Therefore, it is not surprising that both fractions have almost same ΔV^* values, although the inactivation rates of fraction I (k_1) were clearly greater than those of the fraction II (k_2).

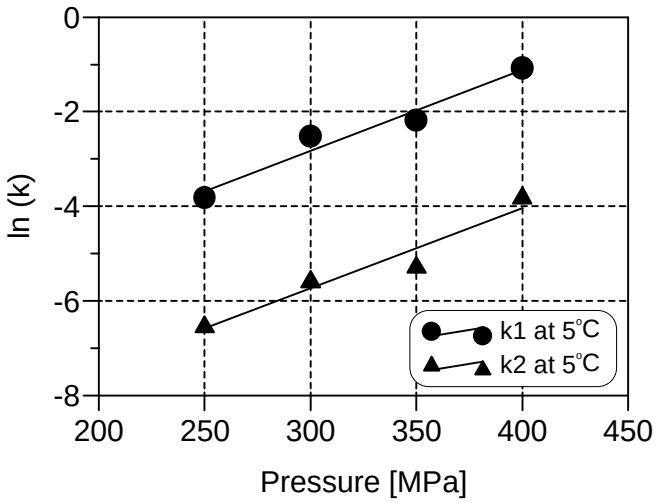

Figure 4.2.3. Effect of pressure on the rate constants at the treatment temperature of 5°C. k_1 and k_2 in s^{-1}

These values are similar to those of previous reported ΔV^* values of $-3.43 \cdot 10^{-5}$ and $-4.17 \cdot 10^{-5}$ m^3 mol^{-1} for *Listeria monocytogenes* and indigenous microflora in pork by HHP treatments at 4 °C (Mussa et al., 1999). The slope for k_2 at 25 °C is 0.033 (plots are not shown) and ΔV^* was calculated as $-8.18 \cdot 10^{-5}$ m^3 mol^{-1}. The slope for k_1 at 25 °C can not be determined. The larger ΔV^* value indicates that the inactivation rate will increase rapidly as the applied pressure increases. Ponce et al.(1998) also reported that inactivation rates at room temperature were smaller than inactivation rates at 2 °C especially at lower pressures, whereas the differences in inactivation rates were compensated at higher pressures.

4.2.5. Inactivation of psychrophiles in liquid whole egg

Three kinds of psychrophiles, *Pseudomonas fluorescens*, *Paenibacillus polymyxa* and *Listeria seeligeri*, formerly known as *Listeria innocua*, were HHP treated at selected critical conditions where rheological properties of LWE were comparable to those of fresh LWE (Table 4.2.2). These bacteria were frequently encountered in food spoilage especially under refrigerated conditions (Ternström et al., 1993). *Pseudomonas* and *Paenibacillus* were sensitive to HHP treatment and viable cell counts were reduced over 5 log cycles in all treatment conditions. However, *Listeria* exhibited a great resistance to HHP as reported by others (Simpson and Gilmour, 1997; Mussa et al., 1999), and almost no inactivation occurred within experimental conditions.

Table 4.2.2. Log reduction of *E. coli*, *Pseudomonas fluorescens*, *Paenibacillus polymyxa* and *Listeria seeligeri* at selected critical processing conditions.

Temp. [°C]	Press. [MPa]	Critical Time [s]	*E. coli*	*Ps. Fluorescens*	*Pae. polymyxa*	*L. seeligeri*
5	250	886	-3.0	<-5.0	<-5.0	-0.1
	300	200	-2.9	<-5.0	<-5.0	-0.4
	350	55	-2.1	<-5.0	<-5.0	-0.2
	400	23	-2.5	<-5.0	<-5.0	-0.4
25	250	438	-0.6	<-5.0	<-5.0	-0.0
	300	109	-0.2	<-5.0	<-5.0	-0.0
	350	35	-0.4	<-5.0	<-5.0	-0.0
	400	I.C.[a]	N.D.[b]	N.D.[b]	N.D.[b]	N.D.[b]

[a]I.C.: Instant coagulation at 25°C, 400 MPa.
[b]N.D.: Not determined.

4.3. Combined Treatments of Nisin and High-Intensity Ultrasound with High Hydrostatic Pressure: Effects on the Microbial Inactivation of Liquid Whole Egg

4.3.1. Introduction

In previous two chapters, processing criteria for the high pressure treatments of LWE were established considering egg protein coagulation and the microbial inactivation kinetics. The selected high pressure processing conditions were 250 MPa for 886 s or 300 MPa for 200 s at the treatment temperature of 5 °C. These processing conditions ensured the minimum changes in the rheological properties of LWE, and reduced the viable cell counts of test microorganisms as effective as the conventional thermal pasteurization. However, the selected high pressure processing conditions were not severe enough to inactivate all kinds of the test microorganisms, and more pressure resistant microorganisms like *Listeria seeligeri* were not at all affected by such processing conditions. One way to overcome this limitation is to combine two or more antimicrobial techniques with different (or sharing) killing mechanisms (Kalchayanad et al., 1994; Hauben et al., 1996; Ter Steeg et al., 1999; Garcia-Graells et al., 2000). Thus, combined processes of HHP with other non-thermal treatments were explored to achieve further microbial inactivation. The two candidates for the combined processes are chosen from the categories of chemical and physical treatment: the natural antimicrobial peptide nisin and the high-intensity ultrasound.

The addition of nisin or high-intensity ultrasound could be conducted before or after the high pressure treatment. Because most of HHP units are batch type system, the use of nisin or ultrasound after HHP implies re-opening of the package of LWE after pressure treatment, and then conducting the nisin or ultrasound treatments. This kind of procedure will lose all advantages of HHP, i.e., the exclusion of post-process contamination or the flexibility of packages. Therefore, only the combined procedure of nisin or ultrasound prior to HHP treatment is investigated in this study.

