Evaluation and Control of Stress Response Functions of Bacterial Cells and Their Utilization for the Integrated Bioprocess

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PREFACE

This dissertation work was carried out under the joint supervision of Professor Isao Komasawa and Professor Ryoichi Kuboi at the Department of Chemical Engineering, Faculty of Engineering Science, Osaka University from 1991 to 1997.

The objective of this thesis is to achieve rational design and development for protein production and purification process by combining both stress-response functions of bacterial cells and aqueous polymer two-phase systems. The interest is focused on the characterization of the stress-response process of bacterial cells, which is induced by exposing them to the environmental stress, from macroscopic and microscopic view points. The author hopes that this research would contribute to the rational design of bioproduction systems by the use of stress-response functions of bacterial cells.

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GENERAL INTRODUCTION

Many useful biomaterials, such as proteins and polypeptides, have been produced by utilizing bacterial cells. The process to produce the target biomaterials and separate them from the cells usually consists of a series of unit operations, such as cultivation, disruption and/or lysis of the cells, solid-liquid separation, protein separation, purification and polishing of target products (Belter *et al*, 1988; Lightfoot *et al*, 1987).

In such bioprocesses there are many potential difficulties. i) Concentration as well as separation is essential to extract the required products, such as proteins and polypeptides, from the large volume of raw materials and by-products because of the low concentration of target products in the recovered mixture (Wheelwright, 1991). ii) In most cases, the target products must be recovered from the cells by using physical (mechanical) disruption methods (Schutte and Kula, 1990) because they are usually not secreted to the surrounding media but produced in the cytoplasm of the cells (Wheelwright, 1991), so that they are highly contaminated with many other products and cell debris at the primary step of the bioprocess. The secretion of some target proteins can be achieved by its modification using recombinant DNA technology (Jacobs and White, 1986, 1987; Mclean *et al.*, 1991) but it is still not effective. iii) The biological activity of proteins, which depends on the specific conformation, is likely to be lost owing to some given change of temperature, ion strength or pH during the process operations (Asenjo and Patrick, 1990). iv) Although recent advances in gene technologies have allowed the production of recombinant proteins and novel engineered polypeptides at high concentration level within host cells, the target products may form an inactive insoluble aggregates (*e.g.* inclusion body) (Bowden *et al.*, 1991; Oeda *et al.*, 1989). The additional operations, such as purification and solublization of the aggregates (Hatefi and Hanstein, 1969;

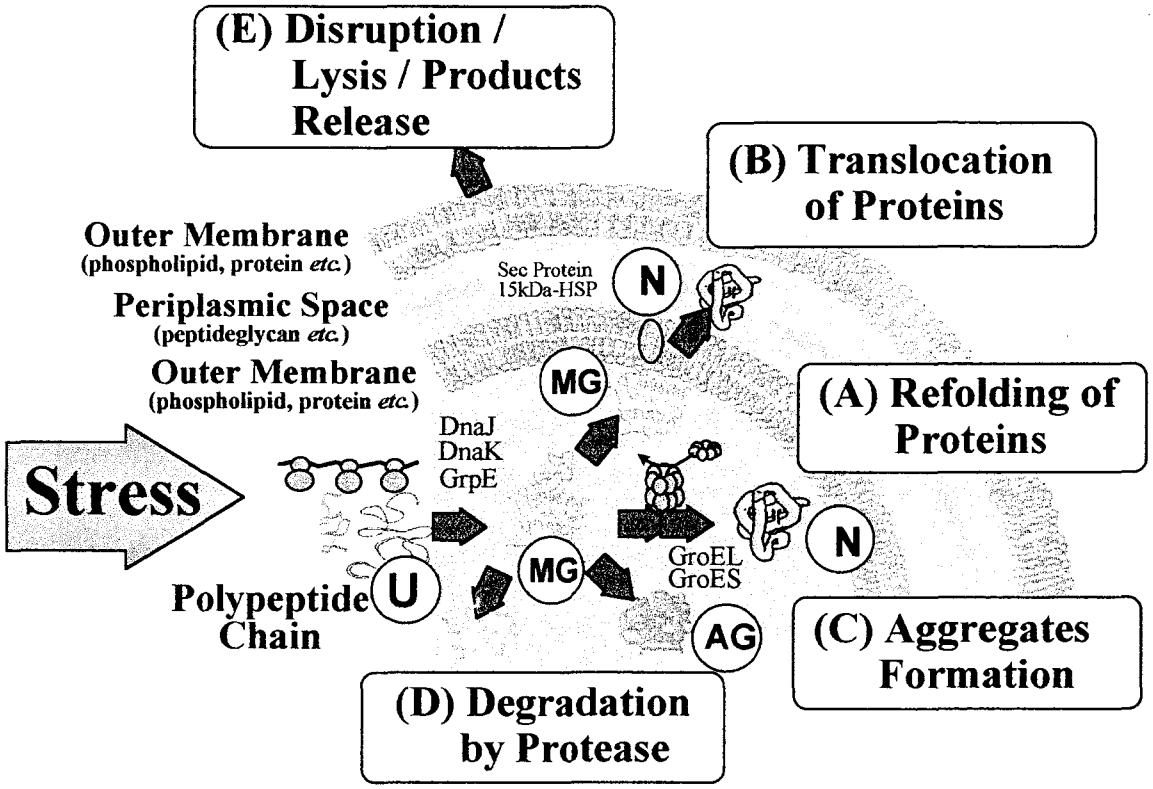


Fig.1 Possible pathways of cell-response against environmental stress

Georgiou *et al.*, 1986) and the refolding of the target (Puri and Cardamone, 1992; Naglak and Wang, 1992), may therefore be needed. The efficiency and selectivity of the bioprocess for the production and recovery of the target are still restricted and should be improved from the economical and operational view points.

This may be caused by the lack of the quantitative understandings of the properties and functions of the bacterial cells. The above difficulties underlying in the conventional bioprocesses may be improved by using the cell functions, which are for example induced under the stress condition (stress-response functions). Bacterial cells normally have a variety of functions, such as intake of environmental nutrients, synthesis of various materials, metabolism, growth, and further, in order to survive in a given and changing circumstance, other important functions such as recognition of environment, response and adaptation to various environmental stresses, what is called,

Table 1 Possibility to solve the problems underlying in conventional bioprocesses by using the 'stress-response' function of bacterial cells

Problems in Conventional Bioprocesses	Possible Utilization of Cell Functions[*1]
·Low Productivity of Target Proteins [*2] ·Denaturation of Proteins[*3]	·Enhanced Production of Target Protein by r-DNA Technology[*2] ·{Enhanced Refolding of Protein followed by Induction of HSPs [**Path A**]; Schein and Noteborn, 1988, Lee and Olines, 1992}
·Contamination with Other Proteins[*2]	·{Control of **Path D**; Schutte and Kula, 1990, Felix, 1971, Dabora and Cooney, 1990} ·{[Enhancement of **Path B**]; Bychkova *et al*, 1988}
·Limited Secretion of Target Protein from Cells[*2]	·Signal-Peptide; Jacobs and White, 1986,1987, Mclean *et al.*, 1991 **[Path B]** ·{Use of Sec-Protein; Collier *et al*, 1988, Liu *et al*, 1989**[Path B]**} ·{Enhancement of Lipid-Protein Interaction [**Path B**]; Bychkova *et al.*, 1988}
·Formation of Inactive Insoluble Aggregates (Bowden *et al*, 1991, Oeda *et al.*, 1989)	·Reduction of **Path C**; {Optimization of culture condition such as temperature, pH, salt concentration Shein and Noteborn, 1988, Lee et al., 1990} ·{Induction of HSPs [enhancement of **Path A**]; Blum *et al.*, 1992, Lee *et al.*, 1992}

* 1 { }; Use of the stress-response function of bacterial cells under the environmental stress
* 2 Wheelwright, 1991
* 3 Asenjo and Patrick, 1990

stress-response function (schematically shown in **Fig.1**). When cells were exposed to heat stress, for example, they respond by transiently inducing various functions of cells, such as (A) rapid synthesis of a set of heat shock proteins (HSPs), such as GroEL and GroES (Ellis and Van der Vies, 1976), and refolding of damaged proteins (Ptysin, 1986; Ayling and Baneyx, 1996), (B) transmembrane translocation of proteins (Bychkova *et al.*, 1988), (C) formation of protein aggregates in an inert insoluble form (*e.g.* inclusion body) (Di Domenco *et al.*, 1989), (D) protein degradation by protease (Goldberg and Dice, 1974). Exposing the cells to lethal stresses, such as mechanical and chemical ones, induces the disruption and/or lysis of cells and the release of intracellular products (E) (Schutte and Kula, 1990; Felix, 1971, Dabora and Cooney, 1990). If quantitative relationships between

'stress factor' and 'cell-response' are studied in details, their functions may be controlled and efficiently utilized for the design and development of bio-processes. In practice, the above problems of the conventional bioprocesses, such as low productivity, aggregates formation, denaturation, and limitted secretion of target protein (**Table 1**), can be solved by controlling and utilizing the stress-response functions of bacterial cells, described as pathways (A)~(E) in **Fig.1**.

The environmental 'stress' is herewith thought to be classified as (1) physical (mechanical), (2) chemical and (3) combined physico-chemical (biological) stresses in relation to the responded behaviors of bacterial cells as shown in **Fig.2**. Among them, physical stress is most destructive for bacterial cells (Schutte and Kula, 1990). This lethal stress usually causes disruption of cell membrane, which consists of outer and inner membranes in *Escherichia coli* cells, the release of cellular products, and cell death. Chemical stresses induced by the addition of ionic and nonionic detergents, organic solvents, chelate-reagents, antibiotics, are relatively mild as compared with the physical one (Felix, 1971; Dabora and Cooney, 1990). The bacterial cells respond in various manners against those chemical stresses.

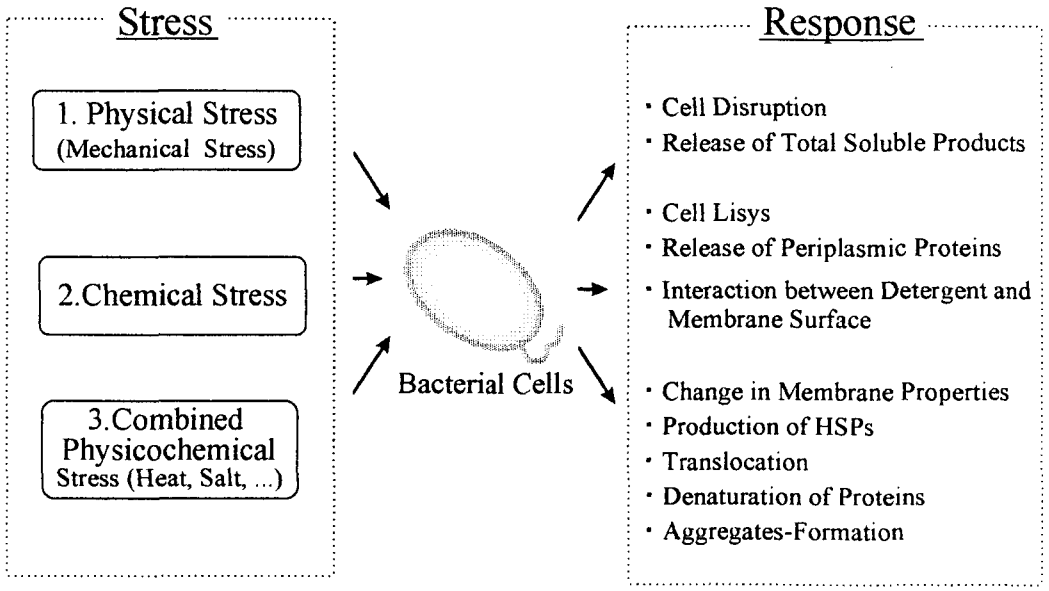


Fig.2 Classification of various stresses and stress-response of cells

For example, the cells, such as *E.coli*, are lysed by the addition of antibiotics, while the addition of nonionic detergent induces the limited solublization of the outer membrane. Further, the bacterial cells respond by inducing a series of and a variety of their functions especially when they are exposed to the combined physicochemical stresses, such as heat stress, cold shock, oxidative stress, starvation, and so on (**Fig.1**). Against these stresses, the latent cell function can be induced because they are not lethal but sublethal one for the bacterial cell survival. The inducing mechanism of such stress-response functions of cells should be studied in each stress level by using both static and kinetic methods.

Various studies on the biological response of bacterial cells against environmental stress have been reported (Hendrick and Hartle, 1993; Gething and Sambrook, 1990; Gething and Smabook, 1992; Horwick *et al.*, 1990; Ellis and Van der Vies, 1991; Ang *et al.*, 1991). However, they are mainly concerned with the functions of HSPs, which are rapidly induced mainly by heat shock and partly by other stresses. Recent studies on the HSPs also emphasize the central role that the HSPs play not only in cellular homeostasis but also in an extraordinary variety of other fundamental cellular processes. For example, the induction of HSPs protects the bacterial cells from the lethality. Some HSPs are also reported to show an important function in the renaturation (refolding) of denatured proteins. They are classified as molecular chaperons (*via* DnaK, DnaJ, and GrpE protein), facilitate the assembly of oligomeric proteins (*via* GroEL and GroES) [corresponding to Path (A) of **Fig.1**], preventing certain transported proteins from folding into transport-incompetent forms (*via* SecB) [corresponding to Path (B) of **Fig.1**], and facilitate the proper folding and assembly of the newly transported proteins (*via* HSP60). However, it is still not completely investigated yet whether those diverse functions of stress response of bacterial cells involve the cooperation of the HSPs or not.

Biological membranes also play important roles in the bacterial cell functions. The compartmentation (localization) of intracellular proteins, followed by their translocation, is a typical example. Many cases are reported in which translocation can be occurred. The difficult enough issue of how a nascent polypeptide traverses a membrane and folds up on the other side has, therefore, given way to the more general and seemingly more difficult problem of how a fully folded, soluble protein can pass the lipid bilayer. To date, most of the data on lipid-protein interactions have been obtained from natural membrane proteins in reconstituted membrane systems from the view point of protein translocation across biological membranes (McElhaney, 1986; George *et al.*, 1990). Current approaches to the study of lipid-protein interactions tend to employ peptide models (Scheele *et al*, 1978) which are designed to interact specifically with either the polar, the interfacial, or the hydrophobic domains of lipid bilayers (Jacobs and White, 1986, 1987; Mclean *et al.*, 1991). Recently, the conformational change of protein, followed by their denaturation, has also been shown to be required for translocation as shown in **Fig.3** (Bychkova *et al.*, 1988). Studying the mechanisms

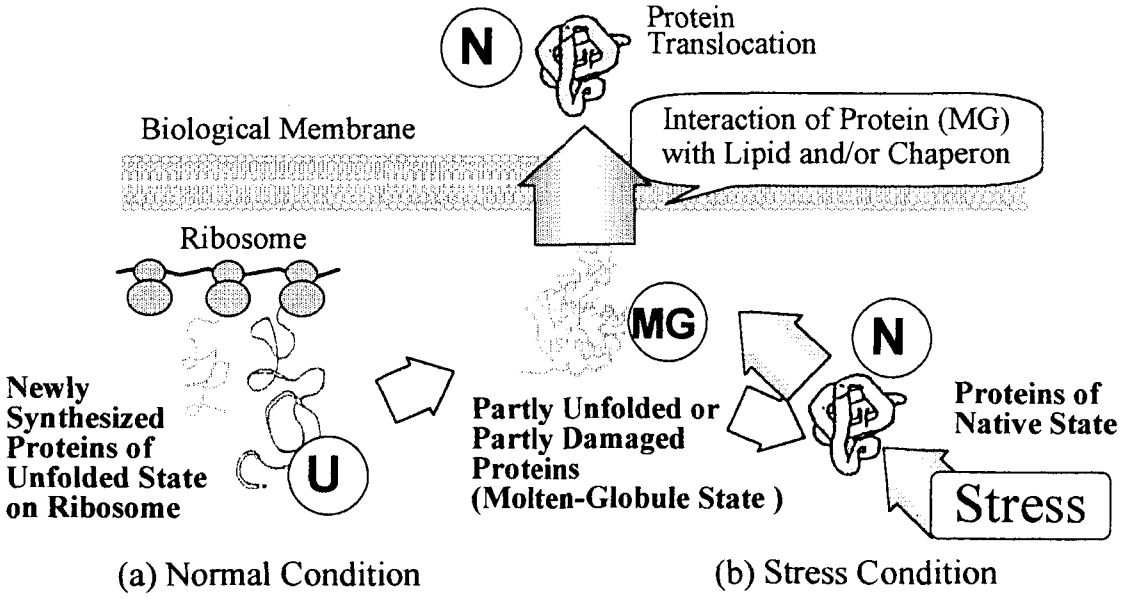


Fig.3 Role of protein conformation and lipid membrane in transport across the membrane (Concept from Bychkova *et al.*(1988))

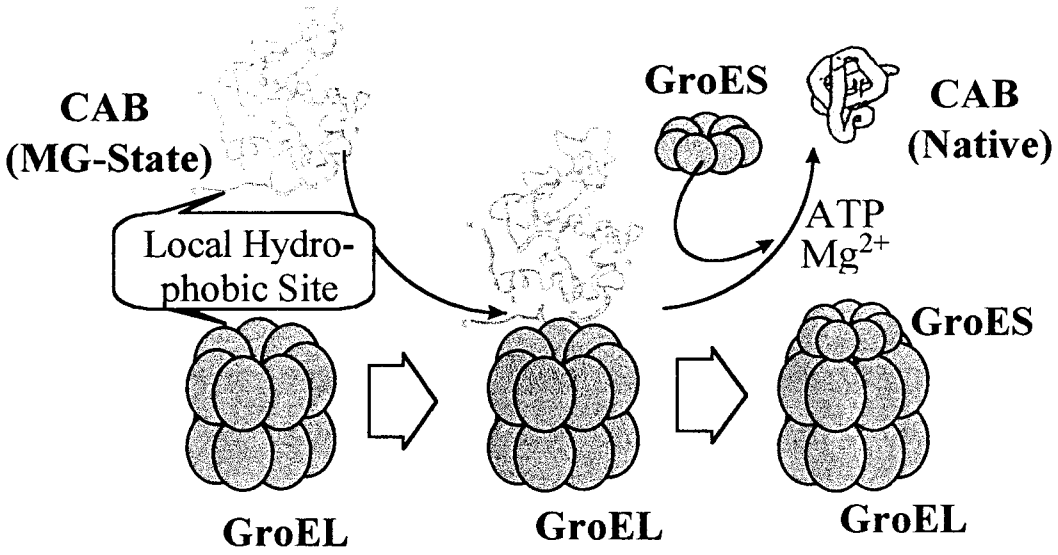


Fig.4 Role of hydrophobic interaction in biological function of chaperon such as GroEL and GroES. (Yano, 1996)

is, therefore, important in order to control protein transport across the membrane.

The biological roles of the above biopolymers, such as chaperons, other HSPs and the phospholipid membranes, are thought to be triggered by the inter- and intra-molecular hydrophobic interaction because both the protein refolding (related to the chaperon) and the protein translocation (related to the lipid membrane) are dependent on whether the local hydrophobic domain exists in proteins and the molecules (Ptisyn, 1986; Bychkova *et al*,1988). Especially in the former case, the role of GroEL in the refolding of unfolded carbonic anhydrase from bovine (CAB) has been quantitatively characterized in relation to the local hydrophobicity of CAB and GroEL (Yano, 1996, **Fig.4**). The folding (A) and transport (B) of intracellular proteins *in vivo* may also be controlled based on such hydrophobic interaction. It is expected that the mechanisms of the bacterial cell function, such as *in vivo* refolding and translocation of proteins, may be clarified by using the quantitative information on hydrophobic properties of such biomolecules.

Various methods have been reported for the characterization of cell surface properties, in particular, on the surface hydrophobicity (van Loosdrecht *et al.*, 1989; Magnusson, 1982; Smyth *et al.*, 1978; Rosenberg, 1980; Rosenberg, 1981; Jonsson and Wadstrom, 1984; Noda and Kanemasa, 1986). Among them, the method using the aqueous two-phase systems (ATPS) has specific advantages for obtaining information on a series of surface properties of cells. i) The surface properties can be analysed through a mild operation (Albertsson, 1986; Walter *et al.*, 1985). ii) The systematic analysis of a series of surface properties can be achieved by varying the mode of partitioning. It has been partly applied by Zaslavsky (1983), Albertsson (1986) and Johansson (1976) as means of determining of the global properties of biomaterials. Tanaka (1993) has recently suggested the characterization scheme of surface properties of various proteins, such as charge, molecular weight, hydrophobicity, and local binding site, by using the ATPS. The surface properties of bacterial cell membrane and phospholipid membrane can be expected to be obtained, employing the ATPS.

Furthermore, the ATPS has been recently applied to various practical processes. For instance, the extractive bioconversion using enzyme (Tjerneld et al., 1984), the extractive cultivation of bacterial cells (separation of produced enzymes from host cells) (Drouin and Cooper, 1992, Tanaka, 1993, Tjerneld et al., 1984), and the extractive protein folding process using molecular chaperon (folded proteins from unfolded ones) (Yano, 1996) are accomplished in the ATPS mainly on the basis of the differences of hydrophobicity of targets and hosts. Consequently, the ATPS can be utilized for the integration of these functions of ATPS and stress-response function of bacterial cells.

It may be interesting to notice the similarity between the inducing mechanism of bacterial cell function (the protein transport and refolding)

and the partitioning of proteins and cells in ATPS, since in both cases intermolecular hydrophobic interaction play an important role. In order to construct the simple process which integrates the functions of bacterial cells and those of ATPS, it may be reasonable to combine and fuse these two functions based on the common hydrophobic interaction.

In this study, the stress-response functions of bacterial cells against various stresses were characterized by using both the static (partitioning) and kinetic methods. The macroscopic properties of biological membrane, such as bacterial cells and model membranes (liposomes), have been systematically characterized at the various equilibrium conditions by using the aqueous two-phase partitioning method. The transient properties of cell membrane under the stressed conditions, such as the physical (mechanical) and chemical stresses, have been also studied by the kinetic analysis. The surface properties of inner membrane of cells was also characterized by using both the partitioning and kinetic methods. The role of hydrophobic interaction in the biological response of bacterial cells, which is induced by sublethal stress, has been analysed by using the biological membrane and liposomes based on the evaluated properties of the membrane and the biomolecules as compared with the stress responded behaviors of cells against the other physical and chemical stresses. The effective and selective bioprocess using bacterial cell functions, which can be estimated by the responded behaviors of bacterial cells against various stresses, has then been established. The flame work and the flow chart of the present study are shown in **Fig.5** and **Fig.6**, respectively.

In chapter 1, characterization methods of surface properties of native (Tanaka, 1993) and unfolded or partly unfolded proteins (Yano, 1996) were developed by using the aqueous two-phase partitioning method and applied to those of bacterial cell membrane and artificial lipid bilayer membrane at

Integration of Stress Response Functions of Bacterial Cells and Use of Aqueous Two-Phase Partitioning Systems

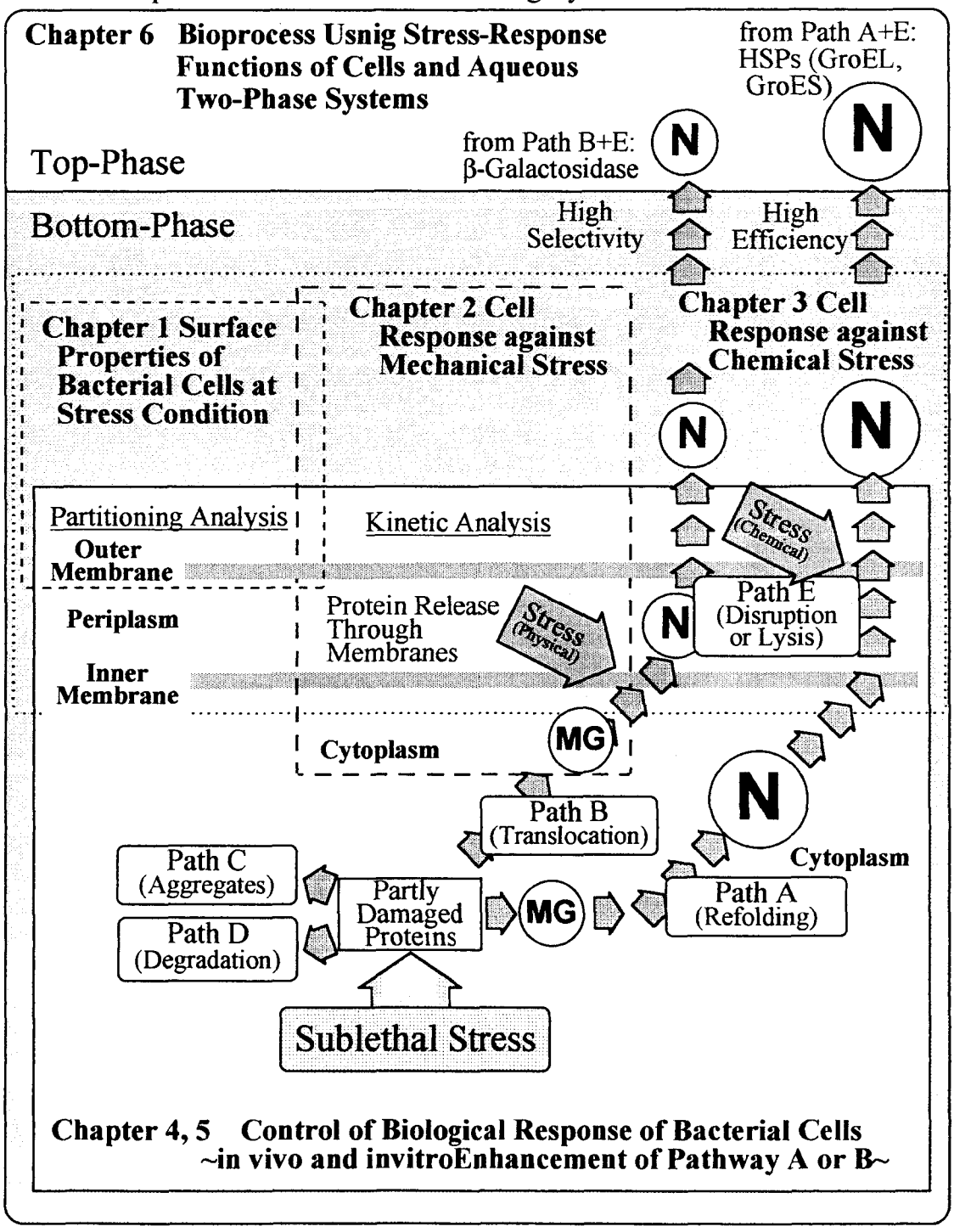


Fig.5 Framework of the present study

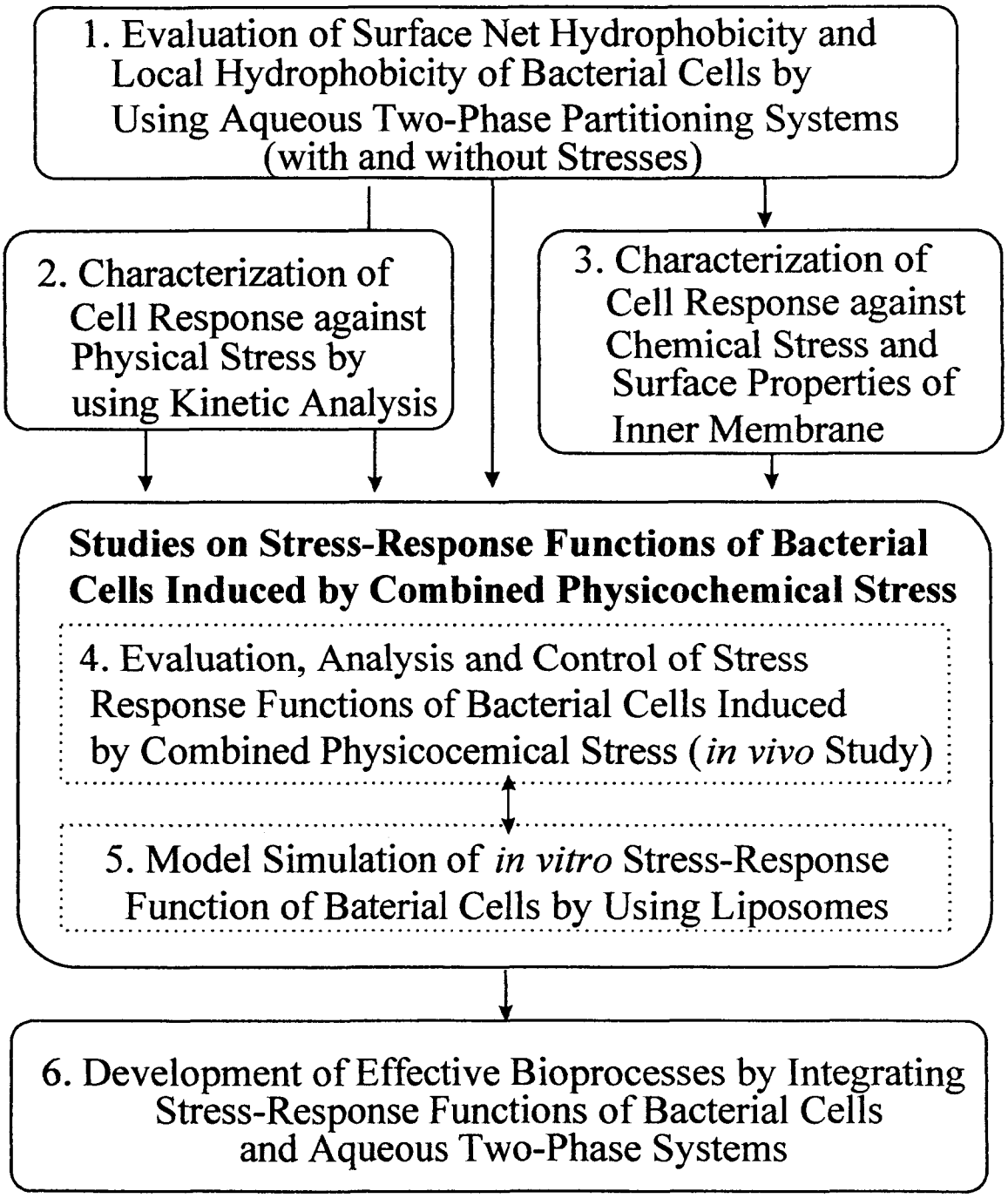


Fig.6 Flow chart of the present study

the equilibrium. Possibility of cell separation processes based on the differences in their surface properties, especially their hydrophobicities, was presented. The surface properties of bacterial cells exposed to various stress conditions were also characterized based on the above methodology.

In chapter 2, the cell response against the mechanical (physical) stress was investigated based on the kinetic analysis of cell disruption and products (proteins) release process. The kinetic parameters, which are dependent on the properties of cells such as physical strength of the whole membrane and the localization of enzymes in the specific compartment of cells surrounded by outer and inner membrane, were then presented by eliminating the effect of various operational conditions. The stress-response of cells was also shown to be controlled by hydrophobic effect. As a case study, the method for effective recovery of periplasmic enzyme was presented by controlling the hydrophobic effect of synthetic polymer on membrane surface.

In chapter 3, for the kinetic analysis of the outer membrane and surface properties of the inner membrane, the response of cells against the chemical stress, which was relatively mild compared with mechanical stress discussed in chapter 2, were investigated. Nonionic detergent, Triton X-100, was used as a chemical stress in order to clarify the hydrophobic interaction between the molecules and the cell membranes. The release kinetics of various enzymes was systematically investigated on the basis of the hydrophobic interaction. The methods to control the cell response and to prepare the partially damaged cells by chemical stress were presented. The surface properties of the inner membrane which affect translocation of proteins were then finally characterized by using the partitioning and kinetic methods.

In chapter 4, the biological response against the sublethal stress, especially heat stress, was investigated in detail in relation to the hydrophobicity of the bacterial cell surface and the intracellular target proteins. The relation-

ship between heat stress and various responses of cells, such as protein folding (A), translocation (B), aggregates formation (C), and degradation by protease(D), was clarified by using the quantitative design parameters. Especially, the transport of cytoplasmic β-galactosidase across the inner membrane, induced by heat stress, was shown to be triggered by the hydrophobic interaction between the proteins and the membrane surface.

In chapter 5, the *in vivo* stress-response functions of cells, especially the heat-induced translocation of proteins across the inner membrane of cells, were verified by using the phospholipid bilayers (liposomes) trapping β-galactosidase. In the *in vitro* model systems, heat-stress was also shown to induce translocation of the enzyme across the lipid membrane. The stress condition for the *in vitro* translocation was well corresponding to that for *in vivo* results. The simple scheme for the possible mechanism of heat-induced translocation of β-galactosidase were presented on the basis of the hydrophobic interaction between the liposome and the proteins. Finally the model simulation for the protein translocation was attempted.

In chapter 6, practical integration was made to apply the results obtained in chapters 1~5 to establish the effective process for the production and separation of bioproducts. A novel and effective bioprocess to produce and separate the i) cytoplasmic β-galactosidase and ii) HSPs (GroEL and GroES) from bacterial cells was developed exploiting the functions and similarity of stress-response of cells and ATPS to enhance both the productivity and selectivity of the target product.

The results obtained in this work are summarized in General Conclusions. Suggestions for the Future Work are described as extension of the present thesis.

Chapter 1

Evaluation of Surface Net hydrophobicity and Local Hydrophobicity of Bacterial Cells and Lipid Membrane by Using Aqueous Two-Phase Partitioning Method

1. INTRODUCTION

The membrane surface of bacterial cells is characterized by various properties such as charge and hydrophobicity, which are due to the composition on their membrane surface of bacterial cells (phospholipid, intrinsic and extrinsic protein, lipopolysaccharide and so on) as shown in **Fig.1-1**. The development of evaluation methods for the determination of these surface properties is therefore important with respect to the following two points. (1) The efficiency and selectivity of product recovery in a practical bioprocess which involves the selection and separation of microorganisms, their cultivations, disruption and/or lysis, and the removal of their homogenates from the target products may be directly affected by the cell surface properties. The recent study on the extractive cultivation process using aqueous two-phase systems (Drouin and Cooper, 1992; Tanaka *et al.*, 1993) also requires the quantitative data of the surface properties in order to design and develop the process efficiently. (2) The cell surface properties may be utilized for clarifying the mechanisms of stress-response of microorganisms because the membrane surface will, at first, recognize the environmental stress, followed by the cell response. For example, *Escherichia coli* cells exposed to temperature stress have been reported to cause the change of lipid composition on the cell membrane (Kito *et al.*, 1973) and the enhancement of penetration of hydrophobic materials to the membrane (Tsuchido *et al.*, 1975,1985). The development of evaluation methods for characterizing cell surface properties is,

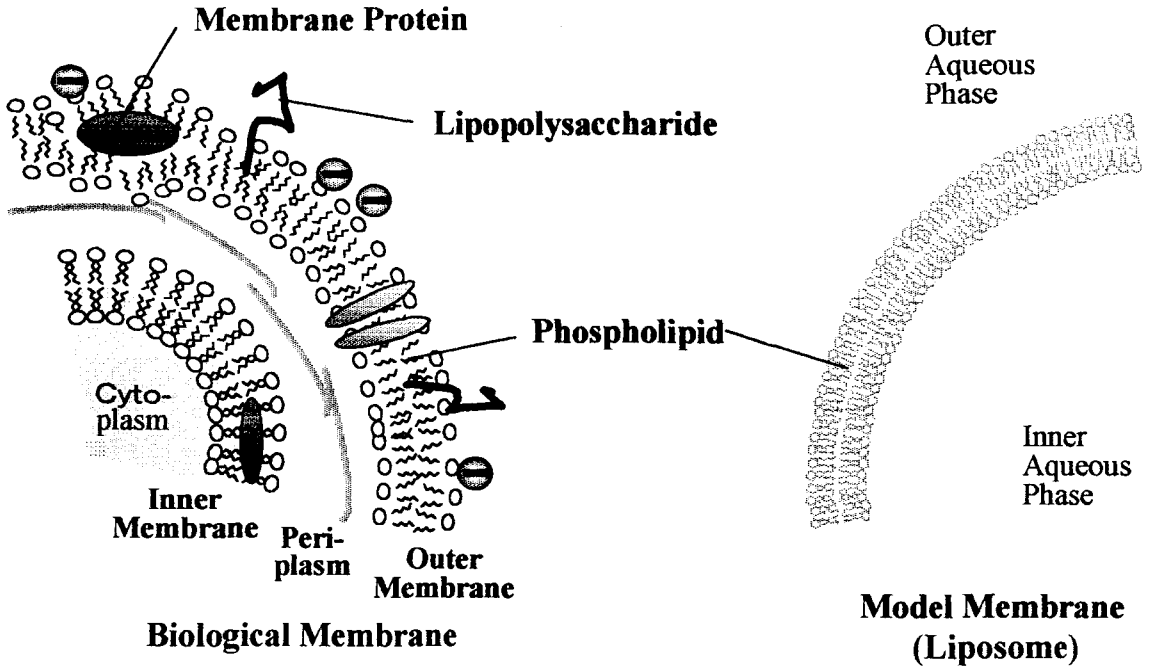


Fig.1-1 Structure of biological membrane (*Escherichia coli*) and model membrane (Liposome)

therefore, needed.

Some researchers previously reported various methods for the characterization of cell surface properties, in particular, on the surface charge (Stan-Lotter *et al.*, 1989; Flegr, 1990) and on the surface hydrophobicity (van Loosdrecht *et al.*, 1989; Magnusson, 1982; Smyth *et al.*, 1978; Rosenberg, 1980; Rosenberg, 1981; Jonsson and Wadstrom, 1984; Noda and Kanemasa, 1986). In the methodologies applied so far, the aqueous two-phase partitioning method has several advantages for obtaining information on the surface properties of cells. i) The surface properties can be analysed through a mild operation because these systems are mainly composed of water (Albertsson, 1986; Walter *et al.*, 1984). ii) The systematic analysis of the surface properties, such as isoelectric points, surface net hydrophobicity, and other specific properties, can be achieved by varying the mode of partitioning. Albertsson (1986) and, recently, Kuboi *et al.*, (1994) investigated the

partitioning behaviors of large biomolecular assemblies such as microorganisms, bacteria, and virus as well as small molecules and biopolymers.

In this chapter, the surface properties of bacterial cells, such as isoelectric point, surface net and local hydrophobicity, have been quantitatively determined by investigating their partitioning behavior in aqueous two-phase systems. The possibility to separate the intracellular proteins and bacterial cells was then discussed on the basis of the evaluated surface properties. The change in the surface properties of the cells exposed to heat treatment was also analysed using the partitioning method. In addition, the possibility to apply the above methodology to the characterization of the surface properties of some kinds of phospholipid membranes (liposomes) as a model membrane was also investigated.

2. THEORETICAL

The analytical methods for the determination of the surface properties of biomolecules using the aqueous two-phase systems (ATPS) containing a polymer, to which charged ligands (such as tetramethylacetate or sulfonate), hydrophobic ligands, (*e.g.* palmitate), or biospecific ligands (for example dye molecules), are attached, have been reported before (Miorner *et al.*, 1982; Miorner *et al.*,1983). If such ligands are not attached to the polymer, the partition coefficient of biomolecules in an ATPS has been found empirically to depend on several factors which act independently. The partitioning coefficient of amino acids, peptides, proteins or cells, may therefore be written as described by Baskir *et al.* (1989)

$$\ln K = \ln K_{\text{electorstatic.}} + \ln K_{\text{hydrophobic}} + \ln K_{\text{salt}} + \ln K_{\text{ligand}} \qquad (1\text{-}1)$$

$K_{\text{electorostatic.}}$, $K_{\text{hydrophobic.}}$, K_{salt}, and K_{ligand} are the contribution to the partition-

ing of the biomolecules from electrostatic, hydrophobic, salt, and ligand effects, respectively. Considering these effects, the surface of proteins can for example be characterized, systematically (Tanaka, 1993; Kuboi *et al.*, 1994). The concept is shown in **Fig.1-2**. Now, it is thought that the ATPS method can also be applied for characterizing the surface properties of bacterial cells, which are highly organized biomolecular assemblies.

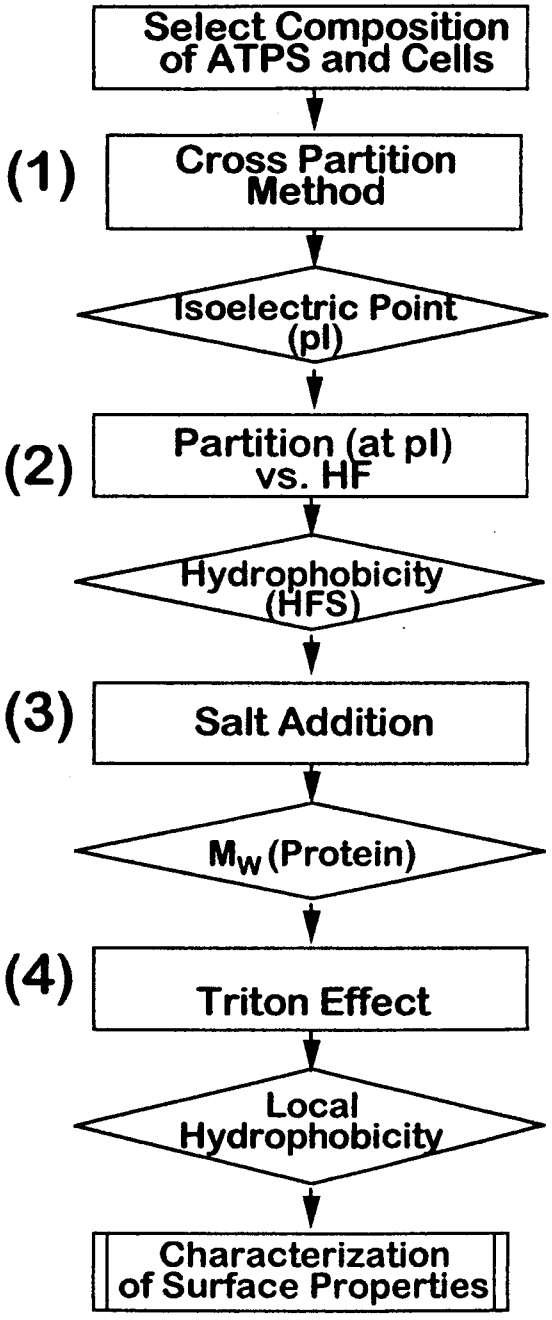


Fig.1-2 Scheme of the characterization of the surface properties of biomolecules

Albertsson (1986) has found that the pH dependence of cell partitioning in some sets of salt containing ATPS showed a common cross point at pH=pI (cross partition method). At pH=pI and low ionic strength, the values of ln $K_{electorostatic.}$ and ln $K_{salt.}$ can be neglected, and thus,

$$\ln K = \ln K_{hydrophob.} \quad (1\text{-}2)$$

Nozaki and Tanford (1971) evaluated the hydrophobicities of several amino acids in water/ethanol and water/dioxane systems. Kuboi *et al.* (1990) have elaborated a relationship between the Nozaki-Tanford values and the partition coefficient of amino acids. They further determined

the hydrophobic differences between the two phases in ATPS (Hydrophobicity Factor, *HF*) and the surface net hydrophobicity of proteins (*HFS*) were determined from the slope of **Eq.(1-3)** using the ATPS.

$$\ln K = HFS \times HF \qquad (1\text{-}3)$$

In analogy, the surface hydrophobicity of bacterial cells can be also determined with the same methodology, following the scheme described above for proteins.

3. EXPERIMENTAL

3.1 Materials. Poly (ethylene glycol) (PEG1540, 4000 and 6000; M_W = 1.5 kD, 3 kD and 7kD, respectively) and dextran 100~200k (Dex; M_w = 100~200 kD) were obtained from Wako Pure Chemicals Ltd. (Osaka, Japan). Nonionic detergents, Triton X-100, X-405, and X-705 (M_W = 0.65 kD, 2.0 kD, and 3.3 kD), were purchased from Sigma (New York, USA). Casamino acid was purchased from Difco Laboratory (Michigan, USA). Baker's yeast (*Saccharomyces cerevisiae*) was purchased from Oriental Yeast Co. Ltd. (Osaka, Japan). Cholesterol (CH) and octyl-β-glucoside (OG), used as modifiers of liposomes, were purchased from Wako. The salts and other chemicals of analytical grade were from Wako.

3.2 Cultivation of Bacterial Cells. Two types of cells were used in this study, namely prokaryotic *Escherichia coli* W3110 and ML308 and eukaryotic *S.cerevisiae* (baker's yeast). The *E.coli* cells were grown in the modified M9 media supplemented with 5g/l glucose or 50g/l glycerol as a carbon source. After the cells were grown in a 300 ml shaking flask containing 100ml media (shaking rate, 130 rpm; growth temperature, 27, 37, and 47^{O}C), they were harvested in the stationary or in the early exponential

growth phase by centrifugation (11000×*g*, 10min, 4°C). They were washed once and were resuspended in 20mM Tris-HCl solution buffered at pH of 7.4. *S. cerevisiae* cells were also suspended in the same buffer.

In the case of bacterial cells which had been stressed by heat treatment, *E.coli* ML308 cells were first grown at 30°C in the M9 media; the temperature of media was then increased to 42°C and the cells were harvested after 0.5~1hr and were resuspended in the Tris-buffer.

3.3 Preparation of Liposome Suspension. The surface properties of model membrane were also characterized. The crude phosphatidylcholine (PC, 93%) was extracted from egg yolk and purified by Singleton's method (Singleton, 1965). Large multilamellar vesicles (LMLV) were prepared by vortex method (Bangham *et al.*, 1965). The LMLV suspension was treated by freeze-thaw in order to stabilize the membrane structure and fuse the small particles each other. The single unilamellar vesicle (SUV) was prepared by the sonication (80W, 10min, 5ml) of the LMLV suspension. The surface of the SUV was modified with hydrophobic cholesterol (CH, from Wako) and hydrophilic octyl-β-glucoside (OG). After the ultrasonic irradiation, SUV (60nm) was separated from the smaller MLV (100nm), by gel filtration (Sepharose 4B, ϕ=15mm×500mm). The SUV, which is containing calcein (CAL) as an entrapping marker, was also prepared in order to analyze the permeability of lipid membrane (Weinstein *et al.*, 1981).

3.4 Partitioning Procedure of Bacterial Cells in Aqueous Two-Phase Systems. The basic compositions of the systems (the total weight is 5g) for the partitioning of cells were 7~13wt% PEG1540, 4000, 6000 and 7~13wt% Dex 60-90k, 100-200k. The ATPSs were prepared by mixing stock solutions of 30wt% PEG and 30wt% Dex with suspensions described above. The pH

of these systems was adjusted by the addition of HCl and NaOH solution of high concentration. After mixing in a 10 ml centrifugal tube by 30-times gentle inversions, a 0.5 mL portion of the total phase systems was sampled and the cell concentration, $C_{Cell,Bulk}$, was measured. The systems were then centrifuged at adequate conditions (10×*g*, 5 min, 25 ^{o}C) so as to enhance phase separation but not to induce sedimentation of the cell particles. This condition has been determined from preliminary experiments of the sedimentation curve of bacterial cells in the single-phase buffer (**Fig.1-3**). The cells were also partitioned in the ATPS containing salts (NaCl, NaSCN, and Na_2SO_4; 0∼400mM) or nonionic detergents (Triton X-100, 405, and 705; 0∼2mM). In all cases, the ratio of $C_{Cell,Bulk}$ to the cell concentration of the suspension was checked from mass balance in order to prevent cell lysis. The cell concentrations of top- and bottom-phase (C_{Top} and C_{Bottom}) were then determined.

The partition coefficient of bacterial cells, K_{Cell}, and liposomes, $K_{liposome}$, was defined as the ratio of concentration of cells and liposomes in the top phase to that of bottom phase, C_{Top}/C_{Bottom}. When the salts or detergents were added to the two-phase systems, the increment change of partition coefficient of cells ($\Delta \ln K_{Cell,Salt}$ ($=\ln (K_{Cell,Salt}/K_{Cell,0})$) or ($\Delta \ln K_{Cell,Triton}$ ($=\ln (K_{Cell,Triton}/K_{Cell,0})$) was used as a measure of the salt or detergent effect detergent effect. $K_{Cell,Salt}$, $K_{Cell,Triton}$, and $K_{Cell,0}$ were, respectively, the partition coefficient of cells with addition of salts, with addition of Triton, and without any addition.

3.5 Measurement. The total concentration of bacterial cells was determined by optical density measurements of diluted samples at 660nm ($0.01 < OD_{660} < 0.2$). The concentration of LMLV and SUV was also determined

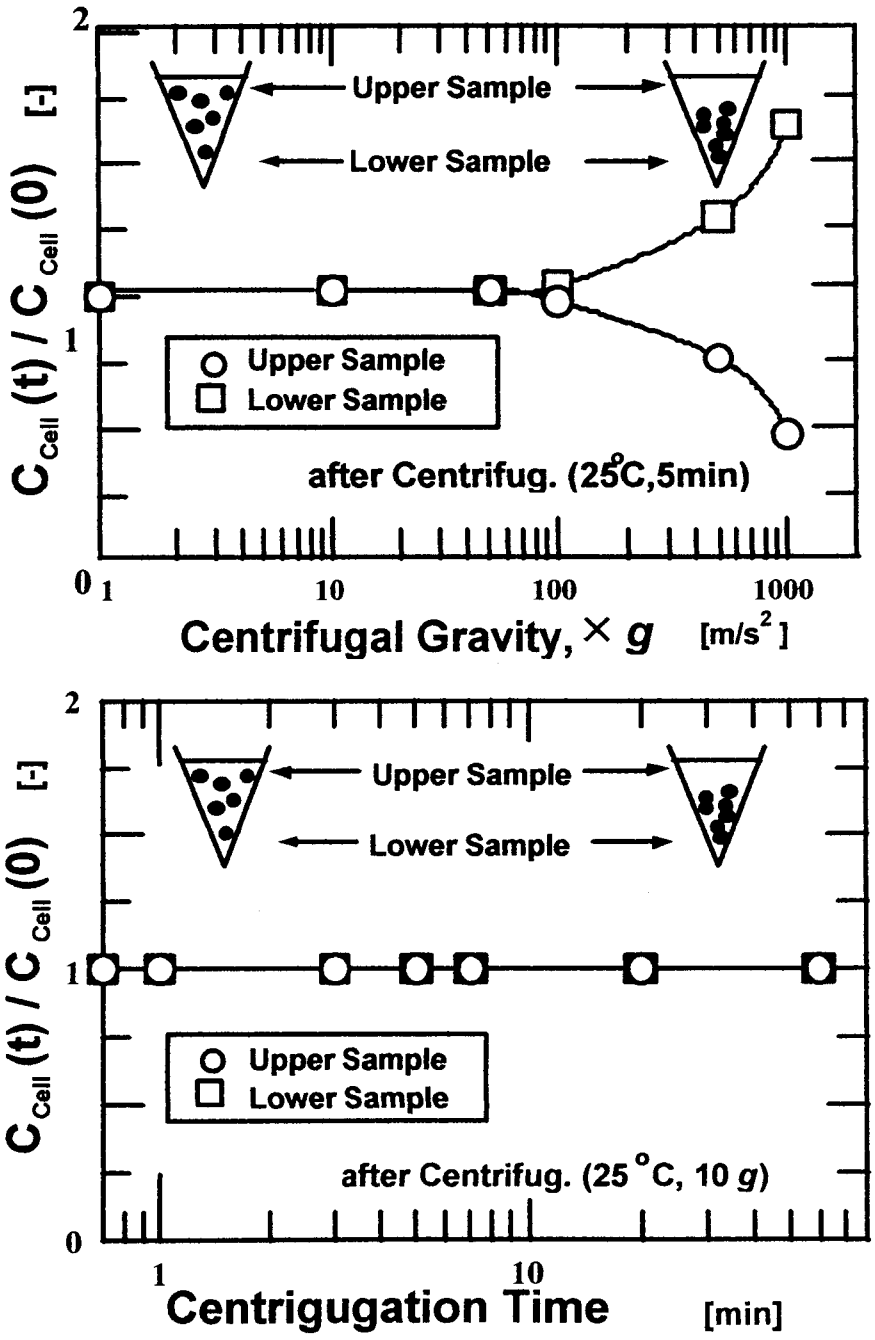


Fig.1-3 Sedimentation behaviors of cell particles in single phase buffer ($C_{Cell}(t)/C_{Cell}(0)$: relative concentration of cells after centrifugation). Effect of (a) centrifugal gravity and (b) time.

from the optical density (OD_{400}) and/or lipid concentration of the liposome containing solution (Weinstein *et al.*, 1981). The *HF* values of the ATPS were determined from the partition coefficient of amino acids, of which the hydrophobicities have been determined, according to Kuboi *et al.* (1990).

4. RESULTS AND DISCUSSION

4.1 Partitioning Behaviors of Bacterial Cells and Characterization of Their Surface Properties in Aqueous Two-Phase Systems. Based on the **Eq. (1-1)** of the theoretical section, four kinds of effects (electrostatic, hydrophobic, salt, and Triton effect) on the partitioning behaviors of bacterial cells in aqueous two-phase systems (ATPS) were investigated by using the same methodology already successfully applied for the characterization of the surface properties of protein, following the scheme for the characterization of the surface properties (**Fig.1-2**). The methods for the control of their partitioning behaviors are, then, discussed on the basis of evaluated values.

4.1.1 Electrostatic Effects on the Partitioning of Bacterial Cells in Aqueous Two-Phase Systems. The effect of pH on the partitioning behaviors of bacterial cells was examined in the ATPS containing various salts. **Figure 1-4** shows the dependence of the partition coefficients of *E.coli* W3110 cells, K_{Cell}, on pH values in three sets of PEG4000 (9%) / Dex (9%) ATPS containing NaCl, NaSCN, or Na_2SO_4 at the same ionic strength of the anion. The addition of NaCl and NaSCN, leading to a negative electrochemical potential (Albertsson, 1986), increases the partition coefficients of *E.coli* cells in the lower pH values, while the addition of Na_2SO_4 is not changed significantly. If cell particles are partitioned at different pH values in a set of systems containing different salts of the same ionic strength, their

curves cross at its isoelectric point, pI (Albertsson, 1986; Mioner *et al.*, 1982; Walter *et al.*, 1985). The pI value of *E.coli* W3110 cells could therefore be determined as being 2.8±0.3.

The isoelectric points (pI) of different kinds of cells were determined by using the above method and the values are summarized in **Fig.1-5**, together with the pI values determined for a variety of proteins, including enzymes. In general, the surface of bacterial cells has an isoelectric point in the

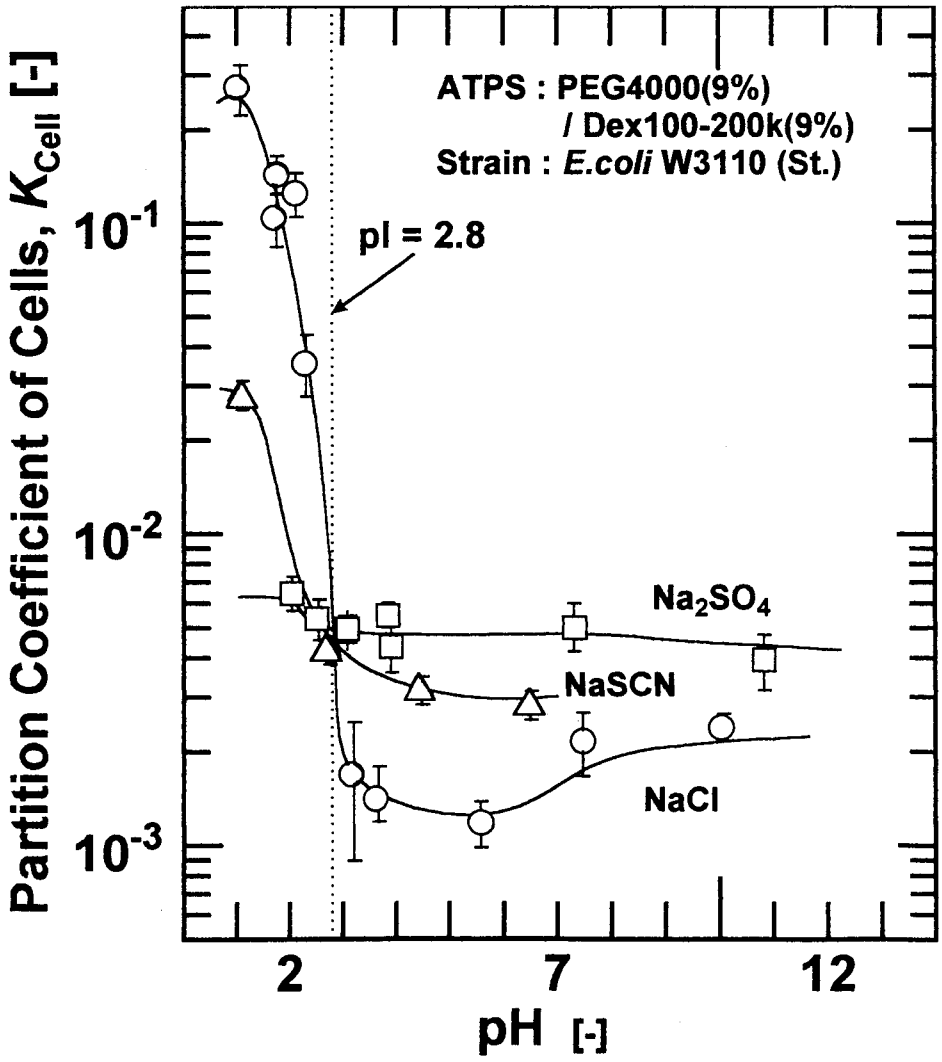


Fig.1-4 Effect of pH on the partition coefficient of *E.coli*W3110 cells, K_{Cell}, in the presence of three kinds of salts (cross partition method (Albertsson, 1986)). Symbols: ○, 400mM NaCl, △, 400mM NaSCN, □, 200mM Na_2SO_4.

lower pH range. These results are in agreement with a previous report (Loder and Liminol, 1985). The pI values of *E.coli* W3110 cells of the stationary growth phase are, then, greater and smaller than that of *E.coli* ML308 cells and *S.cerevisiae* and that of *E.coli* cells of the exponentially growth phase, respectively. The pI values of *E.coli* W3110 cells recovered in the stationary growth phase are not changed although they were grown at the different temperature (27, 37, and 47 °C).

It is thus found that the partitioning behaviors of cells are affected by electrostatic properties, that the isoelectric point of bacterial cells can be determined by the ATPS methodology, and that the pI values are dependent on the type, strain and growth phase, but on the growth temperature.

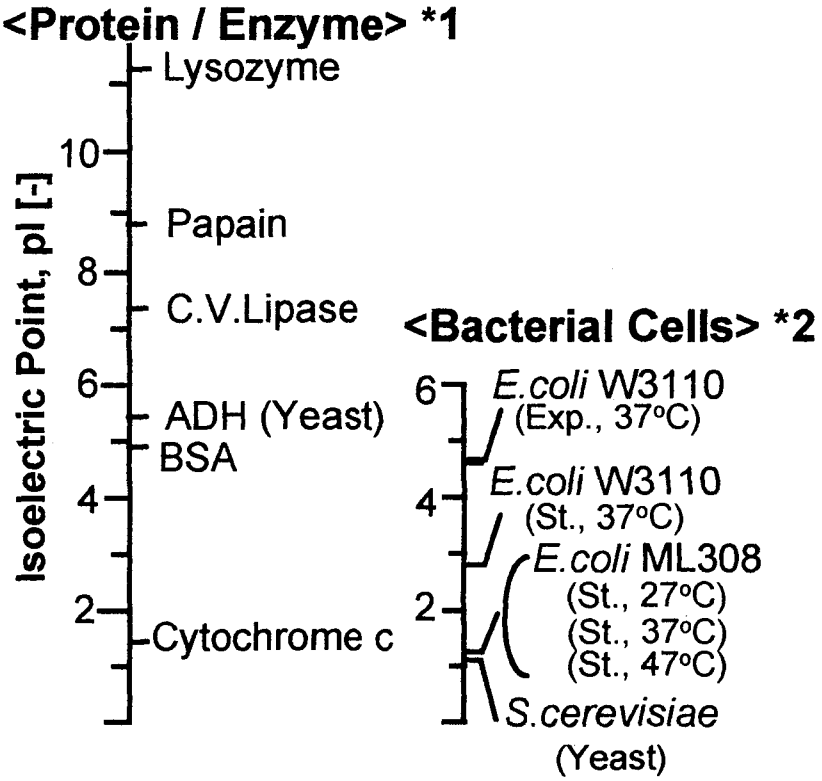


Fig.1-5 pI ladder. *1: pI values of protein and/or enzyme were referred to Kuboi *et al.*(1994-b). *2: St., cells cultivated in stationary growth phase; Exp., in early exponential growth phase. 27, 37, and 47 °C indicate the growth temperature.

4.1.2 Hydrophobic Effects on the Partitioning of Bacterial Cells in Aqueous Two-Phase Systems. At the isoelectric point, pI, of bacterial cells, their partitioning in the ATPS is governed by a hydrophobic combination as mentioned in the theoretical section (**Eq. (1-3)**). Kuboi *et al.* (1990) have recently evaluated the hydrophobic difference between the two-phases as the hydrophobic factor (*HF*) on the basis of the partitioning behaviors of several amino acids, of which hydrophobicities have been already defined (Nozaki and Tanford, 1971). The partitioning behaviors of two kinds of *E.coli* cells (W3110 and ML308) are now investigated in some sets of PEG/Dex ATPS,

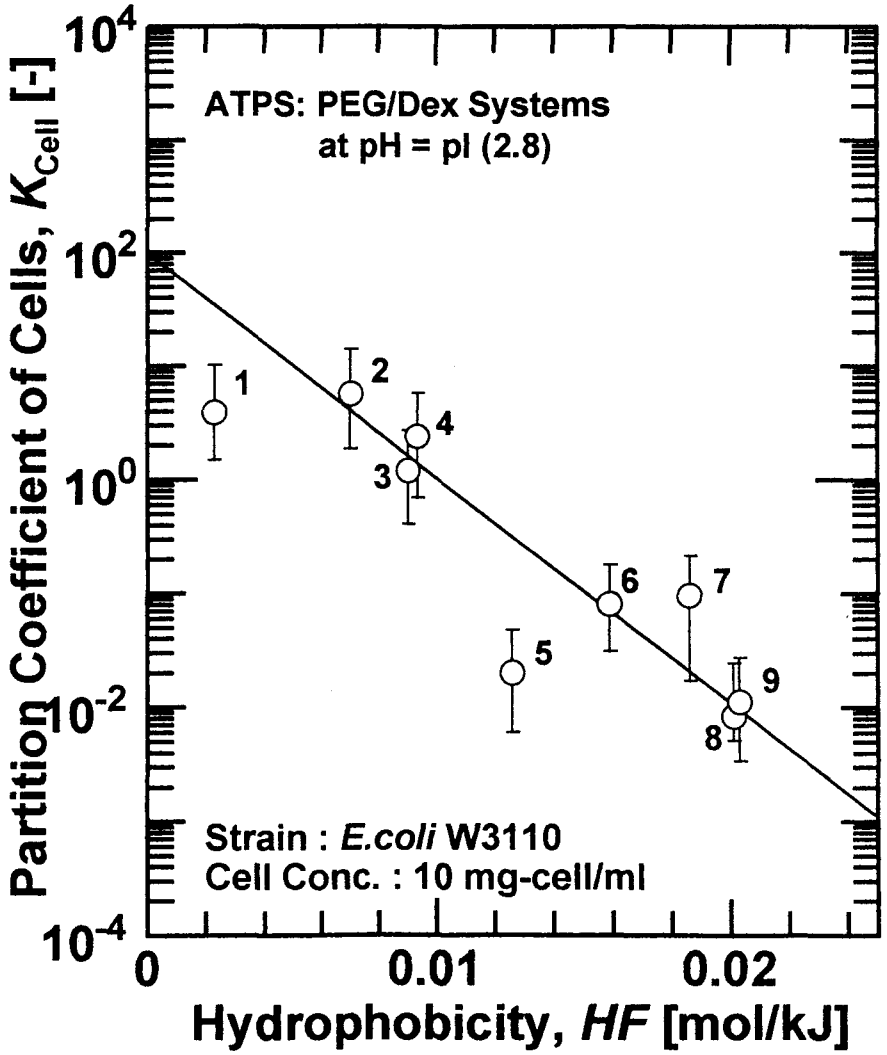


Fig.1-6 Dependence of partitioning coefficient of *E.coli* W3110, K_{Cell}, on the hydrophobic factor, *HF*, at the pI

which have different *HF* values. In **Fig.1-6** the values of partition coefficient, K_{Cell}, of *E.coli* W3110 cells are plotted against the *HF* values at the corresponding pI of the cells. The data of their partition coefficient are clustered on a straight line. The slope of the line (K_{Cell} vs. *HF*) of *E.coli* W3110 then indicates a *HFS* of -537kJ/mol. The *HFS* values can be, herewith, defined as the surface net hydrophobicity (Kuboi *et al.*, 1990).

The surface net hydrophobicity (*HFS*) of different kinds of cells were then determined by using the above method and are summarized in **Fig.1-7**, together with that of amino acids, and proteins (enzymes). In general, the surface of bacterial cells has a relatively hydrophilic nature, compared with that of amino acids, or proteins, and the results are thought to be caused by

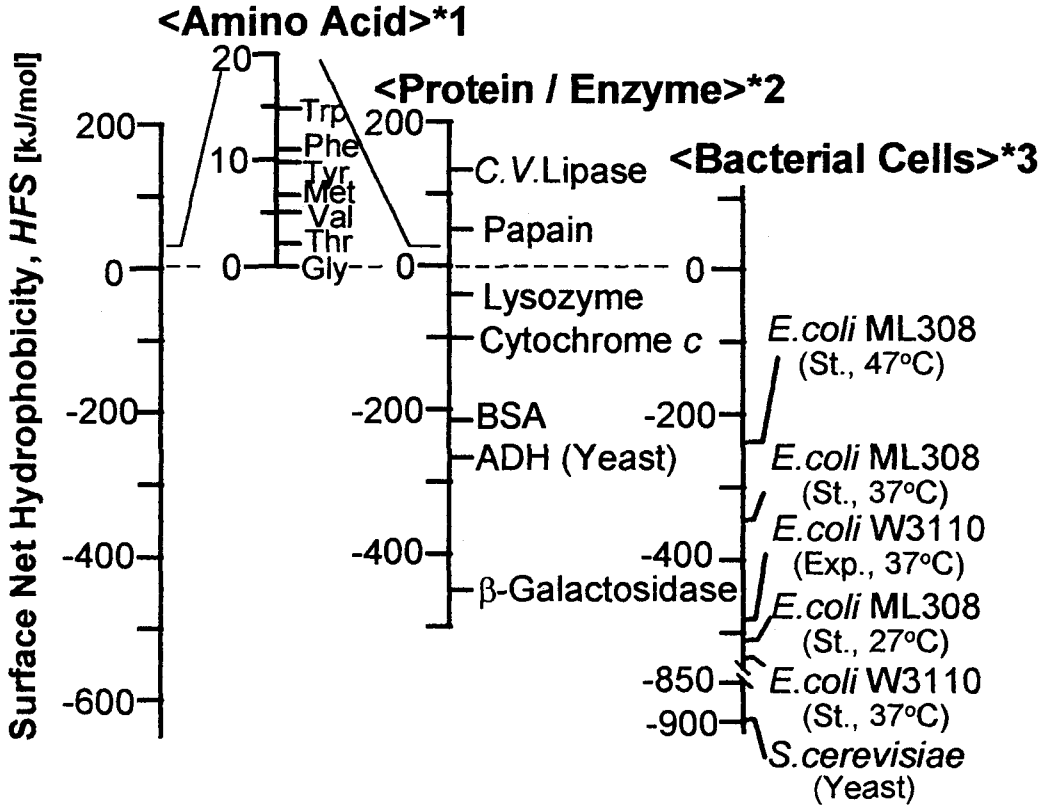


Fig.1-7 *HFS* ladder. *1: *HFS* values of amino acids were referred to Kuboi *et al.*(1990). *2: *HFS* values of protein and/or enzyme were referred to Kuboi *et al.*(1994-b). *3: St., cells cultivated in stationary growth phase; Exp., in early exponential growth phase. 27, 37, and 47 °C indicate the growth temperature.

the hydrophilic head group of phospholipid, which is the main component of membrane surface of cells (Kito *et al.*, 1973). The *E.coli* W3110 cells the hydrophilic head group of phospholipid, which is the main component of have a rather hydrophilic nature than *E.coli* ML308 cells. The *HFS* values of *E.coli* cells of the early exponential growth phase are greater than that of the stationary growth phase. *S.cerevisiae* have the most hydrophilic nature among the cells tested in this study. The increase of the growth temperature cause the increase in the surface net hydrophobicity of *E.coli* cells.

It is found that the surface net hydrophobicity of cell surface, *HFS*, can be determined by the dependencies of cell partitioning on hydrophobic differences of ATPS, *HF*, and the values is dependent on the type, strain, growth phase, and growth temperature of the cells.

4.1.3 Effect of Additional Salt on Partitioning Behaviors of Cells in Aqueous Two-Phase Systems. The effect of additional salt on the partitioning of cells was examined in the PEG4000 (9%) / Dex (9%) systems. **Figure 1-8** shows the dependence of the increment change of the partitioning coefficient of the *E.coli* W3110 cells, $\Delta \ln K_{Cell,Salt}$, on the concentration of additional salts. In most cases, the partitioning of cells is affected by the addition of salt in the lower concentration region (0~5 mM) comparable with the case of proteins (enzymes) (Kuboi *et al.*, 1994) and the values of the $\Delta \ln K_{Cell,Salt}$ reach saturation in the higher concentration range (>200 mM). The partitioning behaviors of bacterial cells, which have a large surface and which contain various kinds of biomolecules on the surface, are greatly affected by the addition of salts because of the salting-out effects in the ATPS. While the addition of sodium phosphate (NaPi) increases the partition coefficients, the

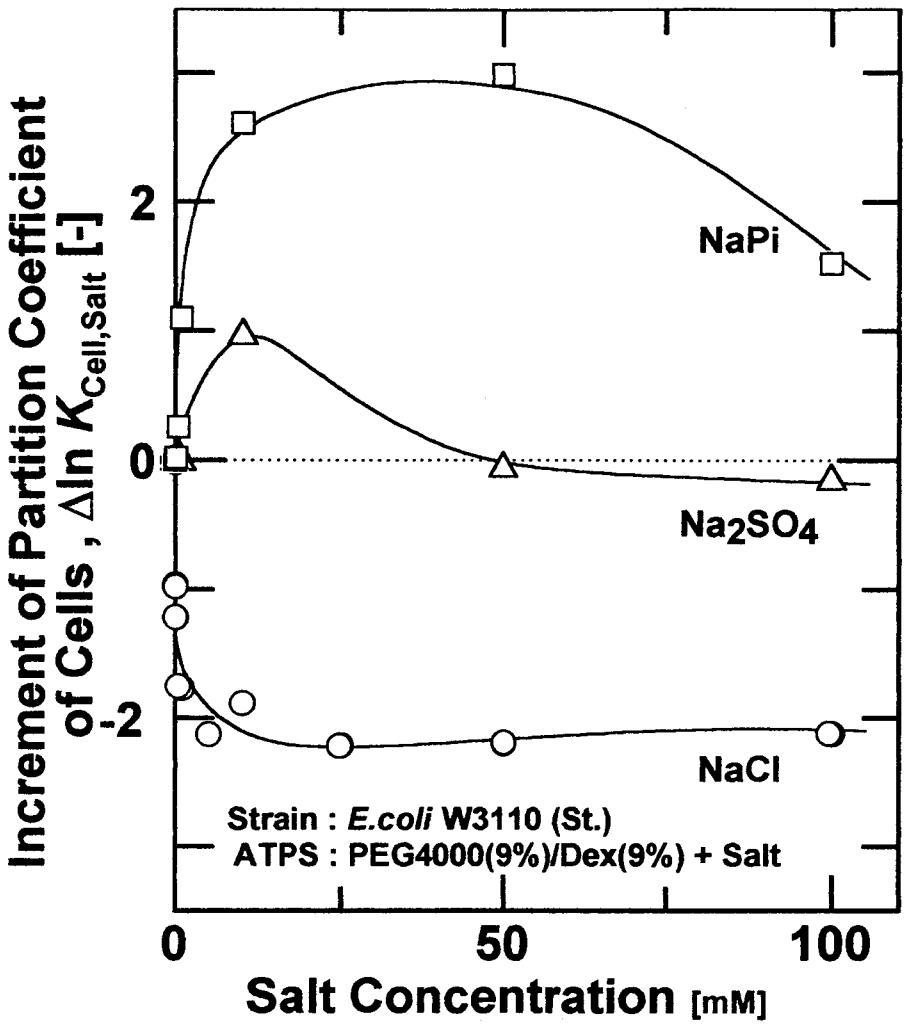


Fig.1-8 Effect of the concentration of three kinds of salts on the partitioning behaviors of *E.coli* W3110 cells in PEG4000 (9%) / Dex 100~200k (9%) two-phase systems

addition of NaCl leads to a decrease of the partition coefficients. In the case of Na_2SO_4, $\Delta \ln K_{Cell,Salt}$ value remains almost unchanged.

The limiting values of $\Delta \ln K_{Cell,Salt}$, of *E.coli* W3110 and ML308 cells at high salt concentration using different salts are summarized in **Table 1-1.** Comparing the effect of the different anion added, phosphate is found to be the most effective for increasing the values of $\Delta \ln K_{Cell,Salt}$. The order of effectiveness is then $Pi > SO_4^{2-} > SCN^- > Cl^-$. There is a slight difference of the effectiveness in cations, too ($Na^+ > K^+$). The efficiency order is corre-

Table 1-1 Effect of addition of several kinds of salts on the partition coefficient of *E.coli*W3110 and ML308 cells

Additional Salt [*1]	$\Delta \ln K_{Cell,Salt}$ [-]
E.coli W3110 (St.)	
Control	$K_{Cell,0} = 1.4 \times 10^{-2}$
NaPi [*2]	2.5
KPi [*2]	2.0
Na_2SO_4	-0.15
K_2SO4	-0.41
NaSCN [*3]	-1.0
NaCl	-2.0
KCl	-2.5
E.coli ML308 (St.)	
Control	$K_{Cell,0} = 2.2 \times 10$
NaPi [*2]	1.2
NaCl [*2]	-0.8

*1 Salts were added to ATPS basically at the concentration of 50mM.
*2 NaPi and KPi indicate sodium phosphate and potassium phosphate, respectively.
*3 The concentration of additional salt is 400mM.

sponding to the Hoffmeyster series which is utilized to explain the effect of salt on the partitioning of macromolecules such as proteins (enzymes).

The partitioning of cells is found to depend on the type of added salts and the types of bacterial cells used in the ATPS.

4.1.4 Effect of Addition of the Nonionic Detergent, Triton X-100, in Aqueous Two-Phase Systems. The effect of the addition of nonionic detergents, Triton X-series, on the partitioning behavior of bacterial cells was investigated at the pI of the cells in the PEG4000 (9%) / Dex (9%) two-phase system. **Figure 1-9** shows the relationship between the Triton X-100 concentration and the increment change of partitioning coefficient, $\Delta \ln K_{Cell,Triton}$, for two kinds of cells. The $\Delta \ln K_{Cell,Triton}$ values of *E.coli* W3110 cells (circles) are dramatically increased to 5.1~5.9 in the presence

of 2mM Triton X-100. On the other hand, the $\Delta \ln K_{Cell,Triton}$ values of *E.coli* ML308 cells are not affected by the addition of Triton X-100 (triangles).

The effect of the average molecular weight, M_W, of Triton X-series on the $\Delta \ln K_{Cell,Triton}$ value of *E.coli* W3110 was also examined as shown in **Fig.1-10**. When Triton X-100 is added to the ATPS, of $\Delta \ln K_{Cell,Triton}$ is maximal, and an increase of M_W of Triton decreases $\Delta \ln K_{Cell,Triton}$. The *HLB* value, which is used as a measure of the hydrophilic lipophilic balance of nonionic detergent (Davies, 1954), is therefore also plotted in **Fig.1-10**. A decrease in $\Delta \ln K_{Cell,Triton}$ is paralleled by an increase in *HLB*.

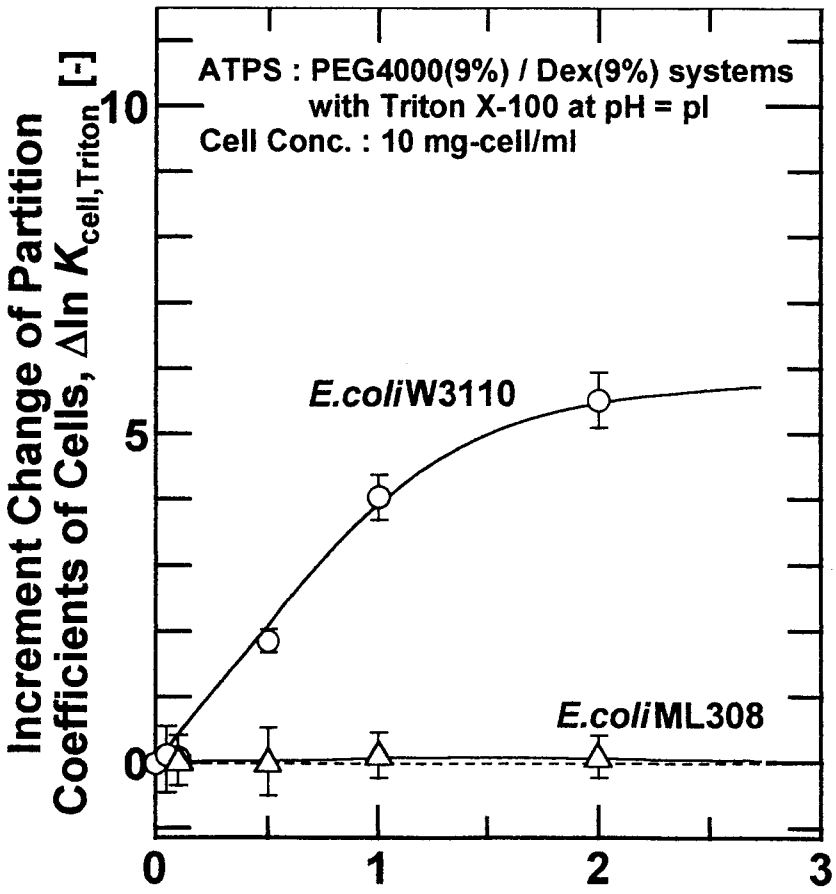


Fig.1-9 Effect of the concentration of Triton X-100 on the partitioning behaviors of *E.coli* ML308 cells in PEG4000 (9%) / Dex 100~200k (9%) two-phase systems

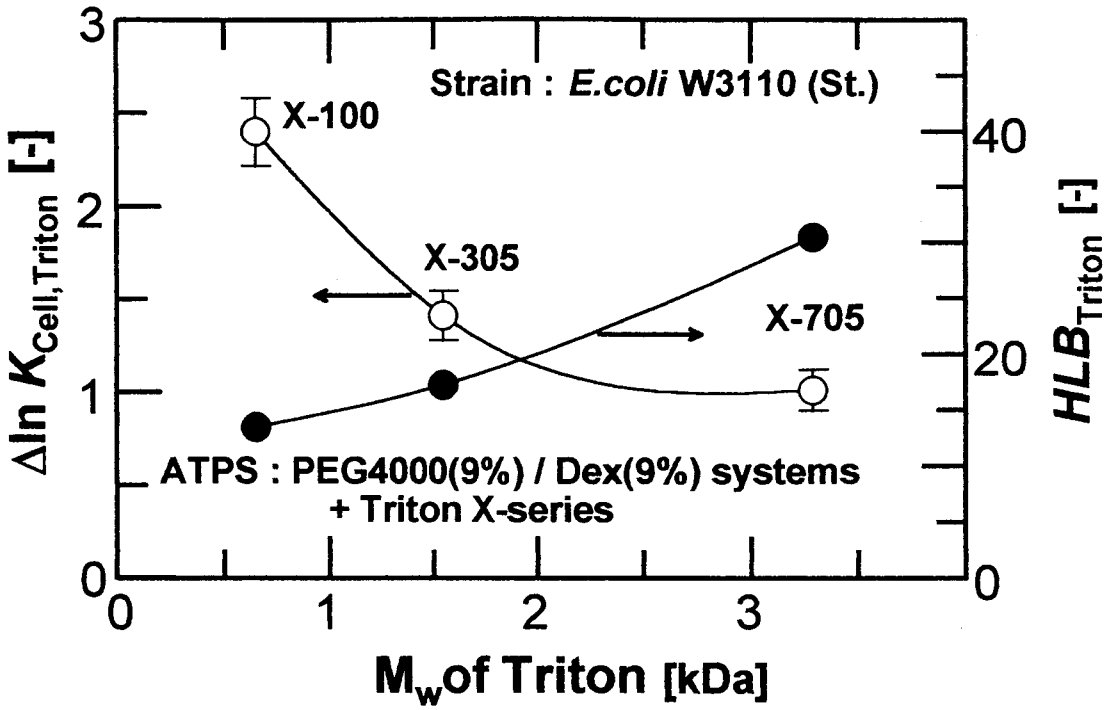


Fig.1-10 Effect of molecular weight of Triton on $\Delta \ln K_{Cell,\ Triton}$ values

The $\Delta \ln K_{Cell,Triton}$ values (2mM Triton X-100) of various cells were analysed and are summarized in **Fig.1-11** together with the values for a few biomolecules. The $\Delta \ln K_{Cell,Triton}$ value of *E.coli* W3110 cells is higher than that of several proteins and that of *E.coli* ML308 cells.

Triton molecule contains a hydrophobic *p-t*-octylphenyl group and a hydrophilic poly (ethylene oxide) chain and preferentially partitions to the top phase of the ATPS. The hydrophobic group interacts with membrane proteins from the fact that Triton have been typically used for the solubliza-tion of membrane proteins (Felix, 1971), so that it can be used as a hydro-phobic probe in order to characterize the hydrophobicity of cell surface (Magnusson *et al.*, 1977). However, there seems to be no relationship be-tween the values of the increment of partition coefficient of *E.coli* cells, $\Delta \ln K_{Cell,Triton}$, (**Fig.1-10**) and the corresponding *HFS* values (surface net hydro

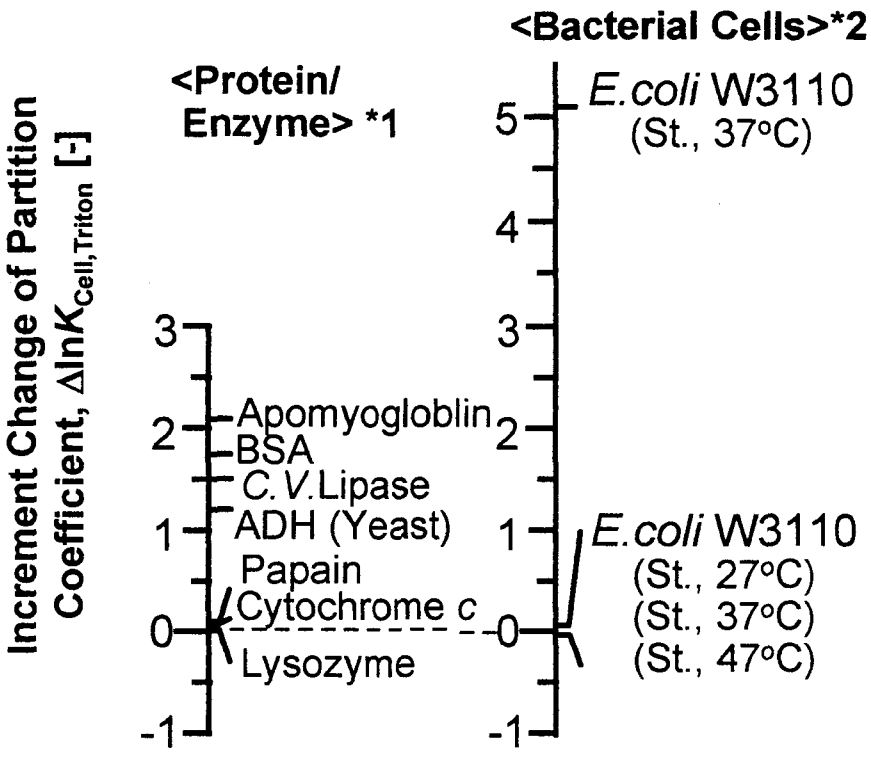


Fig.1-11 Ladder of $\Delta \ln K_{Cell,\,Triton}$ values of cells. *1: pI values of protein and/or enzyme were referred to Kuboi *et al.*(1994-b). *2: 27, 37, and 47 °C indicate the growth temperature.

phobicity, **Fig.1-7**). The $\Delta \ln K_{Cell,Triton}$ value is therefore thought to indicate the local hydrophobicity of the cell surface, which contains some effects such as i) the ability of the hydrophobic interaction of Triton molecules, ii) the types and their composition of membrane proteins, and iii) those of phospholipids on the cell surface. By using the Triton with constant *HLB* in the experiments, the effect of i) can be negligible as shown in **Fig.1-10**. The difference in $\Delta \ln K_{Cell,Triton}$ obtained for various *E.coli* strain is, thus, thought to be caused by the differences in, mainly, the types and composition of membrane proteins and reflect local hydrophobic sites of the cell surface.

4.2 Application of Aqueous Two-Phase Partitioning Method. The possibility of application of the above method to the characterization of (1) stressed cells, which were exposed to heat treatment, and (2) lipid membrane (liposome) as a cell model was investigated

4.2.1 Change in Surface Properties of Bacterial Cells as a Response to Heat Stress. As a case study, the method described above for the characterization of surface properties was applied for the analysis of a possible change in surface properties of bacterial cells stressed to the heat stress. The surface properties (pI, *HFS*, and $\Delta \ln K_{Cell,Triton}$) of *E.coli* ML308 cells, which were exposed to heat treatment (from 30°C to 42°C, 0~1hr), were evaluated by using the methods described above. **Figure 1-12** shows the time course of the surface properties of the cells after exposure to heat stress. While the pI values of the cells are not changed, the values of surface net hydrophobicity,

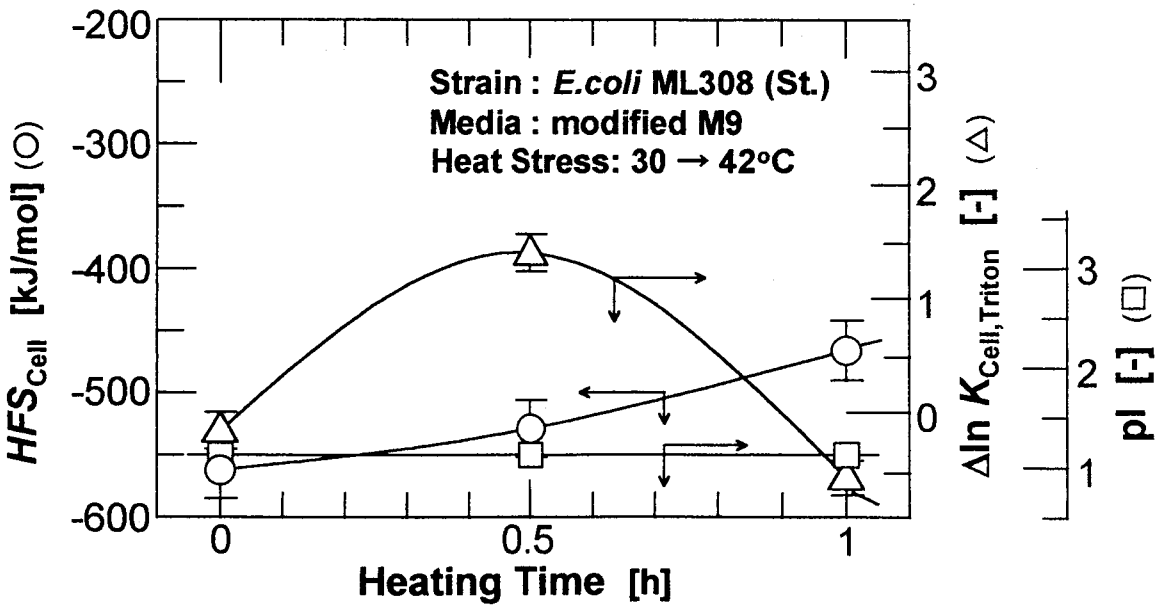


Fig.1-12 Change in the surface properties of cells (pI, HFS_{Cell} and $\Delta \ln K_{Cell,Triton}$) of *E.coli* ML308 cells when cells were stressed by heat treatment at temperature of 42°C

HFS, are gradually increased, and the local hydrophobicity, $\Delta \ln K_{Cell,Triton}$, is initially increased to 2.0 after 0.5 hr and then decreased to the initial value. These results are thought to indicate the following two phenomena. (1) Heat stressing *E.coli* cells induce a change in their surface hydrophobicity but not in their surface charge. (2) The cells response in the membrane surface seems to involve two processes, as a fast response (a change in the composition of membrane proteins) and a slower one (change in surface net hydrophobicity). Tsuchido *et al.* (1986) have reported that the hydrophobic change of the surface plays an important role in the stress-response process of *E.coli* cells and that the surface hydrophobicity of the cells stressed by heat treatment is then increased. The above results are in agreement with these findings.

It is thus found that the characterization methods described in this chapter can be applied for stressed cells and that they can be used for the analysis of the dynamic change of the cell surface properties.

4.2.2 Characterization of Surface Properties of Lipid Membrane (Liposome). The above methodology was applied to the characterization of the liposome surface used as a cell model. The membrane surface of liposomes has no charge in the middle pH range. Therefore, the partitioning behaviors of liposome particles are mainly dependent on the hydrophobic effect at this pH range (**Eq.(1-2)**). The surface hydrophobicity of liposomes were evaluated by using **Eq.(1-3)**. The effect of (1) the composition, (2) the preparation method of liposome, (3) the temperature on the surface hydrophobicity were, then, investigated.

(1) Effect of Membrane Composition on Surface Hydrophobicity of Liposome. The cholesterol (CH) and octyl-β-glucoside (OG) have typically

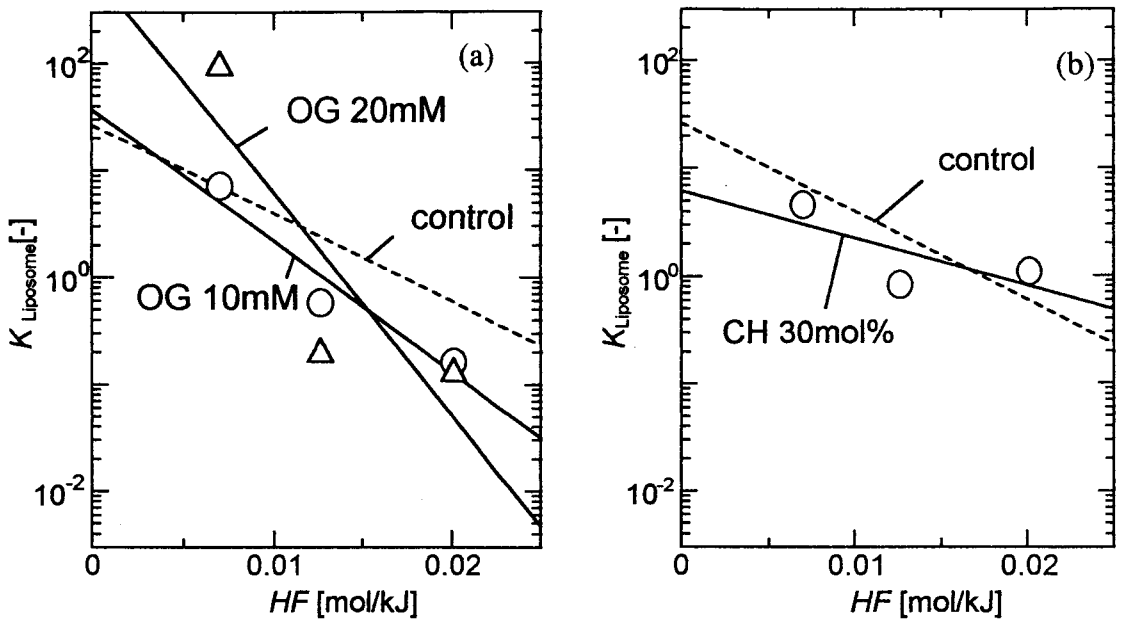


Fig.1-13 Effect of Surface Modification of SUV with (a) octyl-β-glucoside (OG) and (b) cholesterol (CH)

used as modifiers of liposome surface because CH and OG have hydrophobic and hydrophilic group in their molecules, respectively. The *HFS* values of liposomes modified by CH and OG were determined by the ATPS method. **Figure 1-13** shows the relationship between the *HF* values and the corresponding partition coefficients of liposomes, $K_{liposome}$. The data are clustered on a straight line. The *HFS* values of liposomes, which were modified with OG and CH, are respectively, smaller and greater than that of control normal liposomes. These results are summarized in **Fig.1-14**.

The effect of the addition of OG can be explained in terms of apparent increase of hydrophilic group at vesicle surface, which is caused by the incorporation of OG molecules to the fatty acid chain of the lipid bilayer. On the other hand, the effect of addition of CH on surface hydrophobicity is caused by the increase in hydrophilic CH-chain domain in lipid bilayer and the decrease in the phospholipid at the membrane surface.

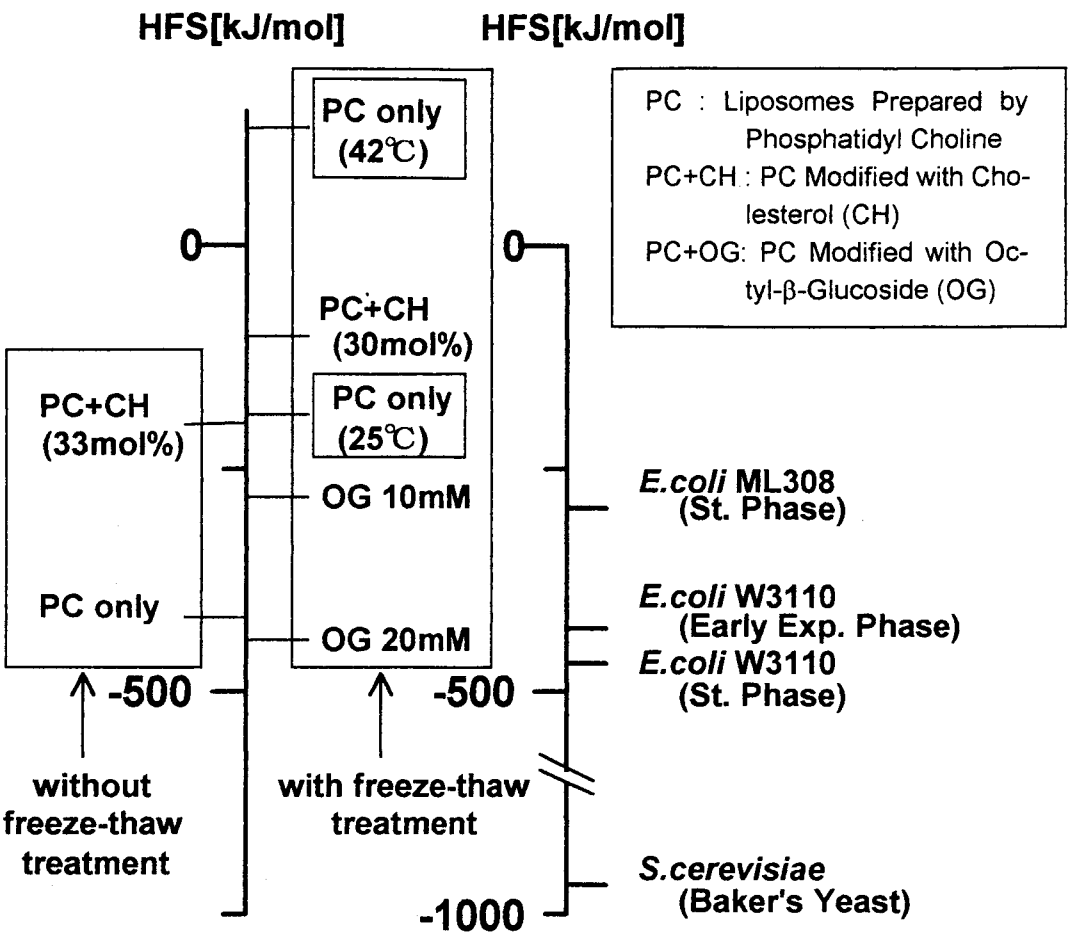


Fig.1-14 *HFS* values of various types of SUV and bacterial cells

(2) Effect of Preparation Methods. The dependence of freeze-thaw treatment on the surface net hydrophobicity of liposome, *HFS*, are also shown in **Fig.1-14**. The hydrophobicity of liposome is increased when the suspension of liposome was treated by the freeze-thaw treatment. This result corresponds to the effect of the treatment on the physical strength, which can be estimated by the kinetic analysis of disruption process (Yotsuyanagi, *et al.*, 1987). It is found that the freeze-thaw process makes the surface of liposome more hydrophobic.

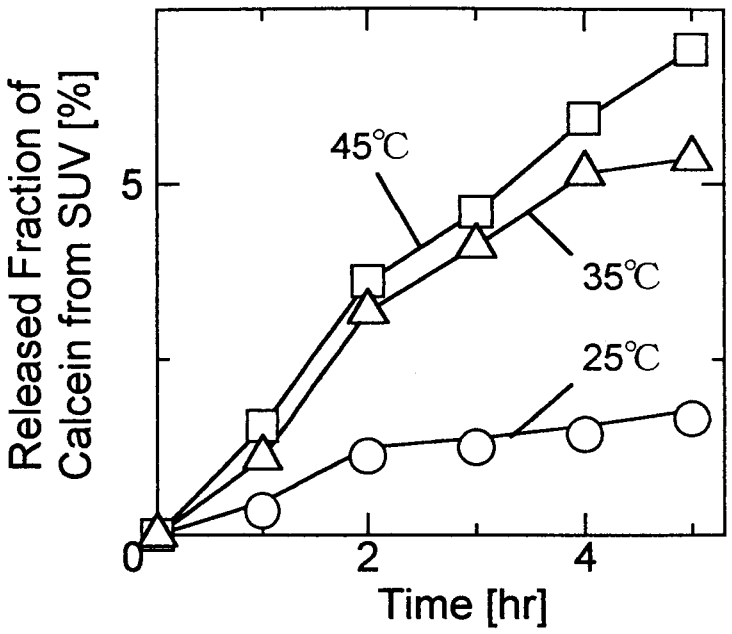


Fig.1-15 Effect of temperature on the change in the permeability of liposome membrane (liposomes (SUV) containing calcein is used)

(3) Effect of Temperature on Surface Hydrophobicity of Liposome. The effect of temperature on the surface hydrophobicity of liposome was investigated. The *HFS* values of liposomes (SUV) at 25°C and 42°C are also shown in **Fig.1-14**. As temperature increase, their hydrophobicity increases. This phenomena may be caused by the increase in the membrane fluidity, which is due to the exposure of hydrophobic phosphatidylcholine to the membrane surface. The dependence of the temperature on the release of calcein from liposomes was examined in order to investigate the change of the membrane fluidity. The results are shown in **Fig.15**. As temperature rises, the release rate of calcein also increases. This result can be considered a kind of stress response of liposomes. This probably indicates that the perturbation of membrane structure, which also reflects in the *HFS* values, was promoting the release of calcein. In analogy, the increase of the surface hydropho-

bicity of bacterial cells (**Fig.1-11**) is suggested to be also caused by the increase of the membrane fluidity.

These functions of cell membranes are, further, thought to be applied for the design and preparation of the artificial biomembranes such as liposomes, which are constructed by phospholipid bilayer. In another aspect, several information obtained from the investigation of liposome can help to study the induction mechanism of the function of bacterial cells. The development of evaluation methods for characterizing surface properties of both bacterial cells and liposome by common parameter is, therefore, needed.

5. SUMMARY

The physicochemical surface properties of bacterial cells and the liposomes with or without the environmental stresses were systematically characterized by using the aqueous two-phase partitioning method.

(1) The surface properties of bacterial cells, such as isoelectric points (pI), surface net hydrophobicity (*HFS*) and local hydrophobicities ($\Delta \ln K_{Cell,Triton}$), could be evaluated by using the same method applied for the characterization of the surface properties of proteins in aqueous two-phase systems. In the practical bioseparation process, which contains the separation of cells and/or their homogenates from the target protein, the operational conditions can be effectively optimized on the basis of the quantitative data both on the protein (enzyme) and on the bacterial cells.