4.3.2. Inactivation of *Listeria* in LWE by the combined treatment of nisin and high hydrostatic pressure

The inactivation of *Listeria seeligeri* in LWE by the combined treatment of nisin and HHP is presented in Figure 4.3.1. Using HHP alone, the reduction of *Listeria* was not detectable(first three bars in Figure 4.3.1). This result is in agreement with previously reported data (Chapter 4.2). Although nisin is known to be an antimicrobial activity to Gram-positive bacteria, nisin treatments at atmospheric pressure reduced the viable cell counts of *Listeria* only slightly up to 10 mg/l of nisin concentration (solid bars in Figure 4.3..1). However, combined treatments of nisin and HHP reduced the viable cell counts of *Listeria* greatly. The extents of inactivation were increased with the concentration of nisin, and pressure treatments with a nisin concentration of 10 mg/l resulted in about 5 log cycle reductions of *Listeria*. Because the net effects of each nisin and HHP on the

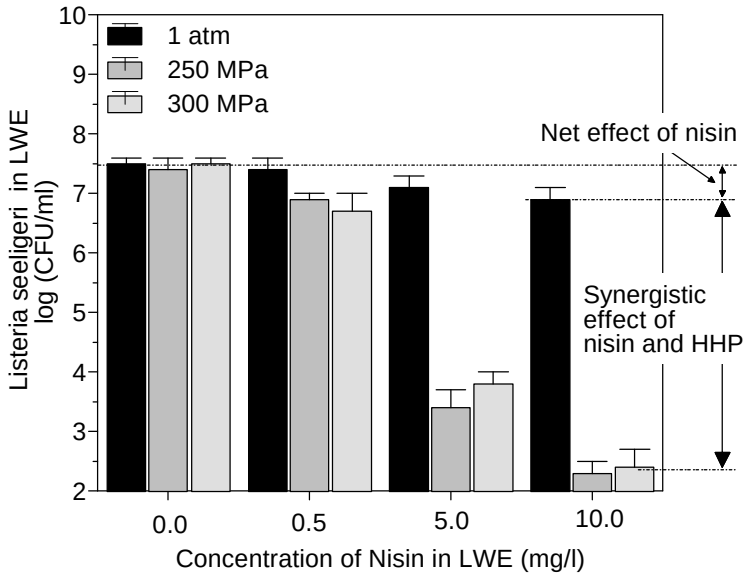

Figure 4.3.1. Combined treatments of nisin and HHP on *Listeria seeligeri* in LWE. Nisin was added about 20 min before HHP treatments. HHP treatments were performed using previously optimized process conditions, i.e., 250 MPa for 886 s at 5 °C, or 300 MPa for 200s at 5 °C.

Listeria inactivation were very small, most of the microbial reductions are considered to be due to the synergistic action of nisin and HHP.

The synergistic effect of nisin and HHP on microbial inactivation can be explained in terms of membrane fluidity. It is known that microorganisms with stiffer membrane would be more sensitive to HHP (Smelt et al., 1994). Because the insertion of nisin into the cell membrane is assumed to induce the local immobilization of phospholipids which results in a decrease in membrane fluidity, it was suggested that the binding of nisin to the cell membrane would directly increase the susceptibility of microorganisms to HHP treatments (Ter Steeg et al., 1999). The synergistic effects would be greater in Gram-positive bacteria than Gram-negative bacteria, since nisin can be directly inserted into the cytoplasmic membrane of Gram-positive bacteria which have no outer membrane. In the case of Gram-negative bacteria, nisin must pass the outer membrane before reaching the targeting cytoplasmic membrane. Indeed, the addition of nisin increased the lethal effects of HHP on the Gram-positive *Listeria* extensively, whereas the additional lethal effects on the Gram-negative *E. coli* were negligible (See next section).

4.3.3. Inactivation of *E. coli* in LWE by the combined treatment of nisin and high hydrostatic pressure

The inactivation of *E. coli* in LWE by the combined treatment of nisin and HHP is presented in Figure 4.3.2. *E. coli* was more susceptible to HHP than *Listeria*, and HHP alone resulted in more than 2 log cycle reductions in viable cell counts. However, *E. coli* was resistant to nisin, and showed almost no effect with the nisin concentrations up to 20 mg/l at atmospheric pressure. Furthermore combined treatments of nisin and HHP resulted in exactly the same extents of *E. coli* reductions as those by HHP alone, and exerted no further additional inactivation.

The synergistic effect of nisin and HHP against Gram-negative bacteria is a still controversial issue. Although the outer membrane of Gram-negative bacteria is believed to prevent the entrance of nisin into the periplasmic space of the cell which is an essential step for the action of nisin, some previous results showed that nisin also increased the sensitivity of Gram-negative bacteria to HHP (Kalchayanand et al., 1994; Hauben et al., 1996). They suggested that HHP transiently disrupts the permeability of the outer