(2) The cells stressed by heat treatment were also characterized quantitatively by using the above method. The surface net and local hydrophobicities of bacterial cells were varied in response to the heat stress although there was no change of the surface charge.

(3) Using the same methodology, the surface hydrophobicity of liposome can be analysed, where the surface hydrophobicity of liposome was found to depend on the composition of liposomes and their preparation methods. The increase of the hydrophobicity on the liposome membrane was found to be caused by the increase in the membrane fluidity. In analogy, it was suggested that the increase in the membrane surface of bacterial cells exposed to the heat stress could be caused by the increase of the fluidity of cell membrane.

Chapter 2

Characterization of Intracellular Properties through Kinetic Analysis of Cell Disruption and Product Release Process against Physical Stress

1. INTRODUCTION

The recovery of intracellular products requires, first of all, the cell disruption with chemical, enzymatic and/or physical (mechanical) methods. The physical methods induce the disruption of cells and release of intracellular proteins and are often favored for usual disruption owing to the economical and operational limitations contained in the other two methods (Schutte and Kula, 1990). The target product recovered with the cell disruption, usually, is contaminated with many other products and cell debris. The recent increase of intracellular bioproducts, which are genetically engineered, stimulates improvement in the design and operation of disruption methods for their effective and selective primary separation (White and Marcus, 1988).

The design and operation of these methods depend very much on the individual cases and hence are difficult to be generalized because of many factors involved in the disruption process. The first-order kinetics has been typically utilized, however, as the model of disruption process using the physical methods such as homogenizer (Hetherington *et al.*, 1971), bead-mills (Currie *et al.*, 1972) and ultrasonic methods (James *et al.*, 1972). The specific energy has been recently employed for the comparison of the energy consumption and the disruption mechanisms for the different disruption methods (Schutte and Kula, 1986) and microorganisms (Matsumoto *et al.*, 1994). The generalization of these disruption methods or the integration of

these concepts is very important.

The cell disruption process is dependent on cell properties such as physical strength of microorganism (Sauer *et al.*, 1989) and intracellular location of enzyme (Follows *et al.*, 1971). A common method for measurement of cell strength has not been reported. Recently the cell strength of mammalian and bacterial cells was measured by Zhang *et al.* (1992, 1994) and estimated by Middelberg *et al.* (1993). Sauer *et al.* (1989) reported that the behaviors of protein release during the disruption were well corresponding to the cell strength. Some qualitative findings for the intracellular location of various enzymes and the release were reported (Marr, 1960). Follows *et al.* (1971) studied the relation of enzyme release and their intracellular location during the physical disruption process. However, the method for the design of effective disruption process for the selective release of a target enzyme has not been systematically investigated.

In this chapter, a systematic and quantitative investigation were carried out on the cell properties in relation to effective and selective release of intracellular products from bacterial cells. Disruption process was kinetically analyzed using a generalized parameter for evaluation of cell properties. The effect of the addition of relatively hydrophobic polymers on the kinetics was also investigated in order to clarify the hydrophobic interaction between the membrane surface and the amphipathic polymer. Based on the evaluated properties and the clarified interaction, the disruption conditions for the recovery of cytoplasmic β-galactosidase from *E.coli* ML308 were optimized as a case study of the effective and selective disruption method.

2. EXPERIMENTAL

2.1 Materials D-Glucose-6-phosphate (G6P) and β-$NADP^+$, used as the substrate and coenzyme for the measurement of D-glucose-6-phosphate dehydrogenase (G6PDH), were purchased from Oriental Yeast Co. Ltd.. o-Nitrophenylgalactopyraniside (ONPG) for the substrate of β-galactosidase (β-gal) was from Sigma. Casamino acid was from DIFCO Laboratory. Baker's yeast (*S.cerevisiae*) was from Oriental Yeast Co. Ltd.. Polyethylene glycols (PEG600, 1540 and 4000) were from Wako Pure Chemicals Ltd. The salts and other chemicals used were all of analytical grade.

2.2 Cultivation and Disruption of Microorganisms *S.cerevisiae*, *B.subtilis* ATCC6633 and *E.coli* OW10/pND5 (harbored plasmid) and mainly W3110 and ML308 were used in this study. The growth media of *B.subtilis* were referred to Nishio *et al.* (1983). The basic media of *E.coli* cells were modified M9 media (pH7.4, in g/l):Na_2HPO_4, 7.0; KH_2PO_4, 3.0; NaCl, 5.0; NH_4Cl, 1.0; casamino acid, 1.0; $MgSO_4$, 0.75; glucose, 5. The 50g/l glycerol in the replacement of glucose was utilized as a carbon source for the production of β-gal using *E.coli* ML308 cells (Gray *et al.*, 1973). Following overnight growth at 37°C in a 100ml meyer (130rpm) with a working volume of 20ml, the culture was used as an inoculum for a 300ml shaking flask using 100ml media. The cells were harvested in the stationary growth phase by centrifugation, washed once in distilled water, and resuspended in treatment buffer (50mM potassium phosphate: KPi, pH7.5) with the various initial cell concentrations (C_0: 1.5~20mg/ml). The effect of the growth phase of *E.coli* cells was also investigated. The PEG solutions of various concentrations and molecular weights were also utilized as the treatment solution.

Disruption of cells was performed using an ultrasonic disrupter UD-200 (Tomy Seiko Co., Ltd; 20kHz) with microtip (TP-040). Ultrasonic wave was irradiated to the cell-suspension in a 10ml test tube with various working volumes (V: 2.5~10ml) and with the various input power (P: 20~80W). The disruption was operated for 30 s periods separated by 60-s intervals in the ice bath. Samples were then centrifuged (7000rpm, 10min, 0°C) and the supernatants were assayed for enzyme activity and protein concentration.

2.3 Analytical Methods The enzymes assayed were acid phosphatase (AcP; EC 3.1.3.2.) as a marker of periplasm (Brockman and Heppel, 1968; Dvorak *et al.*, 1967), glucose-6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49.) and fumarase (Fum; EC 4.2.1.2.) as markers of cytoplasm (Marr, 1960). The activity of AcP, G6PDH, and Fum were measured, respectively, in sodium acetate-acetic acid buffer (pH 4.5), glycylglycine-NaOH buffer (pH7.5), and sodium-phosphate buffer (pH7.5) at a wavelength of 340, 340, and 250nm using p-nitrophenylphosphste (p-NPP), G6P, and L-malate as a substrate (Bergmeyer, 1963). The activity of β-gal (EC 3.2.1.23.) was measured in 50mM Tris-HCl buffer containing 10mM $MgSO_4$ at 420nm using ONPG as a substrate (Steers *et al.*, 1971). The concentration of the total soluble protein was measured by Pyrogaroll Red method (Fujita *et al.*, 1983). All measurements were performed by Shimadzu spectrophotometer UV160-A. The released amounts (R_i) of total soluble proteins (i=T) and enzymes (i=1;AcP, 2;G6PDH, 3;Fum, G; β-gal) were determined as the weight and the activity per unit weight of packed cells, respectively.

Obtainable maxima in activity of AcP, G6PDH and Fum and weight of proteins, $R_{i,m}$ (i = 1, 2, 3, G, and T), for *E.coli*W3110 cells were

0.069U/mg-cell, 0.070U/mg-cell and 0.35mg/mg-cell, respectively. The concentration of the cells was determined with an optical density at 660nm (OD_{660}). The average particle size (d_p) by mass of the cell disintegrates was determined by the dynamic light scattering method (Otuka Electronics, DLS-700Ar). Three variables such as R_i, OD_{660} and d_p were respectively normalized as their remained fractions, D_i ($=(R_{i,m}-R_i)/R_{i,m}$), D_C ($=OD_{660}(t=t)/OD_{660}(t=0)$) and D_S ($=d_p(t=t)/d_p(t=0)$), where $R_{i,m}$ is the obtainable maximum value and t is the disruption time. These three were examined as a possible measure of the degree of disruption.

3. RESULTS AND DISCUSSION

3.1 Effective Parameters for Cell-Disruption Process The degree of cell disruption has been determined from the concentration of proteins (Hetherington *et al.*, 1971) and enzymes (Follows *et al.*, 1971; Melendres *et al.*, 1993), the cell size (Agerkvist and Enfors, 1990; Kula *et al.*, 1990) and

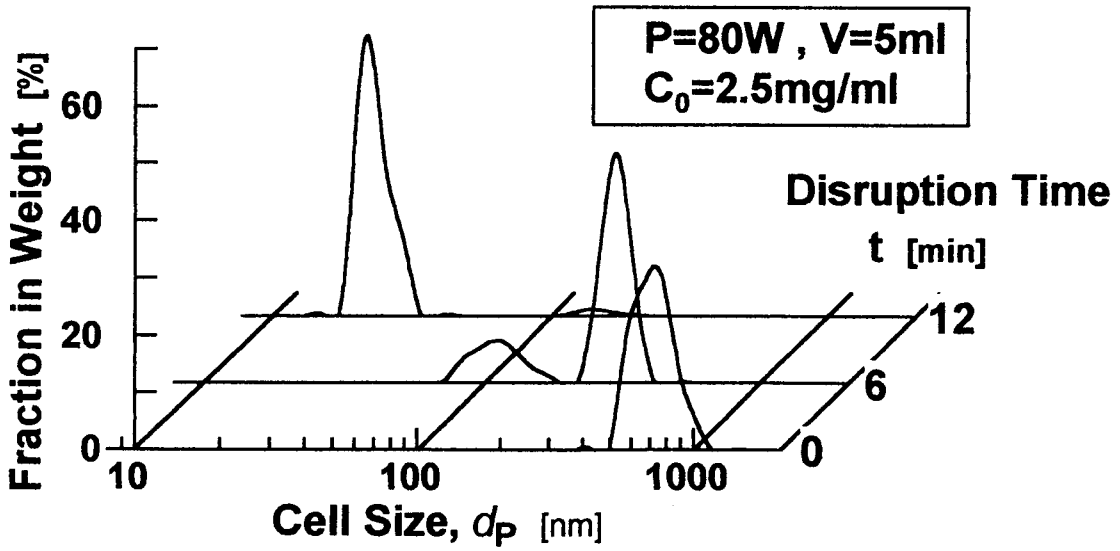


Fig.2-1 Time course of the size distribution of cells in the cell disruption process of *E.coli* W3110

the number of viable cells (Melendres *et al.*, 1993). The effectiveness of the cell concentration measured by an optical density, OD_{660}, was investigated as a basis for a quantitative estimation of the degree of cell disruption. **Figure 2-1** shows the time course of the size distribution of the cell particles for a typical disruption process. It was found that the particle size decreased during the cell disruption process and was an effective measure of disruption. The time course of other normalized fractions are shown in **Fig. 2-2**. These fractions were individually corresponding to the disruption time. At any disruption time, the disrupted fraction from cell concentration, DF_C

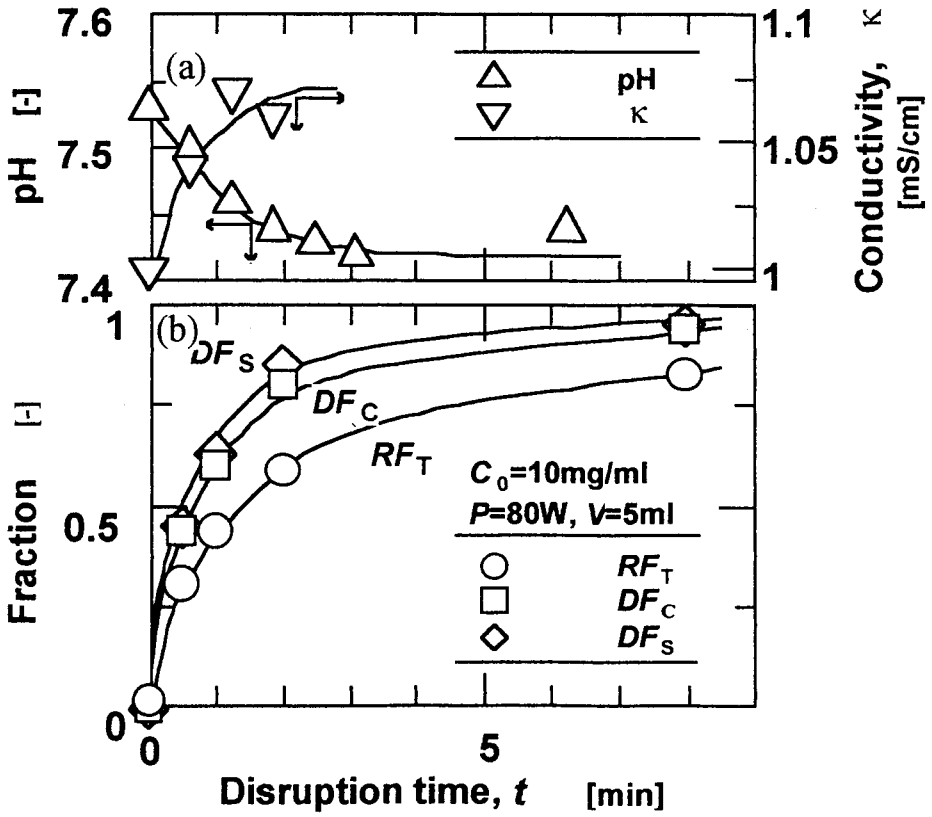


Fig.2-2 Time course of three fractions during the cell disruption of *E.coli* W3110 at C_0=10g/l, P=80W and V=5ml. Symbols: (a) the values of pH (△) and conductivity (▽) ; (b) disrupted fractions determined from cell concentration (DF_C,□) and from average cell size (DF_S,△) and released fraction of total soluble proteins (RF_T,○)

(=1-D_C), was almost corresponding to that from average cell size, DF_S (=1-D_S), whereas these disrupted fractions were slightly greater than the released fraction of total proteins, RF_T (=1-D_T).

Figure 2-3 shows the relationships between the disrupted fraction measured by the cell concentration and the fractions measured by other means. The disrupted fractions from average cell size, DF_S, are compared with corresponding fractions from cell concentration, DF_C, in **Fig.2-3(a)**. The data were obtained in the range of C_0: 1.3~10mg/ml, P: 20~80W and V:5ml. The data are seen to cluster on the diagonal line, suggesting that the cell concentration measured by an optical density can be an effective measure of the cell disruption. The released fractions of total proteins, RF_T, obtained in the range of C_0: 3.5~20mg/ml, P: 20~80W and V:5ml, are compared with corresponding fractions from cell concentration, DF_C, in **Fig.2-3(b)**. The released fractions are seen to be almost proportional to the disrupted fractions up to 0.8. However, the data deviate from the diagonal line, suggesting that the present cells are disrupted prior to the release of the intracellular proteins. The released fractions of the three enzymes RF_i such as AcP(i=1), G6PDH(i=2) and Fum(i=3) are also compared with corresponding fractions from cell concentration in **Fig.2-3(c)**. The data obtained in the range of C_0 greater than 10mg/ml were used, since the reproducible data for the enzyme activity were not obtained in the lower C_0 range. The different released fractions of the three enzymes are likely to be caused by the intracellular locations of the enzymes. Follows *et al.* (1971) have reported the dependence of the release behaviors of enzymes on their intracellular locations, and the present results are in line with their findings.

The cell concentration as measured by OD_{660} is thus proved to be an

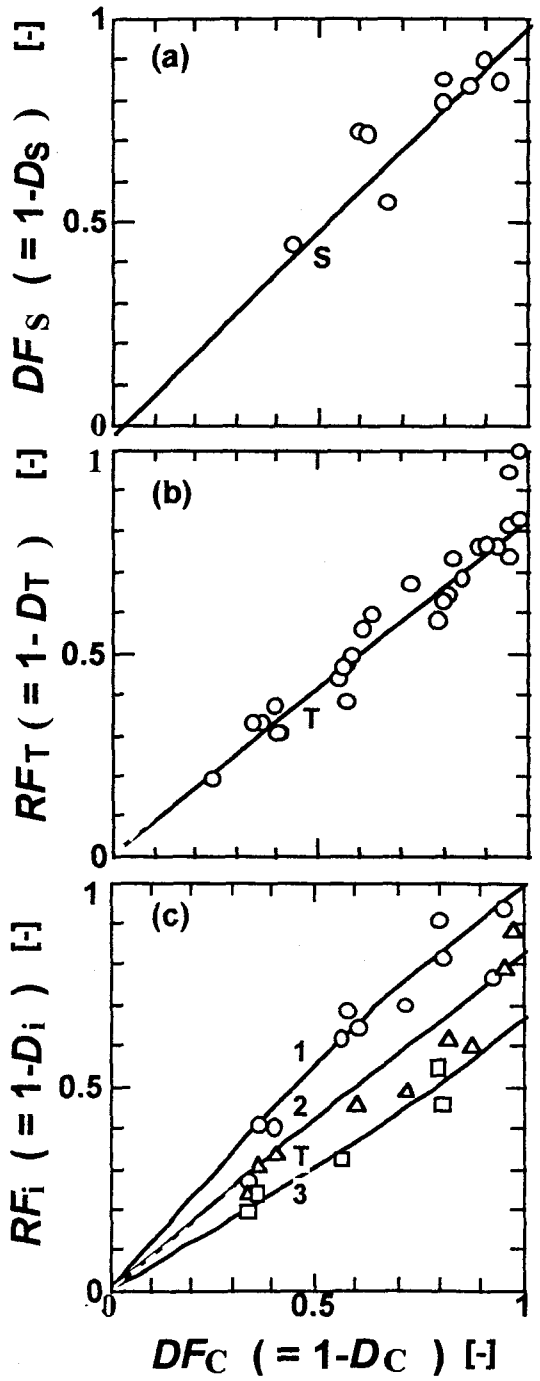


Fig.2-3 Relationship between disrupted fraction measured from cell concentration (DF_C) and (a)average cell size (DF_S), (b)released fraction of total soluble proteins (RF_T), and (c) those of three enzymes (RF_i) from *E.coli* W3110 cells

effective measure for the determination of the extent of the cell disruption which can be measured both simply and quickly.

3.2 Kinetic Analysis of Cell Disruption Process using the Specific Energy. For the cell disruption process, the first-order kinetics are reported to be applied (Melendres *et al.*, 1993).

$$-dC/dt = k_C C \qquad (2\text{-}1)$$

where C is the concentration of undisrupted cells and k_C is the disruption rate constant. Integration of **Eq.(2-1)** with condition of $C=C_0$ at $t=0$ gives,

$$\ln[C_0/C] = \ln D_C^{-1} = k_C t \qquad (2\text{-}2)$$

The product release can also be expressed by the first-order kinetics (Hetherington *et al.*, 1971; James *et al.*, 1972; Schutte and Kula, 1986),

$$dR/dt = k_i(R_{i,m}-R_i) \qquad (2\text{-}3)$$

where R_i is the released amount of total proteins (i=T) and enzymes (i=1,2,3,..), and k_i is their release rate constant. Integration of **Eq.(2-3)** with the conditions of $R_i=0$ at t=0 and $R_i=R_{i,m}$ at $t=$ gives,

$$\ln[R_{i,m}/(R_{i,m}-R_i)] = \ln D_i^{-1} = k_i t \qquad (2\text{-}4)$$

The effects of the initial cell concentration (C_0), acoustic power of ultrasonic wave (P) and the working volume of solution (V) on the cell disruption rate are shown in **Fig.2-4(a)**, **(b)** and **(c)**, respectively. Straight lines are seen in all cases. At fixed values of P=80W and V=5ml, the initial cell concentrations from 3.5 to 20mg/ml are seen in **Fig.2-4(a)** to have no effect on the disruption rate constants. When plotted the slopes of the lines shown in **Fig.2-4(b)** against the acoustic power, the rate constants were found to be linearly increased with the power. The constants were also found to be linearly decreased with the working volume. James *et al.* (1972) found identical dependences with the present results using both batch and flow systems.

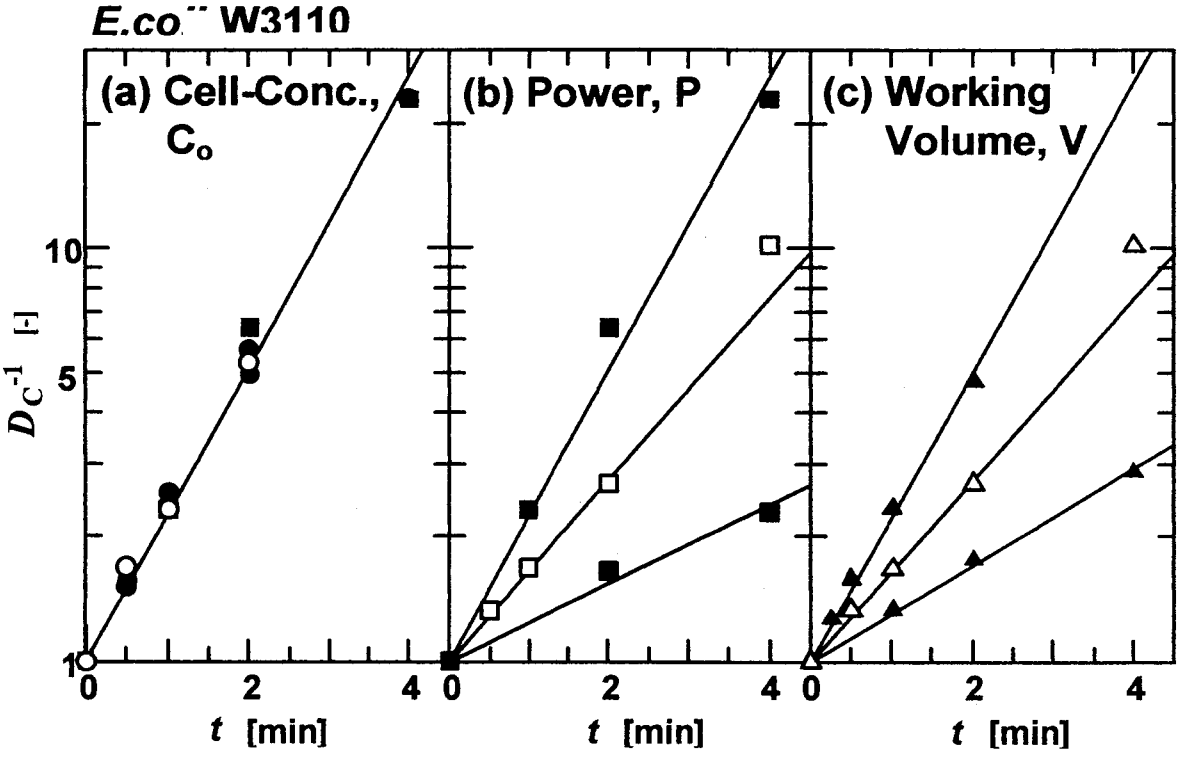


Fig.2-4 Effect of operational conditions on the disruption rate of *E.coli* W3110 cells. Symbols: (a) initial cell concentration, C_0, at P=80W and V=5ml, (○)20g/l, (●) 14g/l, (●)10g/l, (■)3.5g/l; (b)acoustic power, P, at C_0 = 3.5g/l and V=5ml, (□), 40W, (■), 20W, (■),80W; (c) working volume, V, at C_0=2.5g/l and P=40W (△), 5ml, (▲), 2.5ml, (▲), 10ml.

These results suggest that the specific energy supplied to the disruption media, q ($=Pt/V$), is a generalized parameter for the disruption. Actually, the concept of the specific energy input has been used as an effective parameter for the comparison of the energy consumption among the different disruption methods (Matsumoto *et al.*, 1994) and among the different microorganisms (Schutte and Kula, 1986).

The remained fractions during the cell disruption are plotted against q value in **Fig.2-5(a)**. All the data obtained for *E.coli* W3110 are seen to fall on a single straight line. The data for *B.subtilis* and *S.cerevisiae* were on the

respective straight lines and only the resultant lines are shown in **Fig.2-5(a)**. These rate constants, k_C, are found to be dependent on the kinds of microorganisms. For the release constants of the total soluble proteins (i=T) and three enzymes, the identical treatment was carried out. The remained fractions are plotted against q values in **Fig.2-5(b)**. Respective straight lines are seen as expected from the original data shown in **Fig.2-3(c)**. The release rate constant of AcP, G6PDH and Fum from *E.coli* W3110 were greater, equal and smaller as compared to that of total soluble proteins, respectively. The disruption rate constant k_C and the release rate constant k_i are now generalized as follows.

$$K_C = (V/P)k_C \tag{2-5}$$

$$K_i = (V/P)k_i \tag{2-6}$$

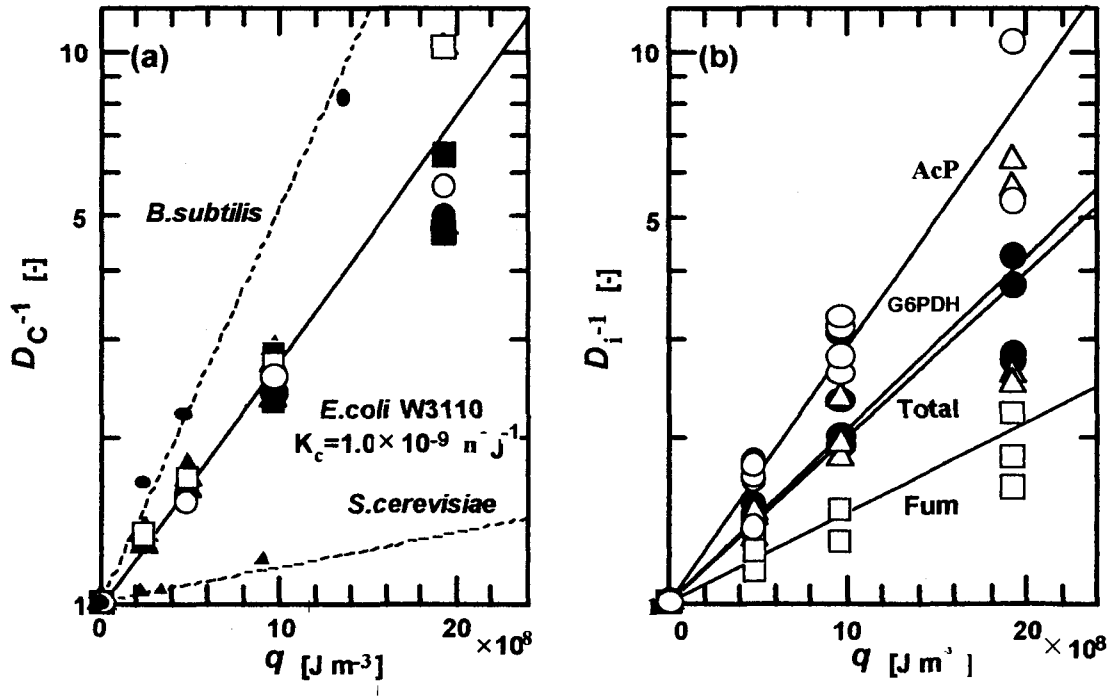


Fig.2-5 Relationship between the specific energy, q, and the remained fraction of (a) cell concentration (D_C) and (b) total proteins (D_T), or intracellular enzymes (D_1, D_2, and D_3) for *E.coli* W3110 cells. i=T; total soluble proteins, 1; acid phosphatase, 2, G6PDH, 3, fumarase

The generalized rate constants, based on this specific energy input, can then be used as effective parameters.

3.3 Evaluation of Cell Properties using the Disruption and Release Rate Constant. The cell properties such as cell strength and intracellular location of enzymes were examined using the generalized rate constants of cell disruption K_C, total soluble protein release K_T, and enzyme release K_i. The inverse value of the cell disruption rate constant, $1/K_C$, which means the amount of energy needed for an unit decrease of the cell concentration, is considered to be dependent on the individual cell wall strength against the disruptive stress. The relationship between the $1/K_C$ values and the types of microorganisms are summarized in **Fig.2-6**. The $1/K_C$ values of the wild type strain (*E.coli* W3110) was 30% smaller than that of the mutant (*E.coli* ML308) and 50% greater than that of the plasmid harbored strain (*E.coli* OW10/pND5). The $1/K_C$ values of the stationary growth phase was about

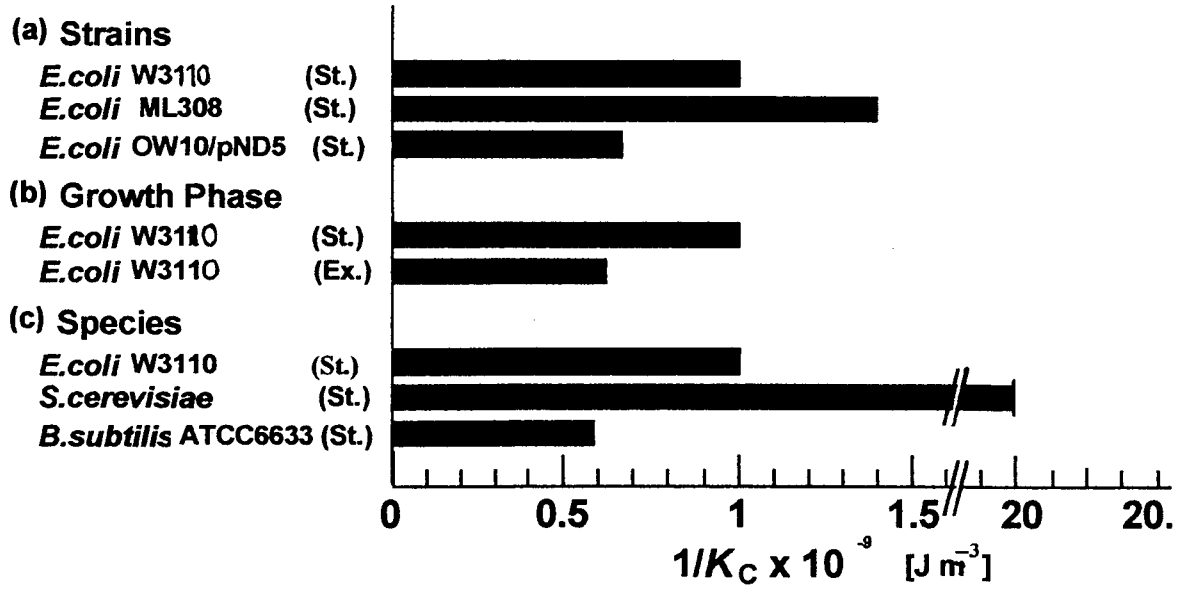


Fig.2-6 Cell disruption characteristics of various microorganisms different (a) strains, (b) growth phases, and (c)species. [(St.), stationary growth phase; (Ex.), early exponential growth phase]

70% greater than that of the early exponential growth phase. Typically, cell wall strength of microorganism was reported to depend on the degree of peptidoglycan cross linkage (Leduc *et al.*, 1989) and the cell size (Doulah *et al.*, 1975). Middelberg *et al.* (1993) presented the relationship between these parameters and the effective cell strength of *E.coli* against the disruptive stress. The $1/K_C$ values shown in **Fig.2-6** may reflect the individual cell properties. The $1/K_C$ value of *S.cerevisiae* is 20 times greater and that of *B.subtilis* is 40% smaller than that of *E.coli* W3110. This large difference may be due to the difference of each cell wall structure. The cell wall strength against the disruptive stress can then be estimated by the generalized disruption rate constant effectively.

The difference in the cell strength affect very much the rate constants for the protein release processes. However, these differences can be elimi nated by the use of the ratio between K_C, K_T and K_i. For example, the ratio of rate constants between total protein release and cell disruption, K_T/K_C, is defined as the release efficiency of total proteins from cells. The release efficiencies K_T/K_C of two different strains (*E.coli* W3110 and ML308) indicate almost the same values of 0.68 and 0.63, respectively. In the same way, the release rate constants of intracellular enzymes are normalized. The release selectivities of each enzymes from *E.coli* cells defined as K_i/K_T are shown in **Fig.2-7**. AcP is known to be in the periplasmic space of cells (Brockman and Heppel, 1968; Dvorak *et al.*, 1967) and G6PDH and Fum are in the cytoplasm (Mar, 1960). The cytoplasmic β-gal overproduced in the mutant strain is accumulated into the periplasmic space both because its release can be induced by the addition of glycine which is effective for inducing release of periplasmic proteins (Ikura, 1989) and because the β-gal

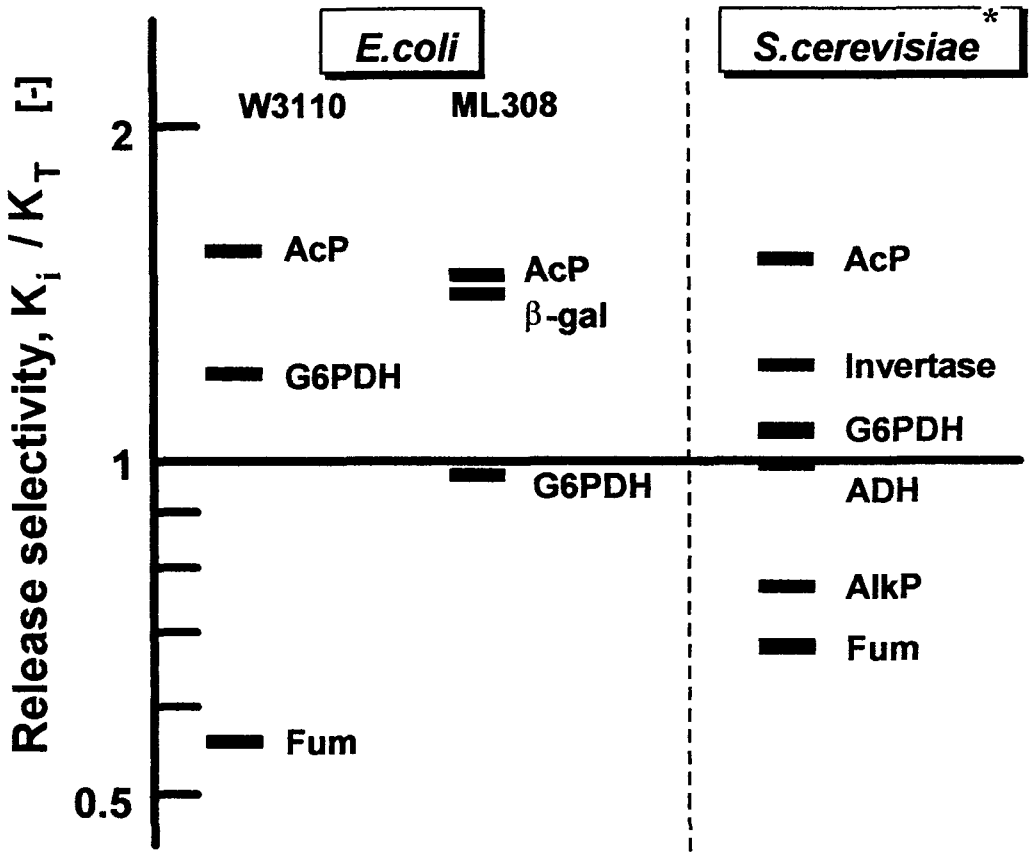


Fig.2-7 Release selectivity of various enzymes contained in three microorganisms. (AcP, AlkP; acid and alkaline phosphatase, ADH, G6PDH; alcohol and glucose-6-phosphate dehydrogenase, Fum; fumarase) *The results of *S.cerevisiae* were from Follows *et al.* (1971)

is translocated across the inner membrane under heat stress (Yatvin *et al.*, 1986, Yatvin, 1987). These values in the ladder of **Fig.2-7** agree well with these of the previous qualitative reports. Follows *et al.* (1971) have reported for the disruption of yeast cells by a high-pressure homogenizer that the enzyme release behavior depends on its intracellular locations of the Yeast cells in the disruption processes using the high-pressure homogenizer. Their results were recalculated according to the present method and are also represented in **Fig.2-7**. Thus the obtained results for enzyme release selectivity and enzyme location are found to be useful and also in good agreement with

previous results in spite of the different microorganisms and disruption methods employed. In the following experiment, the values were used as location factor of enzyme, LF_i ($=K_i/K_T$).

3.4 Characterization of Hydrophobic Interaction between Cell Surface and Polymers by Using the Rate Constants.

(1) Effect of Addition of PEG on the Release Kinetics. Methods to improve the release selectivity of target β-gal from *E.coli* ML308 were investigated based on a knowledge of the rate constants defined in the present

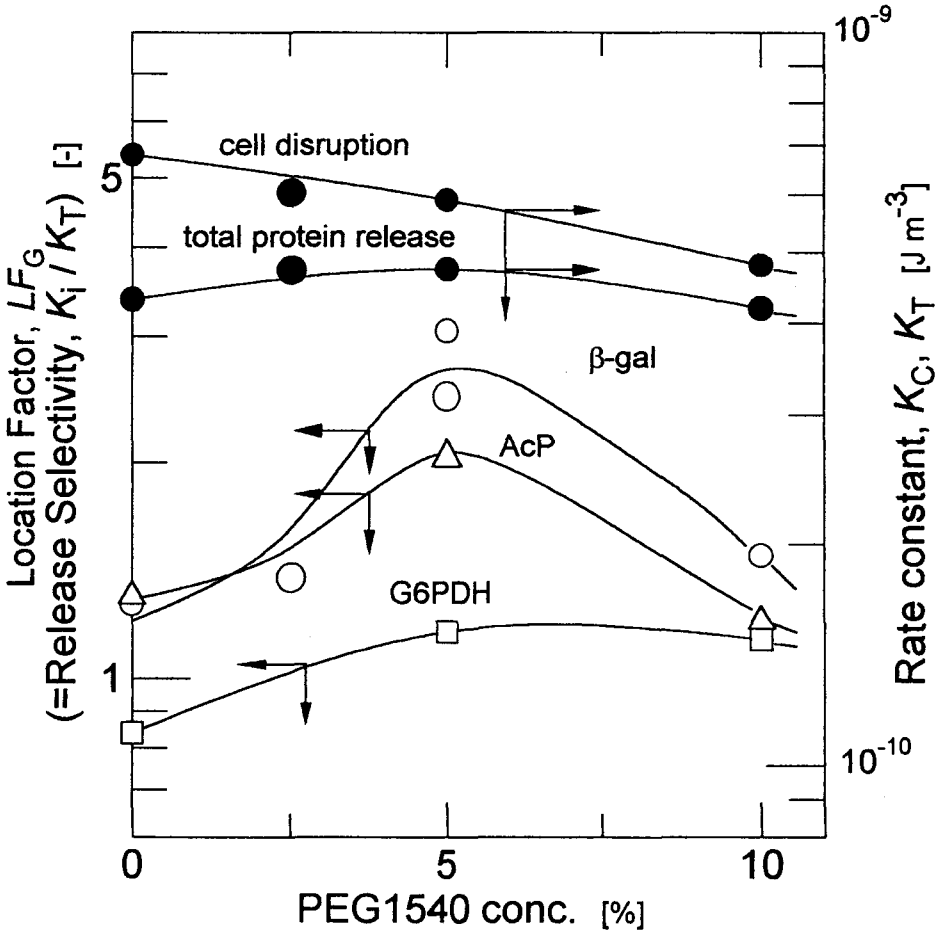


Fig.2-8 Effect of PEG1540 concentration on the cell disruption and release of total proteins and three enzymes for *E.coli* ML308 cells

work. PEG has been known to interact with the cell surface (Honda *et al.*, 1981-a; Honda *et al.*, 1981-b) and can be utilized for the modification of disruption media to improve enzyme recovery (Wang, 1992). The selectivity of the periplasmic β-gal, LF_G, is likely to be improved by the addition of PEG. The effects of addition of PEG1540 on the cell disruption rate constant, K_C, release rate constant, K_T, and their ratio (the release selectivity), LF_G, are shown in **Fig.2-8**. Addition of PEG from 0 to 5% increased the LF_i values of periplasmic AcP and β-gal and further addition of PEG led to a decline in their LF_i values. On the other hand, the release of cytoplasmic G6PDH was not reduced by further addition. The disruption rate constant, K_C, was slightly reduced with the PEG concentration, whilst the release rate constant of the total proteins, K_T, was kept almost constant. From these results, the LF_i values of proteins, especially periplasmic enzyme, was found to be more enhanced by PEG as compared with the cell disruption rate probably because of the hydrophobic interaction between the PEG molecules and the cell wall surfaces (Honda *et al.*, 1981-a; Honda *et al.*, 1981-b). The effect of average molecular weight and concentration of PEG on the location factor of β-gal, LF_G, is summarized in **Fig.2-9**. The addition of 5% PEG1540 is seen to be most effective for the selective release of β-gal.

(2) Effect of Types of *E.coli* Cells on Release of Enzymes in the Presence of PEG. The effect of types of *E.coli* cells was investigated. Two kinds of cells such as *E.coli* W3110 and ML308 were disrupted in 5% PEG1540 solution. **Figure 2-10(a)** shows the relationship between the LF_i values of two enzymes (AcP and G6PDH) and the increased amounts of LF_i values in PEG solution (ΔLF_i) in two kinds of cells. In general, the release of enzyme with larger LF_i values is enhanced by the addition of PEG to the

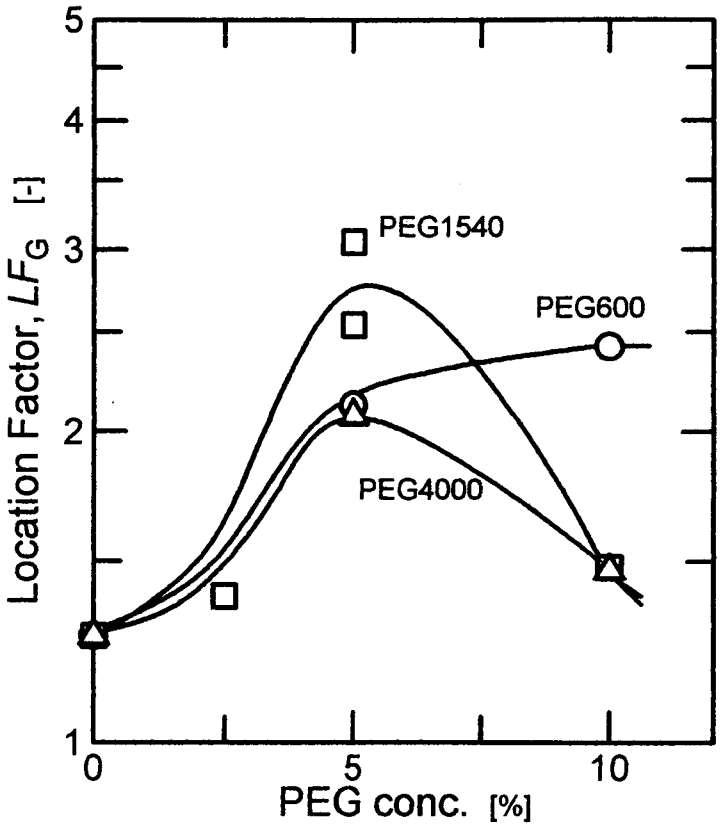


Fig.2-9 Effect of PEGs of various average molecular weights on the release selectivity of β-gal from *E.coli* ML308 cells

disruption media. The slope of the relationship is then almost the same value in two cells. However, the line of *E.coli* ML308 is shifted upward as comparing with that of the *E.coli* W3110 cells. In chapter I, it has been reported that the surface hydrophobicity of *E.coli* ML308 was greater than that of W3110. The upward shift of the line is thought to be caused by the differences in hydrophobicity. As shown in **Fig.2-10(b)**, the LF_i values of *E.coli* ML308 cells are also plotted against the ΔLF_i values. The slope of the line is increased with decreasing the values of molecular weight of PEG. This may be caused by the differences of hydrophobicity of PEG molecules. PEG1540 was the most hydrophobic in the present polymers according to the concept of Davies (1964). The difference of the slope may be dependent on the hydrophobicity. It is therefore expected that the

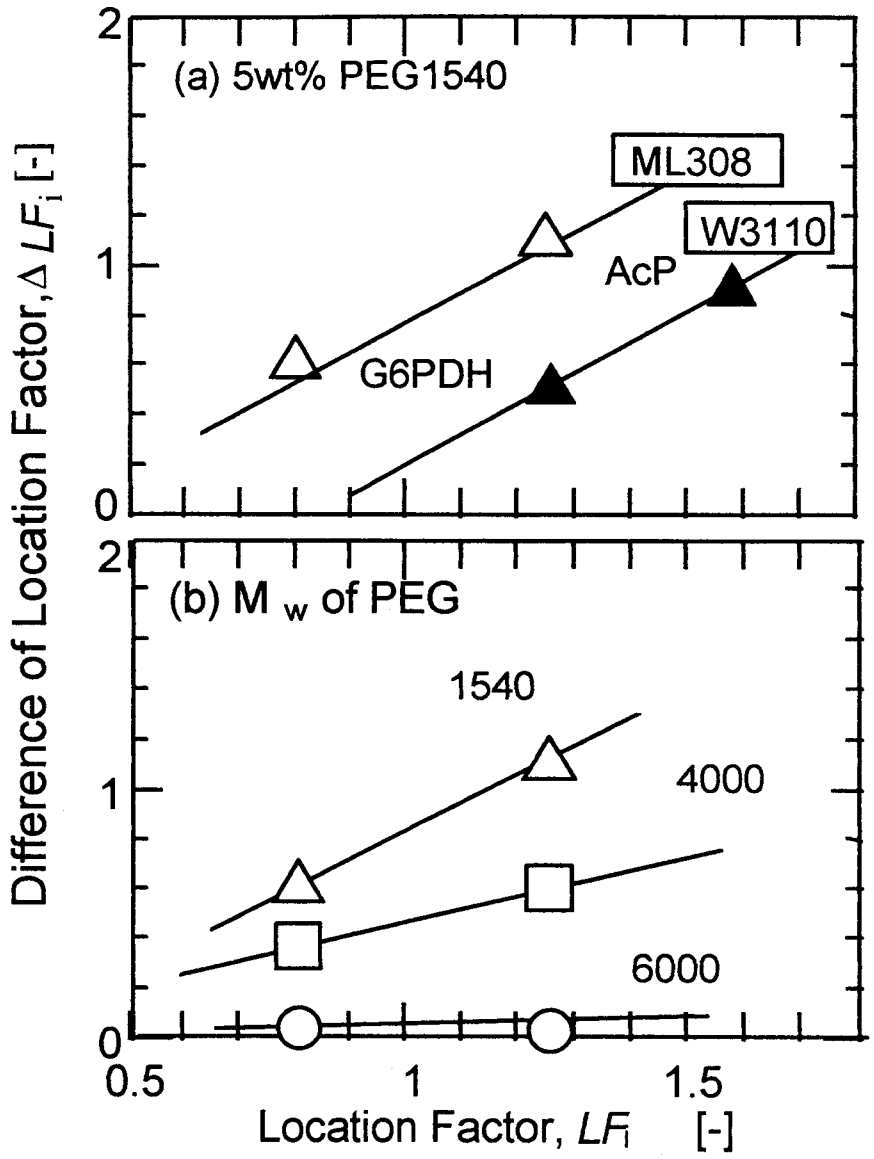


Fig.2-10 Relationship between the LF_i values of two enzymes and the enhanced amounts of the values ($\Delta\ LF_i$) in two kinds of *E.coli* cells (ML308 and W3110)

shifted amounts of ΔLF_i against LF_i can be used as estimation of the hydrophobic interaction between surface and PEG molecules.

(3) Use of Aqueous Two-Phase Systems to Enhance Enzyme Recovery. Aqueous two-phase systems (ATPS) are effective for separation of bioproducts because of their mild and scaleable properties. When these two-phase systems having identical tie-lines are employed, the systems have identical composition for the top and bottom phases but have different phase

volume ratios. The disruption medium was modified with PEG1540 / phosphate ATPS. As shown in **Fig.2-11**, the effect of the volume fraction of top-phase on the disruption and release rate constant was investigated in a same tie-line. At the smaller value of volume fractions, the release selectivity of β-gal, LF_G, was increased up to 1.8 and then the release efficiency, defined as K_T/K_C, was decreased. It was found that the ATPS with the lower volume fraction was the most effective for selective recovery of β-gal.

(4) Optimization of Cell Disruption Process Based on Location Factor. The specific energy for the selective release of target β-gal was optimized in the effective disruption media modified with 5% PEG1540 and PEG1540 / phosphate ATPS. The released fraction, RF_i (=1-D_i), and the specific released fraction normalized by the total soluble proteins, SRF_i (=(1-D_i)/(1-D_T)), indicate the yield and the selectivity, respectively. These values can be

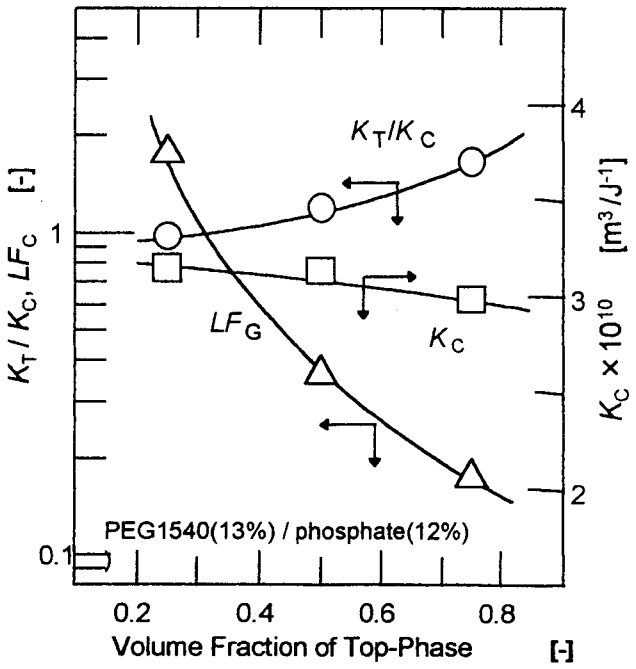


Fig.2-11 Effect of volume fractions of top-phase in PEG/phosphate aqueous two-phase systems on the cell disruption and release rate constants during the disruption process of *E.coli* ML308.

calculated from the release rate constants. These calculated results about the total soluble proteins and the target β-gal in 5% PEG1540 solution are shown in **Fig.2-12(a)** and **(b)** together with the experimental results. The RF_i increased and SRF_i decreased as the disruption proceeded. The difference of released fraction of β-gal and total soluble protein, DRF_i (=RF_i-RF_T), is used for the optimization of the disruption process. The difference is shown in **Fig.2-12(c)**. The specific energy input at which the maximum difference is attained may be the optimal input for the selective release of the target enzyme with a higher yield. The maximum value is obtained by differentiating DRF_i with q as follows.

$$DRF_i = \exp(-K_i q) - \exp(-K_T q) \tag{2-7}$$

$$d(DRF_i) / dq = 0 \tag{2-8}$$

The solution gives,

$$q_{opt} = \ln LF_i / (K_i - K_T) \tag{2-9}$$

The calculated q_{opt} values based on **Eq.(2-9)** and corresponding RF_i, SRF_i and DRF_i values are summarized in **Table 2-1**, together with the

Table 2-1 Summary of the optimal specific energy (q_{opt}), the released fraction (RF_i), the specific released fraction (SRF_i), and the difference of released fractions (DRF_i) of target β-gal from *E.coli* ML308 cells in 5% PEG1540 and PEG1540/phosphate ATPS

Disruption Media		q_{opt} [J m^{-3}]	RF_i [-]	SRF_i [-]	DRF_i [-]
50mM KPi	(Experiment)	3.8×10^9	0.83	1.15	0.10
	(Calculation)	2.0×10^9	0.70	1.20	0.10
5% PEG1540	(Experiment)	1.9×10^9	0.95	1.70	0.38
	(Calculation)	1.2×10^9	0.85	1.75	0.31
PEG/KPi	(Experiment)	1.9×10^9	0.68	1.58	0.25
	(Calculation)	3.6×10^9	0.86	1.28	0.19

experimental data. There are some deviations from the observed data. The deviations may arise from inevitable scatter contained in the data of release constants. Based on the above results, the flow chart for the design of selective and effective recovery of intracellular product in cell disruption was established and shown in **Fig. 2-13** with experimental results.