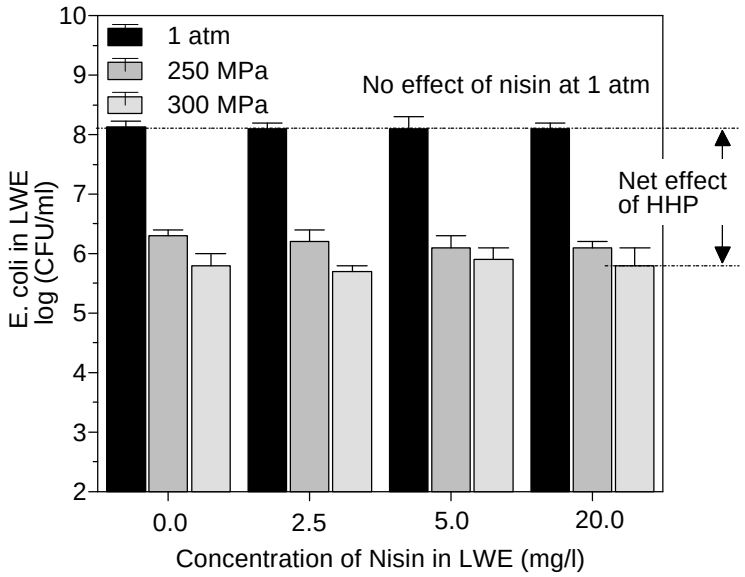

Figure 4.3.2. Combined treatments of nisin and HHP on *E. coli* DH5α in LWE. Nisin was added about 20 min before HHP treatments. HHP treatments were performed using previously optimized process conditions, i.e., 250 MPa for 886 s at 5 °C, or 300 MPa for 200s at 5 °C.

membrane of *E. coli* which may facilitate the access of nisin to the cytoplasmic membrane and makes *E. coli* more susceptible to HHP.

Other researches showed that the addition of nisin during HHP exerted neither synergistic nor additional effect on the inactivation of Gram-negative bacteria (Yuste et al., 1998; Masschalck et al., 2001). The recent model of nisin's configuration within the cell membrane excludes the free passage of nisin through the outer membrane (Breukink et al., 1998), and it seems that more stable pores or even total disruptions of outer membrane are needed if nisin is moved into the cytoplasmic membrane of Gram-negative bacteria. Obviously, HHP can induce such changes in the outer membrane to introduce nisin to it’s site of action, but it is quite questionable whether the target microorganism is still alive with such larger damages. If the target microorganism is already inactivated by HHP, the action of nisin after that event will not show any difference in the viable cell counts.

It is also of interest that the synergistic effects on microbial inactivation were clearly reduced when the microorganism were suspended in real food system such as whole milk or poultry meat (Yuste et al., 1998; Garcia-Graells et al., 1999; Masschalck et al., 2001). One possible explanation is that the food components, especially fats and lipids in food, may protect the HHP induced permeabilization of microbial outer membranes. The inner cytoplasmic membrane which has no direct contact to food constituents might be more susceptible to HHP, and may be disrupted earlier than the outer membrane. The synergism of allyl isothiocyanate and HHP on *E. coli* strains in salted vegetables (Ogawa et al., 2000) could be interpreted by the lack of fats as well as high salt concentration in the food matrix. Another explanation is that the viscous nature of food matrix can prevent the movement of nisin into the, perhaps already permeabilized, cell membrane. The fairly high fat content (10 %) and viscous nature of LWE (μ_{app}=10 mPa·s) fulfil these prerequisites and might explain the reason of no further reductions of *E. coli* by the combination of nisin and HHP.

HHP of 250 and 300 MPa showed almost the same microbial inactivation degrees with or without nisin. These results verify again that these two HHP processing conditions have an equivalent effect on the *E. coli* inactivation.

4.3.4. The combination of ultrasound and high hydrostatic pressure: Selection of ultrasound processing conditions

The high-intensity ultrasound generates heat when it is applied to liquid material. Because ultrasound treatments are intended as a pre-treatment to HHP which has been optimized at the treatment temperature of 5 °C, the temperature increases due to ultrasound treatments should be as minimal as possible. Furthermore ultrasound treatment should induce additional microbial inactivation when it is combined with high pressure treatment.

In order to determine the adequate ultrasound processing conditions to satisfy these requirements, preliminary tests of ultrasound were performed regarding the temperature increase and the *E. coli* inactivation in LWE at various ultrasound intensity levels. Figure 4.2.3 shows the temperature increases of LWE during the ultrasound treatments. The temperature of LWE increased very quickly due to LWE's low specific heats of

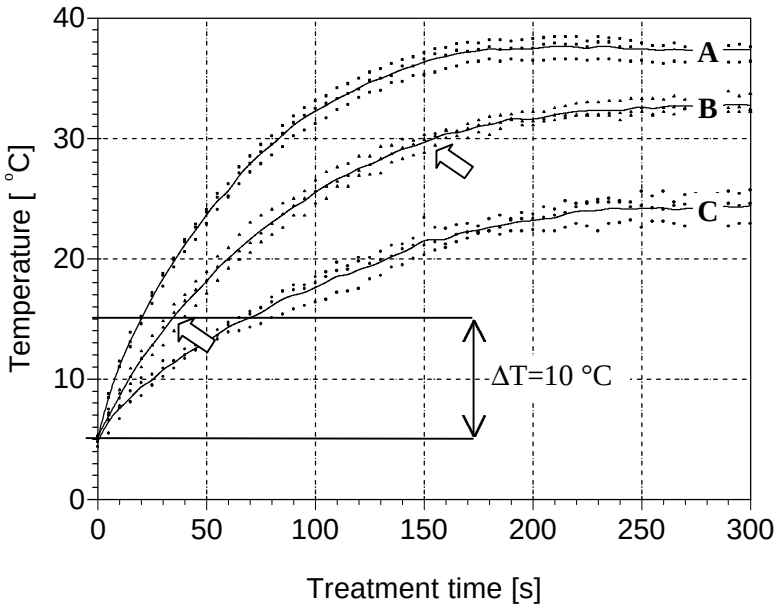

Figure 4.3.3. Temperature increases of LWE by ultrasound treatments. Ten milliliters of LWE were treated. The powers of ultrasounds were 42.0 W (A), 34.6 W (B), or 24.6 W (C) with a constant frequency of 20.0 kHz.

3.68 $kJ \cdot kg^{-1} \cdot K^{-1}$, although the treatment tube was immersed in an iced water bath. The temperature of LWE was stabilized after a few minutes of ultrasound treatments and the equilibrium temperature was correlated with the power of ultrasound.