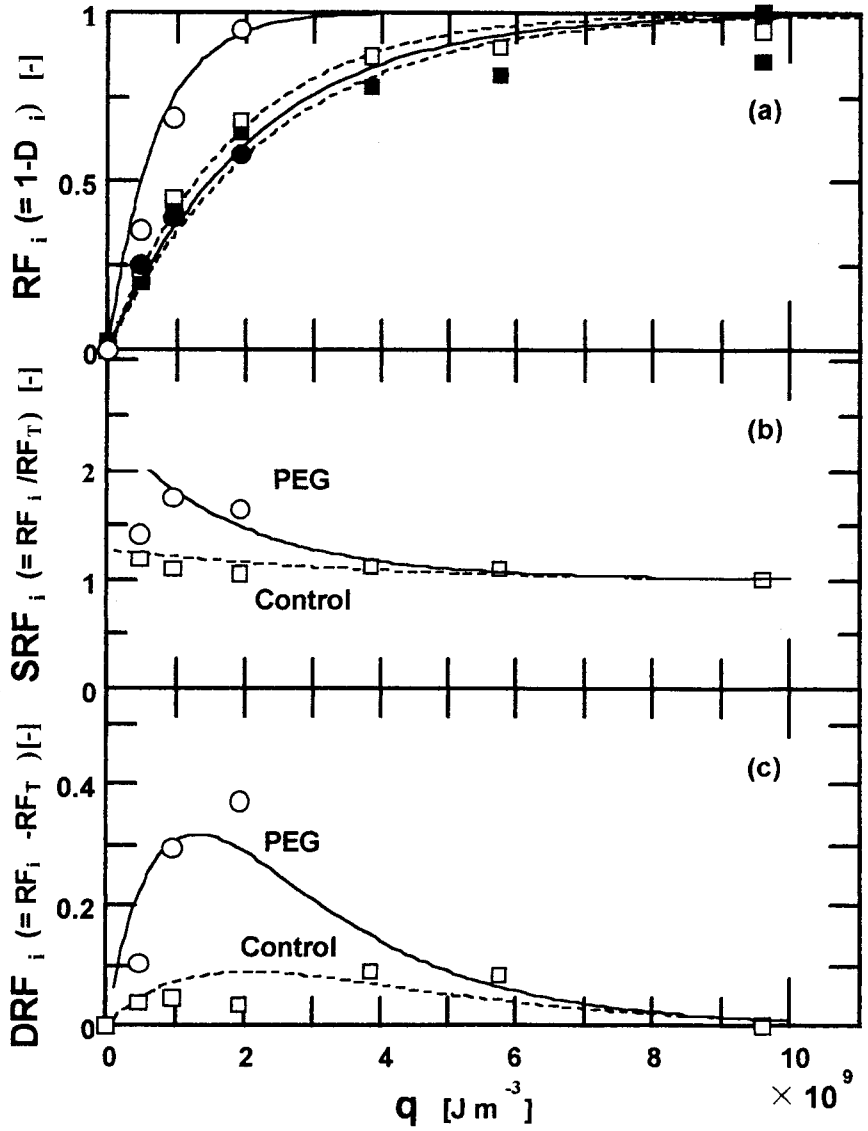


Fig.2-12 Optimization of the specific energy input to obtain the selective release of target β-gal from *E.coli* ML308. Symbols are ○, β-gal in PEG; □, β-gal in control; ●, total protein in PEG; ■, total protein in control.

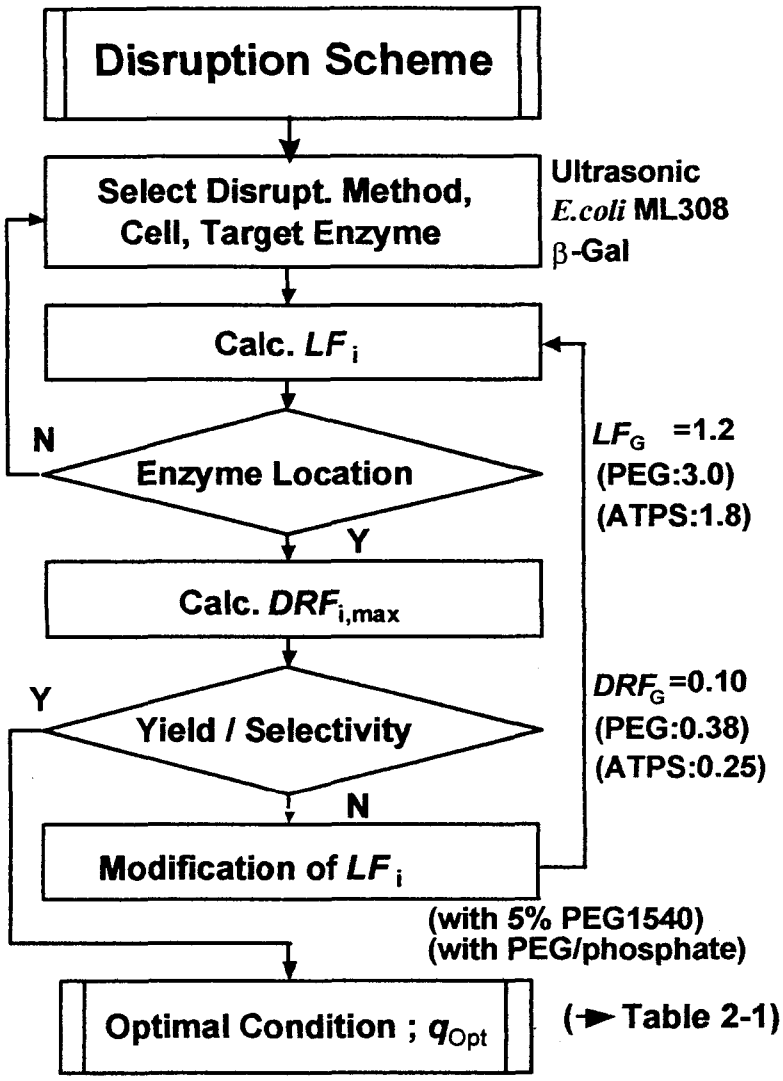


Fig.2-13 Flow chart for selective recovery and effective disruption of intracellular product. The results of modified media are shown in parenthesis

In the same way, the disruption condition in PEG1540/phosphate ATPS was also optimized and summarized in **Table 2-1.** It has been found that target β-gal can be recovered at the smaller energy input with the higher yields and selectivity in the 5% PEG1540 media as compared to the control media.

4. SUMMARY

A method for the optimization of the cell disruption process was investigated in order to achieve selective release and recovery of a target intracellular enzyme.

(1) The cell concentration measured by an optical density was found to be an effective measure for the determination of degree of cell disruption process. Through the kinetic analysis of cell disruption process, the operational parameters were found to be generalized as a specific energy supplied into the disruption media. The generalized values of the disruption rate constant K_C and the release rate constant for each enzyme K_i (i=1,2,..) were well correlated with the cell strength against the disruptive stress and also the intracellular location of each enzyme, respectively.

(2) The evaluated values from the release selectivity of enzyme i against the total proteins, K_i/K_T (defined as location factor, LF_i), were found to show the intracellular location of the enzyme.

(3) LF_i values of periplasmic enzymes were increased when cells were disrupted in the polymer solution. Especially, the maximum value was obtained in 5% PEG 1540 solution because of the hydrophobic interaction between the polymer and the membrane surface of cells.

(4) The disruption condition (specific the selectivity of enzyme recovery was found to be highly dependent on the LF_i values. The flow chart for the design to give both the higher yield and selectivity of target protein was finally established on the basis of the LF_i values.

Chapter 3

Selective Release and Recovery of Intracellular Enzymes from *Escherichia coli* using Nonionic Detergent, Triton X-100

1. INTRODUCTION

Cellular response against chemical stress is highly dependent on the surface properties of cells because the surface must recognize and respond at first to various stresses from their environment. The biological membranes include outer and inner membranes of *E.coli* cells both of which play important roles especially when cells are exposed to and respond against the environmental stress. The development of the method to characterize biological membranes including the outer and inner one is therefore needed in order to understand stress response functions of cells.

The development of characterization method to evaluate surface properties of cell membranes also plays an important role in the practical bioprocess for production and recovery of functional biomaterials. In such processes, the release of recombinant or functional intracellular enzymes and proteins is a first unit operation which give a significant impact on all the following downstream steps. The effective method to achieve the selective and rapid release of the intracellular products from cells is required in commercial operations. Among various methodologies, the cell disruption method using the mechanical stress is usually favored from the operational aspect (Schutte and Kula, 1990) and have been summarized in chapter 2. The method, however, still has some drawbacks, such as potential degradation of enzymes due to shear or heat-mediated inactivation (Engler, 1983) and difficulties in down stream purification of proteins due to contamination with other proteins and cell debris. By characterizing the cell-

response against the weaker chemical stress using chelating agents (DeSmet *et al.*, 1978; Hancock, 1984), chaotropic agents (Ingram, 1981), organic solvent (Murakami *et al.*, 1980), and detergents (Woldringh, 1970; Miozzari *et al.*, 1978; Schnaitman, 1971; Mirelman and Nuchamowitz, 1979), the selectivity and efficiency of intracellular proteins recovered is expected to be improved. Asenjo *et al.* (1990) have recently suggested the possibility of differential product release from microorganisms through the sequential lytic operations. The controlled lysis of *E.coli* cells and selective recovery of intracellular enzymes can further be achieved considering the evalluated values of the surface properties of cells.

The surface properties of the outer membrane of *E.coli* cells have been quantitatively characterized by using the partitioning method in aqueous two-phase systems in chapter 1. The physical characteristics of the outer and inner membranes of *Escherichia coli* cells were also evaluated by the kinetic analysis of release process of intracellular marker enzymes under the mechanical stress in chapter 2. The quantitative information on the cell membrane, especially, the inner membrane of cells is further expected to be obtained both by characterizing the behaviors of cell-response against weaker chemical stress as compared with mechanical one and by combining the characterization method with the partitioning method in ATPS.

The quantitative characterization of the enzyme release process induced by the treatment of the outer membrane with nonionic detergent, Triton X-100 (Schnaitman, 1971; Mirelman and Nuchamowitz, 1979), as a typical example of chemical stress, is the first objective of this chapter. The release of enzymes with different location factors was kinetically analysed concerning with the effect of hydrophobic interaction between the detergent

and the cell surface by using the evaluated parameters such as surface properties of cell surface and location factors of intracellular enzymes. The second objective is, then, to quantify surface properties of the obtained inner membrane of cells by using the partitioning method in ATPS.

2. EXPERIMENTAL

2.1 Materials. D-Glucose-6-phosphate (G6P) and β-$NADP^+$, used as the substrate and coenzyme for the measurement of D-glucose-6-phosphate dehydrogenase (G6PDH) were purchased from Oriental Yeast Co. Ltd. (Osaka, Japan). p-Nitrophenylphosphate (*p*-NPP) for substrate of acid phosphatase (AcP) and alkali phosphatase (AlkP) was purchased from Wako. o-Nitrophenyl-galactopyraniside (ONPG) for the substrate of β-galactosidase (β-gal) was purchased from Sigma (N.Y., USA). Casamino acid was purchased from DIFCO Laboratory (Chicago, USA). Nonionic detergent, Triton X-series (X-45, 100, 165, 305, 405, and 705) and polyethylene glycols (PEG600, 1540 and 4000) were from Wako Pure Chemicals (Osaka, Japan). The salts and other chemicals used were all of analytical grade.

2.2 Cultivation of Bacterial Cells. The strain used in this study is *E.coli* W3110. The basic media of *E.coli* cells were modified M9 media (pH7.4, in g/l):Na_2HPO_4, 7.0; KH_2PO_4, 3.0; NaCl, 5.0; NH_4Cl, 1.0; casamino acid, 1.0; $MgSO_4$, 0.75; glycerol, 50. Following overnight growth at 30°C in a 100ml erlenmeyer flask (130rpm) with a working volume of 20ml, the culture was used as an inoculum for a 300ml shaking flask using 100ml media. The cytoplasmic β-gal was then induced by the addition of 2mM IPTG into the culture at OD_{660} = 0.1~0.2. The cells were harvested in the

stationary growth phase by centrifugation (11000×g, 10min), washed once in 50mM Tris-HCl buffer, and resuspended in the buffer at the cell concentrations, C_0, of 10mg/ml. The suspension of heated cells, which were exposed to heat stress (10min, 42, 47, 50, 55, and 60°C), was also prepared.

2.3 Treatment of Cells with Triton X-series and EDTA. After the suspension of cells and heated cells was diluted by Tris-HCl buffer (50mM, pH 8.0) containing EDTA (final concentration 5mM), adequate amounts of Triton solution with various molecular weight was added to a final concentration of 0.01~20mM. The solution was stirred at 25°C for 0.1~3hr. One-ml portion of the solution was withdrawn and was centrifuged (8000rpm, 5min). The supernatant activity of various enzymes was analysed ($R_{i,Lysis}$ [U-enzyme/mg-Cell]; i represents enzyme types). The precipitates containing the Triton-treated cells were resuspended to the Tris buffer and were disrupted by ultrasonic method (input power, P=40W; solution volume, V=1ml; disruption time, t=2min) in order to determine the total activity of various enzymes per unit weight of packed cells ($R_{i,max}$ [U-enzyme/mg-Cell]). The released fraction of enzyme ($RF_{i,Lysis}$= $R_{i,Lysis}/R_{i,max}$) was then determined as the ratio of released amounts of enzymes ($R_{i,Lysis}$ and $R_{i,max}$) and was used as a parameter for the kinetic analysis .

2.4 Analytical Methods for Cell Properties. The intracellular location of various enzymes and the surface properties of cells were determined by the following method as described in chapter 1 and 2 in details.

(1) Location Factor of Intracellular Enzymes (Kinetic Analysis Method). The released fraction (RF_i) of intracellular enzymes (i; 1, 2, 3,... enzyme types) and total soluble proteins (i=T) can be correlated with the total input energy, q (=Pt/V), during the cell disruption and enzyme release processes

on the basis of the first order kinetics.

$$\ln (1\text{-}RF_i)^{-1} = K_i\, q \qquad (3\text{-}1)$$

Then the release rate constant, K_i, has been reported to depend on the intracellular distribution of enzyme within a cell (chapter 2). A location factor (LF_i) of enzymes can be herewith determined from the release selectivity of intracellular enzymes, which is normalized by using the rate constant of total soluble proteins, K_i/K_T. The LF_i value of enzymes is determined by analyzing the RF_i values during the ultrasonic disruption process and used as a measure of enzyme location within a cell.

(2) Surface Properties of Bacterial Cell Membrane (Aqueous Two-Phase Partitioning Methods). The surface net hydrophobicity (*HFS*) and local hydrophobicity (LH_{Cell}) of cells were determined by the following method using aqueous two-phase systems (ATPS). The partitioning of bacterial cells is mainly dependent on the hydrophobic effect at the isoelectric point (pI) and at the lower ionic strength. The *HFS* values can then be determined from the slope of **Eq.(3-2).**

$$\ln K_{Cell} = HFS \times HF \qquad (3\text{-}2)$$

HF is herewith the hydrophobic differences between the two phases in ATPS (Kuboi *et al.*, 1990) and K_{Cell} is the partition coefficient of cells. The K_{Cell} values were determined in the ATPS (the total weight is 5g), of which compositions were 7~13wt% PEG1540, 4000, 6000 and 7~13wt% Dextran 100-200k at pH=pI, followed by the centrifugation ($10\times g$, 5min, 25°C). The local hydrophobicity of cells, LH_{Cell}, was determined as the increment change of partitioning coefficient of cells in the presence of 1mM Triton X-100, $\Delta\ln K_{Cell,Triton}$ (= $\ln (K_{Cell,Triton}/K_{Cell,0})$; $K_{Cell,Triton}$ and $K_{Cell,0}$ are the partitioning coefficient of cells in ATPS with and without Triton) at pH=pI.

2.5 Measurements. The enzymes assayed were acid (AcP; EC 3.1.3.2.) as marker enzyme for periplasm (Brockman and Heppel, 1968; Dvorak *et al.*, 1967), glucose-6-phosphate and lactate dehydrogenase (G6PDH; EC 1.1.1.49. and LDH; EC 1.1.1.50.) as markers of cytoplasm (Nishio, *et al*, 1983). The activity of AcP was measured in sodium acetate-acetic acid buffer (pH 4.5) at a wavelength of 340nm using *p*-NPP as a substrate (Bergmeyer, 1963). The activity of G6PDH was measured, respectively, in glycylglycine-NaOH buffer (pH7.5) at a wavelength of 340nm using G6P as a substrate. The activity of β-gal (EC 3.2.1.23.) was measured in 50mM Tris-HCl buffer containing 10mM $MgSO_4$ at 420nm using ONPG as a substrate (Steers *et al.*, 1971). The cell concentration was determined with an optical density at 660nm (OD_{660}). All measurements were performed by Shimadzu spectrophotometer UV160-A (Kyoto, Japan).

3. RSULTS AND DISCUSSION

3.1 Release of Intracellular Enzymes from *Escherichia coli* Cells after Triton X-100 / EDTA Treatment. After the *E.coli* cells were treated by Triton X-100 and EDTA, the released behaviors of some intracellular enzymes were investigated. The maximum released amounts of each enzyme are herewith thought to be corresponding to the obtainable maximum values after the exposing by the physical stress for infinite time. The released fractions, $RF_{i,Lys}$, were determined as the ratio of the released amounts of enzymes and the maximum values. **Figure 3-1(a)** shows the typical example of the time course of the $RF_{i,Lys}$ values of some intracellular enzymes ($RF_{i,Lys}$: i=1, AcP: 2, G6PDH: G, β-gal) after the treatment with Triton X-100 / EDTA. These fractions are increased with increasing the incubation

time in exponential manner and saturated to the value corresponding to the specific value of each enzymes. The release rates of periplasmic enzyme (*i.e.* AcP) were quite different from that of cytoplasmic one (*i.e.* G6PDH). This is caused by the membrane structure of *E.coli* cells which consist of outer and inner membrane. If the released behaviors of enzymes can be controlled, the selective release of periplasmic enzyme was expected to be achieved as compared with the behaviors of cell response against the mechanical stress. The process of enzyme release followed by cell permeabilization process induced by chemical stress can, therefore, be explained by the first-order kinetics, which can be represented in the following equation .

$$\ln D_{i,Lys}^{-1} = \ln (1-RF_{i,Lys})^{-1} = k_i t \qquad (3\text{-}3)$$

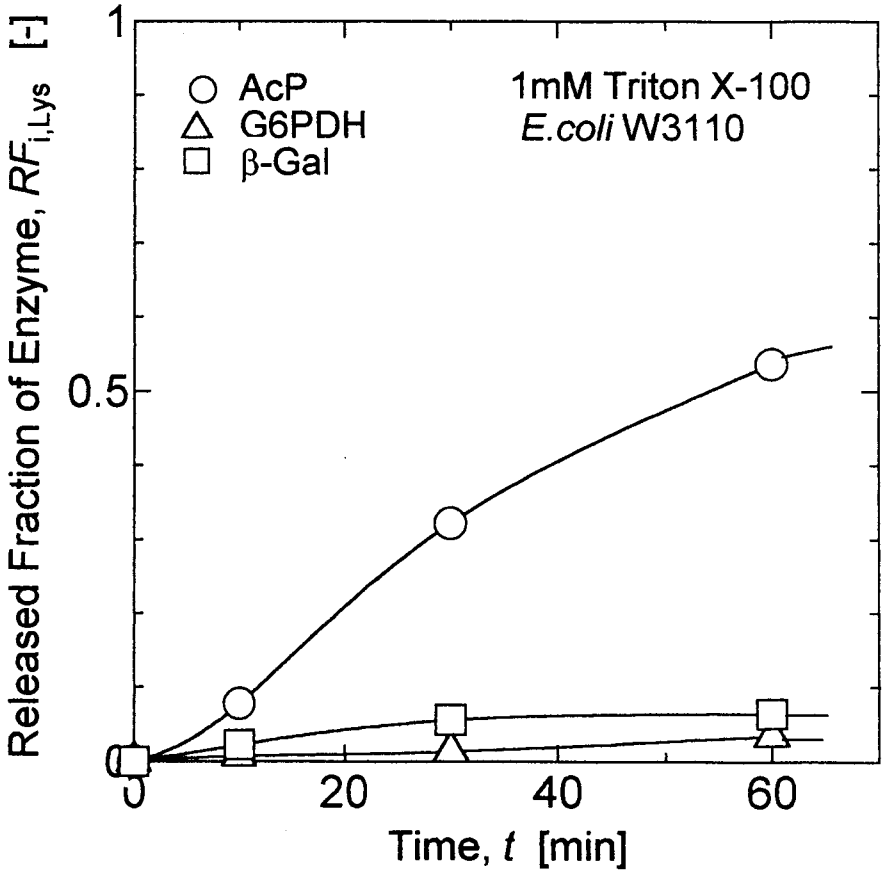


Fig.3-1(a) Time course of released fraction of enzymes by Triton X-100/EDTA treatment

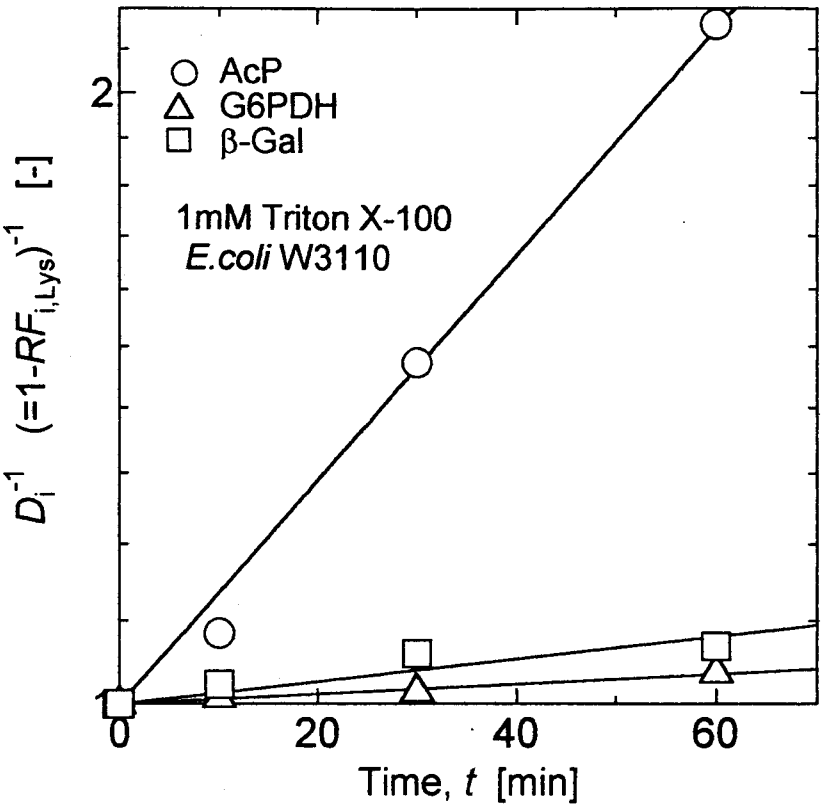


Fig.3-1(b) Time course of released fraction of enzymes by Triton X-100/EDTA treatment

where k_i is a release rate constant of intracellular enzyme i after the Triton treatment. As shown in **Fig.3-1(b)**, the remainder fraction of the above enzymes are plotted against the corresponding disruption time, t. At the lower t values, all the data obtained for each enzymes fall on a single straight line. Such enzyme release process followed by cell permeabilization process with Triton treatment is thought to be mainly dependent on i) the operational conditions of nonionic detergent, Triton X-series, (the concentration, C_{Triton}; the molecular weight, M_W) and (2) the evaluated properties of bacterial cells such as the surface properties of bacterial cells (surface net hydrophobicity, HFS_{Cell}; local hydrophobicity, LH_{Cell}) and the location factor of enzyme (LF_i). By using such parameters, the release rate constant of the enzyme i may be expressed as functions of the following two terms, such as i) the detergent

dependent term, f_{Det}, and (2) the cell dependent term, $f_{Cell,\,i}$ (C_{Triton}, M_w, LF_i, HFS_{Cell}, LH_{Cell}) (**Eq.(3-3)**). The former term is expected to depend only on detergent, while the latter term both on cell conditions and enzyme location in the cells.

$$k_i \propto f_{Cell,\,i}\,(C_{Triton},\, M_w,\, LF_i,\, HFS_{Cell},\, LH_{Cell})$$
$$\propto f_{Det.}(C_{Triton},\, M_w) \times f_{Cell,i}\,(LF_i,\, HFS_{Cell},\, LH_{Cell}) \qquad (3\text{-}4)$$

In the following sections, the release rate constants, k_i, for some intracellular enzymes including β-gal (i=G) were determined at various condition and their relationship, which could be applied to any enzyme, was investigated.

3.2 Relationship between Rate Constant and Detergent Dependent Term. The effect of the operational conditions of nonionic detergent, Triton X-100, such as C_{Triton} and M_w on the rate constant, k_G, was investigated in

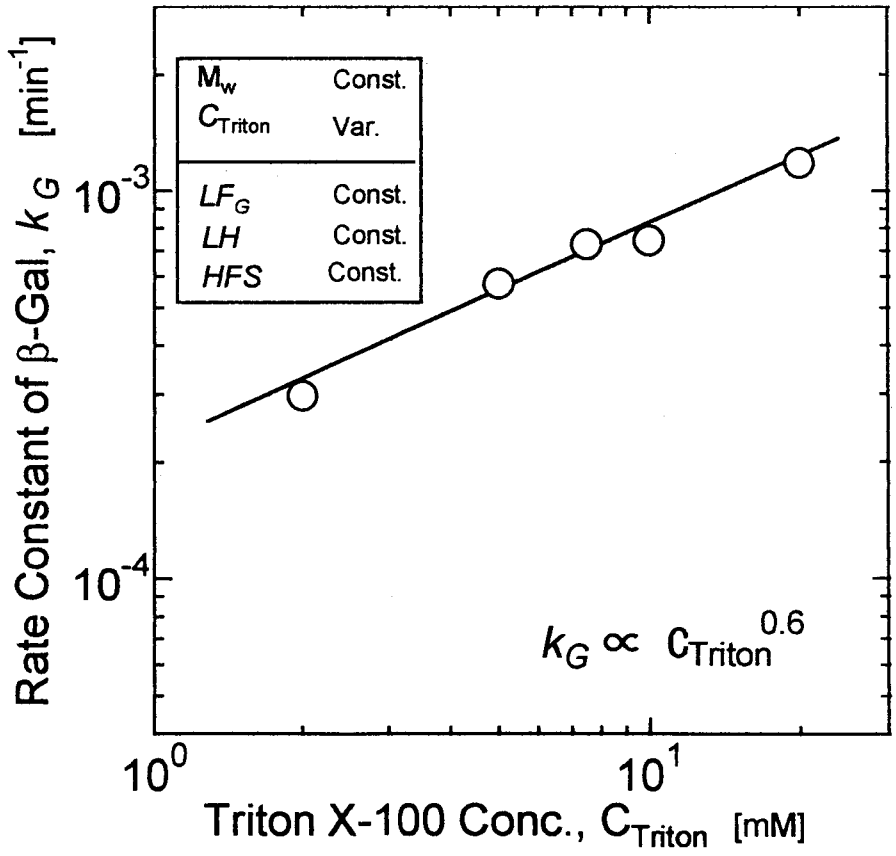


Fig.3-2 Relationship between Triton concentration and rate constant

order to clarify the detergent depending term, $f_{Det.}$ (C_{Triton}, M_w). The controlled release of enzyme is dependent on whether the release of cytoplasmic enzyme can be controlled or not. The release behaviors of the β-galactosidase (β-gal) were investigated in the following.

The effect of concentration of Triton X-100, C_{Triton}, on the release rate constant of β-gal (i=G) was investigated. **Figure 3-2** shows the log-log plot of the rate constant, k_G, against the C_{Triton} values of Triton X-100 during the release process of β-gal from *E.coli* cells. The values of rate constant, k_G, are increased with increasing the C_{Triton} values in this experimental range. The line has a slope of 0.63 and the relationship between k_G and C_{Triton} can thus be expressed as

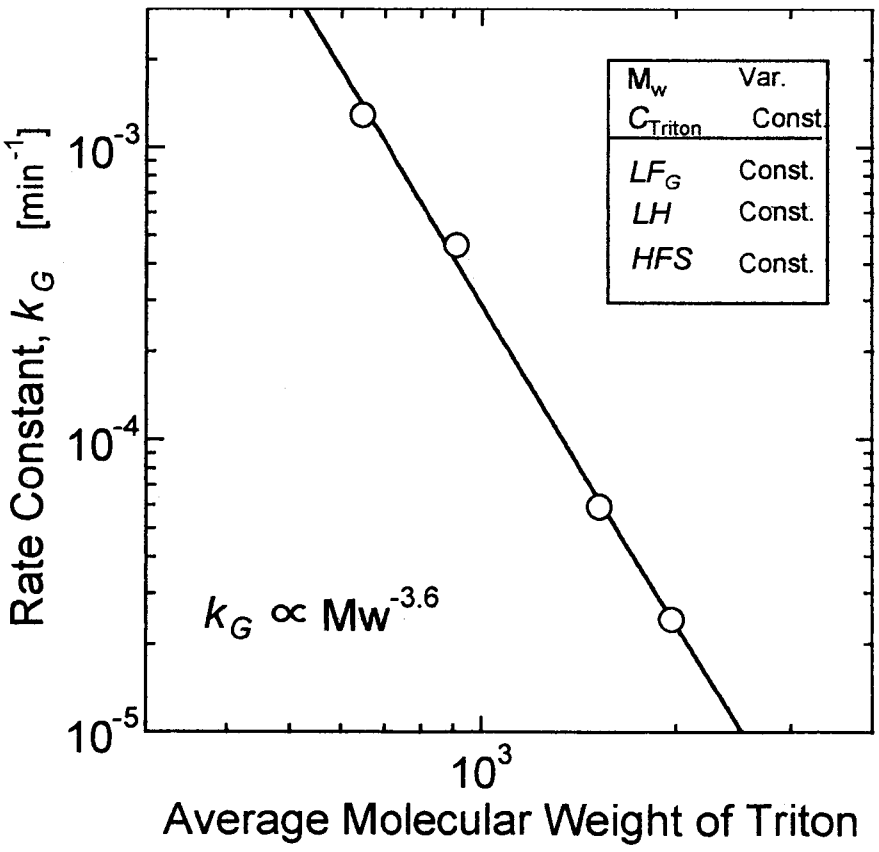


Fig.3-3 Relationship between molecular weight of Triton and rate constant

$$k_G \propto C_{Triton}^{0.63} \quad (3\text{-}5)$$

The effect of molecular weight of additional Triton X-series on the rate constant, k_G, was investigated. **Figure 3-3** shows the dependency of k_G on the molecular weight of Triton, M_w. The k_G values are decreased with increasing the M_w values. The slope of the relationship can be determined as -3.6 and the relationship can be written as

$$k_G \propto M_w^{-3.6} \quad (3\text{-}6)$$

From the above results, the detergent function, $f_{Det.}$, is represented as following equation by using the C_{Triton} and M_w values.

$$\ln f_{Det.} = 0.63 \times \ln C_{Triton} - 3.6 \times \ln M_w \quad (3\text{-}7)$$

The operational parameters of the Triton X-100 were thus generalized as a detergent function, $f_{Det.}$.

3.3 Relationship between Rate Constant and Cell dependent Term. The effect of the cellular conditions, such as the location factor of enzymes (LF_i) and surface properties (HFS_{Cell} and LH_{Cell}) on the rate constant, k_i, was investigated in order to clarify the function of cellular conditions, $f_{Cell,i}$, depending on the cell properties such as HFS_{Cell}, LH_{Cell} and LF_i.

Effect of Location Factor of Intracellular Enzymes on the Release Rate Constants. The effect of location factor of intracellular enzyme on the rate

Table 3-1 Location factor of various enzymes

Enzyme	Location Factor, LF_i [-]
AcP(i=1)	1.62
G6PDH(i=2)	0.63
β-gal(i=G)	0.78

constant was investigated. The *E.coli* W3110 cells were disrupted by ultrasonic irradiation and the location factors, LF_i, for some enzymes were determined by using the **Eq.(3-1)**. The values are summarized in **Table 3-1**. The LF_i values of periplasmic enzyme (AcP; i=1, AlkP; i=2) indicate the value of more than 1 and those of cytoplasmic one (G6PDH; i=3, β-gal; i=G) is less than or nearly equal to 1. These values are corresponding to those described in chapter II. The treatment with Triton X-100 / EDTA has been reported to solublize the outer membrane of *E.coli* cells selectively. The release rate constants, k_i, for various enzymes are therefore expected to depend on the LF_i values. **Figure 3-4** shows the log-log plot of the release rate constants for those enzyme, k_i, against LF_i values. Experimental data are

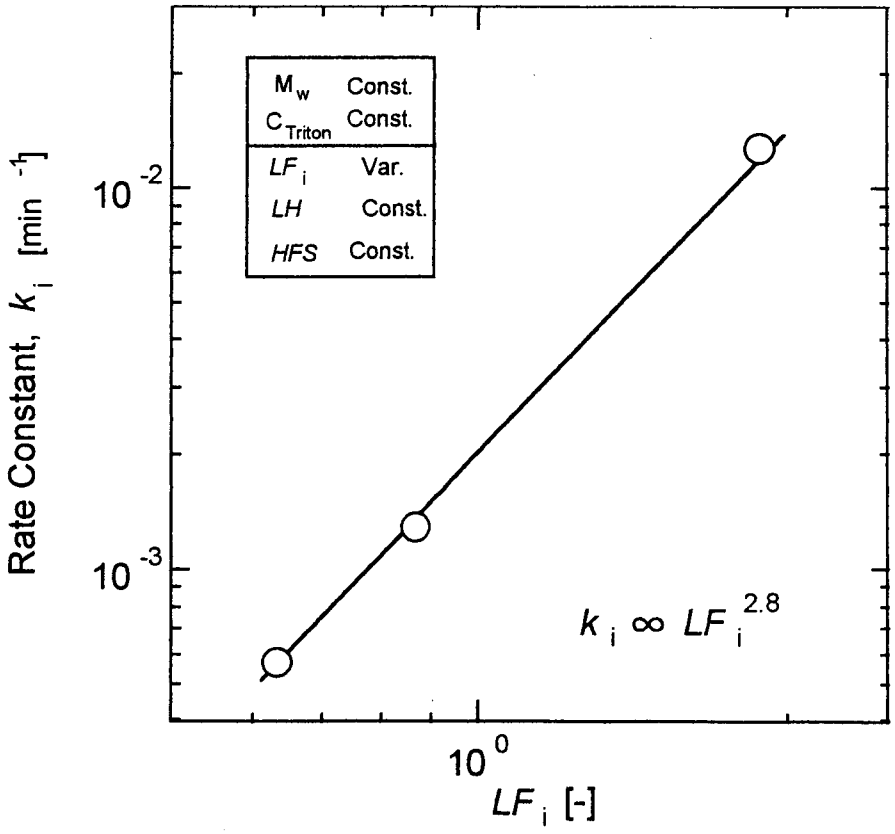


Fig.3-4 Relationship between *LF* values of enzyme and rate constant

Table 3-2 Location factor of various enzymes and surface properties of cells under the heat stress

Heat Stress for Cell	LF_G [-]	HFS_{Cell}	LH_{Cell}
Control	0.78	-435	0.11
47 °C, 10min	1.56	-407	2.19
55 °C, 10min	1.47	-251	3.09

well correlated with the straight line. The following equation can be obtained from the above plot.

$$k_i \propto LF_i^{2.8} \tag{3-8}$$

To date, there have been a lot of qualitative reports on the intracellular location of various enzymes and selective release of intracellular enzyme have been accomplished in trial and error manner by considering the enzyme location in each case. The evaluated LF_i values of enzymes can be correlated with the release rate constant and found to be effective parameters for the design of the selective release process of various enzymes using Triton treatment.

Effect of Surface Properties of Cell Membrane on the Rate Constants. The effect of surface properties of *E.coli* cells, such as surface net hydrophobicity (HFS_{Cell}) and local hydrophobicity (LH_{Cell}), on the release rate constant, k_G, of β-gal was investigated. The evaluated properties such as surface net hydro- phobicity, local hydrophobicity, and location factors for enzymes can be varied by their surrounding condition (chapter 1 and 2). For example, in chapter 1, the surface properties of cells were changed in correspondence with the heat stress condition. The location factor for each enzyme was also shown to depend on the cultivation condition of *E.coli* cells.

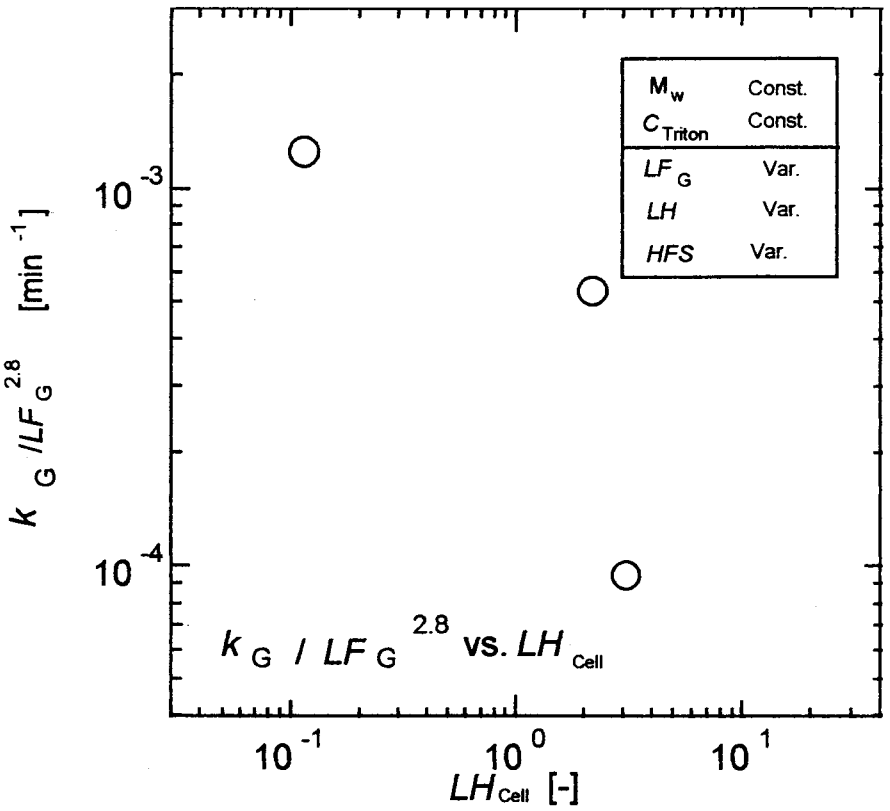


Fig.3-5 Relationship between *LH* values of cell surface and rate constant

After the *E.coli* cells were exposed to heat treatment at various temperature, their surface properties (HFS_{Cell} and LH_{Cell}) and the location factor for β-gal were determined. The values of HFS_{Cell} and LH_{Cell}, and LF_G after heat treatment at various temperature were summarized in **Table 3-2**. The release rate constant for β-gal was then normalized by using the LF_i values, which were determined in each experiment, on the basis of the **Eq.(3-8)** in order to eliminate the effect of the LF_G values. The normalized values of $k_G/LF_G^{1.5}$ of these data are plotted against the LH_{Cell} (**Fig.3-5**) and HFS_{Cell} values (**Fig.3-6**). As shown in **Fig.3-5**, the $k_G/LF_G^{1.5}$ values are scattered against the corresponding LH_{Cell} values. On the other hand, the $k_G/LF_G^{1.5}$ values are well correlated with the corresponding HFS_{Cell} values (**Fig.3-6**). The HFS_{Cell} and LH_{Cell} values indicates the average hydrophobicity and local

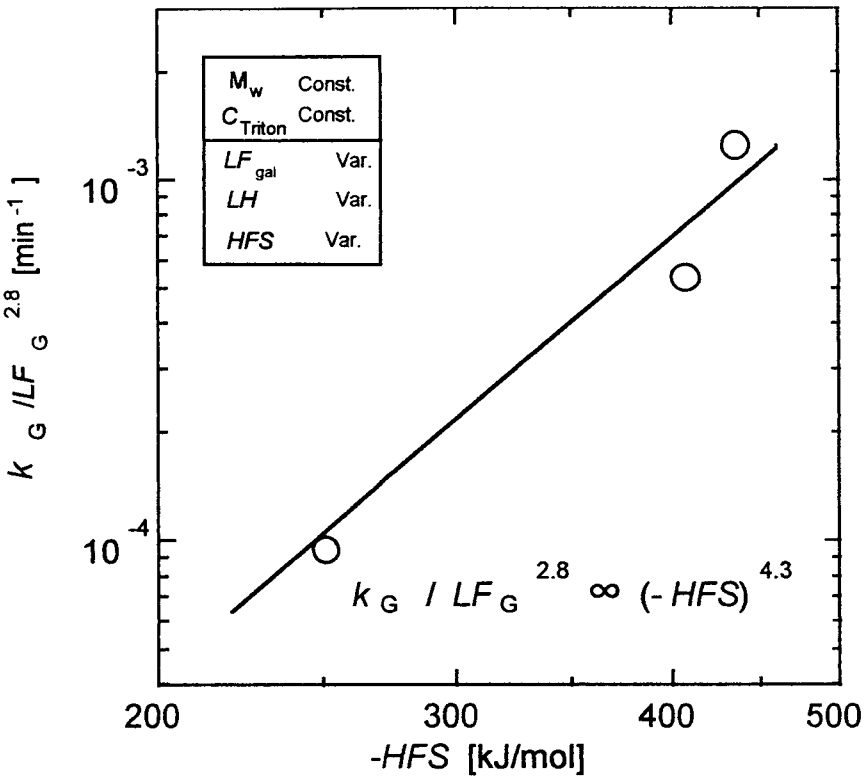


Fig.3-6 Relationship between *HFS* values of cell surface and rate constant

hydrophobicity in membrane surface of cells, respectively. The binding of Triton molecule to such local hydrophobic site, which may indicate number and kinds of membrane proteins, of cell surface is thought to be a trigger factor for solubilization of membrane surface. The HFS_{Cell} value is found to be an important factor for the enzyme release process and the following relationship can thus be obtained.

$$k_G \propto (-HFS_{Cell})^{4.3} \quad (HFS<0) \tag{3-9}$$

The Term Depending on Cellular Conditions. From the above results, the cell dependent term, $f_{Cell.}$, is represented as following equation by using the LF_i and HFS_{Cell} values on the basis of **Eq.(3-8)~(3-9)**.

$$\ln f_{Cell,i} = 4.3 \times \ln(-HFS) + 2.8 \times \ln LF_i \tag{3-10}$$

The dependence of release rate constants, k_i, on the properties of cell

membrane surfaces and the location of enzymes in the specific compartment of cells are thus shown as the above quantitative correlation. Both the surface net hydrophobicities and the location factors of enzymes are thus found to be correlated with their release rate constants.

2.4 Final Correlation for f_i and its Application All the data on the k_i values for various enzymes, determined at various conditions, are plotted against the following function, f_i, based on the **Eqs.(3-7)** and **(3-10)** as shown in **Fig.3-7**. The final correlation can be expressed as follows.

$$\ln f_i = \ln f_{Det.} + \ln f_{Cell,i}$$

$$= (0.63 \times \ln C_{Triton} - 3.6 \times \ln M_w) + (2.8 \times \ln LF_i + 4.3 \times \ln (-HFS)) \quad (3\text{-}11)$$

The release rate constant, k_i, for any enzyme from any cells can be correlated with the generalized equation.

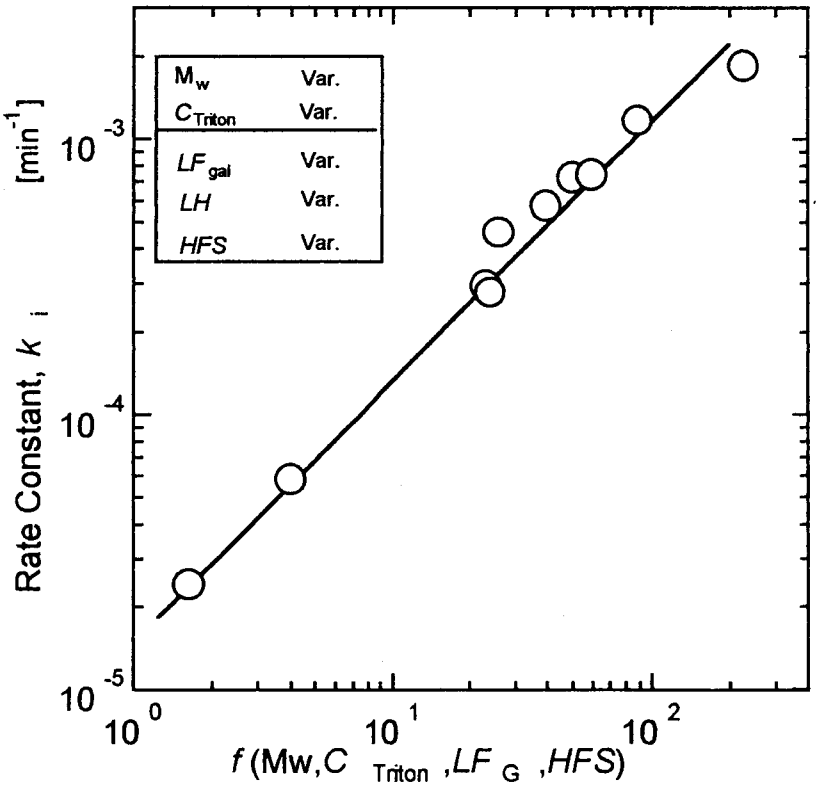


Fig.3-7 Correlation between the rate constant and the generalized function

Conventional method for the selective recovery of intracellular enzymes, the optimal condition was obtained only by trial and error method in each case. In the present method based on **Eq(3-11)**, the release rate constant of any enzyme is correlated as a simple unique function of i) operational condition of selected nonionic detergent and ii) cell properties evaluated. By taking the evaluated properties of bacterial cells into account in the above correlation, the effective design for the selective recovery process of intracellular enzyme can be achieved. For example, the conditions for the selective solublization of the outer membrane can be optimized by using the rate constants of periplasmic and cyto-plasmic marker enzymes (k_P and k_C) as shown in the scheme of **Fig.3-8**. At

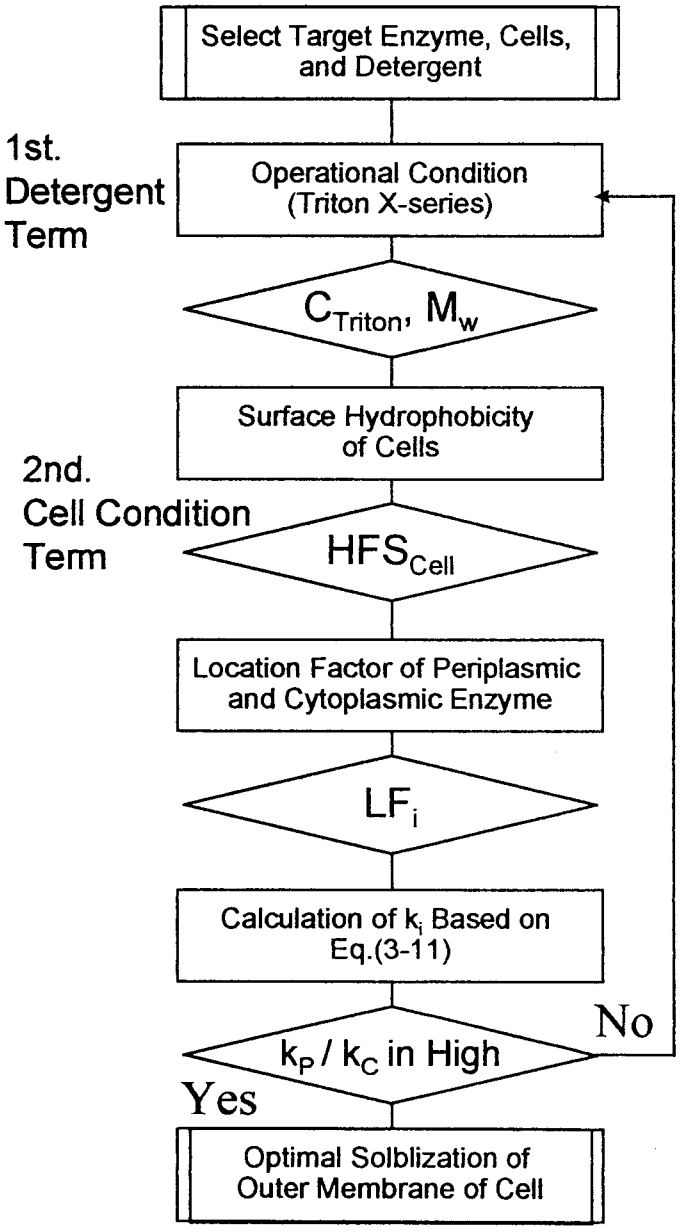


Fig.3-8 Scheme for Selective Recovery of Periplasmic Enzyme and Selective Solublization of Outer Membrane of Cells

the first stage of this scheme, the operational conditions such as concentration and molecular weight of nonionic detergent, Triton X-series, are selected. In the next stage, the surface hydrophobicity of cells and location factor of the above enzymes are determined. The k_P and k_C values can be calculated based on **Eq.(3-11)**. If the difference between the values is high enough for the periplasmic enzyme, k_P, and that for the cytoplasmic one, k_C, the optimal condition for selective release of periplasmic enzyme can be obtained. It can be concluded that the presented method based on the quantitative correlation could be novel and effective method for design of selective recovery of enzymes, accompanying with selective solublization of the outer membrane of *E.coli* cells by nonionic detergent in relation to hydrophobic interaction.

In another aspect, the scheme for the selective recovery of periplasmic enzyme can be used as the characterization of inner membrane of cells by combining with the method described in chapter 1. The controlled release of periplasmic one is thought to be corresponding to the solublization of the outer membrane of cells. The surface properties of *E.coli* cells, which were treated by 10mM Triton X-100 for 30min and 1hr, were determined by

Table 3-3 Surface Properties of Bacterial Cells Treated by Triton X-100 or Lysozyme in the presence of EDTA

Treatment	HFS_{Cell}[kJ/mol]	LH_{Cell}[-]
Control	-435	0.11
10mM Triton X-100, 30min	-420	0.18
10mM Triton X-100, 60min	-410	0.21
1mg/ml lysozyme, 30min	-412	0.19

using aqueous two-phase systems and were summarized in **Table 3-3**. The values of both surface hydrophobicity and local hydrophobicity of the whole and spheroplasted cells are slightly increased and are saturated after 1hr. The behaviors of this change are well corresponding to the release behaviors of enzymes and also to those treated by lysozyme described in chapter 4. The difference of cells before and after the treatment is thought to reflect solublization of the outer membrane. In this way, it has been shown that the surface hydrophobicity of the inner membrane can also be measured by solublizing the outer membrane of cells. The variation of surface hydrophobicity of the inner membrane caused by heat stress is discussed in chapter 4 in relation to heat induced translocation of β-gal across the inner membrane.

4. SUMMARY

The enzyme release process treated with nonionic detergent, Triton X-100, was quantitatively characterized as an example of chemical stress based on the cellular conditions evaluated in chapter 1 and 2.

(1) The enzyme release process treated with Triton X-100 / EDTA could be analysed by the same kinetic analysis in chapter 2. The release rate constant of enzyme was found to be highly dependent on the surface hydrophobicity of cells and location factor of intracellular enzymes.

(2) On the basis of the generalized equation about the enzyme release by using Triton X-100, the selective release of periplasmic enzymes and selective solublization of outer membrane of *E.coli* cells were found to be achieved by considering the cellular conditions, such as surface

hydrophobicities of cells and location factor of intracellular enzymes.

(3) The possibility to analyse the surface hydrophobicity of inner membrane of *E.coli* cells was shown by using both selective solublization method of outer membrane and the characterization method of cell surface using aqueous two-phase systems described in chapter 1. Such quantitative information of the inner membrane surface of cells may contribute to the stress-response functions of cells.

Chapter 4
Evaluation, Analysis and Control of Stress Response Functions of Bacterial Cells Induced by Combined Physicochemical Stress

1. INTRODUCTION

It is a common statement that heat stress impairs cellular functions because some essential proteins are heat-denatured. The heat shock or stress response systems have been universally conserved among microorganisms (Morimoto *et al.*, 1990, Georgopoulus *et al.*, 1990, Gross *et al.*, 1990) and are important adaptation systems for survival of microorganisms at high temperature or under stressed conditions. When the gram-negative bacterium, *E.coli*, is treated with a heat shock, the cell responds by transiently and rapidly inducing various functions of cells, mainly, (i) synthesis of a set of heat shock proteins (HSPs) (Ellis and Van der Vies, 1976) and refolding of damaged proteins (Ptysin, 1986; Ayling and Baneyx, 1996), (ii) formation of inactive protein-aggregates in an inert insoluble form (*e.g.* inclusion body) (Di Domenco *et al.*, 1989), (iii) protein degradation by some HSPs (proteases) (Goldberg and Dice, 1974), (iv) translocation (transmembrane, transport) of proteins (Bychkova *et al.*, 1988). However, the mechanism behind the stress response of bacterial cells is not thoroughly understood because of the lack of unified quantitative information about cell functions and related properties from microscopic to macroscopic view points.

In this chapter, stress response of *E.coli* cells against sublethal stress, especially heat stress, was studied in relation to the hydrophobicity of the bacterial cell surface and that of the intracellular target protein from both macroscopic (cell-basis) and microscopic (molecular-basis) view points,

based on the results obtained in chapter 1, 2, and 3. The quantitative relationship between 'heat stress' and 'various stress response functions of cells' (as represented by several pathways of **Fig.4-1**), such as protein folding (A), translocation (B), aggregates formation (C), and degradation by protease(D), was firstly investigated by using the quantitative parameters. Especially, protein translocation across the inner membrane of *E.coli* cells, induced by heat stress, was shown in this chapter to be triggered by the hydrophobic interaction between partly damaged cytoplasmic enzyme, β-galactosidase (β-gal), and the membrane surface in *Escherichia coli* cells.

In this case study, the purpose is set to design and develop the effective bioprocesses exploiting the above stress response functions of cells for the production and separation of typical cytoplasmic protein, β-gal, as a target. The stress-response functions of bacterial cells such as control of (1) protein-aggregates formation and (2) translocation of β-gal through cell membrane have been firstly investigated. The control mechanism of their

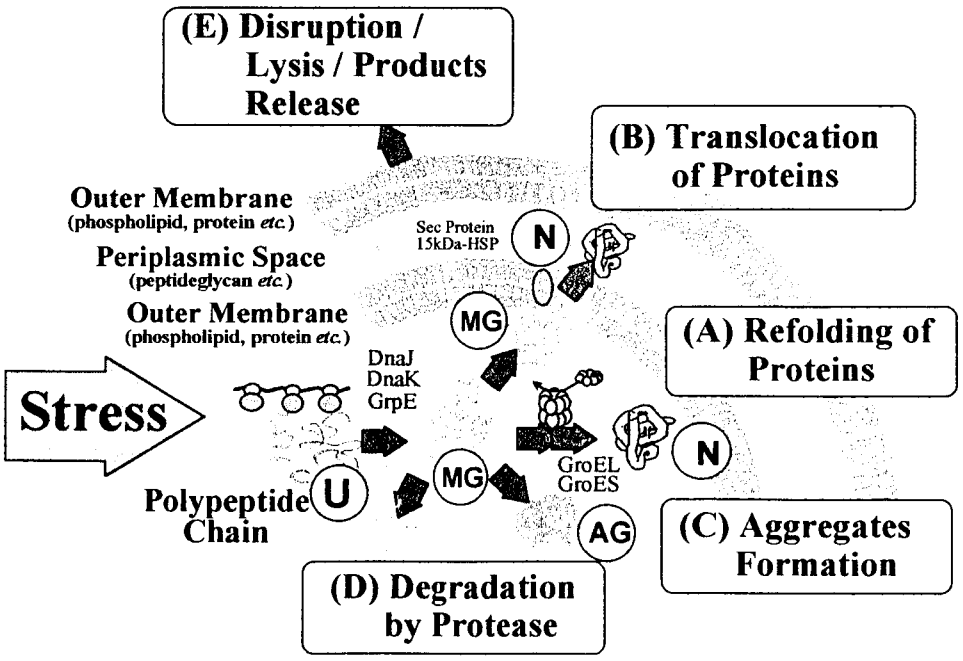


Fig.4-1 Possible pathways of cell-response against environmental stress

functions assisted by the stress proteins (HSPs) were then discussed from microscopic (molecular basis) and/or macroscopic (cell basis) view points.

2.EXPERIMENTAL

2.1 Material. β-Galactosidase (β-gal; from *Escherichia coli* ; E.C. 3.2.1.23) and o–nitrophenyl-β-*D*-galacto pyranoside (ONPG) of the substrate were purchased from Sigma (New York, USA). Poly (ethylene glycol) (PEG1540, 4000 and 6000; M_W = 1.5 kD, 3 kD and 7kD, respectively) and dextran 100~200k (Dex; M_w = 100~200 kD) were obtained from Wako Pure Chemicals Ltd. (Osaka, Japan). Nonionic detergent, Triton X-100 (M_W = 0.65 kD), were purchased from Sigma. Lysozyme (from egg white, E.C. 3.1.1.2) was purchased from Wako. Casamino acid was purchased from Difco Laboratory (Michigan, USA). Isopropyl-β-D-thiogalactopyranoside (IPTG), which is a inducer of β-gal, was purchased from Wako. Ethylene diaminetetraacetate (EDTA) was obtained from Wako. The salts and other chemicals of analytical grade were from Wako.

2.2 Cultivation of Cells and Heat Stress Treatment. The strain used in this study was mainly *E.coli* W3110. The HSPs-defective strains of *E.coli* (*E.coli* SKB178 *gal*E *sup* +*groEL*(Ts) *or* + *groES*(Ts), Georgopolos *et al*, 1973) were also used in this study. The basic media used were modified M9 media (pH 7.4, in g/L): Na_2HPO_4, 7.0; KH_2PO_4, 3.0; NaCl, 5.0; NH_4Cl, 1.0; casamino acid, 1.0; $MgSO_4$, 0.75; glycerol 50. A solution of trace elements (Gray *et al.*, 1973) was also added (1mL/L-media). Stock culture was maintained on 1.5% agar slant of the modified M9 media. Following overnight growth at 30°C in a 100ml flask (130rpm) with a working volume of 20ml, the culture was used as an inoculum for a 300ml shaking flask using nor-

mally 100ml media. Intracellular β-gal was then induced by the addition of 2mM IPTG. The cells were harvested in the late log phase (OD_{660}=1.5~2.0) by centrifugation (4°C, 10000rpm, 10min). After washing by Tris-HCl buffer (50mM, pH 8.0), the cells were resuspended in the buffer or the M9 media. The cell suspension (C_{Cell} =10mg/ml) were prepared and the stored at 0°C for 30min. The cell suspension was heated from 0°C to 55°C or to other temperature in the solution.

2.3 Fractionation of Cellular Components and Solublization of Protein Aggregates. At first, 1 ml portion of the above cell suspension treated with heat stress were irradiated for 3 min by using ultrasonic disrupter UD-200 (Tomy Seiko Co., Ltd.; 20kHz; input power, 40W). The disruption was then operated for 30 s periods separated by 60-s intervals in the ice bath. The solution was centrifuged at 15,000 × *g* for 15min to separate the protein aggregates and other insoluble material from the soluble one. The supernatant was recovered as a soluble-protein fraction, [SP], and the other pellet was resuspended in 0.5 ml buffer I (50mM Tris-buffer, 2wt% Triton X-100 and 5wt% EDTA; pH 7.5) after washing the pellet. After 1-hr incubation at 37°C, the sample was centrifuged at 15,000 × *g* for 15min. The pellet was once washed by 50mM Tris-HCl buffer and was solublized by buffer II (50mM Tris-buffer, 1wt% SDS; pH 7.5) at 37°C for 1-hr and recovered as aggregate-protein fraction, [AP].

2.4 Intracellular Location of Enzymes. Subcellular distribution of β-gal was determined by the following two methods.

(1) Lysozyme/EDTA Treatment. Activity of β-gal in periplasm was determined by solublizing the outer membrane of *E.coli* cells. The cells suspension were added to 5M sucrose solution containing 5% ethylenediami-

netetraacetate (EDTA) for 5min at 0°C and then the lysozyme (final concentration; 0.01mg/mL) was added to the solution. The spheroplast formation of *E.coli* cells was confirmed both by observing the morphological change of the cells using optical microscopy and by analyzing the release behaviors of cytoplasmic and periplasmic enzymes (Chapter 3). After centrifugation (8000rpm, 5min), the resultant supernatants were analysed as periplasmic fraction, [PP]. The pellet was resuspended in 50mM Tris buffer and was further disrupted by ultrasonic method at the above condition. The β-gal activity of the supernatant was measured as total activity of β-gal. The percentage of periplasmic β-gal was determined from the ratio between the periplasmic activity and the total one.

(2) Location Factor Using Enzyme Release Rate. The distribution of intracellular protein was also evaluated from the kinetic analysis of release process of the enzyme and total soluble proteins (Chapter 2), followed by cell disruption process. The cells were then disrupted in a 1ml eppen tube by ultrasonic method in the 1ml Tris buffer at the input power of 40W for 0~5min. The productivity of total soluble proteins was determined as their maximum released amount per unit weight of cells. The resultant supernatants recovered after various disruption times were utilized for characterization of intracellular distribution. For the disruption process of cells and the release process of intracellular proteins, the relationship between the released fraction of intracellular soluble protein (RF_i) and total input energy (q) can be obtained in the following equation.

$$\ln (1-RF_i)^{-1} = \ln (1-R_i/R_{i,max})^{-1} = K_i q \qquad (4\text{-}1)$$

where R_i ($R_{i,max}$) is (maximum) released amount of intracellular proteins per unit cell weight and i indicates either the total soluble proteins or each spe-

cific protein. The ratio of release rate constant of target product over that of total proteins, K_i/K_T, indicate the intracellular location of enzymes (described in Chapter 2) and can be defined as location factor of enzyme i, LF_i.

2.5 Surface Properties of Bacterial Cells and Spheroplast. The surface properties such as surface net hydrophobicity (*HFS*) and local hydrophobicity (LH_{Cell}) of *E.coli* cells and spheroplasted ones (treated by lysozyme / EDTA) were analysed by using the aqueous two-phase partitioning method (Chapter I). When pH is then selected at the pI at lower ionic strength, the partition coefficient of cells, K_{Cell}, was mainly dependent on the hydrophobic effect and the following relationship can be obtainable.

$$\ln K_{Cell} = HFS \times HF \qquad (4\text{-}2)$$

where hydrophobic factor (*HF*), which have been defined from the partition coefficient of amino acids, is an indicator of the hydrophobicity of two-phase systems and the *HFS* values can be defined as net hydrophobicity of cell surface. The saturated values of $K_{Cell,\,Triton}$ values in the presence of Triton X-100 can be determined as an indicator of local hydrophobicity of cell surface. The LH_{Cell} ($=\ln K_{Cell,Triton}/K_{Cell,\,0}$) values can herewith be defined as the increment of the partitioning coefficient of cells in the presence ($K_{Cell,Triton}$) and absence of 1mM Triton X-100 ($K_{Cell,\,0}$). By using the same methodology, the values of *HFS* and *LH* of β-gal were also determined.

2.6 Measurement. The activity of β-gal was determined from the initial rate of hydrolysis reaction of ONPG. The concentration of total soluble proteins was determined by Bradford method (Bradford, 1975). The various proteins in fractionated samples were separated by SDS-polyacrylamidogel electrophoresis (SDS-PAGE) using PhastGel System (Pharmacia, Uppsala, Sweden) for analysis. The gel (PhastGel Gradient 3-25, Pharmacia) was stained

by coommassie brilliant blue (CBB) and the content of HSPs (GroEL and GroES) in the above sample was determined from the densitometry analysis of CBB-stained gel.

3. RESULTS AND DISCUSSION

3.1 Typical Response of Intracellular Proteins in *Escherichia coli* cells after Heat Treatment. The behaviors of heat stress-response of *E.coli* cells were investigated. After the cells were stressed at various heat-condition, the percentage of each fraction such as soluble proteins (SP) and protein aggregates (AP) over the protein weight per dry weight of control no-stress cells was determined and the remained percentage was defined as the amounts of degraded proteins (DP). **Figure 4-2** shows the effect of (a) heating time and (b) temperature on the percentage of SP, AP and DP fractions. The amounts of SP are decreased and AP and DP fractions are increased with increasing the heating time at the temperature of 55°C (**Fig.4-2(a)**). Then, the values are saturated in a short-time heating (1~10min). The SP and AP fractions are respectively decreased and increased especially when temperature is increased to more than 55°C (**Fig.4-2(b)**).

Heat stress has been reported to induces the conformational change of intracellular proteins and cells then respond by transiently inducing various functions of cells, such as (A) the synthesis of a small set of heat shock proteins (HSPs; GroEL, GroES) to refold heat-denatured proteins (Ellis and van der Vies., 1976), (B) the formation of insoluble protein-aggregates (Di Domenico *et al.*, 1982), (C) the protein degradation by protease (Goldberg and Dice, 1974), (D) the translocation of proteins across the inner membrane (Yatvin *et al.*, 1986; Yatvin, 1987; Kitagawa *et al.*, personal communication) and so on. In this experimental range, the paths (B) and (C) are

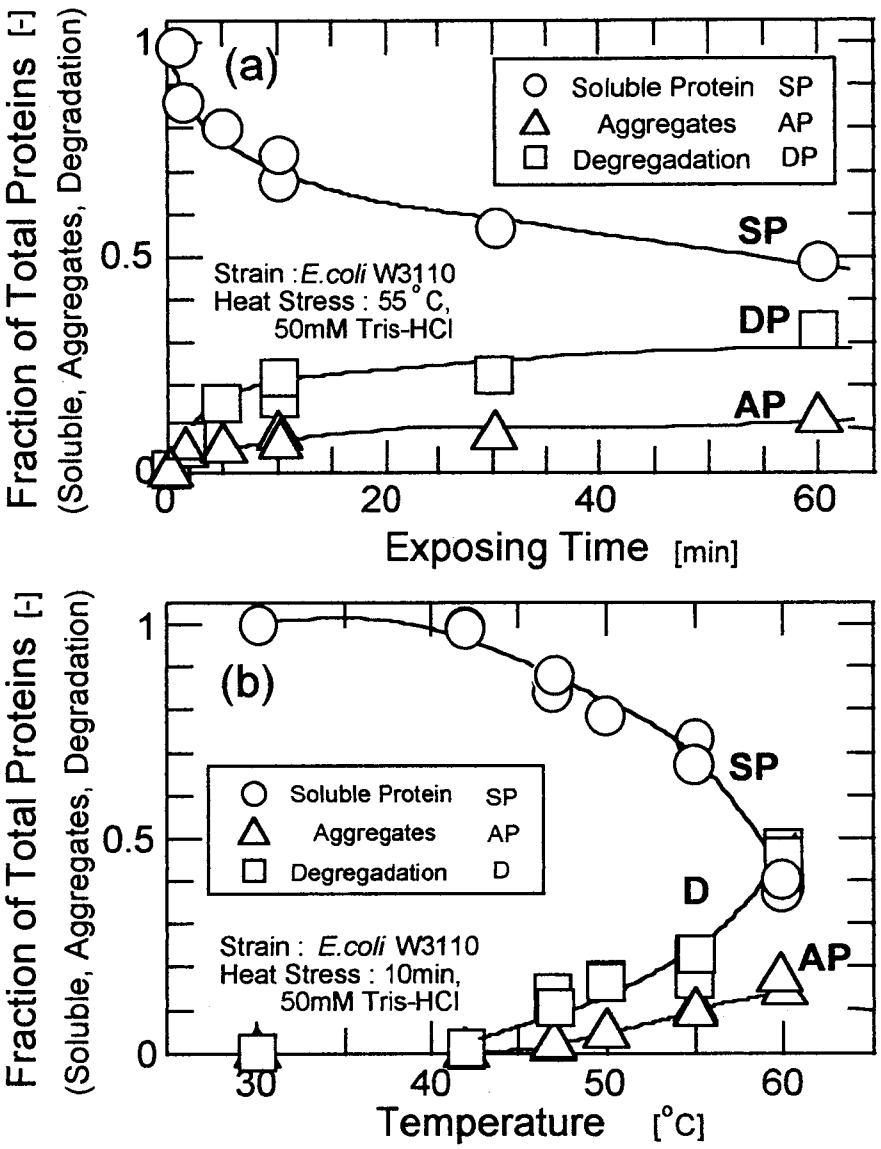


Fig.4-2 Effect of (a)Heating Time and (b) Temperature on Fraction of soluble proteins (SP), protein aggregates (AP), and degradation of proteins by protease (D)

observed especially at higher temperature (>55°C). The CBB-stained images of SDS-PAGE gel of SP and AP fractions of cells heated at various temperature (47, 55 and 60°C) are also shown in **Figure 4-3**, together with that of PP fractions after the lysozyme/EDTA treatment in a separate experiment. The amounts of β-gal which is a kind of cytoplasmic enzyme are increased

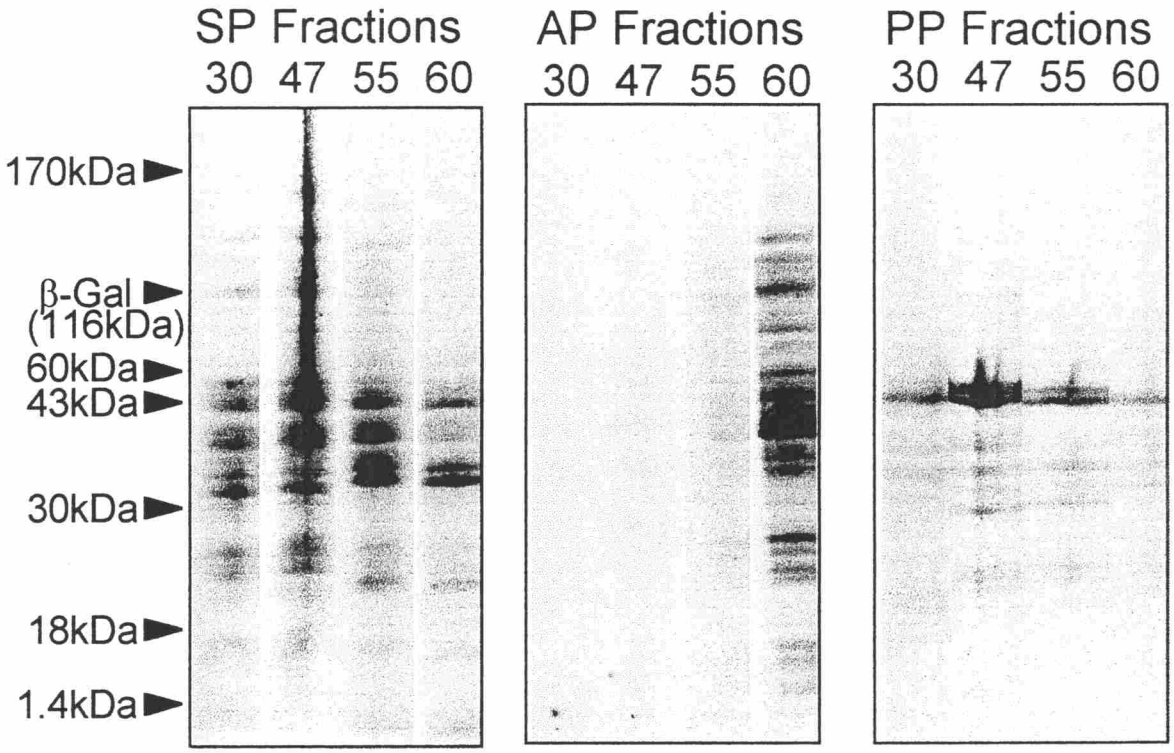


Fig.4-3 CBB-stained SDS-PAGE gel showing the protein contents of (a) soluble, (b)aggregate and (c) periplasm fraction at various temperature.

in AP fractions and are decreased in SP fraction with increasing the heating temperature. The cytoplasmic β-gal is also detected in PP fraction and suggested to be translocated to periplasm by exposing cells to heat treatment. In the following, the responded behaviors of *E.coli*, especially, the behaviors of protein translocation are investigated by using cytoplasmic β-gal as a reporter enzyme.

3.2 Effects of Heating Time and Temperature on Location Factor of Cytoplasmic β-Galactosidase. It has been reported that the LF_i values are well corresponding to the subcellular distribution of intracellular soluble proteins and, especially, that of periplasmic enzyme is more than 1.0 (described in chapter II). The effect of heating time at 55°C on the subcellular location

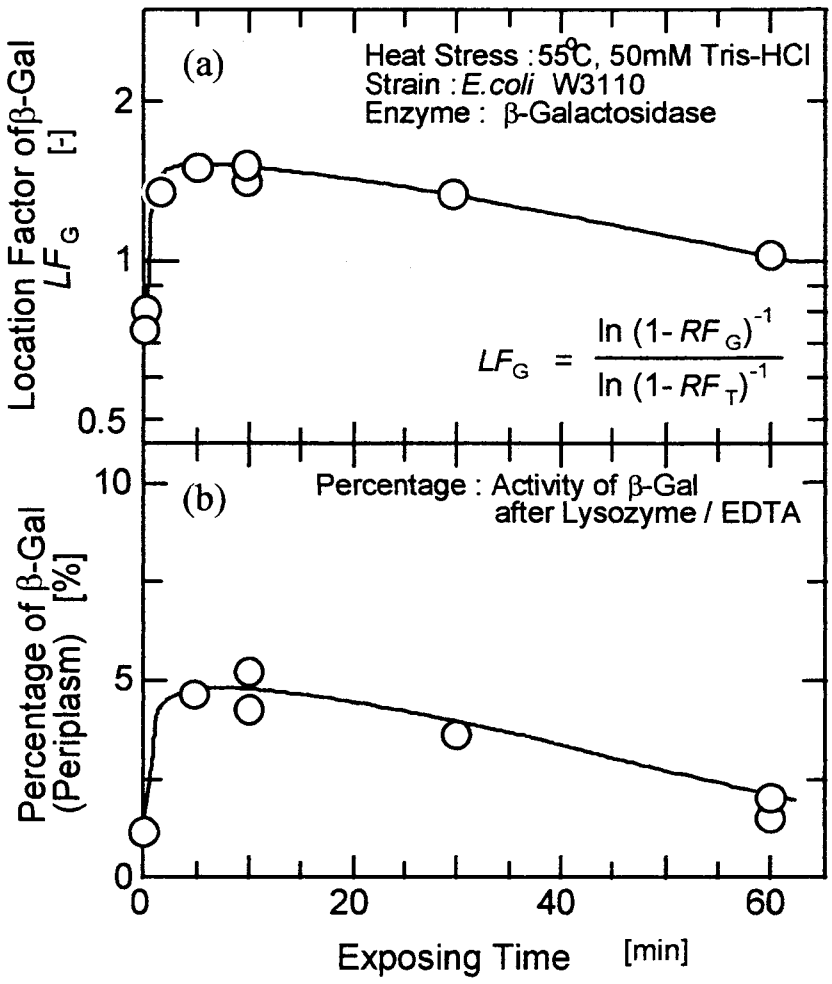


Fig.4-4 Effect of Heating time on the location factor and the percentage of periplasmic activity of β-gal

of β-gal was investigated by using LF_G values, which could be determined by **Eq.(4-2)** as a measure of the location (**Fig.4-4**). **Figure 4-4(a)** shows the relationship between the heating time and the LF_G values. The LF_G values dramatically increase from 0.78 to 1.5 by heat treatment for 1-10min and, then, decrease with increasing time. The activity of β-gal in periplasm was also analysed by the conventional method using lysozyme / EDTA. **Figure 4-4(b)** shows the time course of percentage of periplasmic activity of β-gal. The values are well corresponding to the LF_G values and the same results can be obtainable. 5% of total cytoplasmic β-gal was translocated to perip-

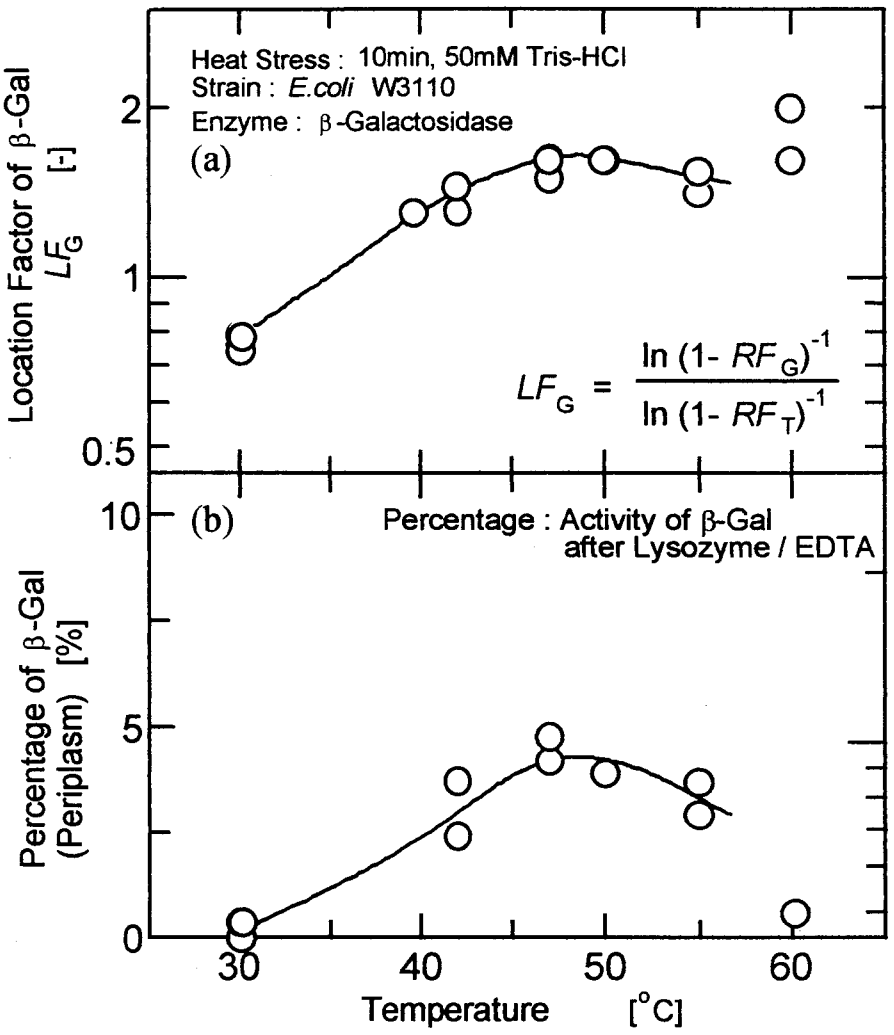


Fig.4-5 Effect of Heating temperature on the location factor and the percentage of periplasmic activity of β-gal

lasm and the heat-exposing to cells for 1-10min are found to be optimal for the increase in the LF_G values, that is, the translocation.

The effect of temperature on the translocation of cytoplasmic β-gal was further investigated. *E.coli* cells were exposed to heat stress for 10min at various temperature and then the LF_G values in each cell stressed were determined. **Figure 4-5(a)** shows the dependence of LF_G values on temperature. The LF_G values are increased with increasing temperature at less than 47~50°C and are slightly decreases at more than 50°C. The percentage of

periplasmic activity of β-gal was also analysed after lysozyme/EDTA treatment and was shown in **Fig.4-5(b)**. The same behaviors can also be obtainable. The percentage of β-gal translocated to periplasm is then maximum at the temperature of 47~50°C. It has been reported that the heat stress induce the translocation of non signal sequence protein (Yatvin *et al.*, 1986, Yatvin, 1987) in *E.coli* cells. Recently, Kitagawa *et al.* (private communication) have reported that the translocation of cytoplasmic β-gal is induced by exposing *E.coli* cells to heat treatment and is maximal at 45°C. The obtainable phenomena on the translocation of cytoplasmic β-gal are well corresponding to their results.

In the translocation of β-gal across inner membrane, there are thought to underlie several phenomena in relation to the protein-lipid interaction. Among of them, the change in physicochemical properties of cellular biopolymers, such as (a) the change in surface hydrophobicity of inner membrane of *E.coli* cells, (b) the conformational change (denaturation) of β-gal, and (c) restabilization of denatured proteins by GroE proteins, are thought to be a main factor affecting the translocation process. In the following, the surface properties of cell membrane and the conformational change of target β-gal after heat treatment and the effect of GroE protein on the translocation were investigated.

3.3 Change in Surface Hydrophobicity of Outer and Inner Membrane of *E.coli* Cells after Heat Stress. Effect of heat stress on the surface hydrophobicity of *E.coli* membrane was firstly investigated by using the aqueous two-phase partitioning method (chapter 1). After the cells were exposed to heat stress at various temperature, the surface properties, such as surface net hydrophobicity (HFS_{Cell}) of the spheroplasted cells (treated by ly-

sozyme/EDTA) were analysed. Their surface properties of the spheroplasts are shown in **Fig.4-6** as a function of the stressing temperature, together with those of whole cells. The HFS_{Cell} values of the spheroplasts are significantly increased at the temperature of more than 42°C although those of whole cells are gradually increased with increasing temperature and are the highest at 60°C.

The values of HFS_{Cell} are reported to be caused by the contribution of the composition of phospholipid and hydrophobic membrane proteins on the cell surface, respectively (described in chapter 1). Other results in our laboratory show that the membrane surface hydrophobicity of small unilamella vesicle (SUV) prepared by palmytoyloleilphosphochorine (POPC) is increased with increasing temperature and then the membrane fluidity is also increased (data not shown). It is found that the surface properties of the outer and the inner membrane is independently changed and, especially, the increase in the surface hydrophobicity at the inner membrane of cells at rela-

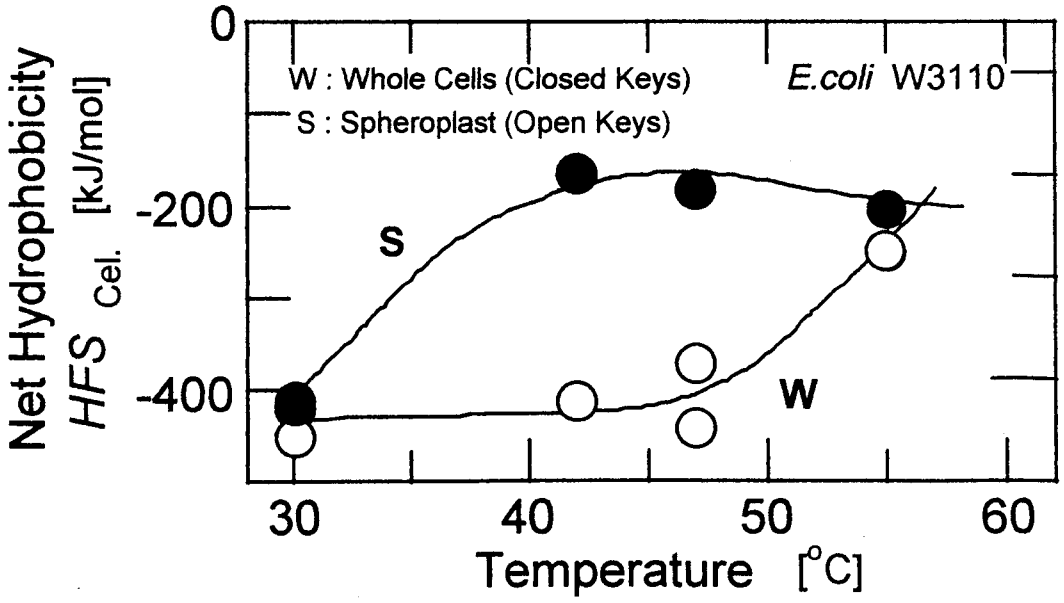


Fig.4-6 Effect of heating temperature on the surface net hydrophobicity (*HFS*) of whole and spheroplasted cells

tively lower heat-stress (42 and 55°C) are well corresponding to the translocation phenomena (**Fig.4-5**). Typically, it has been reported that the membrane fluidity could be the critical factor in the outer membrane and periplasmic proteins reaching their final compartment (Ito *et al.*, 1977). Here, one possible explanation for the triggering event of translocation of cytoplasmic β-gal is thought to be a heat induced disordering of the lipid membrane.

3.4 Conformational Change of β-Gal Induced by Heat Treatment. The behaviors of thermal inactivation of β-gal were investigated. The percentage of β-gal activity remained after heat treatment was plotted against the heating time in **Fig.4-7**. In general, the β-gal activity decreases with increasing heating-time. The inactivation rate of *in vitro* β-gal is then greater than that of *in vivo* β-gal. It is possible that the heat treatment induce conformational change of β-gal.

The conformational change of β-gal after heat treatment was analysed *in vitro* by using this method and the results were summarized in **Table 4-1**. The *HFS* values, defined as the surface net hydrophobicity of the β-gal, are

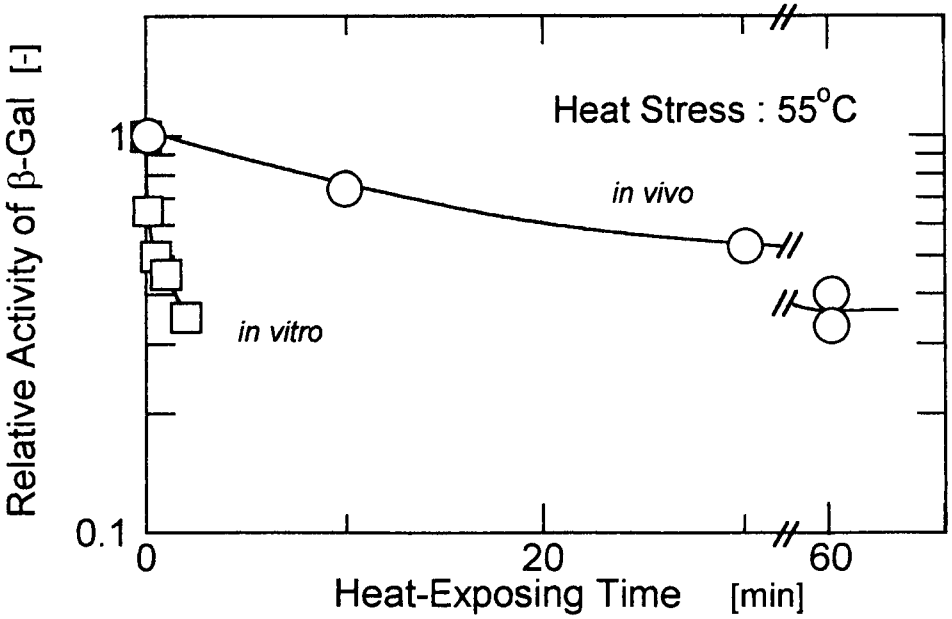


Fig.4-7 Time course of the relative activity of β-gal under the heat stress

Table 4-1 Surface properties of β-gal under the heat stress

Kinds of Stress	HFS_G[kJ/mol]	LH_G[-]	λ_{max}[nm]
Control	-450	0.78	339
50oC, 1hr	-17.7	1.27	339
60oC, 1hr	96.2	0.27	339

increased with increasing the temperature. The LH_G values, defined as a local hydrophobicity, are maximum at relatively lower temperature (50°C). On the other hand, the λ_{max} values, which is an typical indicator of conformational change of β-gal (Ayling, A. and Baneyx, F., 1996), are not changed after heat treatment. These results indicates that heat-stress at relatively lower temperature may induce the only limited conformational change of oligomeric β-gal and transform the structure to partially dissociated and/or unfolded structure having strong local hydrophobic site. Bychkova *et al.* (1988) have recently discussed that the conformational change of protein from native state to the partially-folded state is required in the protein translocation process. The local hydrophobicity of monomer protein carbonic anhydrase from bovine (CAB) has been reported to be increased sharply when the conformation is transformed to the partially-folded state (Kuboi *et al.*, 1993). The change in local hydrophobicity (LH_G) of β-gal is well corresponding to the previous findings although it is an oligomeric protein. The enhancement of hydrophobic interaction between the partially unfolded protein and lipid, followed by the heat-induced conformational change, is thus thought to be the trigger of the β-gal translocation. **Figure 4-8** shows the relationship between the local hydrophobicity of β-gal, LH_G, and the corresponding location factor, LF_G under the heat stress. The

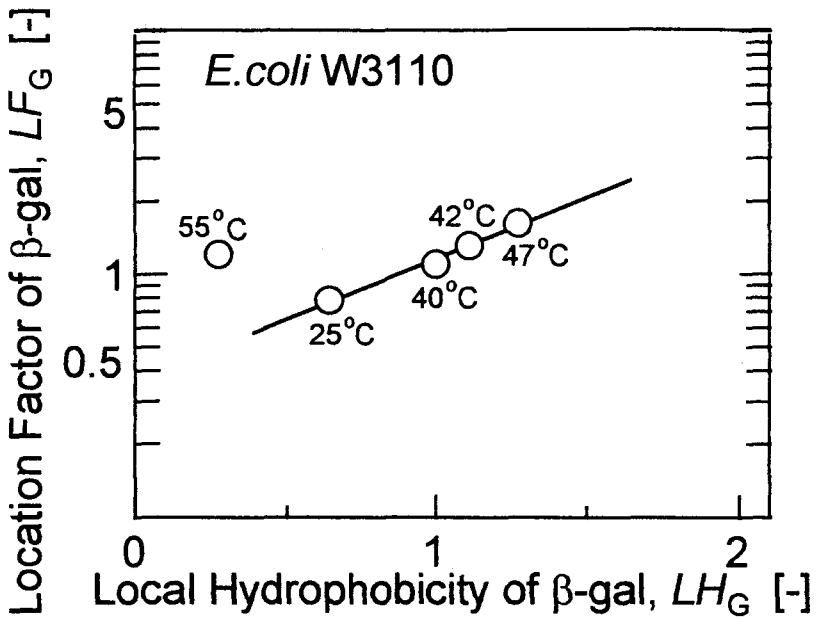


Fig.4-8 Relationship between local hydrophobicity of β-gal, LH_G, and their location factor, LF_G, under the heat stress

translocation phenomena is herewith found to depend on the conformational change of β-gal under the heat stress. This concept is also supported by the result that the heat stress (42~47°C) induces the *in vitro* translocation of β-gal across the POPC membrane of SUV as described in chapter 5.

3.5 Inhibition of β-Gal Translocation by Induction of HSPs. The effects of the induced quantity of GroEL on translocation of β-gal are investigated. **Figure 4-9** shows the dependence of i) quantity of GroEL in SP fraction and ii) surface hydrophobicity, *HFS*, of spheroplasts on the corresponding LF_G values in the stressed *E.coli* cells. In general, the LF_G values increase when the induced quantity of GroEL is relatively low and when the surface hydrophobicity of inner membrane indicates the higher values.

In order to clarify the inhibitory effect of GroEL, the location factor of β-gal, LF_G, before and after the heat treatment of the cells at 47°C for 10min was investigated in the GroES- or GroEL-defective *E.coli* cells. The LF_G

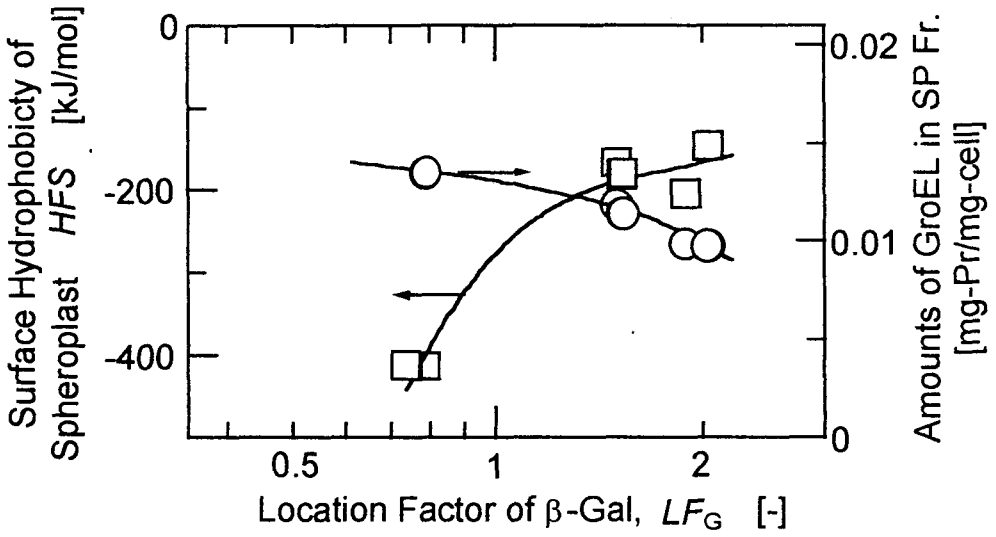


Fig.4-9 Relationship between location factor of β-gal and corresponding the *HFS* values of inner membrane of *E.coli* cells and the induced amounts of GroEL

values are shown in **Fig.4-10**, together with the amounts of AP fraction and the SP fraction of intracellular total proteins. **Figure 4-10(a)** shows the control values of each strains without heat stressing. As shown in **Fig.4-10(b)**, the LF_G values in the HSPs-defective strains are greater than that in wild-type strains. The amounts of protein-aggregates in the GroES-defective strain are the largest. The amounts of soluble proteins in GroES-defective strain are greater than those in other strains. The β-gal translocation is thus found to depend weakly on the induced amounts of GroEL and strongly on the hydrophobicity of inner membrane.

GroE proteins such as GroEL and GroES which are induced by heat stress have been reported to facilitate the assembly of oligomeric proteins (Gouloubinoff, 1988). It has been recently reported that the GroEL interacted with the folding-intermediate and the refolding was inhibited in the presence of GroES and ATP in the *in vitro* refolding processes of guanidine-hydrochloride denatured β-gal (Ayling and Baneyx, 1996). The

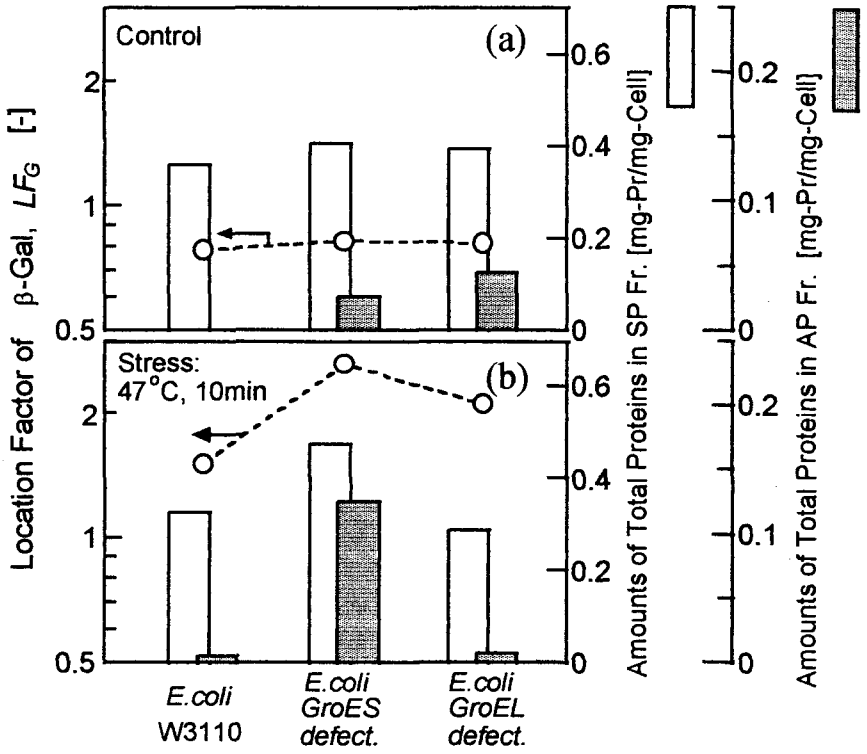


Fig.4-10 Location factor of β-gal, the amounts of total proteins SP and AP fractions in HSPs defective *E.coli* cells

hsp60, including GroEL, has been reported to show following two-activities : 1) enhancement of protein folding and 2) maintaining proteins at the unfolded or partially refolded state to facilitate their channeling through the machineries for the transport across the inner membrane of mitochondria (Koll *et al.*, 1992). The above results postulated the heat-induced translocation model of β-gal which includes thermal denaturation and partial conformational change of β-gal, increase in membrane fluidity and hydrophobicity of inner membrane of cells, enhancement of hydrophobic interaction between lipid and the stressed β-gal, and inhibitory stabilization of the protein with HSPs (GroE proteins) induced by heat stress.

3.6 Combined Effects of Other Stresses under the Heat Stress. The combined effects caused by the addition of salts on the stress-response of

Table 4-2 Effect of addition of salts on location factor of β-gal under the heat stress (55°C, 60min)

Solution Types	Location Factor, LF_G
Control (50mM Tris-HCl)	1.02
M9 media	2.07
M9 media + 10wt% KPi	2.43
M9 media + 20wt% KPi	2.23

bacterial cells can not be neglected because cells have been also reported to respond against such osmotic stresses in the same way, for example, by producing a kind of heat shock proteins (Meury and Kohiyama, 1991). The location factor of β-gal, LF_G, in *E.coli* cells was examined after the cells were exposed to heat stress (55°C, 60min) in i) M9 media and ii) potassium phosphate (KPi) solutions of various concentrations. The results are summarized in **Table 4-2**. The values of LF_G are increased in the M9 solution even though the cells have been exposed to relatively strong heat stress (55°C, 60min). This is mainly due to the presence of the nutrition salts in the media. In the case of the addition of 10wt% KPi salts, the LF_G value is also increased. In the presence of KPi at higher salt concentration (20wt%), the LF_G value is still improved (although it is less than that in the case of 10wt% KPi solution). These results of the further enhancement of heat-induced translocation of β-gal in the presence of KPi salts or nutrition imply that the cells may induce the enhanced response against the combined heat and osmotic stresses. It suggests that the translocation curves, shown in **Fig.4-4,** shift upwards by the combined effects of nutrition and the additional KPi salts under the heat stress conditions, *ex.* Stress induced translocation of β-gal can be improved and controlled by the addition of salts (osmotic stress).

3.7 Process Design for Effective and Selective Recovery of Intracellular β-Gal by Using Its Heat-Induced Translocation. In this chapter, the possibility of selective release and recovery of the cytoplasmic β-gal from *E.coli* cells has been presented based on the results of the stress-response function of cells (*i.e.* stress induced translocation of the partly damaged protein.). In chapter 2, the design scheme for the selective release of intracellular enzymes has been reported by using LF_i ($=K_i/K_T$) as a design parameter. In the case of periplasmic enzymes ($K_i/K_T>>1$), it has been shown that they can be selectively recovered not only by mechanical stresses (chapter 2) but also by the chemical stress by solublizing the outer membrane of *E.coli* cells in chapter 3.

As a conclusion, the scheme for the optimization of selective release and recovery of intracellular proteins by using stress-response function of cells can be shown in **Fig.4-11**. The stress-response function of cells can be utilized even if the target protein is neither secretary nor periplasmic and existing in cytoplasm by using ΔLH as a design parameter (**Table 4-1**, **Fig.4-8**). Here, if the increment of local hydrophobicity (ΔLH) of the target protein is high enough under the stress (heat) condition, the target cytoplasmic protein can be translocated into the periplasmic space accross the inner menbrane by using stress-response function of cells and can be selectively recovered by using the selective disruption method (Chapter 2) or selective solublization method of the outer membrane of cells (Chapter 3).