The inactivation of *E. coli* in LWE by ultrasound treatments was shown in Figure 4.3.4. Ultrasound treatments of 24.6 W resulted in one log cycle reduction after 300 s of treatment time. Two log cycle reductions of *E. coli* were achieved after the same time of radiation when the power of ultrasound was increased to 42.0 W. However, when the same data were redrawn in the axis of total energy input (Figure 4.3.4.B), the inactivation curves converged together, and little difference was seen with respect to the maximum powers of ultrasound.

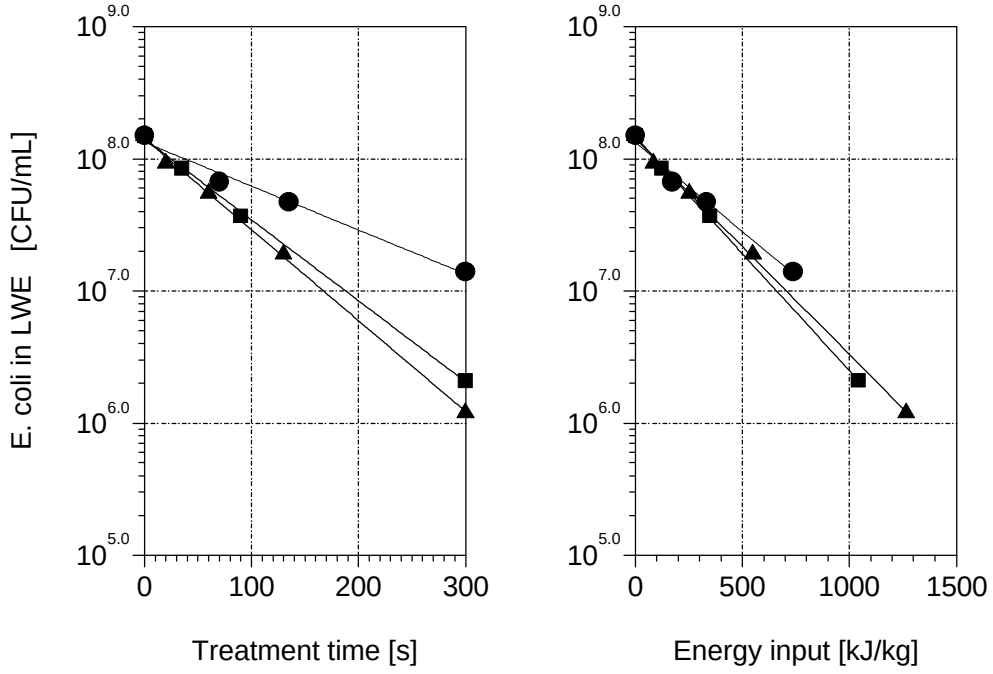

Figure 4.3.4. Inactivation of *E. coli* DH 5α in LWE by ultrasound treatments. Initial temperature of LWE was about 5°C. Figure A and B represent the same results with the different unit of x-axis. The powers of ultrasound are as follows: –▲–, 42.0W; –■–, 34.6 W; –●–, 24.6 W.

Considering the increase of LWE temperature and the degree of *E. coli* inactivation, two processing conditions of ultrasound were selected for the further combination experiments: 34.6 W for 30 s and 34.6 W for 150 s. These ultrasound processing conditions increased the temperature of LWE from 5 °C to 15 °C and 30 °C, with the *E. coli* reductions of 0 and 0.5 log cycles, respectively.

4.3.5. Inactivation of *E. coli* and *Listeria seeligeri* in LWE by the combined treatment of high-intensity ultrasound and high hydrostatic pressure

It was reported that the lethality of microorganism was increased not only by a simultaneous combination of ultrasound and heat (i.e., thermosonication), or ultrasound, heat

and pressure (i.e., manothermosonication), but also by a consecutive combination of ultrasound and heat (Sala et al., 1995). The increased lethality by the consecutive combined treatments indicated that ultrasound treatment would induce some non-transient damages to the target microorganisms. The possible sites of these damages could be cell wall, cytoplasmic membrane or DNA (Earnshaw et al., 1995), although the exact nature of inactivation mechanism is still unknown.

A consecutive combination of ultrasound and HHP resulted in no significant effects on *E. coli* and *Listeria seeligeri* in LWE within the experimental conditions (Figure 4.3.5). *E. coli* was more susceptible to both ultrasound and HHP than *Listeria seeligeri*, and the combined treatments of ultrasound and HHP caused a slightly increased extent of *E. coli* inactivation, which is attributed to the additional effect of ultrasound. *Listeria seeligeri* was resistant to both ultrasound and HHP treatments, and no inactivation was observed within the experimental conditions regardless of individual, or combined treatments.

The lack of combination effect on microbial inactivation in these results can be explained by the low temperature (5 °C) of LWE. Indeed, most microorganisms showed greater sensitivity to ultrasound only at increased temperature, generally over 50 °C (Hurst et al., 1995; Zenker et al., 1999; Villamiel and de Jong, 2000). It could be inferred that the applied ultrasound of our experiment was too weak to induce any change in target microorganism, and therefore no further microbial inactivation could be occurred by the subsequent high pressure treatment. However, the total energy inputs of the applied ultrasound treatments ,which were approximately 104 $kJ \cdot kg^{-1}$ for 30 s, or 519 $kJ \cdot kg^{-1}$ for 150 s were quite high when compared to those of the continuous ultrasound treatments with short residence time (Zenker et al., 1999). The critical role of temperature on the ultrasound treatments seems contradictory to the microbial inactivation mechanism of ultrasound, because the degree of cavitation vanishes as temperature increases, and no cavitation occurs above the boiling point of the medium. Probably the increased heat and mass transfer due to the microstreaming phenomenon of ultrasound improved the efficiency of thermal effects and thermal effects might play important role in the inactivating process by ultrasound.