In practice, the stress condition for selective recovery of (1) β-gal and (2) HSPs (GroEL and GroES) from *E.coli* cells can be optimized followed by this scheme. At the normal condition for cultivation of cells (at lower temperature of 30°C), both β-gal and HSPs are existing in cytoplasm of

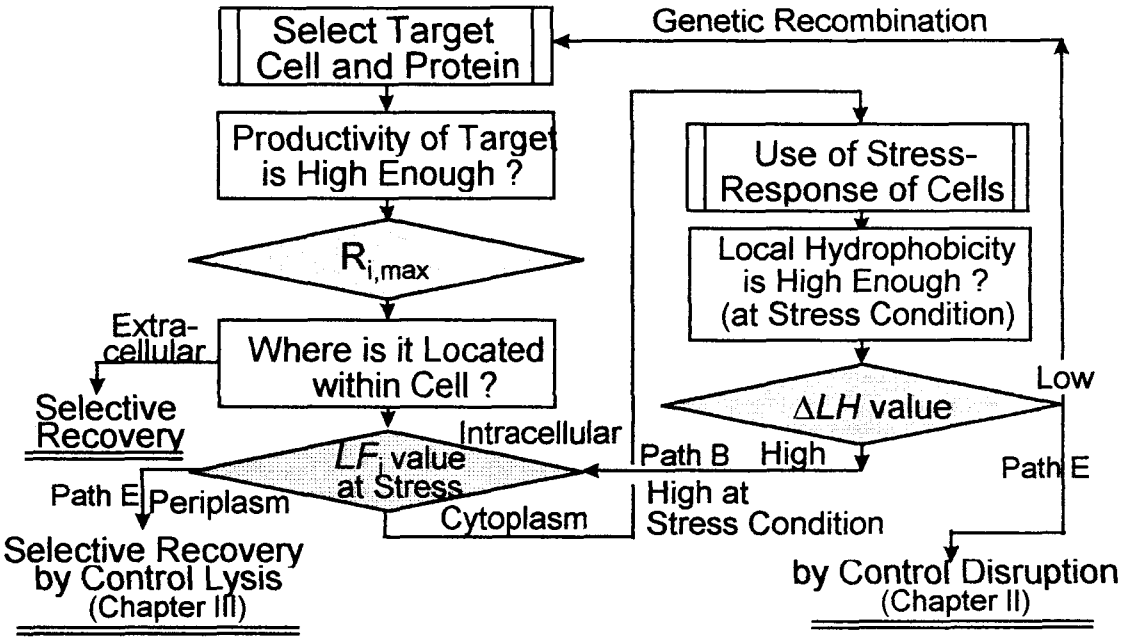


Fig.4-11 Scheme for optimization of selective release and recovery intracellular proteins by using stress-response function of cells (the translocation phenomena across inner membrane of *E.coli*)

E.coli cells. The possibility of the translocation of the former cytoplasmic enzyme, β-gal, to periplasm across the inner membrane of *E.coli* cells is positive as indicated in this chapter, while that of the latter HSPs is negligible. Because the LF_i value for the former is increased while the latter is not as described in chapter 6 when *E.coli* cells were exposed to heat stress (**Fig.4-2**, **Fig.4-4** and **Fig.4-5**). This was shown to be caused by the increase of local hydrophobicity LH_G of the target β-gal under the heat stress and the enhancement of hydrophobic interaction between the target and the inner membrane of *E.coli* cells. In this experimental range, the LF_i is optimal at 47 °C for 10min in GroEL defective strain. On the other hand, *LF* of HSPs is not increased so much in the same temperature range because of their high stability against heat stress as expected from their own functions. In this way, the selective recovery process of cytoplasmic proteins can be rationally de-

signed regardless of kinds of proteins and cells by using the stress-response functions of bacterial cells.

The combined effects of osmotic stress under the heat stress should be further considered when the cells are reutilized to the further cultivation. The heat stressing to cells at relatively high temperature also induce the cell death because of the thermal denaturation of intracellular proteins (**Fig.4-7**) and so on. In the conventional process containing cultivation, disruption and followed various unit operations, the use of the above phenomena on the heat-induced translocation of target protein may improve the efficiency and selectivity of target recovery. Because the death of the bacterial cells is a serious problem in the process using the living cells, such as an extractive cultivation process using aqueous two-phase systems (described in chapter 6), where the cells must be reutilized for the further cultivation in order to achieve continuous operation. The results on the heat-induced translocation of β-gal enhanced by using the osmotic stress under the heat stress (**Table 4-2**), therefore, imply following advantages, *i.e.* the stress-response function of cells, especially the translocation of β-gal can be induced at the relatively weak heat stress conditions (for example 37~40°C) which cause both the reduction of cell death and the enhancement of translocation of β-gal. In the following chapter related to the extractive cultivation process the optimal condition was determined based on this results.

4. SUMMARY

The stress response functions, such as protein folding (A), translocation (B), aggregates formation (C), and degradation by protease (D), were investigated by using the quantitative parameters.

(1) The heat stress to *E.coli* cells at relatively high temperature (more than 55°C) was found to induce the formation of insoluble aggregates and the degradation of intracellular total proteins.

(2) Heat-stress to *E.coli* cells at relatively lower temperature range (40~50°C) was found to induce the translocation of cytoplasmic enzyme, β-gal, across the inner membrane of *E.coli* cells. This behavior of β-gal was analysed by using parameters determined from the kinetic analysis of enzyme release processes. The maximal value was obtained when the *E.coli* cells were heated at 47 °C for 10min.

(3) The surface hydrophobicities of cells and spheroplasts, *HFS*, with various heat stress were determined by using aqueous two-phase systems. The values of inner membrane of cells were increased with increasing temperature. The local hydrophobicity of β-gal, LH_G, was maximized at the temperature of 40~50 °C. The mechanisms of β-gal translocation are thus suggested to be triggered by the enhancement of hydrophobic interaction between the inner membrane of cells and β-gal molecules.

(4) The design scheme for the selective recovery of intracellular enzymes using heat-induced translocation of β-gal was presented on the basis of hydrophobic interaction between the cell membrane and the protein to show the possibility to apply the optimization method to the other proteins. The use of combined stress (heat and osmotic stresses) was also found to induce the translocation phenomena more efficiently with reducing its impact against the bacterial cells.

Chapter 5

Model Simulation of Heat-Induced Translocation of Entrapped β-Galactosidase Across Lipid Bilayer Membrane

1. INTRODUCTION

Among various functions of biological membranes, the transport of protein across the lipid membrane is one of the most important function. From the *in vivo* results in chapter 4, the cytoplasmic β-galactosidase (β-gal) has been shown to be translocated to periplasm across the inner lipid membrane of *E.coli* cells by exposing them to heat stress, depending on the hydrophobicity of inner membrane, the local hydrophobicity of target β-gal, and the induced amounts of HSPs (GroEL). Liposomes, which are self-assemblies of closed phospholipid bilayers, have been widely studied as a model for the biological membrane and for many other purposes, in particular, as drug delivery systems (Ho *et al.*, 1986) and artificial cells (Oberholzer *et al.*, 1995). The *in vivo* heat-induced translocation of cytoplasmic β-gal can be applicable to such artificial membrane and verified by using the liposomes as a cell membrane model.

To date, most of the data on lipid-protein interactions have been obtained from membrane and/or periplasmic proteins in both natural and reconstituted membrane systems from the view point of protein translocation across biological membranes (McElhaney, 1986; George *et al.*, 1990). Current approaches to the study of lipid-protein interactions tend to employ peptide models (Blobel and Dobberstein, 1975) which are designed to interact specifically with either the polar (the surface), or the hydrophobic domains of lipid bilayers (Jacobs and White, 1986, 1987; Mclean *et al.*, 1991). In the other approach, the transport of many precursor proteins have been reported

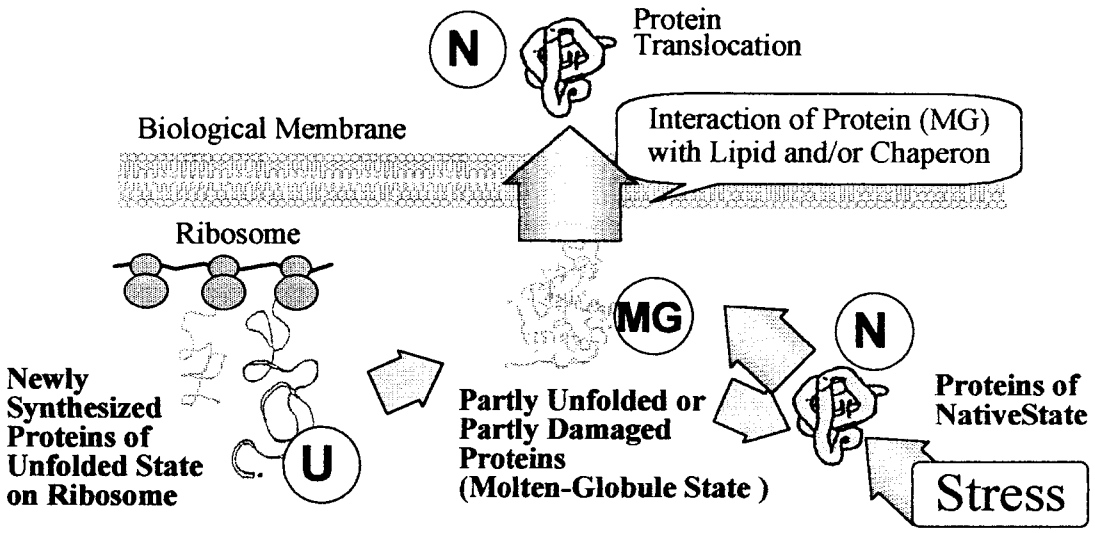


Fig.5-1 Interaction between protein and lipid membrane in transport across the membrane (Concept from Bychkova *et al.*(1988))

to involve interaction with a cytoplasmic chaperon (Collier *et al.*, 1988; Crooke *et al.*, 1988). Recently, the conformational change of protein, followed by their denaturation, has also been shown to be required for translocation (Bychkova *et al.*, 1988) as shown in **Fig.5-1**. Among possible protein conformations, the protein of the partially unfolded state having the strong hydrophobic binding site is thought to be appropriate for binding to or insertion into lipid bilayers and play an important role in biological systems, especially, in binding to heat shock proteins (HSPs) (Martin, 1991; Zahn, 1994), which stabilize the protein structure or promote protein folding. In both cases, translocation of proteins is thought to be triggered by the hydrophobic interaction between the target protein and lipid surface. It is expected that the mechanisms of protein translocation may be clarified and controlled by using the quantitative information on hydrophobic properties of such biomolecules.

The objective in this chapter is the verification of *in vivo* response of

cells under the physicochemical stresses, especially focusing on 'heat-induced translocation of cytoplasmic β-gal' found in chapter 4. The liposomes entrapping β-gal were firstly prepared. Secondly, the liposomes were exposed to the heat stress in the same manner as *in vivo* in order to investigate the heat-induced translocation phenomena. Based on these results, a possible model for the *in vivo* heat-induced translocation of β-gal was presented and compared with the experimental results.

2.EXPERIMENTAL

2.1 Materials. 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) was purchased from Avanti Polar Lipids Inc. β-Galactosidase (β-gal) and o-nitrophenyl-β-D-galactopyranoside (ONPG) as the substrate were purchased from Sigma (New York, USA). Poly (ethylene glycol) (PEG1540, 4000 and 6000; M_W = 1.5 kD, 3 kD and 7kD, respectively) and dextran 100~200k (Dex; M_w = 100~200 kD) were obtained from Wako Pure Chemicals Ltd. (Osaka, Japan). Fluorescent probe, diphenyl-1,3,5-hexatriene (DPH) was also purchased from Wako. Nonionic detergent, Triton X-100 (M_W = 0.65 kD), were purchased from Sigma. The salts and other chemicals of analytical grade were from Wako.

2.2 Preparation of Liposome. Multilamellar vesicles (MLVs) were firstly prepared by hydrating a POPC thin film with 100mM Tris-HCl buffer (pH 7.5) followed by freeze-thaw treatment (five times) with dry ice / ethanol. Large or small unilamellar vesicles (LUVs or SUVs) composed of POPC were prepared by extrusion (15 times) of MLVs through polycarbonate filters with various pore size (50, 100, and 200nm) using an extrusion device (Lipofast; Avestin Inc.). Liposomes containing β-gal were also prepared by

the same procedure described above except that the lipid film was hydrated with buffer containing 4mg/ml β-gal. The lipid concentration of each sample was 15 μ M.

2.3 Gel Permeation Chromatography. Prepared liposomes solutions were fractionated to each sample of 1ml by using the gel permeation chromatography (Sepharose 4B; ϕ, 15mm; length, 150mm). The optical density at 660nm (OD_{660}) and β-gal activity (dA_{420}/dt) of each fractions were then monitored. In each fractions, the β-gal activity in the solution treated by 2mM Triton X-100 for 1min was also measured. **Figure 5-2** shows the elution profile of gel permeation chromatography for the solution containing liposome with entrapped β-gal and untrapped free β-gal. The liposomes and

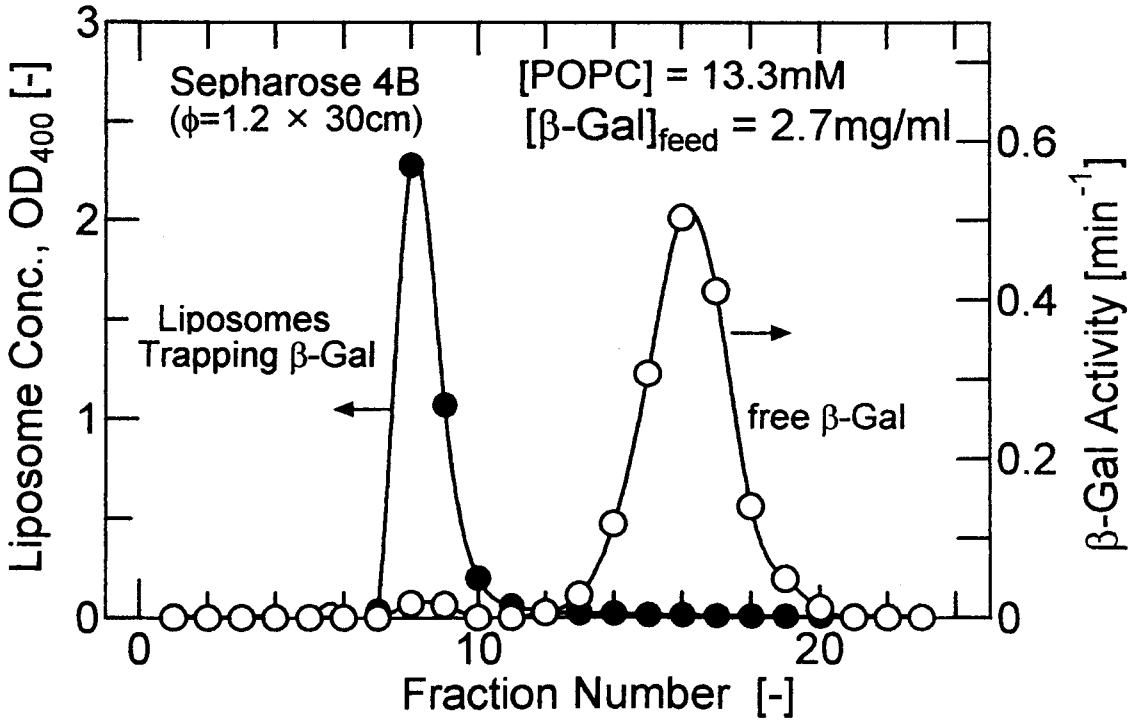


Fig.5-2 Elution profile of gel permeation chromatography (Sepharose 4B) by Monitoring optical density at 400nm and activity of β-gal after entrapping β-gal in POPC liposomes.

the free β-gal were, respectively, detected at the fraction of number 7~10 and at the fraction of 13~18. The percentage of entrapped β-gal was estimated as about 38% from mass balance of the activity. In the following experiment, the liposome fractions (number 7~10) were recovered and used as the solution of liposomes entrapping β-gal. Then, the total amount of β-gal initially entrapped inside the liposomes (100 %) was measured after solubilization of the liposomes membrane with 2 mM Triton X-100, again. All measurement for the activity of β-gal was determined from the initial rate of hydrolysis reaction of ONPG (Gray *et al.*, 1973).

2.4 Analytical Methods for Characterization of Liposome Membrane.

(1) Surface Hydrophobicity of Liposome and β-Gal. ~ Aqueous Two-Phase Partitioning Method ~ The surface net hydrophobicity (*HFS*) of liposomes and β-gal was analysed by using the aqueous two-phase partitioning method (described in chapter 1). When pH is then selected at the pI at lower ionic strength, partition coefficient of liposomes and proteins was mainly dependent on the hydrophobic effect and the following relationship can be obtainable.

$$\ln K = HFS \times HF \qquad (5\text{-}1)$$

where hydrophobic factor (*HF*), which have been defined from the partition coefficient of amino acids, is an indicator of the hydrophobicity of two-phase systems and the *HFS* values can be defined as surface net hydrophobicity of liposome membrane. Further, the local hydrophobicity of proteins was also determined as LH_{pr} (= $\Delta \ln K_{Pr} = \ln K_{Pr,Triton} - \ln K_{Pr,0}$; $K_{Pr,Triton}$, $K_{Pr,0}$, the partition coefficient of protein with and without 1mM Triton X-405). In the case of local hydrophobicity of liposome, the values of surface net hydrophobicity *HFS* may reflect the *LH* values because the *HFS* values of

liposomes can be well correlated with the conventional values of local hydrophobicity which are determined by the fluorescence intensity of 8-anilino-1-naphthalene-sulfonate (ANS; data not shown).

(2) Membrane Fluidity. Membrane fluidity of liposome was determined as fluorescence polarization (*P*) using the fluorescent probes, DPH (Macdonald *et al.*, 1988). The fluorescent probe was oriented into lipid bilayer as following way. The solutions of DPH (in ethanol) were added to the liposome suspension to maintain the lipid/probe molar ratio at 250 ($[DPH]_{final}$=2μM). The mixture was then incubated at least for 1hr at room temperature with gentle stirring. Probes remained in outer aqueous phase of liposomes were negligible since they have little fluorescence in water. The fluorescence polarization of samples was measured with a spectrofluorometer to which excitation and analyzing polarizers (FP 2010, JASCO) were fitted. The sample was excited with vertically polarized light (360nm) and then the emission intensity at 430nm of both parallel ($I_{/\!/}$) and perpendicular ($I_{\perp}$) to the excited light was recorded. Then, polarization of DPH was calculated from the following equation.

$$P = (I_{/\!/} - I_{\perp}) / (I_{/\!/} + I_{\perp}) \qquad (5\text{-}2)$$

The membrane fluidity of liposomes was determined as the inverse values ($1/P$) by using the *P* values.

3. RESULTS AND DISCUSSION

3.1 Change of β-Gal Activity in Outer Aqueous Phase of Liposome after Heat Treatment. After the prepared liposomes trapping β-gal were exposed to heat stress at various temperature for various time, the β-gal activity in outer aqueous phase was then measured. **Figure 5-3** shows the effect of the heating time on the β-gal activity in the outer aqueous phase. The

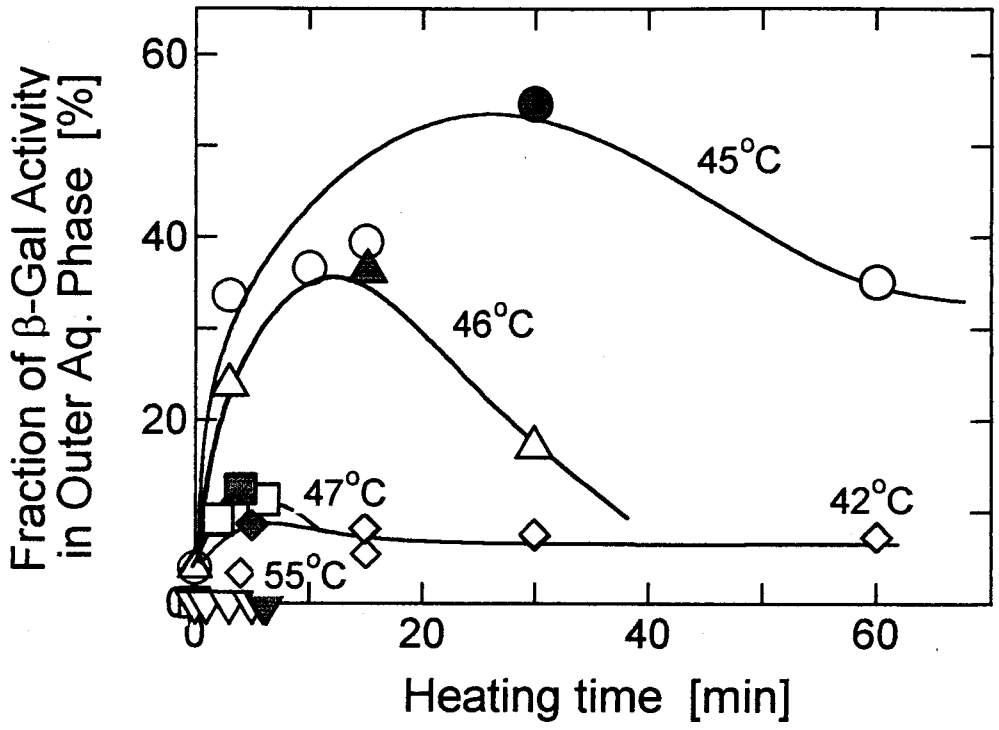


Fig.5-3 Effect of heating time on the released fraction of β-gal entrapped in liposome

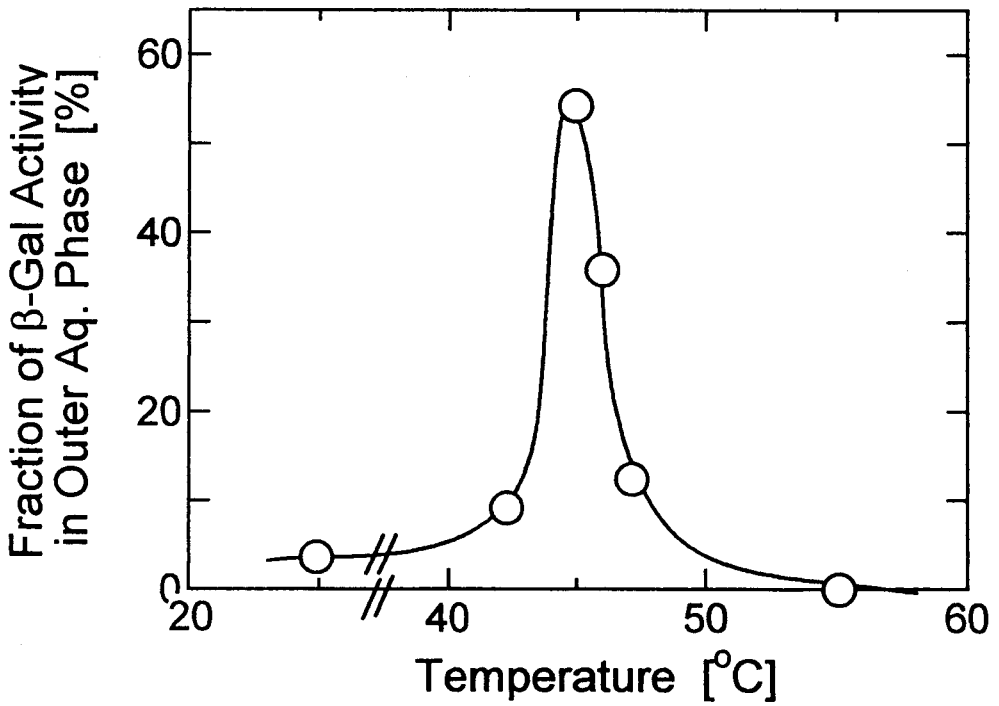


Fig.5-4 Effect of heating temperature on the released fraction of β-gal entrapped in liposome

β-gal activity is not increased when the liposomes are heated less than 40°C. At the temperature of 42 and 47 °C, the value increases in a few minutes and then gradually decreases. Especially, when the liposomes were heated at the temperature of 45°C for 30min, β-gal activity in the outer aqueous solution is dramatically increased to 50~60%. On the other hand, β-gal activity is not detected at more than 55°C. The obtainable maximal activity of β-gal in the outer aqueous phase is plotted against the heating temperature as shown in **Fig.5-4.** Rather interesting bell-shaped curve with narrow width is observed, where the width of the profile is sharply limited between 40 and 50°C.

β-Gal is a typical example of cytoplasmic proteins that have no signal sequence and reported to translocate to periplasm across inner membrane of Gram-negative *Escherichia coli* cells *in vivo* by heat treatment (Yatvin *et al.*, 1986; Yatvin, 1987; Kitagawa *et al.*, private communication). By exposing the liposomes with entrapped β-gal to heat treatment, the similar results on the *in vitro* heat-induced translocation could be obtained *in vitro* (**Fig.5-3** and **Fig.5-4**). These phenomena on the increase in β-gal activity in the outer aqueous phase may be caused by the following phenomena. i) Not only outer β-gal but also inner one may catalyze the reaction because of the transport of substrate due to the enhancement of the membrane permeability. ii) The liposome structure may be destroyed and the inner β-gal is leaked from liposomes. In the following experiments, these possibilities of i) and ii) were investigated.

3.2 Other Possibility in Heat-Induced Increase in β-Gal Activity in Outer Aqueous Phase

(1) Stability of Liposome during Heat Treatment. Heat stability of liposome structure was investigated at the 45°C in order to confirm whether

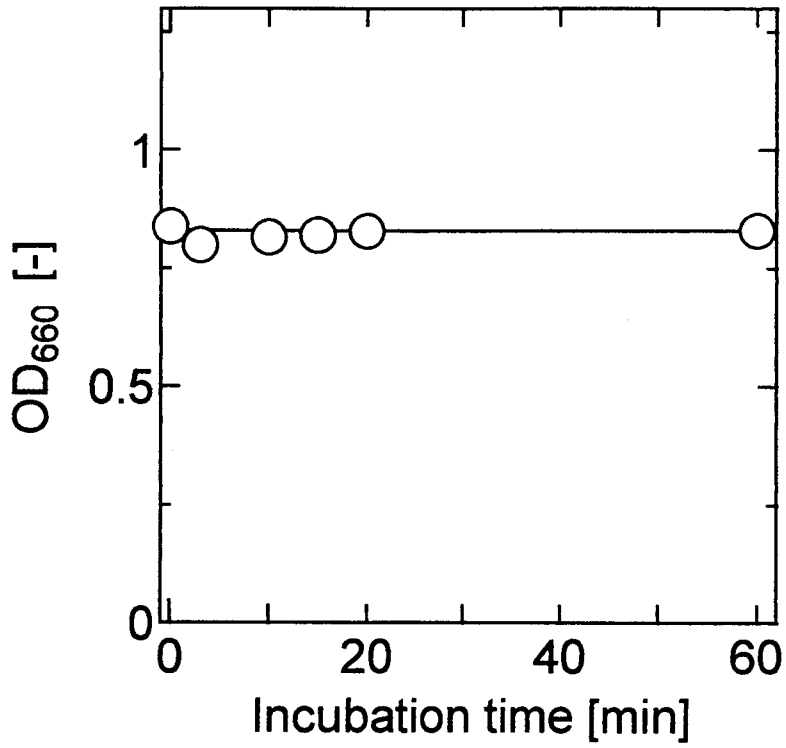


Fig.5-5 Stability of POPC liposomes under heat stress (45°C)

the liposome structure was destroyed by heat treatment or not. **Figure 5-5** shows the time course of optical density at the wavelength of 660nm, OD_{660}, of liposome solution at 45°C. Notable change is not detected in OD_{660} values in this experimental condition (25~60°C). The change in liposome size of such samples was also not detected by the DLS methods, so that the size was maintained at almost the same value (particle diameter = 175±21nm). Kikuchi *et al.* (1991) has also reported that the liposome size was not changed and further there is no leakage of encapsulating materials although they were exposed to heat stress at relatively high temperature (121°C, 20min). In this experimental range (heat treatment at 40~60°C), it is confirmed that the liposome was stable during the heat treatment. In our experimental temperature (40~50°C), therefore, destruction of liposomes can be neglected.

(2) Gel Permeation Profile of Heated Solution with Entrapped β-Gal.

In order to clarify whether the increase of the β-gal activity in outer aqueous phase is caused by the enhancement of membrane permeability of substrate or not, the components containing the heated solution of β-gal-entrapped liposome were analysed after separation using the gel permeation chromatography. Samples of each fractions were detected from optical density at 660nm (OD_{660}), absorbance at 280nm (A_{280}), and the activity of β-gal (dA_{420}/dt). **Figures 5-6** shows the elution profiles of (a) control (without heating) and heat-stressing liposome (b) at 45°C for 30min and (c) at 55°C for 30min. In the control profile, only one peak is detected in fractions of number 4~7. On the other hand, there are two major peaks which are corresponding to liposomes and free β-gal released from liposomes when the liposomes entrapped β-gal were exposed to heat stress at 45°C for 30min (**Fig.5-6(b)**). The fractions of free β-gal can be further separated to two types. One is a fraction which is detected from both A_{280} and dA_{420}/dt (number 19~22). The other fractions are detected by only A_{280} (number 23~26). The results imply that two kinds of β-gal, which are active and inactive, are existing in the surroundings of liposomes after heat treatment. As shown in **Fig.5-6(c)**, there is no change in the profile of OD_{660} and A_{280} after heat treatment at 55°C for 30min as compared to control profiles. The activity of β-gal is not detected at all in all fractions. It is found that the β-gal is translocated by the adequate heat conditions (45°C), that two kinds of β-gal are existing in the outer aqueous solution at the condition, and that the overheating induce the inactivation and no translocation of β-gal entrapped in the inner aqueous phase. In this way the β-gal is shown to be translocated by heat treatment.

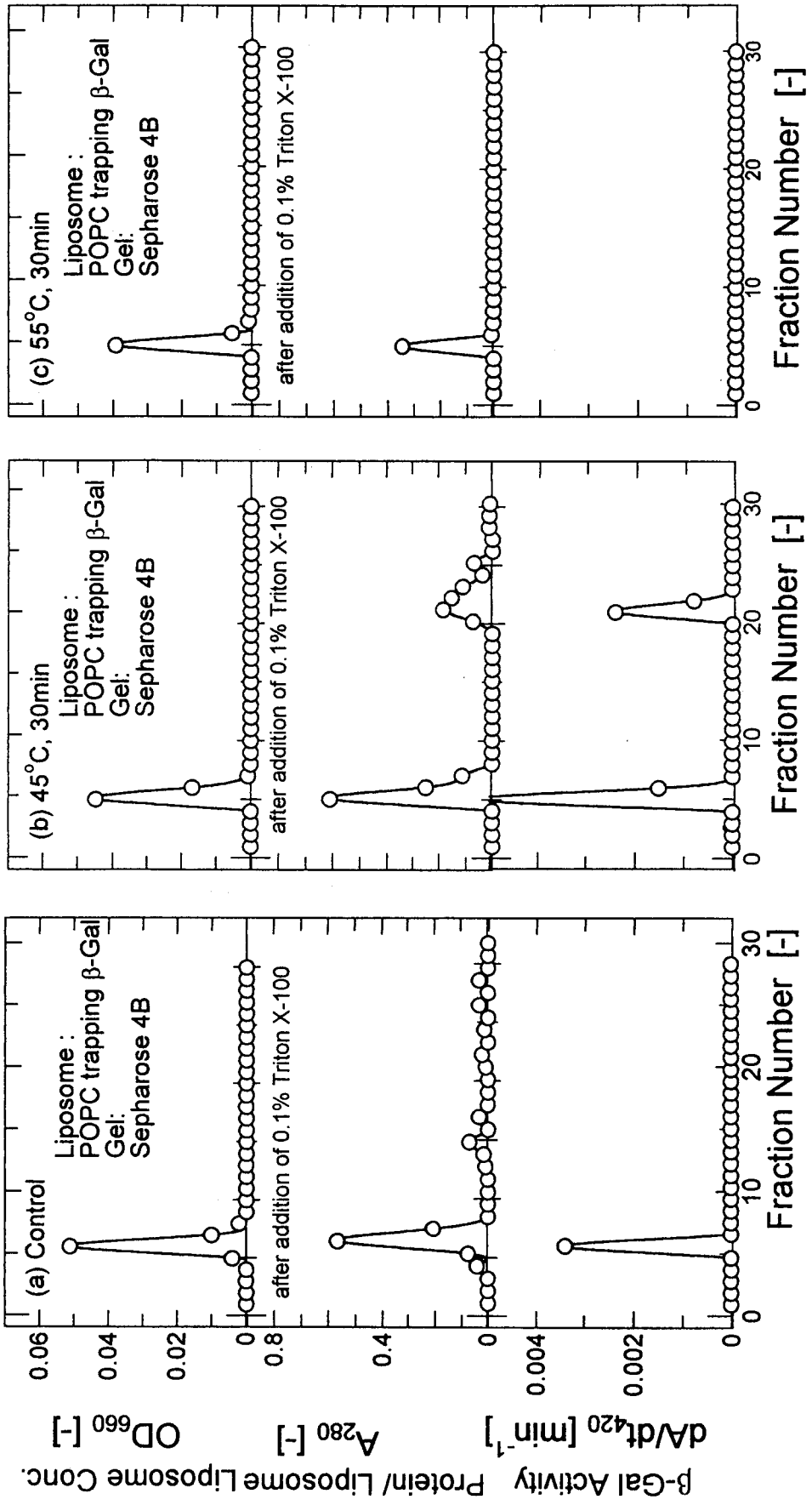


Fig.5-6 Gel permeation profiles ((a); control, (b) with heat stress (47°C, 30min), (c) with heat stress (55°C, 30min))

3.3. Variation of Surface Properties of Liposomes and β-Gal

(1) Variation of Membrane Fluidity and Surface Hydrophobicity of Liposome after Heat Treatment. As a first possibility, β-gal translocation is thought to be caused by the change in general properties of phospholipid membrane by exposing them to heat treatment. The physicochemical properties of liposome membrane, such as membrane fluidity ($1/P$) and surface hydrophobicity ($HFS_{Liposome}$), were investigated during the heat treatment (**Fig.5-7**). The membrane fluidity of POPC liposomes was determined by

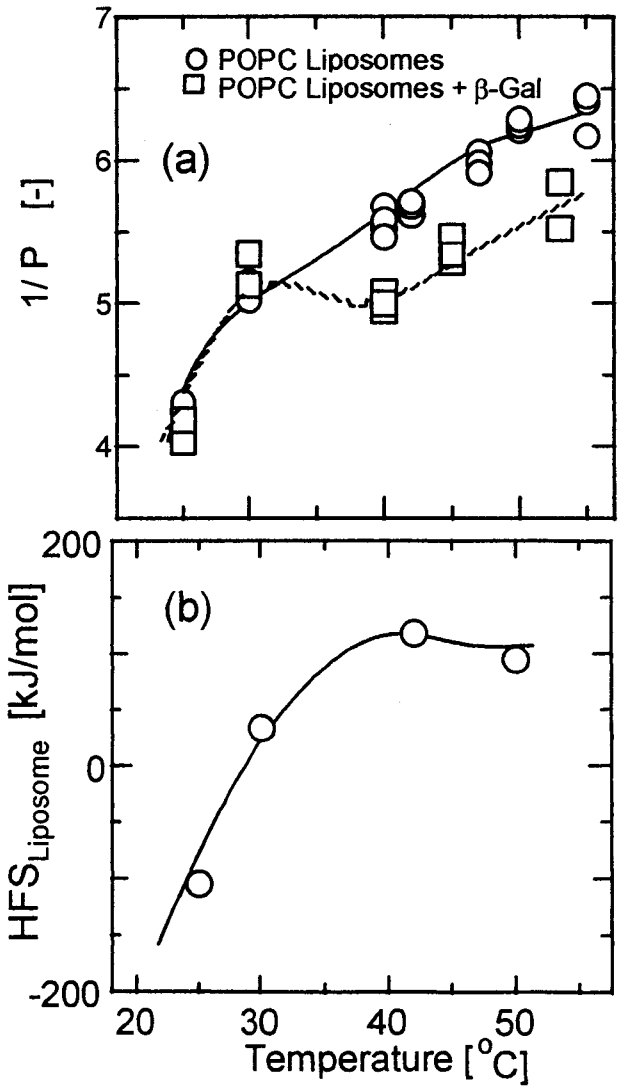


Fig.5-7 Dependence of $1/P$ and HFS values on temperature

using the fluorescent probe DPH, which was bound to the hydrophobic site of membrane surface. **Figure 5-7(a)** shows the inverse values of polarity ($1/P$) as a function of heating temperature. With increasing the temperature, the values are increased, indicating that the fluidity of POPC membrane is then increased. By using the above method, the change in membrane fluidity of liposome with entrapped β-gal after heat treatment was also investigated. In this condition, the similar curve of $1/P$ against temperature is obtained except for 40~50 °C. However, the values of the latter liposomes indicate smaller values than that of control, so that the β-gal is thought to stabilize the membrane fluidity in the range from 40 to 50°C. (Especially in the range from 40 to 50°C, the $1/P$ values indicate the smaller values as compared with that of the control.) These results indicate that the heat-exposing to liposome encapsulating β-gal may enhance the interaction between the inner membrane surface and the β-gal existing in inner aqueous phase because of the enhancement of membrane fluidity and local hydrophobicity of liposomes, depending on the heating temperature.

The hydrophobicity of liposome exposed to heat treatment at various temperature was also determined by using the aqueous two-phase partitioning method (Kuboi *et al.*, 1991, chapter 1). **Figure 5-7(b)** shows the relationship between heating temperature and surface hydrophobicity of liposomes, HFS_{Liposome}, which can be determined by the above method. The HFS_{Liposome} values are increased with increasing the temperature and these values are saturated at the temperature of more than 50°C. Further, the HFS_{Liposome} value is found to be well corresponding to $1/P$ value.

The *in vivo* heat-induced translocation of β-gal across inner membrane

of *E.coli* also indicates that the change in the lipid composition of inner membrane cause such translocation (Yatvin, 1987). In our *in vivo* experiments using *E.coli* cells exposing to heat treatment, surface hydrophobicity of inner membrane of cells has increased with increasing the heating temperature (chapter 4). In the case of POPC liposomes, the surface hydrophobicity of the membranes was increased with increasing the temperature (**Fig.5-7(b)**) and then the membrane fluidity of liposomes, determined by using fluorescent probe, DPH, was also increased (**Fig.5-7(a)**).

These results imply that the heat stress changes the physical state of liposomes membrane, especially, in the membrane fluidity and that the increase in membrane fluidity is well corresponding to the increase in surface hydrophobicity of membrane surface.

(2) Thermal Denaturation of β-Gal Accompanying with Change in Conformation and Surface Properties after Heat Treatment. Second possibility for heat-induce translocation of β-gal across the lipid membrane is the heat induced denaturation of β-gal, accompanying with the conformational change. The behaviors of denaturation of β-gal and their conformational change were investigated after the β-gal was exposed to the heat stress. **Figure 5-8** shows the time course of the relative activity of β-gal at the various heating temperature. The values are exponentially decreased with increasing the heating time. The rate of denaturation is then increased with increasing heating temperature. Edwards *et al.* (1990) have previously indicated the change of β-gal conformation during their thermal denaturation process and the inactive tetramer of β-gal existed during the thermal denatu-

ration process. The surface properties (*e.g.* surface net hydrophobicity, *HFS*, and local hydrophobicity, *LH*) have previously reported to be caused by such conformational change and analysed by aqueous two-phase partitioning method (Kuboi *et al.*, 1992). The surface properties of β-gal (HFS_G and LH_G) were analysed after the β-gal was exposed to heat treatment. The results are summarized in **Table 5-1**. The HFS_G values are gradually increased in proportion to the temperature. On the other hand, LH_G values, which indicate the local hydrophobicity of protein, are maximal in exposing the β-gal to heating. The dependence of LH_G values on the temperature was investigated in details and the results are shown in **Fig.5-9**. The bell-shaped profile of LH_G values, of which range is from 40 to 50°C, can be obtainable. The surface properties, especially, the local hydrophobicity of β-gal are thus found to maximal at the heating temperature of 40~50°C.

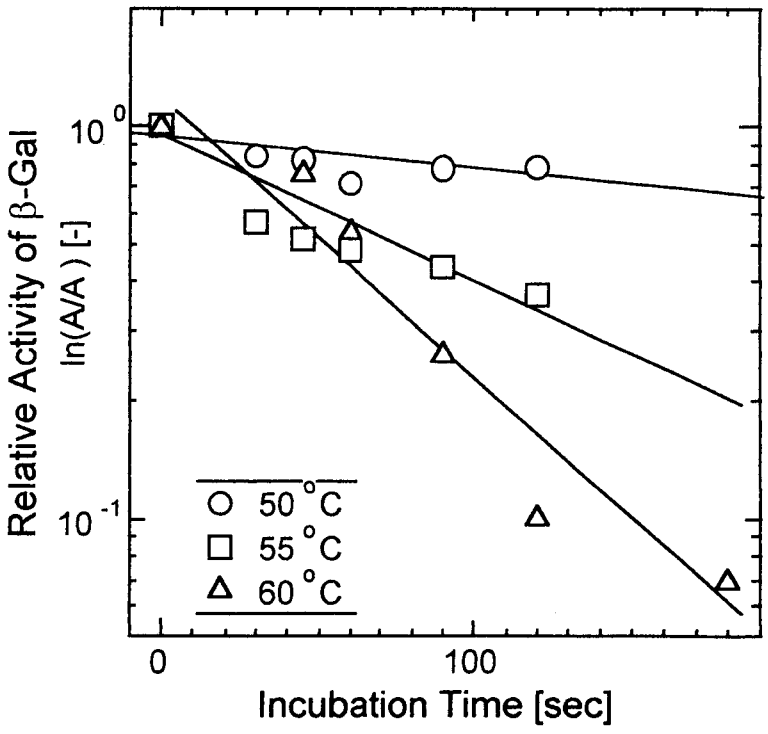


Fig.5-8 Time course of relative activity of β-gal under the heat stress

Table 5-1 Surface Hydrophobicity of β-gal after heat treatment at various temperature

Kinds of Stress	HFS_G [kJ/mol]	LH_G [-]
Control	-450	0.78
50°C, 1hr	-17.7	1.27
60°C, 1hr	96.2	0.27

Typically, it has been reported that the translocation of proteins (*e.g.* extracellular α-lactalbumin (Herreman *et al.*, 1981), periplasmic β-lactamase (Minsky *et al.*, 1986), and so on) across a variety of membranes involves a non-native or denatured conformational states. The denaturation of protein has also shown to be required for translocation from some evidences (Bychkova, *et al.*, 1988). The denaturation of the proteins must be a first step of translocation process.

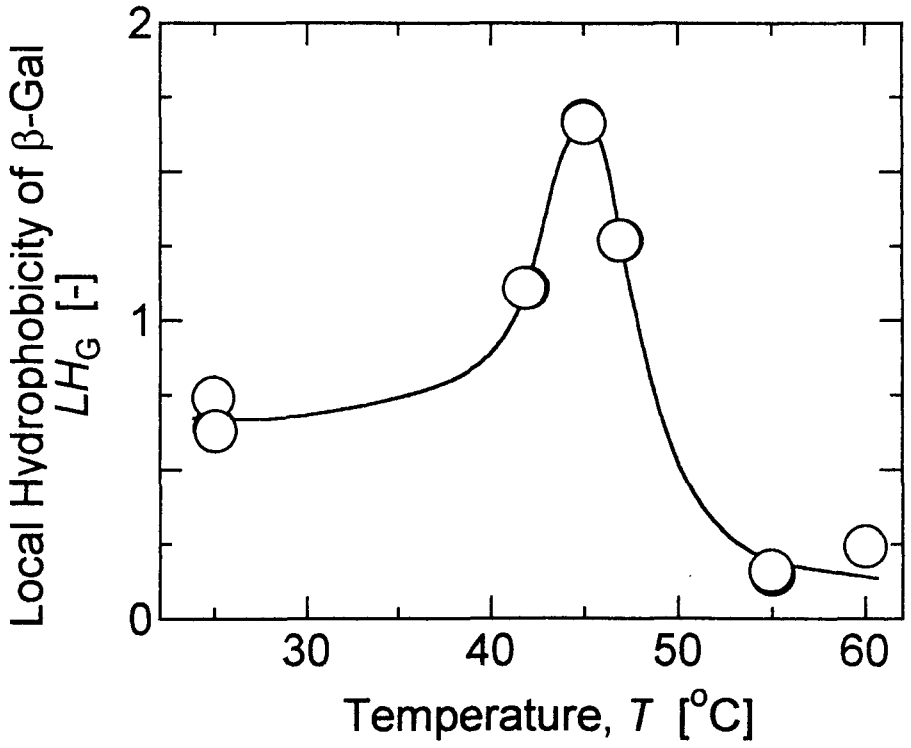


Fig.5-9 LH values of β-gal after heat treatment at various temperature

Ayling and Baneyx (1996) have recently reported about the conformational change of β-gal during various denaturation methods, such as acid, GuHCl, Urea, and the refolding of them. In the case of thermal denaturation of β-gal, a minimal scheme for the thermal denaturation of β-gal has been presented and shown the existence of reversible inactive tetramers during the thermal denaturation (Edwards *et al.*, 1990). The denaturation of β-gal has also reported to continue to occur after activity is lost but must proceed through intermediate partially unfolded structure as is expected for protein denaturation (Shortle, 1989). Such intermediate of β-gal is thought to be formed during the thermal inactivation of β-gal (**Fig.5-8**). It has been re-

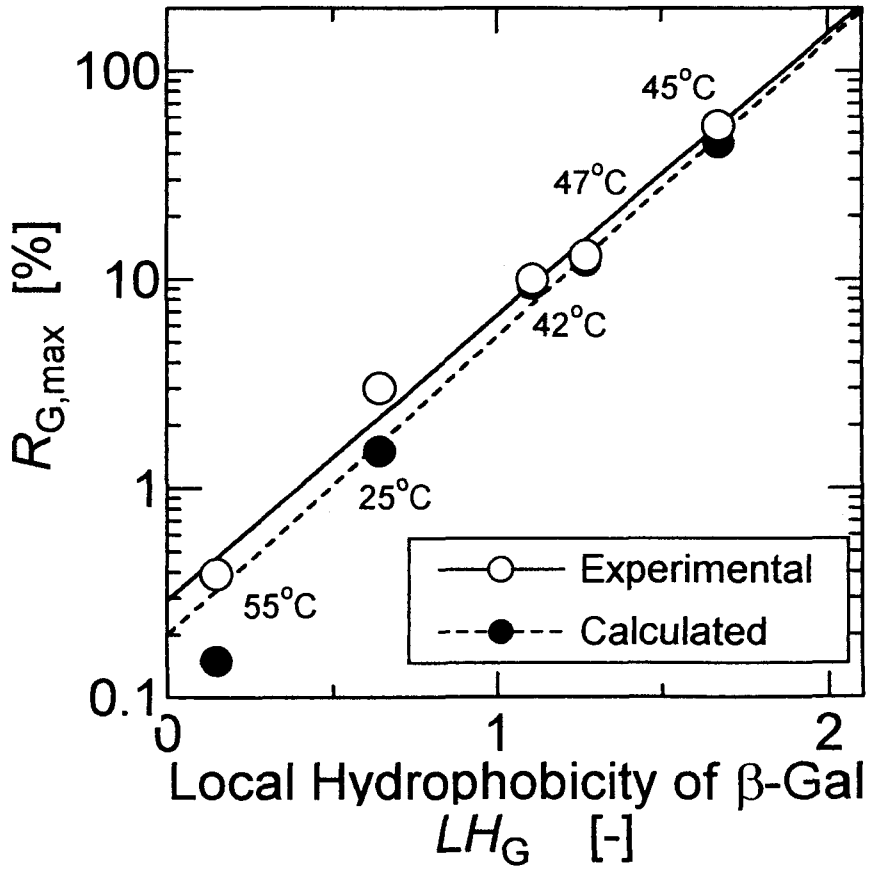


Fig.5-10 Relationship between LH_G value and obtained maximum values of β-gal activity in outer phase

ported that the *LH* values of carbonic anhydrase (from bovine, CAB) having various conformations were investigated and the local hydrophobicity, *LH*, values of CAB of partial unfolded states indicated relatively high values (Yano, 1996). In the case of β-gal, the LH_G value of the intermediate-states is much higher than that of other states (**Fig.5-9**) and the β-gal of such state is therefore thought to interact easily with the hydrophobic binding sites of liposome surface which increase the surface hydrophobicity (**Fig.5-7**). The relationship between LH_G values (**Fig.5-8**) and corresponding activity of translocated β-gal (**Fig.5-4**) after heat treatment at various temperature was summarized in **Fig.5-10**, so that the behaviors of heat-induced translocation of β-gal were found to be well corresponding to the increase in their local hydrophobicity under the heat condition.