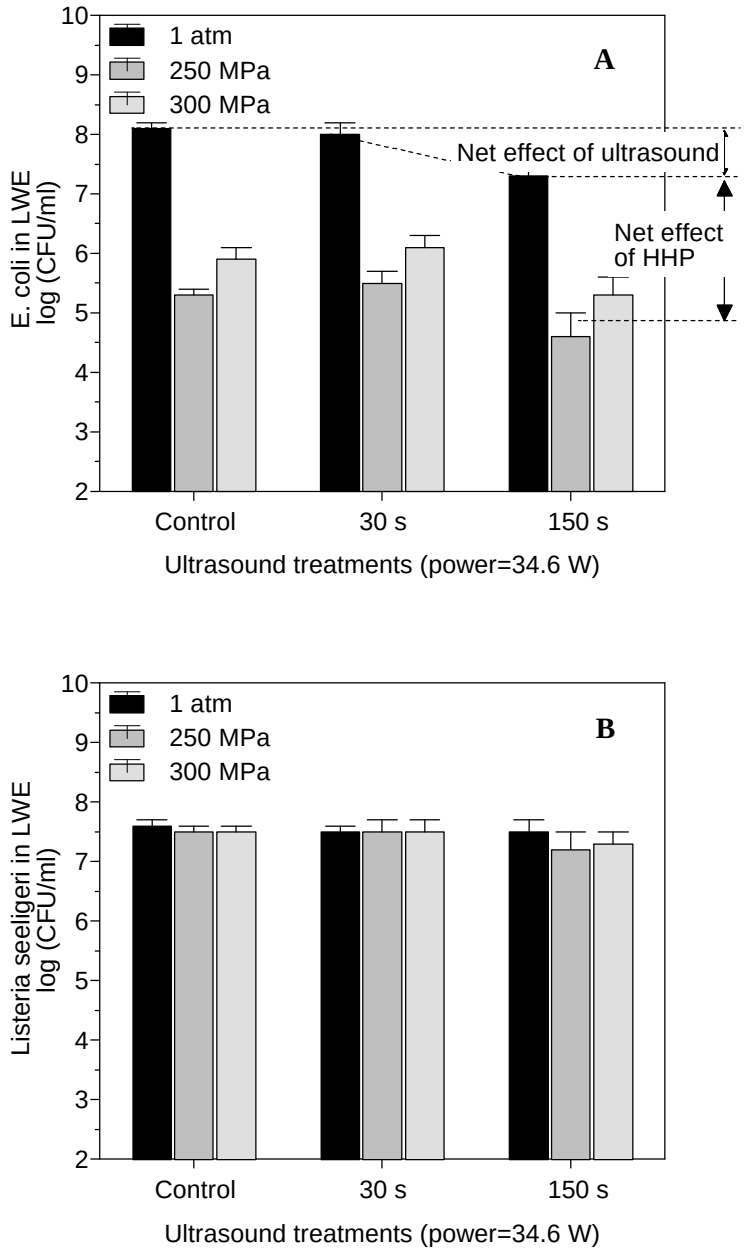

Figure 4.3.5. Combined treatments of ultrasound and HHP on (A) *E. coli* DH 5α and (B) *Listeria seeligeri* in LWE. Increases of temperature by ultrasound treatments were about 10 °C by 30 s and 25 °C by 150 s. HHP (250 MPa, 886 s and 300 MPa, 200 s) were performed at 5 °C.

4.4. Combined Treatments of Nisin and High-Intensity Ultrasound with High Hydrostatic Pressure: Effects on the Functional Properties of Liquid Whole Egg

4.4.1. Introduction

The ultimate objective of liquid egg processing would be to ensure the safety of the consumer and provide sufficiently long shelf life for the manufacturer while the functional properties of fresh liquid eggs are retained. Although the conventional heat treatments of LWE are carefully conducted on the critical temperature-time conditions where the egg protein denaturation is minimized, parts of LWEs are frequently over-processed during thermal treatments, and changes in functional properties due to pasteurization or subsequent frozen storage have been reported (Herald and Smith, 1989; Cunningham, 1995; Yang and Baldwin, 1995; Kiessling and Franke, 1999).

To overcome the limitations of conventional heat treatment attempt was made to employ the non-thermal treatments in LWE processing. In previous chapters, the HHP processing conditions were optimized for LWE treatment considering egg protein coagulation and microbial inactivation kinetics (Chapter 4.1; Chapter 4.2). The selected high pressure processing conditions ensured the minimum changes in the rheological properties of LWE, and reduced the viable cell counts of test microorganisms effectively as the conventional thermal pasteurization. However, the selected high pressure processing conditions could not inactivate all kinds of the test microorganisms. Therefore, combined processes of HHP with other non-thermal treatments, such as nisin and the high-intensity ultrasound, were explored to achieve further microbial inactivation (Chapter 4.3), and the Nisin-HHP combination gave more promising results, especially increased microbial reductions against high pressure resistant *Listeria*.

The objective of this research was to verify the effects of combined processes on the functional properties of LWE. The processing conditions of HHP (300 MPa for 200s at 5 °C), nisin (10 mg/l), and ultrasound (34.6 W for 30 s at 5 °C) were adopted from previous study.

4.4.2. Foaming Property

Foaming is the incorporation of air into a food matrix, usually by whipping. The foaming power of LWE is attributed to the egg white proteins which produce a rigid membrane structure surrounding the entrapped air bubbles. The egg yolk is considered as an inhibitor to foaming power by competing with the egg white proteins in the air bubble interfaces, but the stability of foam is strengthened by the presence of egg yolk (Matringe et al., 1999).

The effects of combined processes on the foaming property of LWE are shown in Table 4.4.1. It can be seen that the application of HHP at the optimized process condition does not affect the foaming property of LWE. HHP alone and the combined process of nisin and HHP induced slight increases both in foaming power and foaming stability, but they were not statistically significant. This maintenance of functional properties after HHP treatment was not unexpected, because the HHP processing condition had been selected where minimal rheological changes had occurred. However, the significant increase in foaming power was observed by the combined process of ultrasound and HHP.

Table 4.4.1. Effects of combined processes on the foaming property of liquid whole egg.

Process	Foaming power [% Overrun]	SD[1)]	Foaming stability [% Stability]	SD
Control	479[a]	13.7	52[a]	5.1
HHP[2)]	490[a]	10.0	56[a]	3.1
Nisin-HHP[3)]	484[a]	12.3	55[a]	1.9
Ultrasound-HHP[4)]	638[b]	14.4	50[a]	2.6

[1)] SD: Standard deviation.

[2)] HHP: 300 MPa for 200 s at 5 °C.

[3)] Combined process of nisin (10 mg/l) and HHP.

[4)] Combined process of ultrasound (34.6 W for 30 s at 5 °C) and HHP.

[a,b)] The columns with no common superscript differ significantly ($P<0.05$).

No publication was found concerning the foaming property of pressurized LWE. However, studies on egg white showed that the foaming power can be either decreased or increased by the extent of applied pressure (Richwin et al., 1992; Iametti et al., 1999). These contrary effects on foaming property were observed not only by high pressure treatment but also by thermal treatment. The foaming property of LWE was either decreased (Cunningham, 1995; Herald and Smith, 1998) or not affected or even increased (Ball, Jr., 1987; Yang and Baldwin, 1995) depending on the pasteurization temperature and treatment time. Changes in the solubility of specific LWE proteins during thermal treatments were regarded to be the cause for the loss of functionality, whereas the dissociation of the triacylglycerol-ovomucin complex by mild heat treatments, which allows ovomucin to participate in foam formation, was believed to be the cause for the improved foaming property. Although it is not known whether these events would occur during high pressure treatments, small changes in protein structures either by heat or pressure seem to exert a positive effect on foaming property of LWE.

The larger increases of foaming power observed in the case of US-HHP combination might be explained by the homogenization effect of ultrasound. Mechanical homogenization process tended to increase the foaming power of LWE. The homogenization effect of ultrasound usually disperse the protein and fat particles in LWE more evenly, which may improve the foaming property of LWE. However, the physical property of US-HHP treated LWE showed larger differences from that of fresh LWE as indicated by significantly lowered viscosity of LWE (about 6.0 mPa·s instead of 10.0 mPa·s; from preliminary experiment). During ultrasound treatment proteins of LWE probably became partially unfolded in structure which accompanies the increase in foaming power.

4.4.3. Emulsion Property

The principal problem of food emulsions is the instability of emulsion which is caused by coalescence of droplets. Because the coalescence is affected by the properties of the interfacial film and the droplet size of emulsion (Das and Kinsella, 1993), the average droplet diameter and the size distribution provide useful information for the emulsifying property. Generally, smaller droplets are favored regarding emulsion stability.