3.4 Possible Mechanisms for Heat Induced Translocation of β-Gal Across Lipid Membrane. It is clarified that the β-gal translocation is highly dependent on i) the increase in surface hydrophobicity of liposomes together with the membrane fluidity and ii) the increase in the local hydrophobicity of β-gal followed by thermal denaturation. The possible mechanism for heat-induced translocation of β-gal can be now presented on the basis of the heat-induced enhancement of hydrophobic interaction between the liposomes and the β-gal. The possible scheme for the mechanism is shown in **Fig.5-11**. The first step for translocation is the denaturation of β-gal by heat stress. Then, the conformation of β-gal is changed and the local hydrophobicity is increased (**Table 5-1** and **Fig.5-9**). When β-gal is excessively exposing to heat stress, the β-gal tetramer is further dissociated to four subunits and cannot interact with membrane surface of liposomes (**Fig.5-6(c)**) (Edwards *et al.*, 1990). At the same time, the heat stress also induce

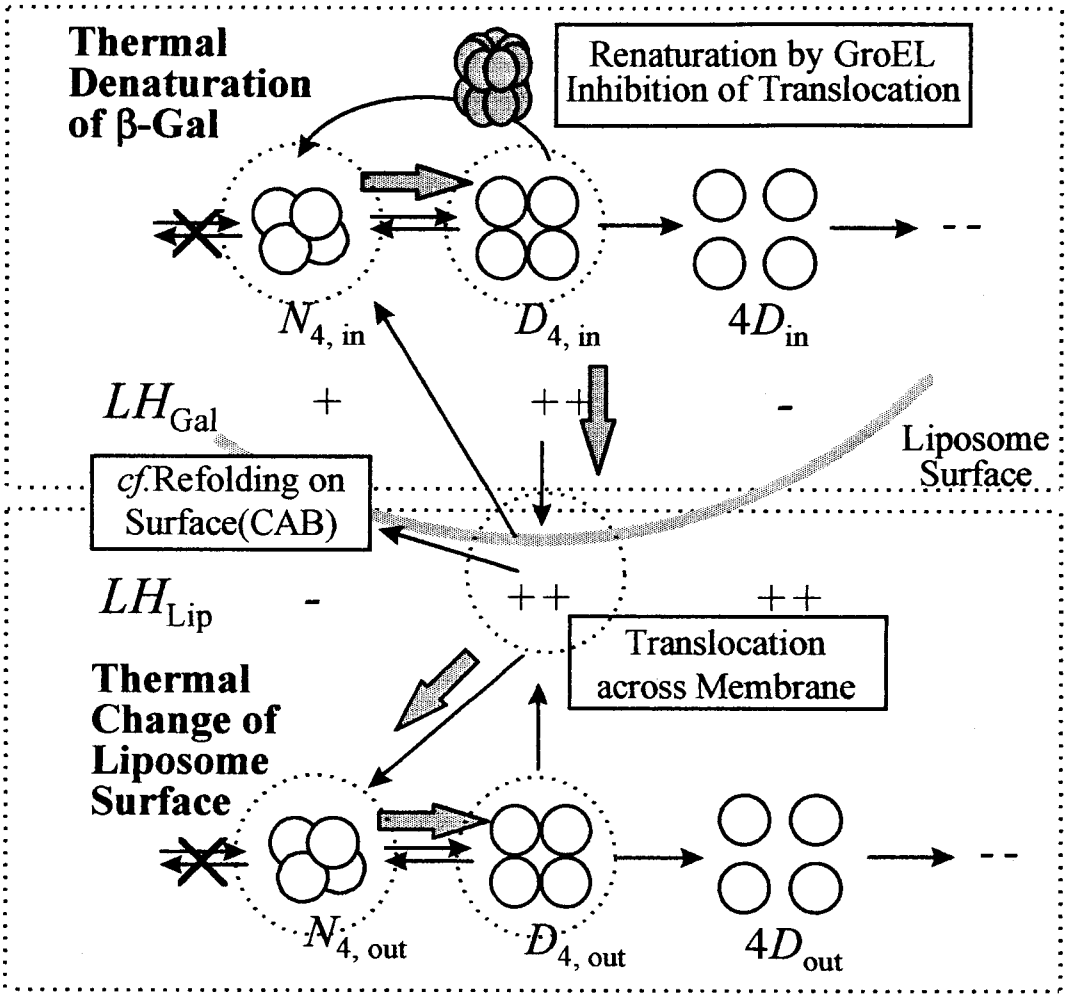


Fig. 5-11 Possible pathways in heat induced translocation of protein across lipid membrane of liposomes

the increase in membrane fluidity and local hydrophobicity of POPC liposomes (**Fig.5-7(a)** and **(b)**). The increase in the local hydrophobicity of both proteins and liposomes enhances the hydrophobic interaction between them (**Fig.5-7(a)**) and induces the translocation of β-gal (**Fig.5-3**, **5-4**, and **5-6**) or the release to the same phase with active state. The translocated β-gal is again exposed to the same heat stress and the inactivation of β-gal is occurred in the outer aqueous phase. In such mechanism, the β-gal is translocated by heat treatment. Among of all pathways, the dissociation

pathways of inactive tetramer in inner and outer aqueous phase and the release pathways to the same phase can be negligible at the adequate heat condition because the kinetic rates of the pathways are thought to be very slow as compared with that of other pathways. The simplest pathway can here be represented as

$$\underset{\text{Region}\ \cdots\ \text{Inner Phase}}{N_{4,\text{in}}} \rightarrow \underset{\text{Inner Phase}}{D_{4,\text{in}}} \rightarrow \underset{\text{Membrane}}{I} \rightarrow \underset{\text{Outer Phase}}{N_{4,\text{out}}} \rightarrow D_{4,\text{out}} \qquad (5\text{-}3)$$

In the following, the heat-induced translocation of β-gal, especially, at the condition of 45°C stressing was simulated based on the simplest model.

3.5 Simulation of Translocation of β-Gal Based on the Simplest Model.

Based on the above discussion, possible pathways which underlie in the heat-induced translocation process of β-gal can be modeled as schematically shown in **Eq.(5-3)**. $N_{4,\text{out}}$ and $N_{4,\text{in}}$ are β-gal of active state (4 denote tetrameric), which are respectively existing in outer and inner phase of liposomes. $D_{4,\text{out}}$ and $D_{4,\text{in}}$ indicate the β-gal at the inactive state in the outer and inner phases, respectively, and I represents the intermediates of β-gal bound and entrapped into the lipid membrane. These pathways can be herewith expressed as a series of rate equations for the concentration of each species. The decrease rate of the active tetrameric β-gal at inner phase ($-d[N_{4,in}]/dt$) may be expressed as

$$-d[N_{4,in}]/dt = k_d\,[N_{4in}] \qquad (5\text{-}4)$$

Here, the initial concentration of the unfolded protein is equivalent to the activity of β-gal ($[N_{4,in}]_I$). In the same way, the rate of decrease of $D_{4,\text{in}}$, I,

$N_{4,out}$, $D_{4,out}$ can be expressed as

$$d[D_{4,in}]/dt = k_d [N_{4in}] - k_1 [D_{4,in}] \quad (5\text{-}5)$$

$$d[I]/dt = k_1 [D_{4in}] - k_2 [I] \quad (5\text{-}6)$$

$$d[N_{4,out}]/dt = k_2 [I] - k_d [N_{4,out}] \quad (5\text{-}7)$$

$$d[D_{4,out}]/dt = k_d [N_{4out}] \quad (5\text{-}8)$$

The concentration profile of the native β-gal in the inner phase was calculated with varying the kinetic parameters by using Runge-Kutta method. **Figures 5-12** show the time course of the concentration of β-gal translocated to the outer phase of liposomes. A series of lines are the calculated values of the β-gal concentration when kinetic parameters such as (a) k_d, (b) k_1, and (c) k_2 were varied as shown in this figure. Generally, the shape of the curves are well corresponding to that of expected curves from the experimental values. As shown in **Fig.5-12(a)**, the time for maximal percentage of β-gal concentration of each curves transfer from the 80 to 2 min when the k_d values are varied from 0.01 to 1. The most suitable curve for the experimental data is obtained at the k_d value of 0.025. When the k_1 values are varied from 0.01 to 15, the curve which is similar to the experimental curve can be obtained at k_1 values of nearly 10 (**Fig.5-12(b)**). Furthermore, the increase in k_1 values increase the height of curves, so that the optimal curves can be obtained at the k_1 values of nearly 10 (**Fig.5-12(c)**). The values of the above kinetic parameters at the condition of 45°C were determined by trials and errors and the obtained optimal values are shown as follows.

$$k_d = 0.025 \text{ min}^{-1}, \quad k_1 = 11.9 \text{ min}^{-1}, \quad k_2 = 9.5 \text{ min}^{-1} \quad (5\text{-}9)$$

The denaturation constant of β-gal obtained from the experimental data,

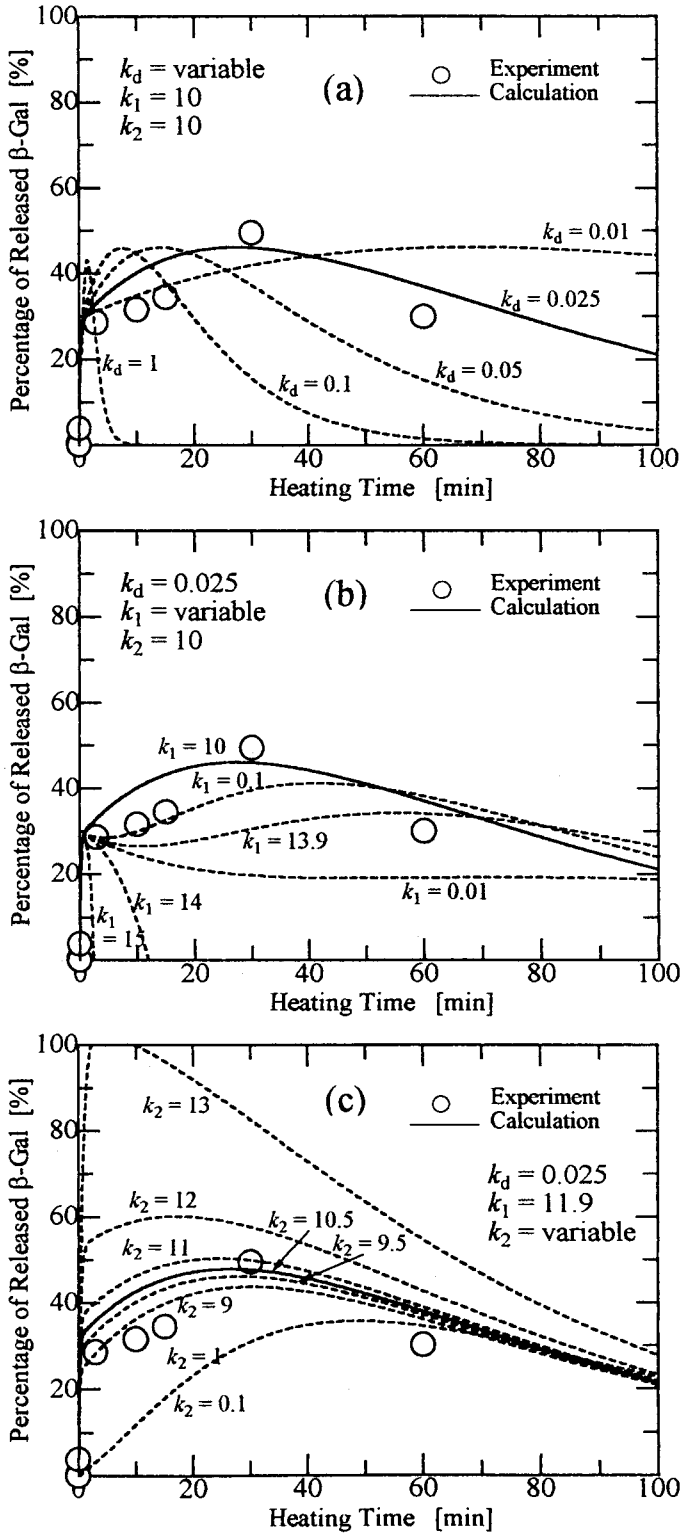


Fig.5-12 Effect of variation of kinetic parameters ((a) k_d, (b) k_1, and (c) k_2) on the concentration profiles of β-gal

k_d(exp), indicate 0.042 min^{-1}. The difference between these values may be caused by neglecting the other pathways (**Fig.5-11**) in the simulation. The values of the above kinetic parameters under the other stress conditions were determined by using the same method. The rate constant of β-gal denaturation, k_d, is gradually increased with increasing temperature. **Figure 5-13(a)** shows the dependence of the calculated values, k_1 and k_2, on heating temperature. The calculated values are well corresponding to the experimental values which have been obtained in **Fig.5-8**. The calculated values of both k_1 and k_2 indicate the higher values than that of k_d. They are increased at the temperature of less than 45°C and decreased at the higher temperature range. These shapes are well corresponding to that of temperature dependence of maximum values of translocated β-gal (**Fig.5-4**). As shown in **Fig.5-13(b)**, the k_1 and k_2 values can be correlated with the local hydrophobicity of β-gal under the above heat stress. The β-gal translocation is suggested to be achieved mainly by means of such fast process, which involves (i) the inter-

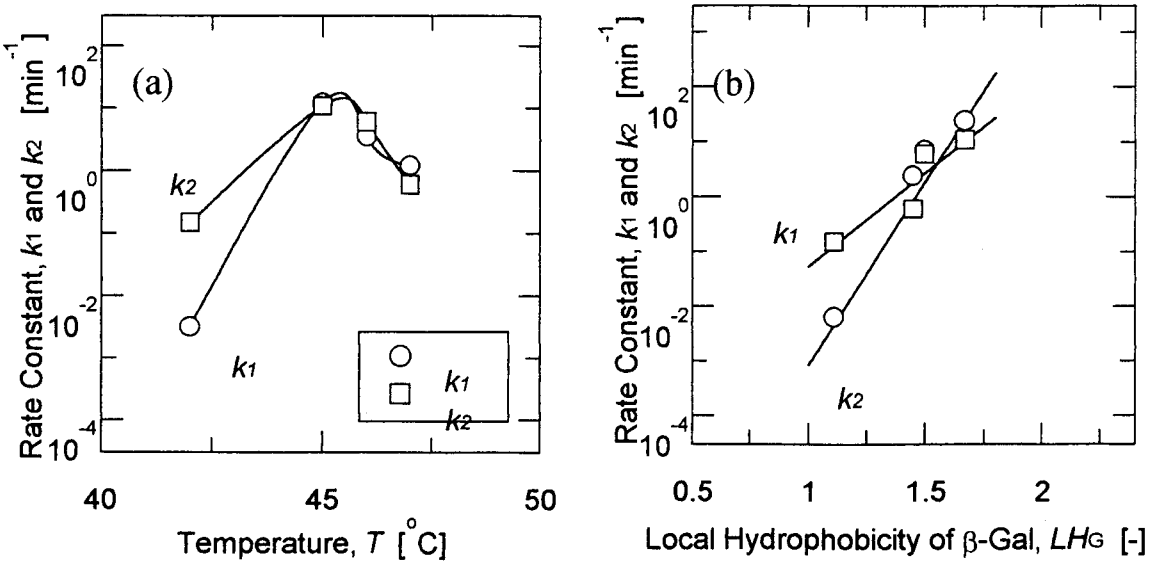


Fig.5-13 Dependence of calculated rate constants (k_1 and k_2) on (a) temperature and (b) local hydrophobicity of β-gal, LH_G

action between the partly damaged β-gal having high local hydrophobicity and the local hydrophobic sites of fluidized membrane and (ii) the translocation of β-gal across the liposome membrane with accompanying the conformational change on the membrane. The translocated amounts of β-gal is therefore saturated in a short exposing-time. As shown in **Fig.5-10**, the maximum values of translocated β-gal have been also dependent on the local hydrophobicity of β-gal. The phenomena is confirmed by comparing the results obtained by model simulation and the experimental results.

The concentration profiles of β-gal were calculated by using these parameters and shown in **Fig.5-14**. Generally, calculated curve simulates experimental results fairly well. From the figure, following tendency can also be noticed. The concentration of native β-gal in the inner aqueous phase is decreased exponentially. The concentration of the inactive tetramer and lipid-bound intermediates of β-gal are not varied because k_1 and k_2 values

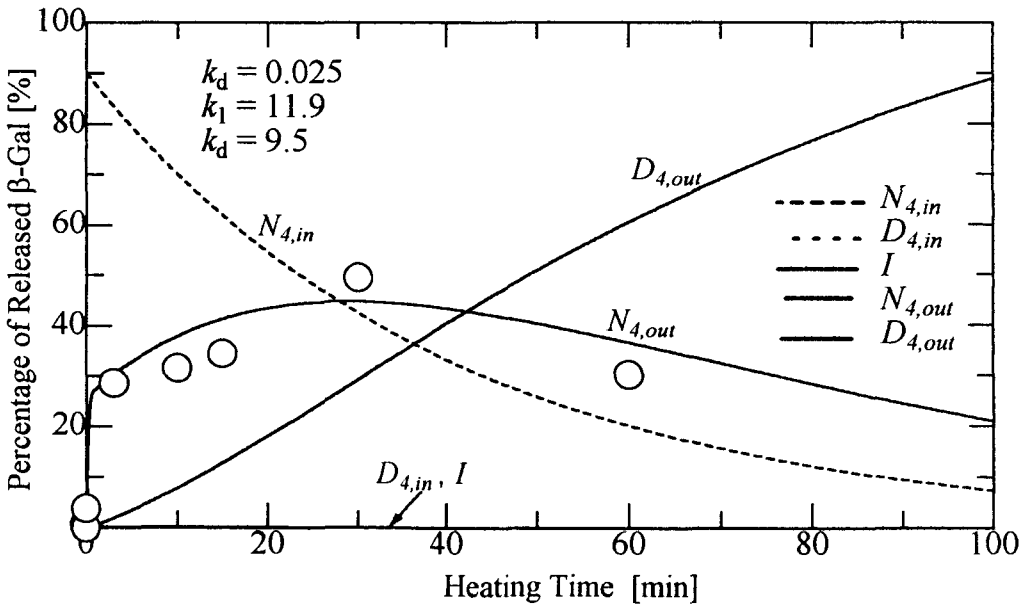


Fig.5-14 Calculated concentration profiles with optimal kinetic parameters compared with experimental values

are much higher than k_d value. The concentration of native β-gal translocated to the outer aqueous phase are increased at the initial treatment of heat stress (less than 30min) and then decrease owing to the heat denaturation in the outer aqueous phase. Accompanying with a series of phenomena, the concentration of translocated inactive β-gal increases with increasing the heating time. Although further study on model simulation is needed in future, the heat-induced translocation process of β-gal can be explained and simulated even by the simplest model expressed in **Eq.(5-3)**, suggesting that heat induced translocation *in vivo* (described in chapter 4) can be analysed by the similar model.

4. SUMMARY

The mechanism of the *in vivo* heat-induced translocation of β-gal were verified by using the liposomes entrapping β-gal as a cell model system.

(1) Heat-stress against liposomes entrapping cytoplasmic enzyme, β-gal, was found to induce its translocation across the liposome membrane. The translocated activity of β-gal in the outer solution was maximized when the liposomes entrapping β-gal were heated at 45°C for 30min.

(2) The surface and local hydrophobicity of liposome membrane, HFS_{Liposome} and LH_{liposome}, increased with increasing temperature and the local hydrophobicity of β-gal, LH_G, was maximized at the temperature of 40~50 °C. The mechanisms of β-gal translocation were thus suggested to be triggered by the enhancement of hydrophobic interaction between the liposome surface and β-gal molecules.

(3) The simple scheme for the possible mechanism of heat-induced tran-

slocation of β-gal were successfully presented on the basis of the hydrophobic interaction between the liposome and the proteins. The heat-induced translocation process of β-gal could be explained and simulated even by the simple model expressed.

Chapter 6

Process Integration of Stress-Response Function of Bacterial cells and Aqueous Two-Phase Systems

1. INTRODUCTION

In Chapter 4, the method for the utilization of the stress-response function of cells was investigated and it was found that the optimized heat (and osmotic) stresses against the bacterial cells could induce i) translocation of cytoplasmic β-gal and ii) enhancement of productivity of cytoplasmic heat shock proteins (HSPs; GroEL and GroES). While extractive cultivation in aqueous two-phase systems (ATPS) has been shown to provide effective bioprocesses to produce and separate product biomolecules from the recombinant microorganisms using a continuous operation. And thus, much research has been devoted to extractive bioconversion, such as the production of biosurfactant (Drouin and Cooper, 1992) and intracellular enzymes (Tanaka, 1993-a) from *Bacillus subtilis* and glucose from cellulose enzymes (Tjerneld *et al.*, 1984). The integration of the stress-response functions of cells and the extractive cultivation process using ATPS can be expected to result in further efficient and simple bioprocesses.

Some heat shock proteins (HSPs; DnaK, GroEL and GroES) from *Escherichia coli* are classified in terms of the molecular chaperones, which are involved in mediating the folding of denatured proteins (Ellis and van der Vies, 1976). The HSPs are induced as the response to the lethal stresses against cells, such as temperature shift (Miyake *et al.*, 1993), carbon starvation (Groat *et al.*, 1986-a) and others (Groat *et al.*, 1986-b; Hightower, 1980). Recently, the osmotic stress by the addition of salts has been reported to trigger the production of HSPs, especially DnaK (Meury and Kohiyama,

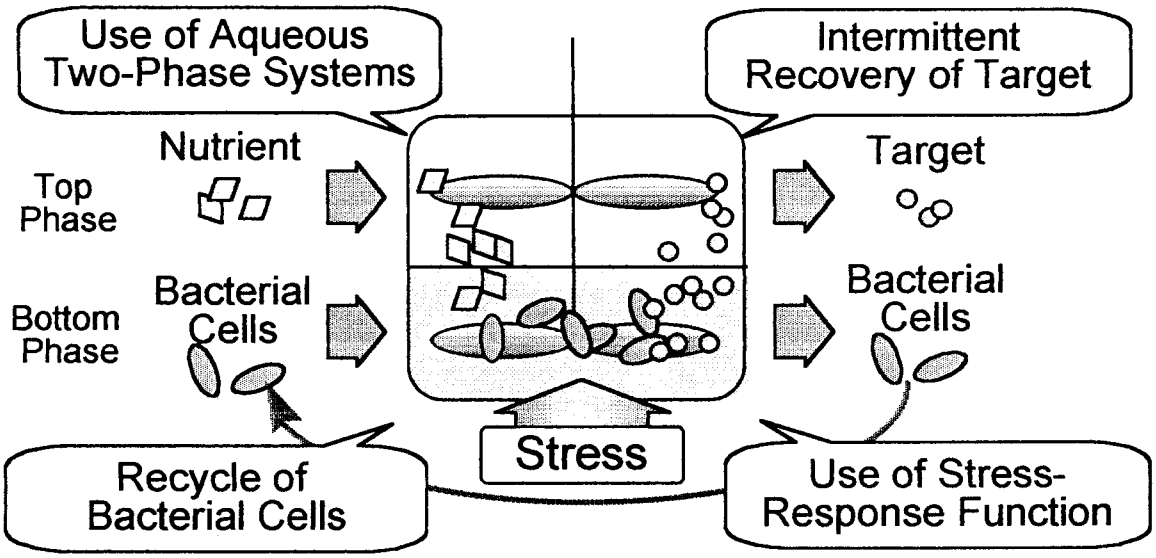


Fig. 6-1 Extractive cultivation process by combining both the stress-response function of bacterial cell and aqueous two-phase systems

1991). It is expected that the independent or combined use of these cell's own functions can be exploited for the improvement of the efficiency of the bioproduction process.

The HSPs have been, typically, purified using multi-step separation methods such as the combination of ion-exchange chromatography, gel-exclusion chromatography and salting-out (precipitation) (Hendrix, 1979; Schoner *et al.*, 1985). ATPS are effective for the quantification of surface properties of proteins and cells (Kuboi *et al.*, 1994-a), and can be utilized to the production and separation of bioproducts, such as amino acid, proteins and bacterial cells, because of their mild and scaleable properties (Albertsson, 1986). Surface properties of GroEL and GroES from *E.coli* have been quantitatively measured and proved to be utilized for the regulation of their partitioning behavior in aqueous two-phase systems (Yano *et al.*, 1994). It is expected to establish more simple and effective separation processes by integrating these stress response functions of cells and the ATPS, based on the quantitative properties of HSPs and *E.coli* cells.

In this chapter, the scheme for the design and development of the stress-mediated extractive cultivation process using such stress-response functions of cells in the ATPS was firstly presented. As case studies, (i) cytoplasmic β-gal and (ii) HSPs from *E.coli* cells were selected as target prteins. The optimal condition for the extractive cultivation of each target proteins has been determined following the presented scheme. Finally, semi-continuous stress-mediated extractive cultivation processes have been developed, where the top-phase containing target proteins is recovered and bottom phase containing the surviving cells is recycled.

2. SCHEME FOR INTEGRATION OF STRESS-RESPONSE OF CELLS AND ATPS

Strategy for the integration of stress-response functions of cells and aqueous two-phase systems (ATPS) can be presented by integrating each sub-scheme for partitioning of bacterial cells and proteins (chapter 1), cell disruption (chapter 2), lysis (chapter 3), and utilization of stress-response functions (chapter 4) as shown in **Fig.6-2**. Major importance is in the sub-scheme for the utilization of stress-response. Firstly in this sub-scheme, the release methods are selected on the basis of the enzyme location factor within the cells as shown in chapter II in details. The production, release and recovery method of the target protein can be improved by using the stress-response function of cells. The growth condition of cells are then examined in ATPS after suitable ATPS are selected. Finally, the partitioning condition to separate the target protein from cells is optimized, following the separation sub-scheme of bacterial cells (chapter 1) and protein (Tanaka, 1993). At the optimal condition, the stress-mediated extractive cultivation

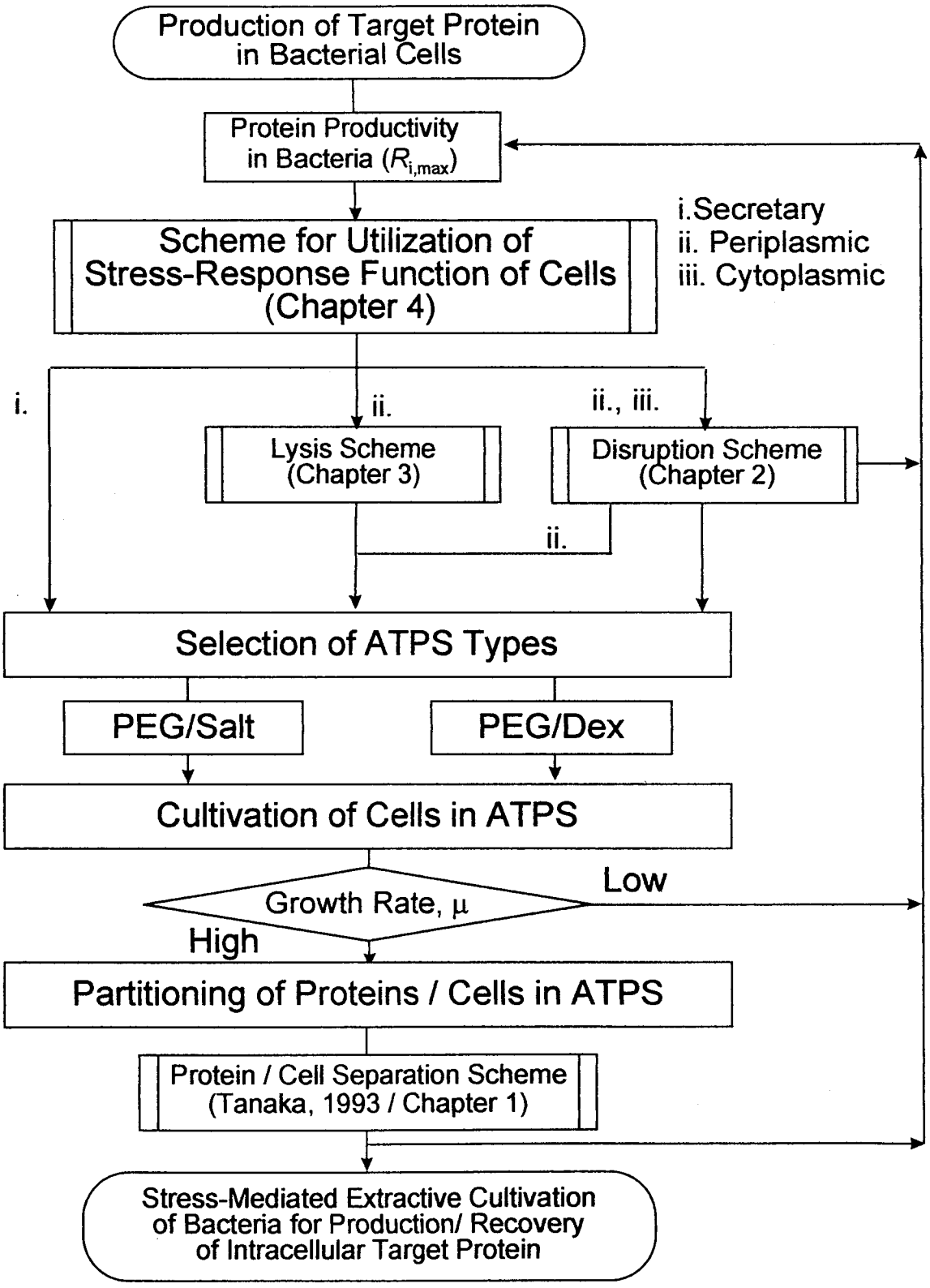


Fig.6-2 Scheme for the integration of stress-response function of cells and aqueous two-phase systems

processes of bacterial cells for the production and recovery of the target intracellular protein can be constructed.

Two examples, as case studies, using stress-response function of cells were examined following the presented strategy. The one is the extractive cultivation of *E.coli* cells for the selective recovery of cytoplasmic β-gal, which is widely produced for the commercial use and the fused marker enzyme for the production of engineered novel enzymes. In this case, the process is expected to be optimized by utilizing stress induced translocation of cytoplasmic β-gal to periplasmic space across inner membrane of cells (enhancement of pathway B in **Fig.4-1**). The other is the extractive cultivation for the production of cytoplasmic heat shock proteins (HSPs), such as GroEL and GroES, which are needed to produce for various medical and analytical uses. In this case, instead of translocation, refolding and productivity of HSPs are expected to be enhanced by utilizing heat and osmotic stresses against the cells (pathway A in **Fig.4-1**).

3. EXPERIMENTAL

3.1 Materials. Poly (ethylene glycol) (PEG600, 1540, 4000, 6000 and 20000) of various molecular weight and dextran (Dex 100~200k) were obtained from Wako Pure Chemicals Ltd. Casamino acid was obtained from DIFCO Laboratory (Michigan, USA). o-Nitrophenyl-β-D-galactopyranoside (ONPG) was obtained from Sigma Chemical Company. Coommassie Brilliant Blue G-250 was purchased from Fluka. The salts and other chemicals used were of analytical grade.

3.2 Cultivation. The bacterial strains used were gram-negative *E.coli* ML308 (β-gal constitutive) and OW10/pND5 (GroEL and GroES constitu-

tive). The details of this microorganism and plasmid were referred to Miyake *et al.*(1993). The basic media for *E.coli* were modified M9 media (pH 7.4, in g/l): Na_2HPO_4, 7.0; KH_2PO_4, 3.0; NaCl, 5.0; NH_4Cl, 1.0; casamino acid, 1.0; $MgSO_4$, 0.75; glycerol 50. In the case of recombinant strain, the 5g/l glucose was used as a carbon source in the place of glycerol and the antibiotics, ampicillin (50mg/ml) and tetracycline (25mg/ml), were supplemented to the media to select for and maintain drug resistance. A solution of trace elements (Gray *et al.*, 1973) was also added(1ml/l-media). Stock culture was maintained on 1.5% agar slant of modified M9 media. Following overnight growth at 37 °C (for ML308) or 30 °C (for OW10/pND5) in a 100ml flask (130rpm) with a working volume of 20ml, the culture was used as an inoculum for a 300ml shaking flask using normally 100ml media. In the case of *E.coli* OW10/pND5, the temperature of the culture broth was increased from 30 °C to 42 °C at an optical density at 610nm, OD_{610}, of 1.0 in the logarithmic growth phase in order to give the heat stress. After the heat treatment, the cells were harvested by centrifugation (10000rpm, 10min) (Yano *et al.*, 1994). In the same way, the *E.coli* cells were also grown in aqueous two-phase media containing water, PEG, Dex or phosphate and the above salts at various concentration.

3.3 Ultrasonic Disruption. *E.coli* cells were disrupted by ultrasonic irradiation, during the late exponential growth phase *i.e.*, at an optical density at 660nm (OD_{660}) of 1.0 (for ML308 strain) and after the heat treatment of cells (OD_{610}=1.5~2.0; for OW10/pND5 strain). The 100ml culture-broth containing *E.coli* cells was irradiated by ultrasonic wave with the input power of 80W, using an ultrasonic disrupter (Tomy Seiko Co. Ltd; UD-200, 20kHz). Five-ml of the media was then sampled and centrifuged (5000rpm,

10min), and the supernatant assayed.

The crude extract solution containing i) β-gal or ii) HSPs together with other soluble proteins were obtained by ultrasonic disruption (273K, 80W, 4min, against 10ml cell-suspension in OD_{660} of 1) and centrifugation (7000rpm, 10min), and were used for the investigation of the partition behavior of the target β-gal and the total soluble proteins in aqueous two-phase systems.

3.4 Aqueous Two-Phase Systems. Both PEG/Dex and PEG6000 / phosphate aqueous two-phase media were utilized in the cultivation of the *E.coli* cells. PEG, Dex or phosphate salts for the two-phase media, the M9 minimal salts and water were measured in flask before sterilization (394K, 20min). The composition of the salts in the two-phase systems is represented by the total input concentration ((w/v)%). Two kinds of phosphate salts, KH_2PO_4 / Na_2HPO_4 (Phosphate(I)) and KH_2PO_4 / K_2HPO_4 (Phosphate (II)) in a weight ratio of 3/7, were used in most cases as the forming component. In all partition experiments, ATPS were separated with centrifugation (5000rpm, 10min) and the top and bottom phases analyzed.

3.5 Behaviors of Production and Partitioning of Target Proteins in Aqueous Two-Phase Systems. The cells harvested in the modified M9 media were resuspended in 50mM Tris-HCl buffer (pH8.0) / 10mM $MgSO_4$ (TM-buffer,5ml) and were disrupted in a 10ml test tube by ultrasonic method (80W,8min). The cell-free extract containing i) β-gal or ii) HSPs and other proteins was used for the characterization of their partitioning behaviors. The cells cultivated in the aqueous two-phase media with the excess salts were also disrupted in the 5ml TM-buffer at the input power of 80W for 0~8min. The productivity of total soluble proteins and i) β-gal or

ii) HSPs were determined as their maximum released amount per unit weight of cells ($R_{i,max}$ [mg-protein/mg-cell]). The resultant supernatants recovered after various disruption times were utilized for characterization of cell strength and the productivity of HSPs through the kinetic analysis of cell disruption and protein release process (described in chapter 2).

The partitioning behaviors of i) β-gal or ii) HSPs and total soluble proteins were, respectively, examined in PEG6000 (8%) / phosphate (8%) and in PEG4000(9%) / Dex100-200k(9%) ATPS containing various salts at various concentrations by feeding the above cell-free extract. The two-phase systems were separated to top and bottom phases by centrifugation (5000rpm, 10min) and were analyzed for partition coefficients of β-gal ($K_{\beta\text{-gal}}$), HSPs (K_{GroES} and K_{GroEL}) and proteins, K_{Pr}.

3.6 Integrated Processes using Aqueous Two-Phase Systems. The extractive cultivation of *E.coli* cells, which was including the cultivation and disruption of cells and the release and separation of intracellular target proteins, was operated intermittently as follows. The flowchart of a series operation is shown in **Fig.6-3,** especially, in relation to the intermittent operation to produce and separate the HSPs.

i) Case I: β-Galactosidase The extractive cultivation of *E.coli* cells was studied in the following manner. Firstly the *E.coli* ML308 cells were grown at 37 °C in PEG/phosphate aqueous two-phase media (100ml) and the culture broths were irradiated by the ultrasonic wave under the conditions described above. The aqueous two-phase systems were then separated into top and bottom phases by centrifugation. The top phase (45ml) was recovered and the bottom phase containing the surviving *E.coli* cells was reutilized for cultivation together with a new top phase (45ml). This operation was re-

peated three times. In each operation, the new top phase was prepared by separating the similar aqueous two-phase systems with the components of the modified M9 media but without the presence of *E.coli* cells.

ii) HSPs (GroEL and GroES) In the case of extractive cultivation process for the recovery of HSPs, the recombinant *E.coli* OW10/pND5 cells were firstly grown in PEG/Dex aqueous two-phase media (100ml) and the culture broths were heated from 30 to 42 °C at the OD_{610} of 1.0 to produce HSPs (Miyake *et al.*, 1993). In batch process, they were then irradiated by the ultrasonic wave (100W,0~16min) and were recultivated at the temperature of 30 °C. In a semi-continuous one, the aqueous two-phase systems were then separated into top and bottom phases by centrifugation (5000rpm, 10min). The top phase was recovered (20~30ml) and the bottom phase containing the surviving *E.coli* cells was reutilized for the cultivation together with a new top phase (20~30ml). In this operation, the new top phase was prepared by separating the similar aqueous two-phase systems with the same components of the above media but without the presence of *E.coli* cells. The series

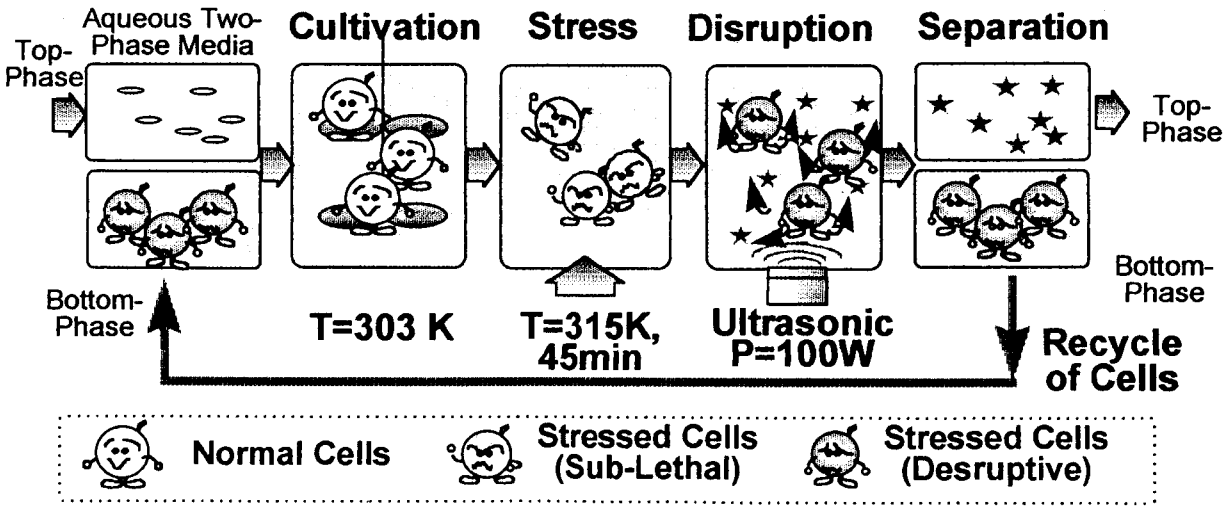


Fig. 6-3 Intermittent operation of extractive cultivation of bacterial cells in aqueous two-phase systems

of operations were repeated five times.

3.7 Assay. The cell concentration was determined by optical density at 660nm and 610nm. The activity of β-gal was assayed by using ONPG as substrate (Gray *et al.*, 1973). Proteins were assayed by the Bradford method (Bradford, 1976). GroEL and GroES contained in cell free extracts were analyzed by transmittance of SDS(sodium dodecyl sulfate)-polyacrylamide gel after electrophoresis. Transmittance was measured by using a Dual-Wavelength Flying-Spot Scanner CS-9000 (Shimadzu Corp.).

4. RESULTS AND DISCUSSION

4.1 Selective Recovery of Cytoplasmic β-Gal by Using Combined Heat and Osmotic Stresses. As a case study for the process integration of ATPS and stress-response functions, especially, heat-induced translocation across inner membrane (Pathway B in **Fig.4-1**), cytoplasmic β-gal was selected as the first target enzyme for the stress-mediated extractive cultivation process.

4.1.1 Optimization of Stress Conditions for the Integrated Process. The stress condition for translocation of β-gal were firstly optimized following the scheme shown in **Fig.6-2**. In chapter 4, the heat-induced translocation was shown to be maximized at the range of 40~50°C (**Fig.4-4**) because of the partial unfolding of target β-gal, followed by the *in vivo* thermal denaturation. However, the heat stress of this temperature range is known to give serious damage to the bacterial cell for their survival owing to the induction of the thermal denaturation of intracellular proteins and the formation of the insoluble molecular aggregates. In the above integrated process especially in a continuous (or intermittent operation), the cell death, caused by excess stress, is a serious problem since the cells are often reutilized to the further

cultivation after the recovery of intracellular proteins. In chapter IV, combined osmotic stress with heat stress was shown to induce more effective translocation of β-gal (**Table 4-1**), so that such translocation phenomena was induced at the relatively weak heat stress condition (37~40°C). The optimal condition can, therefore, be determined at lower temperature range by considering the combined effects of heat and osmotic stresses.

Secondly, the ATPS, which were suitable for the extractive cultivation process, were selected. Previous workers have studied mainly polymer phase systems such as PEG-Dex for the extractive cultivation in ATPS. PEG/Dex systems however appear to involve certain economical and operational limitations due to high viscosity. It can be overcome by the use of PEG/salt phase systems (Walter *et al.*, 1984). PEG/salt systems have a wide range of hydrophobic differences between the two phases of these systems (Kuboi *et al.*, 1994). The selectivity in partitioning target enzymes can, therefore, be enhanced by use of these phase systems (Veide *et al.*, 1983). Little attention has been given, however, to the extractive cultivation in PEG/salt media, since the high salt concentration for this system is thought to inhibit the growth of normal cells such as *Escherichia coli* (Stanier *et al.*, 1979). In some cases, the addition of salts in quantities greater than the minimal salt concentration leads to strong inhibition of growth (Stanier *et al.*, 1979).

The use of PEG/phosphate ATPS is, however, found to be useful from the view points of the effective separation of the target from cells and the effective induction of its translocation, even though the growth rates of cells were reduced in such ATPS. For example, the growth curves of *E.coli* cells in a control culture and also in aqueous two-phase systems are shown in **Figure 6-4**. In the PEG/Dex systems, composed of two polymers, the cell

growth, as expected, was almost the same as that in the control. *E.coli* cells were able to grow in PEG/phosphate two-phase media although their growth rates were lower than that in above two culture.

The culture condition at relatively low temperature (37°C) in the PEG/phosphate ATPS media, which are expected to enhance the translocation of target β-gal due to their combined effects, was selected as the basic conditions. In the following, the optimal condition was determined by considering growth of cells, translocation, productivity and partitioning of the target enzyme on the basis of the scheme shown in **Fig.6-2.**

4.1.2 Cultivation of *E.coli* Cells in PEG/Phosphate ATPS

(1) Effects of Two-Phase Systems Forming Components on the Cell Growth. The effects of the concentration of phosphate salts (Phosohate(I)) and PEG on cell growth were examined using a single phase media containing Phosphate(I) or PEG4000 of various concentrations. The observed specific growth rates are summarized in **Table 6-1**. Growth rate decreased continuously with increasing Phosphate(I) and PEG4000 concentration. **Table 6-2** shows the growth rate of *E.coli* cells in single phase media to which PEG of various molecular weights and Dex of 100~200k were added at the concentration of 20%. A slight reduction of specific growth rate was observed in the solutions that contain PEG20000 and Dex. In solutions containing a polymer such as PEG600, 1540, and 4000, the growth rate is dramatically reduced. In **Table 6-2**, the viscosities of the polymer solution were also shown so as to discuss them. In the former case, inhibition of cell growth can be caused by dissolved oxygen limitation owing to the inadequate aeration in the high viscous media. However, the latter inhibition of

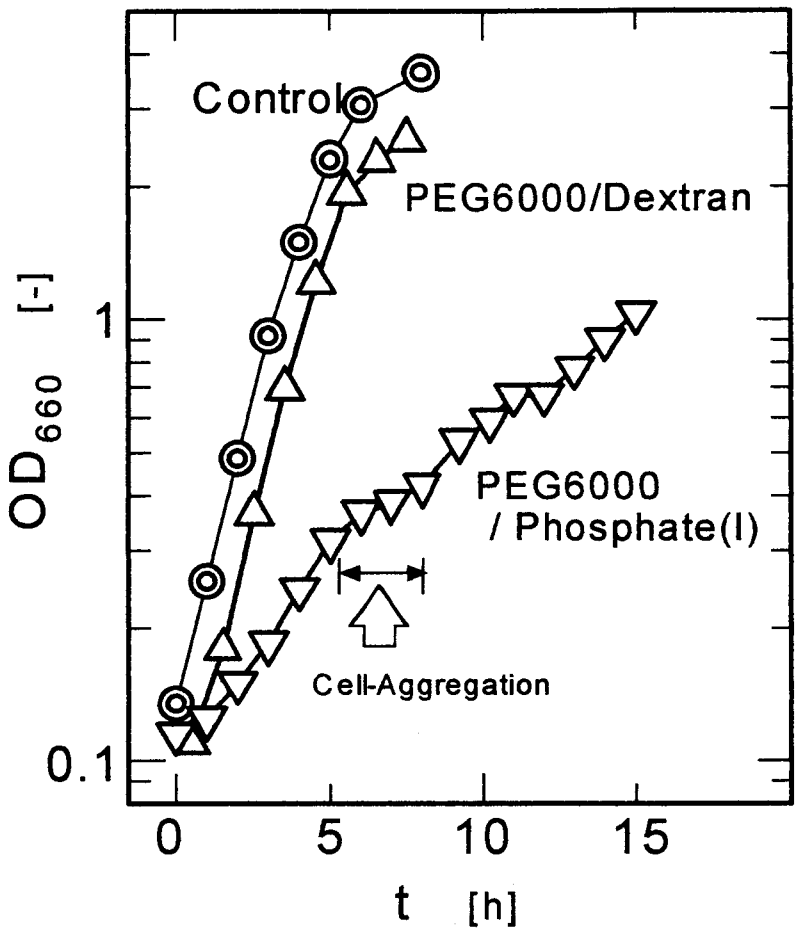


Fig. 6-4 Growth behaviors of *E.coli* ML308 cells in two kinds of aqueous two-phase systems

Table 6-1 Effects of phosphate(I) and PEG4000 concentration on the specific growth rate of *E.coli* cells in the single solutions

Kinds of ATPS Forming Components		Specific Growth Rate [hr^{-1}]
Control		0.640
Phosphate (I)	5%	0.504
	10%	0.298
	20%	0.092
PEG4000	10%	0.642
	20%	0.251

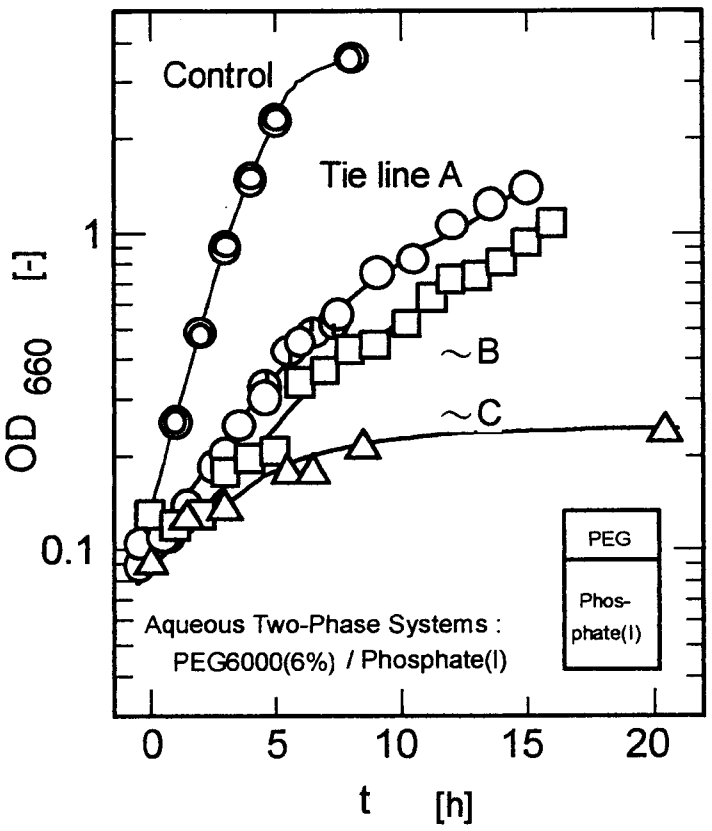
Table 6-2 Relationship between the specific growth rate and the viscosity of various polymers (polymer concentration is 20%)

Kinds of Polymer	Specific Growth Rate [hr^{-1}]	Viscosity $\times 10^{-3}$ [Pa·s]
Control	0.640	0.89
Dextran 100-200k	0.462	50.9
PEG600	0	2.30
PEG1540	0	3.39
PEG4000	0.251	5.71
PEG6000	0.637	15.3
PEG20000	0.462	50.6

cell growth in the low viscous media is likely to be caused by the interaction between the cell wall and PEG molecules (Honda *et al.*, 1981-a,b). Based on these results, the use of PEG6000 as ATPS forming component was found to be suitable for the cultivation of *E.coli* cells in aqueous two-phase media.

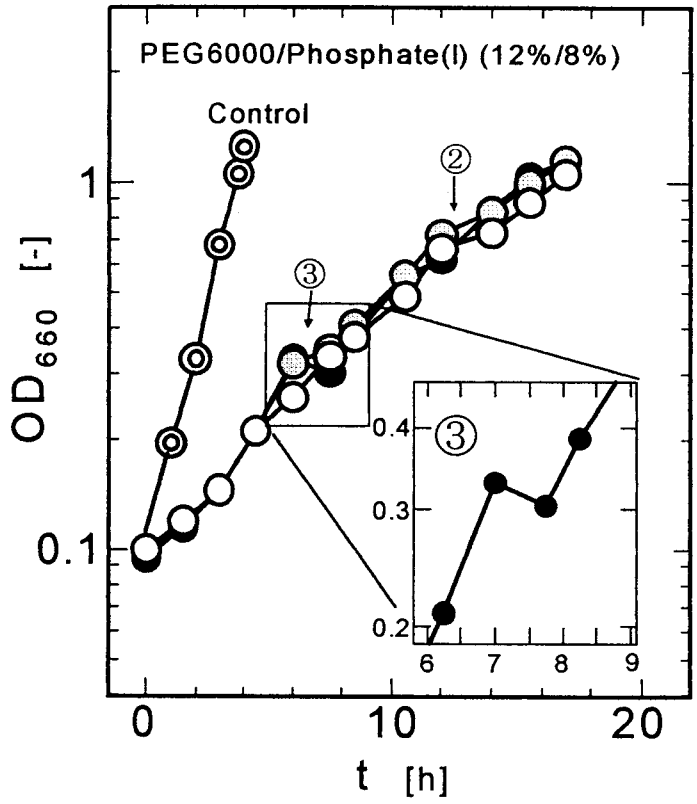
(2) **Effect of Tie-Line Length and Phase Volume Ratio on Cell Growth in PEG/phosphate Systems**. The tie-line length is increased with increasing phosphate concentrations. The effects of tie-line length were examined in various PEG6000/Phosphate(I) aqueous two-phase systems, in which Phosphate(I) concentrations were varied, but in which PEG concentrations were maintained at 6%. **Figure 5-4** shows the growth curve of *E.coli* cells for these systems; the results indicating that the cell growth is inhibited by increasing Phosphate(I) concentration. This inhibition is caused by the increase in the Phosphate(I) concentration in the bottom phase where the *E.coli* cells are partitioned.