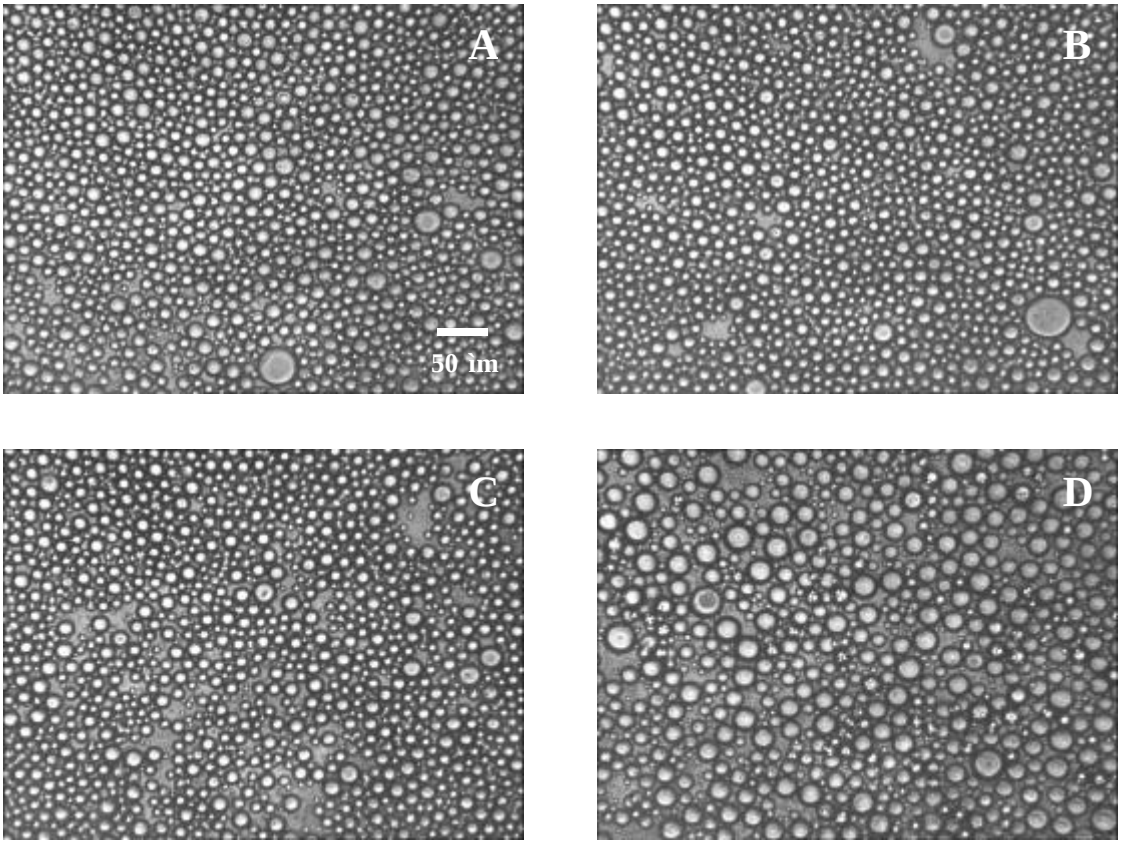

Figure 4.4.1. Effects of combined treatments on the emulsifying property of liquid whole egg (LWE). Emulsions are made with untreated LWE (A); HHP treated LWE (B); Nisin-HHP treated LWE (C); US-HHP treated LWE (D). The bar is 50 ìm.

Representative microphotographs of emulsions which were made with combined processes and control LWE are shown in Figure 4.4.1. The droplet sizes of an emulsion made with high pressure treated LWE were similar to those with untreated LWE. The presence of nisin during HHP treatment also did not affect the droplet sizes. This results verify again that the application of HHP at the optimized process condition does not affect the functional property of LWE.

In contrast, the emulsion made with US-HHP treated LWE showed an increased tendency in droplet sizes. This increase in droplet size of an emulsion can be explained by the lowered viscosity of US-HHP treated LWE. It was reported that the droplet diameters of an emulsion increase with a decrease of viscosity in continuous phase (Das and

Kinsella, 1993). Because the ultrasound treatment of LWE had reduced the apparent viscosity of LWE from 10.0 to 6.0 mPa·s, the less viscous continuous phase would increase the speed of droplet motion immediately after creation, thereby increasing the rate of recoalescence. It was reported that oil-in-water emulsions prepared with previously pressure-treated protein solutions (up to 800 MPa) of either whey protein concentrate or β-lactoglobulin as emulsifier had a broader droplet-size distribution than emulsions prepared with the native untreated proteins (Messens et al., 1997). These results were interpreted in terms of modification of the structure of protein, such that it leads to a loss of emulsifying efficiency owing to protein aggregation, despite the increase in surface hydrophobicity. However, no significant change in the average droplet size of emulsion was also observed if the whey proteins were pressurized at 200 or 400 MPa (Galazka et al., 1995).

4.4.4. Color

The effects of combined processes on the color of LWE are shown in Table 4.4.2. The L-values were almost constant among control, HHP and nisin-HHP samples. However, the combination of ultrasound and HHP resulted in a slight increase in L-value. In the case of thermal treatments, the increase in L-value, i.e., the whitening process, was observed in the very early stage of thermal treatments and considered to be due to the denaturation of heat labile proteins (Rhim et al., 1988). It has been reported that high pressure also induces the increases of L-values especially in protein-rich food samples (Shiggehisa et al., 1991; Carlez et al., 1995). However, this whitening process of HHP was not followed by a rapid shift of color toward the yellow and brown due to the Maillard reaction as reported by thermal treatments (Kessler and Fink, 1986).

A more significant change in color was observed in a- and b-value of LWE. The increase in a value might be interpreted by the formation of Fe-conalbumin complex. The conalbumin yields a red color when it forms a complex with Fe^{3+} ions (Tan and Woodworth, 1969). Actually, the egg white in which conalbumin is suspended contains no Fe^{3+} ions, but the Fe^{3+} ions can be supplied from the egg yolk during the preparation of LWE.

Table 4.4.2. Color changes of liquid whole egg by combined processes.

Process	L (Whiteness)	a (Redness)	b (Yellowness)
Control	60.88[a]	+5.90[a]	+33.06[c]
HHP[1)]	60.92[a]	+8.32[b]	+30.10[ab]
Nisin-HHP[2)]	61.04[a]	+8.32[b]	+30.45[b]
Ultrasound-HHP[3)]	61.73[b]	+8.25[b]	+29.32[a]

[1)] HHP: 300 MPa for 200 s at 5 °C.

[2)] Combined process of nisin (10 mg/l) and HHP.

[3)] Combined process of ultrasound (34.6 W for 30 s at 5 °C) and HHP.

[a,b,c)] The columns with no common superscript differ significantly ($P<0.05$).

However, it is not known whether HHP enhances the formation of Fe-conalbumin complex. The yellow color of LWE which is indicated by b-value is contributed by the pigments in egg yolk such as xanthophyll, lutein and zeaxanthin (Yang and Baldwin, 1995). Combined treatments resulted in slight decreases in b-value. However, whether this decrease is caused by the destruction of pigments or by the masking effects due to the increase of L- and a-value is not clear.

Chapter 5. Conclusion

Non-thermal food processing technologies, such as high hydrostatic pressure, high electric field pulses, high intensity ultrasound or the utilization of antimicrobial bacteriocins, have received great attentions by food scientists due to their potential as a new method to preserve food with minimal loss of organoleptic and nutritional quality of fresh products. These non-thermal processing technologies may be applied to produce wholly new food products, or substitute the conventional thermal processes which could induce significant losses in product quality due to the occurrence of cold point or over-processed zone. Liquid whole egg (LWE) is one of the typical food examples that the thermal process has only a narrow margin of processing possibility. Since egg proteins are easily coagulated by heat, the conventional thermal pasteurizations are conducted on 60-65 °C for 2-3 min, which reduce only one or two log reductions of viable cell counts, thereby the commercial pasteurized LWE often contains 10^2 or more than 10^3 microbial cells/g (Schmidt-Lorenz, 1983).

The objective of this thesis was to investigate the applicability of non-thermal technologies to the LWE processing. High hydrostatic pressure (HHP) was focused as the main unit operation among the non-thermal technologies, and the utilization of nisin or high-intensity ultrasound was also studied as additional non-thermal treatments.

For the application of emerging technologies to LWE processing, changes of physico-chemical properties induced by such processes should be considered along with the microbial inactivation. Therefore, our efforts were concentrated to accumulate the kinetic data both on physical properties and microbial inactivation which are essential for the process designing.

The processing criteria for high pressure treatment of LWE considering egg protein coagulation was investigated as a first step for process optimization. The LWEs were subjected to a wide range of pressure (100–400 MPa), temperature (5–45 °C), and time (0–3600 s) combinations in order to determine the boundary conditions of the high pressure treatments. The degree of egg protein coagulation due to pressure treatment was presented in terms of the excess work of structure breakdown (ΔW) which was calculated from a rheological measurement. Obviously, the pressure-induced coagulation of LWE was strongly dependent on the treatment temperature and pressure. For example, no coagulation could be observed at 100 and 150 MPa until 3600 s at 5 and 25 °C, whereas significant level of coagulation was observed at the same pressures if the treatment temperature was increased to 45 °C. Under isothermal conditions, the starting point of coagulation (lag phase) was shortened with increasing pressure.

The equivalent lines of pressure-induced coagulation at three different temperatures (5, 25 and 45 °C) were constructed in the pressure-time plan, and those figures could successfully depict the phase changing process of LWE from liquid state via transition state to semi-solid state. The ΔW value of 5.0 kJ/m^3 was considered as a critical value for HHP processing, where the rheological properties of processed LWE were similar to those of fresh LWE. The evaluated processing criteria indicated that pressure treatments at low temperature (5 and 25 °C) had clearly better opportunities to conduct high pressure treatment without egg protein coagulation.

Kinetic studies on the isothermal HHP inactivation of *E. coli* in LWE were performed at 5 and 25 °C in the pressure range of 250 to 400 MPa. The characteristic tailing inactivation curves were described by a first order biphasic model. As compared to a previous rheological study, it was suggested that the phase of LWE influences the inactivation kinetics of *E. coli* as well as the treatment pressure and temperature. Furthermore the degree of *E. coli* inactivation at isothermal pressure condition was independent of applied pressures if the physical characteristics of LWE are considered, i.e., between 2.0 to 3.0 log reductions at 5 °C, and less than 1.0 log reductions at 25 °C in the range of 250 to 400 MPa. Therefore, HHP at 5 °C is more favorable than at 25 °C. Within treatment temperature of 5 °C, HHP treatments at 250 and 300 MPa are regarded as more realistic processing conditions than 350 and 400 MPa that have very short proc-

essing times. From the results of approximately 3 log reductions of *E. coli* and over 5 log reductions of *Pseudomonas* and *Paenibacillus*, HHP treatment of LWE at 5 °C is regarded to be as effective as conventional thermal pasteurization. The existence of sublethally injured portions in the surviving cells indicates that further microbial inactivation could take place with additional physical or chemical stresses.

Thus, the consecutive combinations of nisin with high hydrostatic pressure (Nisin-HHP) and ultrasound with high hydrostatic pressure (US-HHP) were explored to achieve enhanced microbial inactivation in LWE processing. The HHP processing conditions were fixed to either 250 MPa for 886 s at 5 °C or 300 MPa for 200s at 5 °C which have been determined as the optimized HHP processing conditions.

The US-HHP combination caused an increased lethal effect on *E. coli* in an additive manner, but no reduction of *Listeria* was observed within the experimental conditions. The lack of increased microbial inactivation effects in this ultrasound-HHP combination can be explained by the low temperature of LWE, because ultrasound treatments have shown enhanced microbial inactivation only at higher temperature region.

More promising results were obtained by the combined treatment of nisin and HHP. The addition of nisin prior to pressure treatments significantly increased the lethal effects of HHP against *Listeria seeligeri*. Since the net effects of each nisin and HHP on the *Listeria* were very small, the increased *Listeria* reductions, up to 5 log cycles, are considered to be due to the synergistic action of nisin and HHP. It is supposed that the added nisin was inserted in cytoplasmic membrane of *Listeria*, which itself is not sufficient for bactericidal effect, but further stress from HHP on the cell membrane leads to the cell death of *Listeria*. The marginal effects of Nisin-HHP combination on *E. coli* can be explained by the membrane structure of Gram-negative *E. coli* or by the protective effects of LWE. These results demonstrated that the Nisin-HHP combination would effectively reduce the microbial loads of Gram-negative *E. coli* mainly by pressure effects, and those of Gram-positive *Listeria* by the combined action of nisin and HHP.

The ultimate objective of liquid egg processing would be to ensure the safety of the consumer and provide sufficiently long shelf life for the manufacturer while the functional properties of fresh liquid eggs are still retained. Therefore, the effects of the HHP and combined non-thermal processes on the functional properties of LWE, such as

foaming property, emulsifying property and color were compared to those of fresh LWE.

Little changes in foaming and emulsifying properties were observed by the application of HHP at the optimized process condition. The presence of nisin (10 mg/l) during HHP treatment also did not affect the foaming and emulsifying property. The slight changes in color values by HHP alone and nisin-HHP combination were correspondent to the effects from the very early stage of thermal treatments. This maintenance of functional properties after HHP alone or Nisin-HHP combination treatment agreed with our expectation, because the HHP processing condition had been selected where minimal rheological changes had occurred. In contrast, the US-HHP treated LWE showed a significant change in foaming and emulsion property indicating some structural changes might have occurred during this treatment. The initial increase of L-value by US-HHP combination indicated the denaturation of heat labile proteins. These results suggest that the LWE product with superior quality could be produced by the application of appropriate (combined) nonthermal treatments.

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