When aqueous two-phase systems having identical tie-lines are employed, the systems have identical compositions for the top and bottom phases but have different phase volume ratios (Albertsson, 1986). The effect of the phase volume ratio on the growth in PEG/Phosphate(I) systems was investigated in a same tie-line. **Figure 6-6** shows the growth analysis of *E.coli* cells for systems with different volume ratio along a single tie-line in PEG6000/ Phosphate(I) (12%/8%) systems. In this, the measured growth rates are nearly identical for all volume ratios. The aggregation of the *E.coli* cells was not observed when the volume of the bottom phase was greater than that of top phase. Under the other conditions, however, the *E.coli* cell aggregates were observed when an OD_{660} based on the bottom



Keys	PEG6000 [w/w%]	KNaPi [w/w%]	Tie-line	Aggre-gation
◐	6.	5.0	—	-
○	6.	8.0	A	-
□	6.	10.0	B	-
△	6.	13.0	C	-

Fig. 6-5 Effect of tie-line length on the growth of *E.coli* ML308 cells in the PEG6000 /KNaPi aqueous two-phase systems (tie lines A~C refer to **Figure 6-7**)



Run	Keys	V_{Top}/V_{Bottom}	Tie-line	Aggre-gation
①	○	0.23	B	-
②	◎	1.04	B	+
③	●	8.60	B	++

Fig. 6-6 Effect of phase volume ratio on the growth of *E.coli* ML308 cells in the PEG6000/KNaPi aqueous two-phase media

phase volume reached approximately a value of 2.

The results in PEG6000/Phosphate(I) systems are summarized in **Fig.6-7**. The key in this Figure indicates the growth behavior and the shading of key indicates the degree of aggregation. The increase in tie-line length, that is, the increase in phosphate concentration in the bottom phase is related to the reduction of growth rate. The cause of cell aggregation, which are identical for growth rate, seems to be related with the phase volume ratio in a single same tie-line. Summarizing the effects of the controlla-

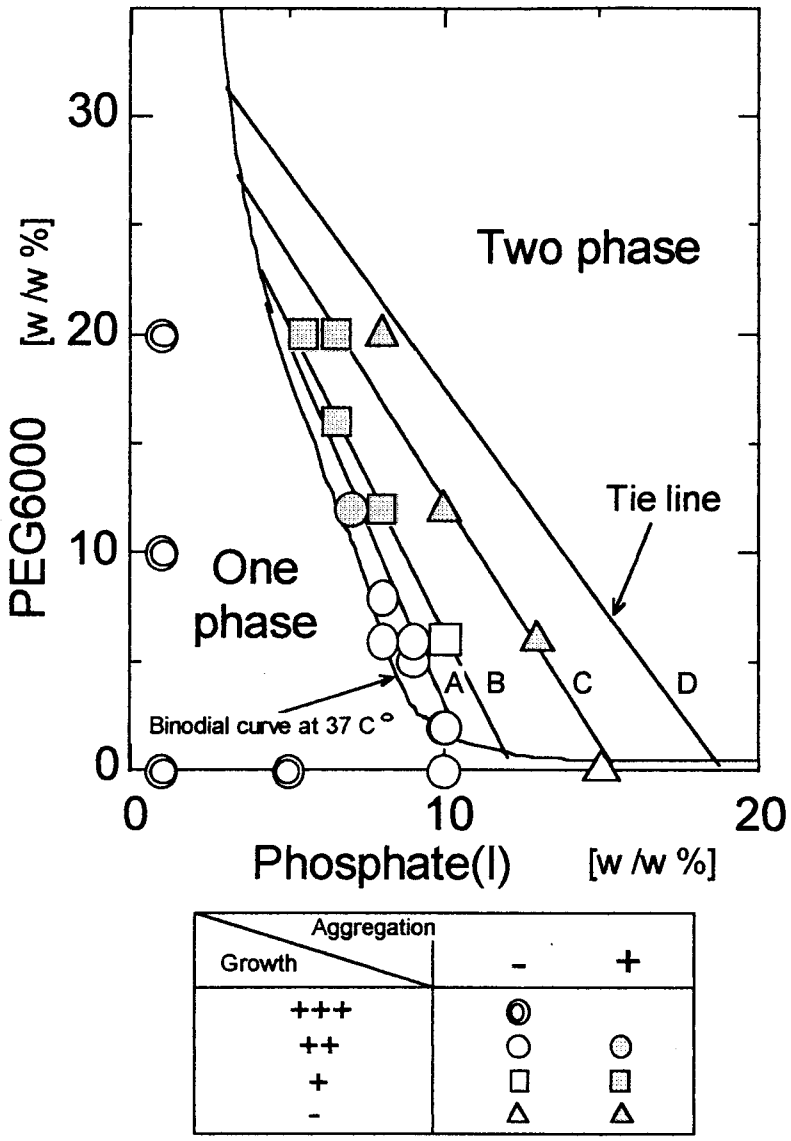


Fig. 6-7 Summary of growth behavior of *E.coli* ML308 cells in the PEG6000/KNaPi aqueous two-phase media with the phase diagram.

ble factors as shown in **Fig.6-7**, cell growth is shown to be most suitable in the phase systems near the plait point.

4.1.3.Release and Partitioning of the Target β-Gal from *E.coli* Cells.

(1) Release of β-Gal from *E.coli* Cells. The culture broths of *E.coli* cells, cultivated in single phase media, were irradiated by the ultrasonic wave with the input power of 80W for various time length at the OD_{660} of 1.0 and the

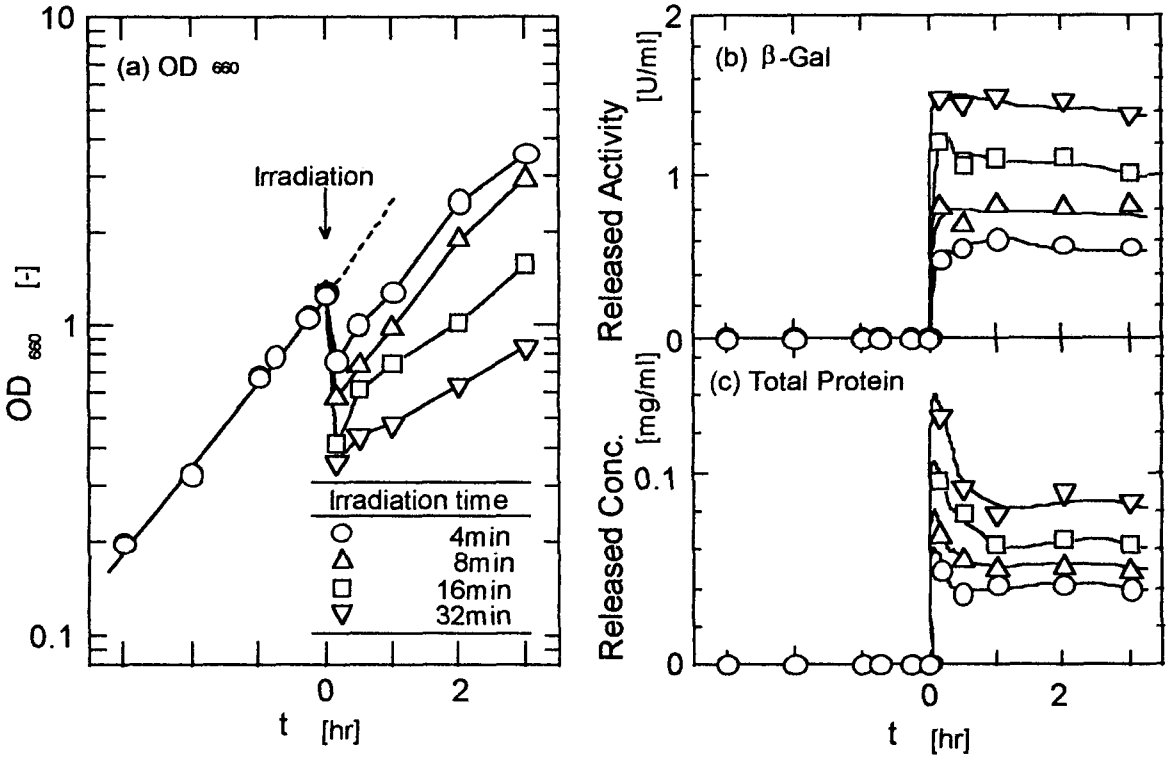


Fig. 6-8 Time course of (a) OD_{660} , (b) activity of β-gal, and (c) concentration of total soluble proteins after ultrasonic irradiation in the single phase solution.

surviving cells were then utilized for the recultivation as shown in **Fig.6-8**. At the greater disruption times, the values of cell concentration (OD_{660}) decreased (**Fig.6-8(a)**). Similarly, the larger amount of β-gal (**Fig.6-8(b)**) and total soluble proteins (**Fig.6-8(c)**) were then released to the surrounding media. Here both the decreased amounts of cell concentration and the released amounts of β-gal and total proteins were proportional to the logarithm of the disruption time. *E.coli* cells were found to grow again immediately after disruption in any condition as shown **Fig.6-8(a)**. However, the growth rate was slightly reduced when disrupted for longer than 16min. As shown in **Fig.6-8(b)**, the amount of β-gal released was maintained at the same for a long incubation period following disruption. However, the total

soluble protein decreased to 60~70% of the initial value as shown in **Fig.6-8(c)**. Some of the proteins released were therefore probably hydrolyzed by the protease released together. The specific activity of β-gal after 0.5~1hr, thus, increased to higher values of 15~18U/mg as compared to values of 10~13U/mg, obtained immediately after disruption. Based on these findings, the condition of the ultrasonic irradiation for 8min was effective for the greater growth rate of cells and the greater amounts of released β-gal.

(2) Partitioning of β-Gal in PEG/Phosphate ATPS. In this extractive cultivation, target β-gal is preferred to be recovered from the top phase of two-phase systems. Using the crude extract from *E.coli*, the effects of additional salts on the partition coefficients of β-gal ($K_{\beta\text{-gal}}$) and total protein (K_{Pr}) were examined in PEG/phosphate systems. **Table 6-3** shows the effects of the addition of M9 salts on the partition coefficients. The values of $K_{\beta\text{-gal}}$ in PEG/Phosphate(II) systems were 7 to 8 times greater than those in PEG/Phosphate(I) systems. The values of $K_{\beta\text{-gal}}$ were reduced in almost all conditions by the addition of M9 salts. The values of $K_{\beta\text{-gal}}$ decreased dramatically especially when NaCl was added. **Figure 6-9** shows the effect of NaCl or Na_2SO_4 salt concentration on the $K_{\beta\text{-gal}}$ values and on the K_{Pr} values in PEG/Phosphate(I) systems. The addition of NaCl of 10g/l minimized the $K_{\beta\text{-gal}}$ values, as shown in **Fig.6-9(a)**. The M9 media for cultivation of *E.coli* cells contains 5g/l NaCl. The partition coefficient for the total proteins, K_{Pr}, however remained almost at the same level. The effect of other sodium salts with a strong salting out effect, i.e. Na_2SO_4, was also examined and this is shown in **Fig.6-9(b)**. The partition coefficient of β-gal was dramatically increased to about 70, by the addition of 5 to 10g/l Na_2SO_4, while

Table 6-3 Effects of salts containing M9 media on the partitioning behaviors of β-gal and total soluble proteins

Kinds of Salt	$K_{\beta\text{-gal}}$ [-]	K_{Pr} [-]
PEG6000 / Phosphate(I)		
-	7.58	0.285
NaCl (5g/l)	0.135	0.369
NH_4Cl (1g/l)	2.71	0.533
Casamino Acid (1g/l)	7.23	0.477
Glycerol (50g/l)	3.09	0.618
$MgSO_4$ (0.25g/l)	7.09	0.134
PEG6000 / Phosphate (II)		
-	52.5	0.124

while that of total protein was varied only slightly. Thus the partitioning behavior can be improved by replacing NaCl by Na_2SO_4. The partitioning behavior of β-gal have been found to be controlled by the addition various salts (Kuboi *et al.*, 1994; Tanaka, 1993-b). The above effect of NaCl and Na_2SO_4 thus corresponds to these findings.

4.1.4 Application to the Integrated Process.

(1) Selection of Optimal Conditions for the Integrated Process. The results obtained for growth rate, productivity and the partitioning behavior of β-gal in the two PEG/phosphate systems are summarized in **Table 6-4**. The *E.coli* cells were cultivated in aqueous two-phase media containing NaCl or Na_2SO_4, and the culture broths were irradiated by an ultrasonic wave for 8min when at an OD_{660} values of 1.0. The growth rate and the productivity of β-gal were slightly reduced with increasing salt concentration. In PEG/ Phosphate(I) systems, the partition coefficients for β-gal were less than 1

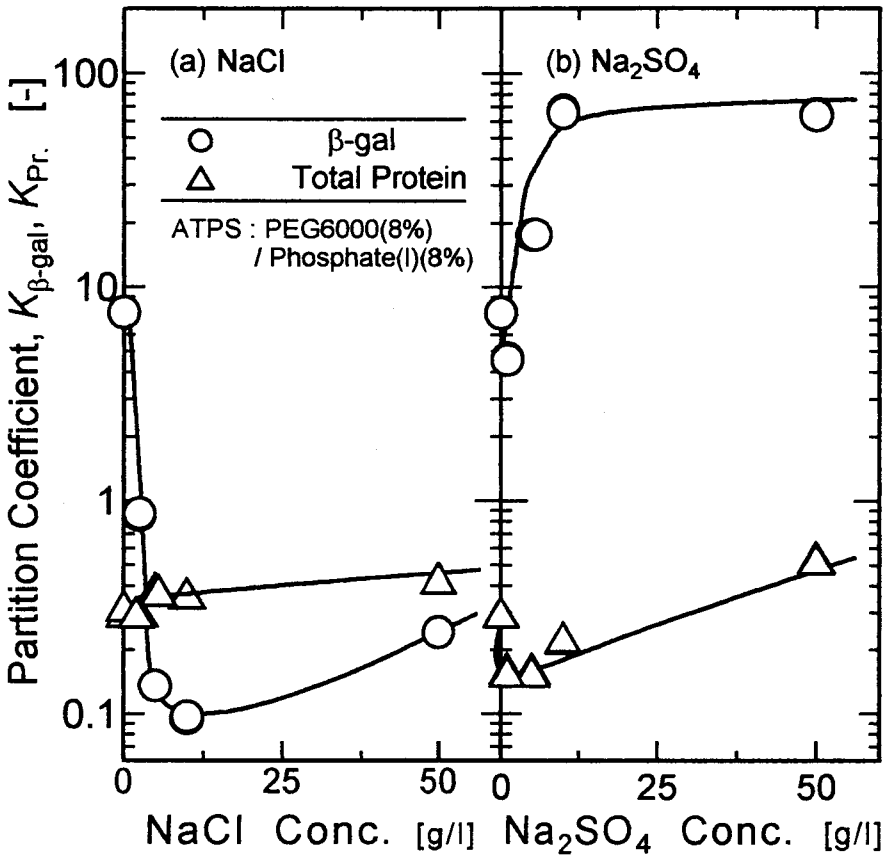


Fig. 6-9 Partitioning behavior of β-gal and total soluble proteins in PEG6000(8%) / phosphate (I) (8%) aqueous two-phase systems when (a) NaCl or (b) Na_2SO_4 was added.

less than 1, but were greater than 1 for the PEG/Phosphate(II) systems. These partition coefficients can be increased to values greater than 10, by the addition of 5g/l Na_2SO_4. Consequently, PEG/Phosphate(II) systems containing 5g/l Na_2SO_4 and not NaCl was found to be optimal for the integrated process.

(3) **Intermittent Operation**. The integrated process was extended finally to an intermittent mode. The *E.coli* cells were cultivated in PEG/ Phosphate(II) media containing not NaCl but 5g/l Na_2SO_4. β-Gal was released by ultrasonic irradiation with an input power of 80W for 8min against 100ml media. The top phase was recovered and the bottom phase containing surviving

cells was recycled for further cultivation with a new top phase. This operation was repeated three times. The results are shown in **Fig.6-10(a)~(d)**. **Figure 6-10(a)** shows the growth curve of *E.coli* cells through the above intermittent operation. The upper arrows indicate the time when the top phase was replaced and the lower arrows the time of ultrasonic irradiation. The cells were grown three times at the same growth rate. **Figure 6-10(b)** shows the time course in the partition coefficients of the β-gal and the total soluble proteins. The total soluble proteins were partitioned mainly into the bottom phase and β-gal could, thus, be recovered continuously from the top phase. The activity of β-gal in the recovered top phase was increased stepwise by the repeated ultrasonic irradiation, and the specific activity of the recovered solution was kept more than 20U/mg as shown in **Fig.6-10(c)**.

Table 6-4 Effect of the addition of NaCl or Na_2SO_4 on the growth rate, productivity of β-gal and partitioning behaviors of proteins in PEG6000(8%) / phosphate(I) (8%) and in PEG6000(9%) / phosphate (II) (9%)

Additional Salts [g/l]		Growth Rate	Productivity	Partition Coefficient [-]	
NaCl	Na_2SO_4	[hr^{-1}]	[U/mg-Cell]	$K_{\beta\text{-gal}}$	K_{Pr}
PEG6000 / Phosphate (I) (8% / 8%)					
5.0	-	0.187	4.48	0.0392	0.0808
-	-	0.202	5.52	0.277	0.116
-	5.0	0.164	5.20	0.281	0.130
-	10.0	0.156	5.08	0.242	0.111
PEG6000 / Phosphate (II) (9% / 9%)					
5.0	-	0.218	4.40	1.16	0.221
-	-	0.224	5.30	2.22	0.213
-	5.0	0.191	4.26	10.3	0.127
-	10.0	0.160	4.08	17.6	0.198

The total soluble proteins remained at constant level as shown in **Fig.6-10(d)**. Consequently, the present integrated process using PEG/phosphate aqueous two-phase systems can be utilized for the effective extractive bio-separation of the intracellular bioproducts.

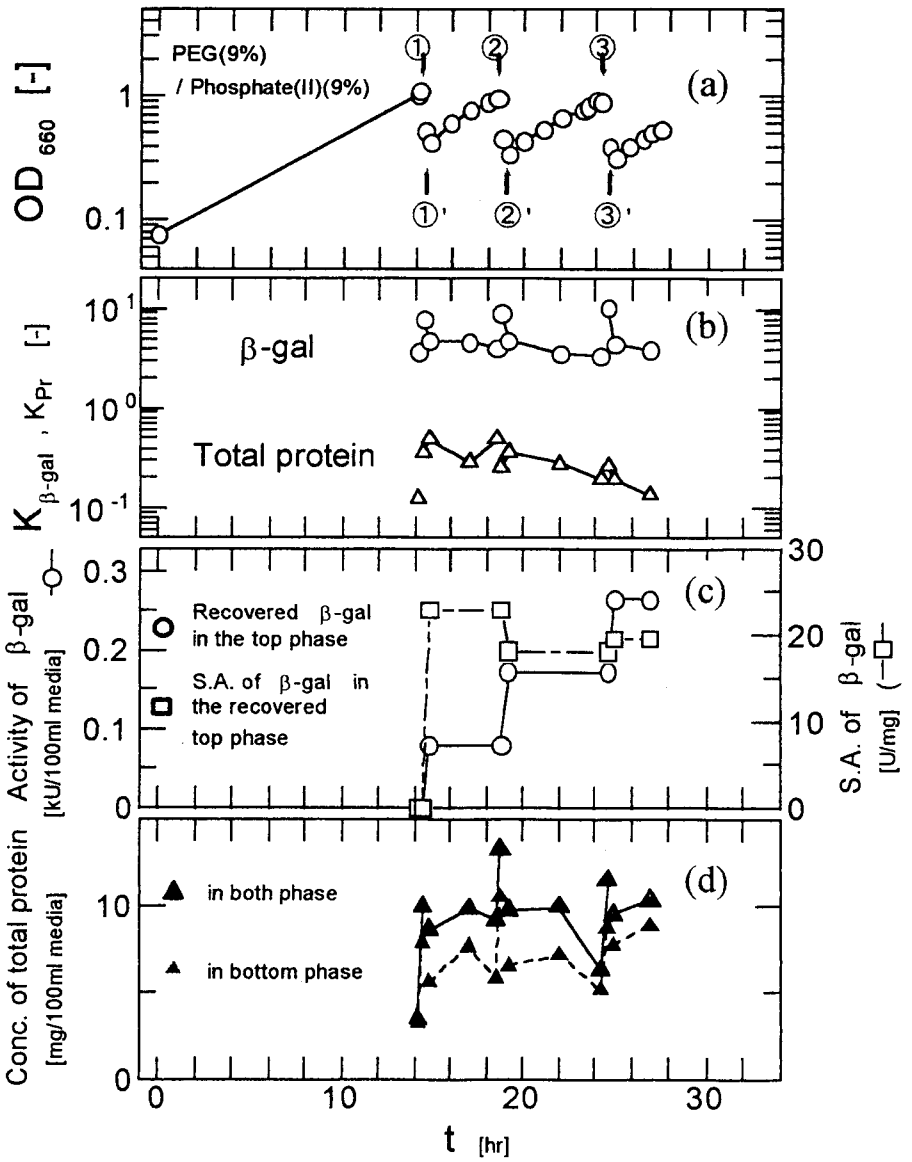


Fig. 6-10 The intermittent operation : the time cource of (a)OD_{660}, (b) partition coefficient of β-gal and total proteins, (c)activity and specific activity of β-gal, (d)total proteins in the both and bottom phase (upper and lower arrows shown in (a) indicate the time when ultrasonic was irradiated and upper phase was replaced, respectively)

4.2. Enhanced Production of Cytoplasmic HSPs by Using Heat and Osmotic Stresses. As the second case study for the process integration of ATPS and stress-response functions, especially, the enhancement of productivity of heat shock proteins (HSPs; GroEL and GroES) (Pathway A in **Fig.4-1**), the cytoplasmic HSPs were selected as target proteins for the second stress-mediated extractive cultivation process.

4.2.1 Growth Behaviors of Recombinant *E.coli* Cells in Aqueous Two-Phase Systems. Figures 6-11 show the growth behaviors of *E.coli* OW10/pND5 in the two-phase systems. As shown in **Fig.6-11(a)**, the growth rate of the cells was reduced when the average molecular weight of PEG decreased. In PEG1540/Dex systems, the cell growth was strongly inhibited, although they were grown in PEG4000, 6000/Dex systems at almost the same growth rate as that in control culture. Honda *et al.* (1981) have

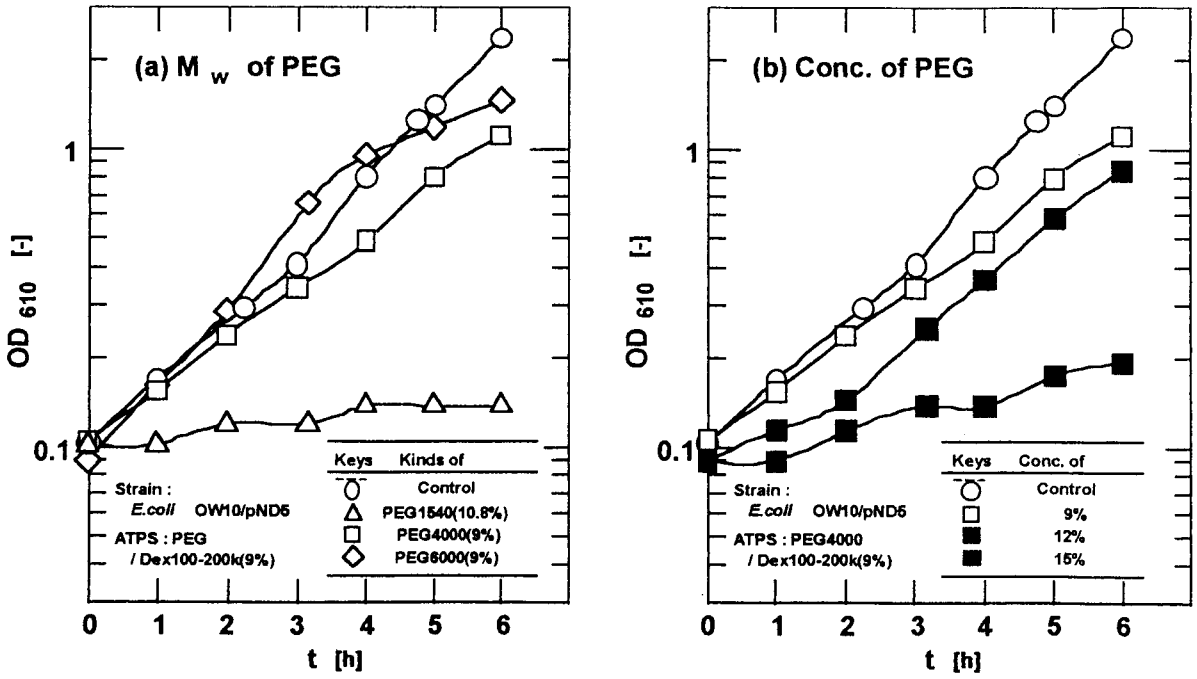


Fig. 6-11 Effect of (a)molecular weight and concentration of PEG on the growth behaviors of recombinant *E.coli* cells (OW10/pND5) in PEG/ Dextran aqueous two-phase media.

reported that PEG molecules interact with the cell walls. In the cell disruption process, the addition of PEG1540 to the media enhance the release rate of the intracellular enzyme, especially periplasmic one (Kuboi *et al*, 1995-b). This growth inhibition of the cells encountered is possibly caused by the interaction between the PEG molecules and the cell walls.

The effect of tie-line length on the growth behaviors was examined in various PEG4000/Dex systems, varying PEG4000 concentrations. As shown in **Fig.6-11(b)**, the cell growth was inhibited with PEG concentration, *i.e.*, with increasing tie-line length. The increase of tie-line length has been reported to increase the interfacial potential between two-phases, interfacial tension (Albertsson, 1986) and hydrophobicity (Kuboi *et al.*, 1994-a). The cell growth may be dependent on these differences. Although the mechanism has not been clearly understood yet, the cell growth was optimal near the binodial curve, especially, near the plait point.

The *E.coli* cells were grown in PEG4000(9%)/Dex(9%) two-phase sys-

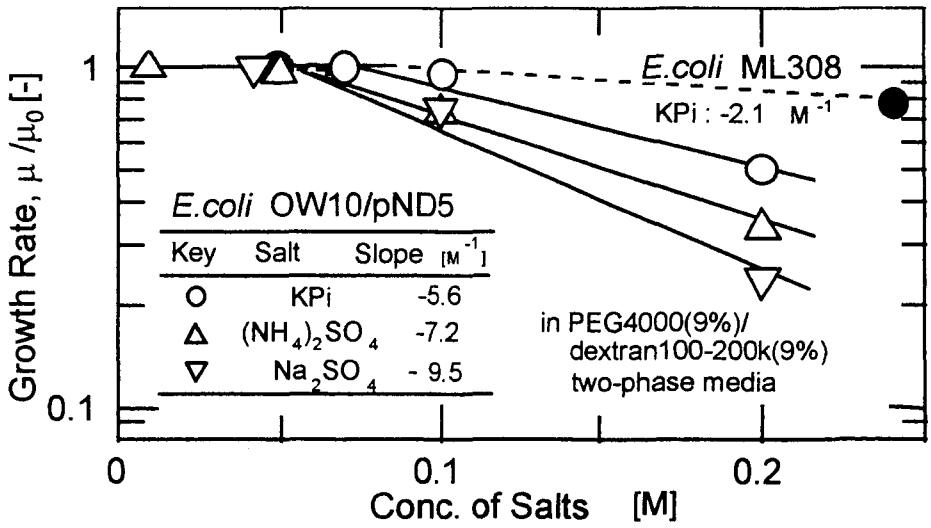


Fig. 6-12 Dependence of the growth rate of *E.coli* cells (OW10/pND5) on the concentration of various salts supplemented to PEG/Dex two-phase media.

tems which contained additional salts at various concentrations. The growth rates, μ, normalized by that in control culture, μ_0, were plotted against salt concentration in **Fig.6-12**. The specific growth rates, μ/μ_0, of *E.coli* cells were found to be exponentially reduced with increasing salt concentration. The sensitivity of cells against various salts was, then, determined by the slopes of these lines in each condition. When KPi salt was added to the two-phase media, the values of the slopes of *E.coli* OW10/pND5 and ML308 were -5.6 and -2.1 M^{-1}, respectively. Clearly, the recombinant *E.coli* cells (OW10/pND5) were more sensitive against KPi salt than the normal cells (ML308) which were able to grow in PEG/KPi systems. It is found that the polymer-polymer aqueous two-phase systems were suitable for the cultivation of the recombinant strains. The effects of the addition of KPi, $(NH_4)_2SO_4$ and Na_2SO_4 on the growth rate of *E.coli* cells were examined in the same way. The value of the respective salts were -5.6, -7.2 and -9.5 M^{-1}. The addition of KPi was found to be the smallest effect on the growth rate and to be acceptable for the cultivation from the view point of cell growth.

4.2.2 Protein Localization and Productivity within the Cells. Through the kinetic analysis of disruption process of the *E.coli* cells, the physical strength of bacterial cells and the intracellular location and productivity of a target protein can be quantitatively evaluated (described in chapter 2).

For the cell disruption process, the remainder fraction measured from cell concentration, D_C, can be correlated to the total input energy supplied into the disruption media, q, as expressed by the next equation (described in chapter 2).

$$\ln D_C^{-1} = \ln (C/C_0)^{-1} = K_C q \qquad (6\text{-}1)$$

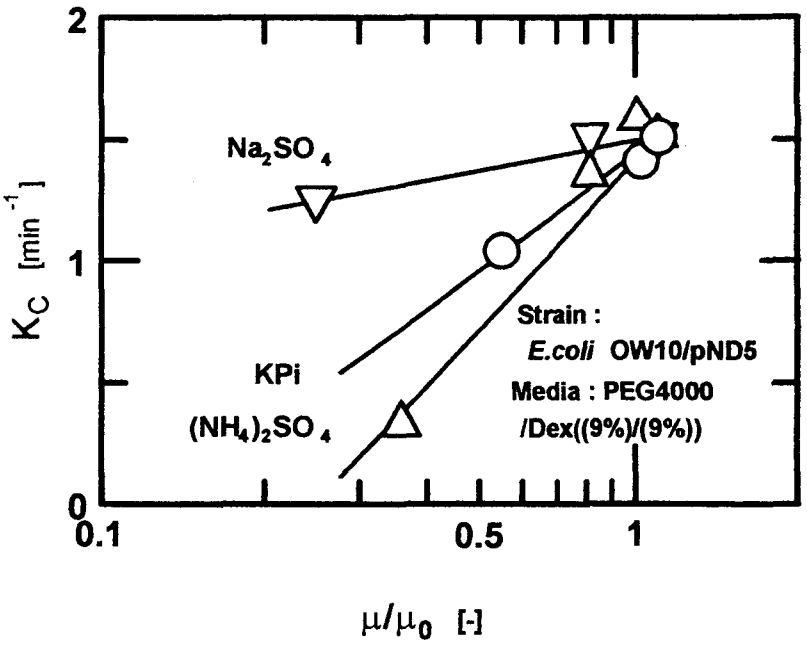


Fig. 6-13 Relationship between the specific growth rate and disruption rate constant from **Eq.(6-2)**. The disrupted cells were cultivated in PEG / Dex two-phase systems containing various salts.

where the disruption rate constant, K_C, is dependent on the type of cells and the growth phase and can be utilized for the quantitative estimation of the change of cell wall strength; K_C values become small when cells have relatively strong walls. As shown in **Fig.6-13**, the disruption rate constants, K_C, of *E.coli* cells, cultivated in the media containing the various salts, were plotted against their normalized growth rates. In general, the K_C values decreased, that is, the cell strength increased with the decreasing growth rate. The specific growth rate, μ/μ_0, of cells is exponentially decreased with increasing the salt concentration. The cells cultivated in the media containing salts at high concentration may, therefore, construct the strong cell wall. It has been reported that the slower growing cells can construct the stronger cell walls (Sauer *et al.*, 1989). The above results are also corresponding to their findings.

For the release process of intracellular proteins, the similar relationship between the remainder fraction of protein, D_i, and total input energy, q, can be obtained as expressed in the following equation.

$$\ln D_i^{-1} = \ln \{1-R_i/R_{i,max}\}^{-1} = K_i q \qquad (6\text{-}2)$$

where R_i ($R_{i,max}$) is (maximum) released amount of intracellular protein per unit cell weight and i indicate either the total soluble proteins or each specific protein. The ratio of release rate constant of target product over that of total proteins, K_i/K_T, can be estimated from this equation and indicate the

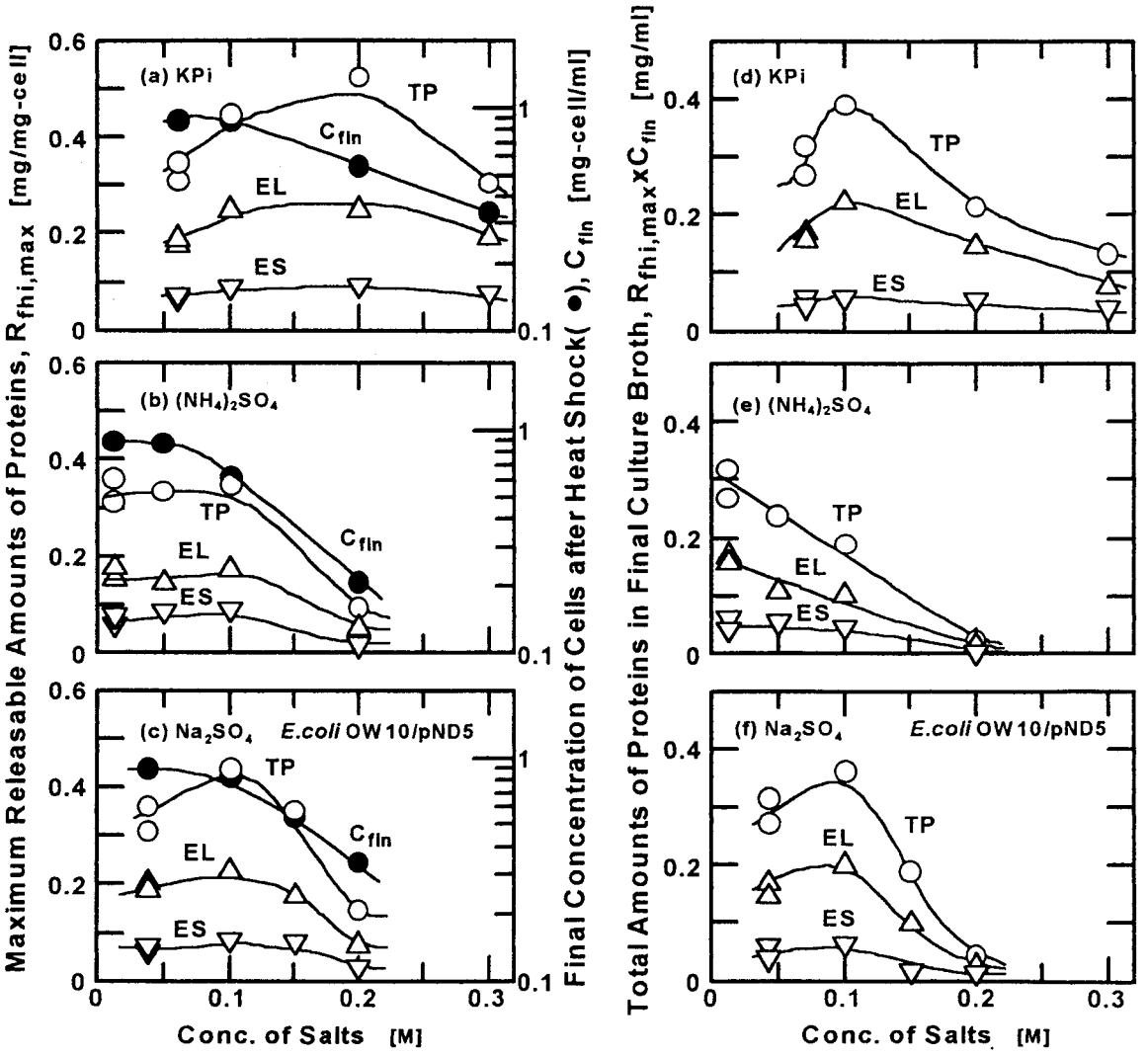


Fig. 6-14 Summary of the productivity of total soluble proteins and HSPs. The dependence of the productivity of HSPs within cells, the final concentration of cells, and net productivity of HSPs on the salt ((a), (d) KPi, (b), (e) $(NH_4)_2SO_4$, and (c), (f) Na_2SO_4) concentration.

indicate the intracellular location of the target protein (Kuboi *et al.*, 1995-b). The values of the target HSPs can here be defined as location factor, LF_i. The LF_i values of HSPs (GroEL and GroES) were examined. The LF_{GroEL}-values were about 1.0 and the LF_{GroES} values were from 0.7 to 1.0 in any condition; it is evaluated that the HSPs, such as GroEL and GroES, located in cytoplasm under the stress condition. These results are corresponding to the previous findings. In these protein release processes, $R_{i,max}$ value for the maximum amounts of the released total proteins per unit weight of cells (Kuboi *et al.*, 1995-b; Hetherington *et al.*, 1971) was used as the total amount of produced proteins. The $R_{i,max}$ value for each protein and the final concentration of cells after the heat treatment, C_{fin}, were plotted against the salt concentration as shown in **Figs.6-14(a)~(c)**. The productivities of total proteins and HSPs were increased with increasing KPi concentration up to 0.2M and were reduced at the greater concentration (**Fig.6-14(a)**). As shown in **Fig.6-14(b)** and **(c)**, these productivities were similarly increased with the increasing $(NH_4)_2SO_4$ and Na_2SO_4 concentrations up to 0.1M. They were, then, decreased when $(NH_4)_2SO_4$ (**Fig.6-14(b)**) and Na_2SO_4 (**Fig.6-14(c)**) were added into the media at the concentration of more than 0.1M. The osmotic stress caused by the addition of salts has been reported to induce the production of the heat shock protein, DnaK (Groat *et al.*, 1986-a). The enhancement of the production of the GroE proteins may also be caused by the osmotic stress. The final cell concentration of various culture broths was corresponding to that of the growth rate shown in **Fig.6-12**. Thus, the net productivity of HSPs in each systems can be determined by the product of them as shown in **Fig.6-14 (d)~(f)**. The maximum values

the PEG/Dex media supplemented with KPi and Na_2SO_4 at the concentration of 0.1M (**Fig.6-14(a)** and **(c)**) although productivity was gradually reduced in the addition of Na_2SO_4 (**Fig.6-14(b)**). It can be concluded that the addition of salts such as Na_2SO_4 and KPi to the growth media can improve the productivity and the optimal concentration is 0.1M.

4.2.3 Partitioning of Heat Shock Proteins in PEG/ Dex Aqueous Two-Phase Systems. In the extractive cultivation, the target HSPs are desirably to be recovered from the top phase of the two-phase systems. Phosphate and sulfate salts effectively increase the partition coefficient of proteins with high molecular weight (Kuboi *et al.*, 1994-a). The effect of addition of various salts, such as KPi, $(NH_4)_2SO_4$ and Na_2SO_4, on the partition coefficients of total proteins and HSPs inPEG4000(9%) / Dex100-200k (9%) two-phase systems was shown in **Fig.6-15**. As shown in **Fig.6-15(a)**, the partition coefficients of total proteins were increased up to 1.0~2.0 when KPi or $(NH_4)_2SO_4$ was added at the concentration of more than 0.05M. **Figure 6-15(b)** shows the dependence of the partition coefficient of GroEL on the salt concentration. These values were increased when KPi and $(NH_4)_2SO_4$ salts were added. Then, the addition of KPi made the partition coefficient increase up to 4~20. The effect of the addition of salts on partition coefficient of GroES was also shown in **Fig.6-15(c)**. The above two salts, especially $(NH_4)_2SO_4$ were effective for the increase of partition coefficient of GroES. Whilst, there is no effect of addition of Na_2SO_4 on the partition coefficient of above proteins as shown in **Fig.6-15(a)~(c)**.

In the presence of other salts for bacterial nutrition, combined effects of the addition of the above salts on the partitioning of GroEL, GroES and

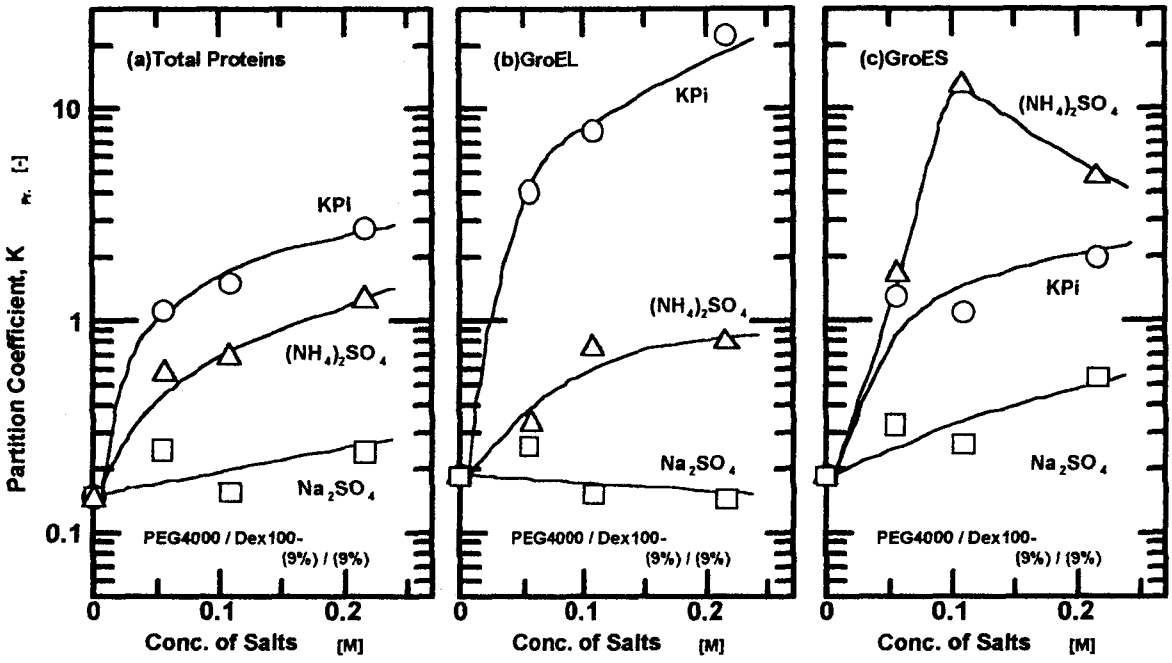


Fig. 6-15 Partitioning behavior of (a) total soluble proteins, (b) GroEL, and (c) GroES in PEG4000(9%)/Dex (9%) two-phase systems when KPi, $(NH_4)_2SO_4$, and Na_2SO_4 were added.

total proteins were examined in PEG4000/Dex two-phase systems and the results were summarized in **Table 6-5**. With increasing the concentration of additional KPi salt, the values of the partition coefficients of target GroEL and GroES were selectively increased to about 1.0 although that of total proteins was only increased to about 0.5. By the addition of $(NH_4)_2SO_4$, the partition coefficients of both total proteins and two HSPs increased to about 2.0. Similarly, the values of the partition coefficient of these proteins were also improved to more than 1.0 by the addition of Na_2SO_4. Although the results of the partition behaviors of these proteins in the presence of other salts were not directly corresponding to the results which were shown in **Fig.6-15**, it was confirmed that the addition of KPi was effective for the

Table 6-5 Summary of partition coefficients of total soluble proteins and HSPs in PEG4000(9%)/Dex(9%) two-phase systems containing KPi, $(NH_4)_2SO_4$, and Na_2SO_4 with other salts (0.25g/l $MgSO_4$, 2 g/l glucose, 1g/l casamino acid, and 5g/l yeast extract)

Additional Salts [M]			Partition Coefficient, K [-]		
KPi	$(NH_4)_2SO_4$	Na_2SO_4	Total Pr.	GroEL	GroES
0.07	0.01	0.04	0.22	0.23	0.76
0.10	0.01	0.04	0.68	0.84	1.5
0.15	0.01	0.04	0.53	0.97	1.3
0.07	0.05	0.04	0.53	0.59	0.97
0.07	0.10	0.04	1.3	1.8	1.0
0.07	0.15	0.04	2.5	2.0	1.5
0.07	0.01	0.10	0.50	0.51	1.3
0.07	0.01	0.15	1.3	1.3	1.8

selective recovery of HSPs from the top-phase.

4.2.4 Effect of Ultrasonic Irradiation on Cell Growth and Products Release in PEG/Dex Media As indicated in the previous chapter, GroEL and GroES were produced in cytoplasm of bacteria, and therefore, cells should be disrupted to release those proteins. The culture broths of cells, cultivated in PEG/Dex systems at 303K, were irradiated by ultrasonic wave with the input power 100W for various time length after heat shock treatment at 315K for 45min and the surviving cells were then utilized for the recultivation at 303K as shown in **Fig.6-16**. When the longer disruption time was used, the values of cell concentration (OD_{610}) decreased at the greater disruption times (**Fig.6-16(a)**) and larger amount of total soluble proteins (**Fig.6-16(b)**) with GroEL and GroES (**Fig.6-16(c)**) were released to the sur

rounding media. As shown in **Fig.6-16(a)**, the recultivated cells were found to grow again right after disruption in any conditions at almost the same growth rate as that before ultrasonic irradiation. As shown in **Fig.6-16(b)** and **(c)**, the amount of total soluble proteins and HSPs decreased and their decreased amounts were greater at the longer disruption time, especially, at 16min. Some of the released proteins were probably hydrolyzed by the protease released together. From these results, irradiation for 8min is found to be effective for the release of HSPs (GroEL and GroES) and also the repeated growth of *E.coli* cells.

4.2.5 Intermittent Operation for Production and Separation of Heat Shock Proteins Finally, the integrated process for the production and recovery of HSPs was extended to an intermittent mode. The *E.coli* OW10

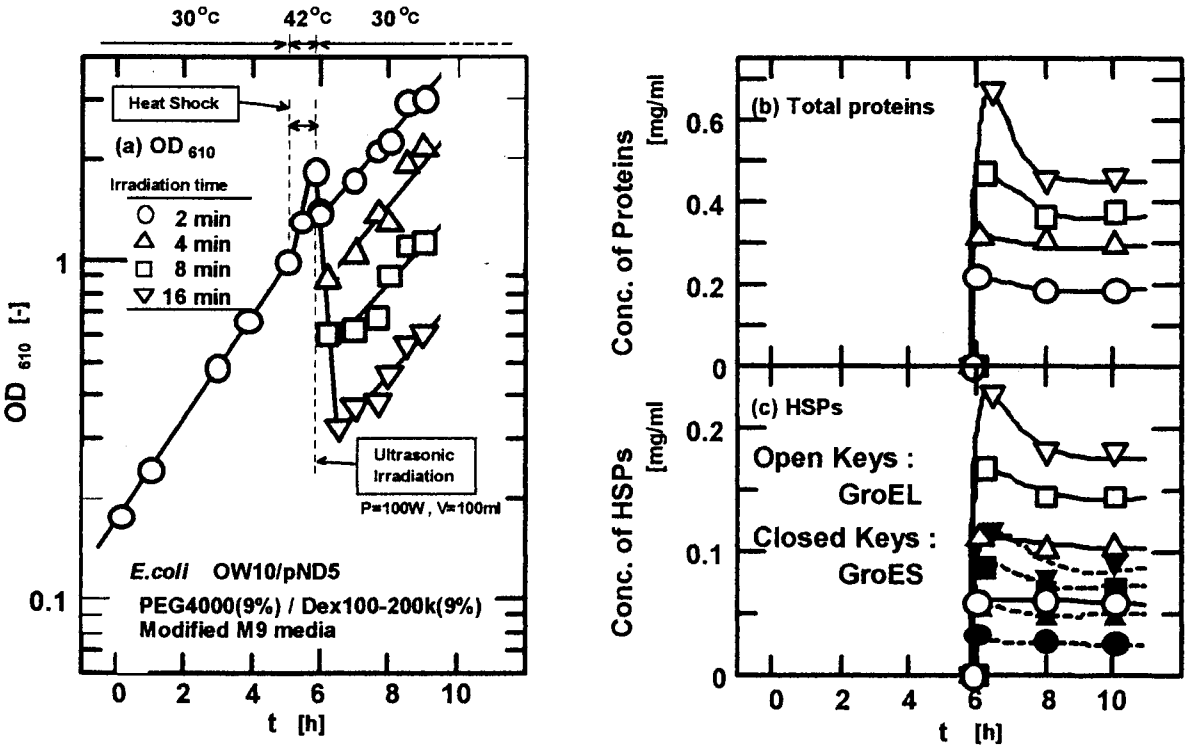


Fig. 6-16 Time course of (a)OD_{660}, (b)concentration of total proteins, and (c) concentration of HSPs after ultrasonic irradiation in PEG4000 (9%) / Dextran (9%) two-phase media.

/pND5 cells were cultivated in PEG/Dex media containing 1M potassium phosphate salt at 303K. At OD_{610} of 1.0, the HSPs was induced by the heat shock treatment (315K, 45min) and then was released by ultrasonic irradiation with an input power of 100W for 8min against 100ml media. The top phase containing the HSPs was recovered and the interface and the bottom phase containing the surviving cells was recycled for further cultivation at 303K with a new top phase. This operation was repeated five times and their operations were indicated in **Fig.6-17(a)**. The results are shown in **Fig.6-17(b)~(e)**. **Figure 6-17(b)** shows the growth curve of the *E.coli* cells through the above intermittent operation. The cells were grown five times at almost the same growth rate. **Figure 6-17(c)** shows the time course of partition coefficients of the total soluble proteins, GroEL and GroES. The partition coefficient of the total soluble proteins was maintained at the same value. Then those of GroEL and of GroES were slightly greater and smaller than 1.0, respectively. As this operation proceeded, these values approached to 1.0. This may be because of the effects of the impurified materials released with proteins and accumulated in the systems. **Figure 6-17(d)** shows the time course of the contents of GroEL and GroES and that of total soluble proteins in the top phase. The fraction of GroEL and GroES in the total soluble proteins were maintained at the values of 50% and 10%, respectively. The concentration of the total soluble proteins was maintained within the range from 0.10 to 0.18mg/ml. As a result, the accumulated weight of each protein recovered from the top phase was increased stepwisely by the repeated ultrasonic irradiation and fraction of GroEL and GroES remained at the values of about 50% and 10%, respectively, as shown in **Fig.6-17(e)**.

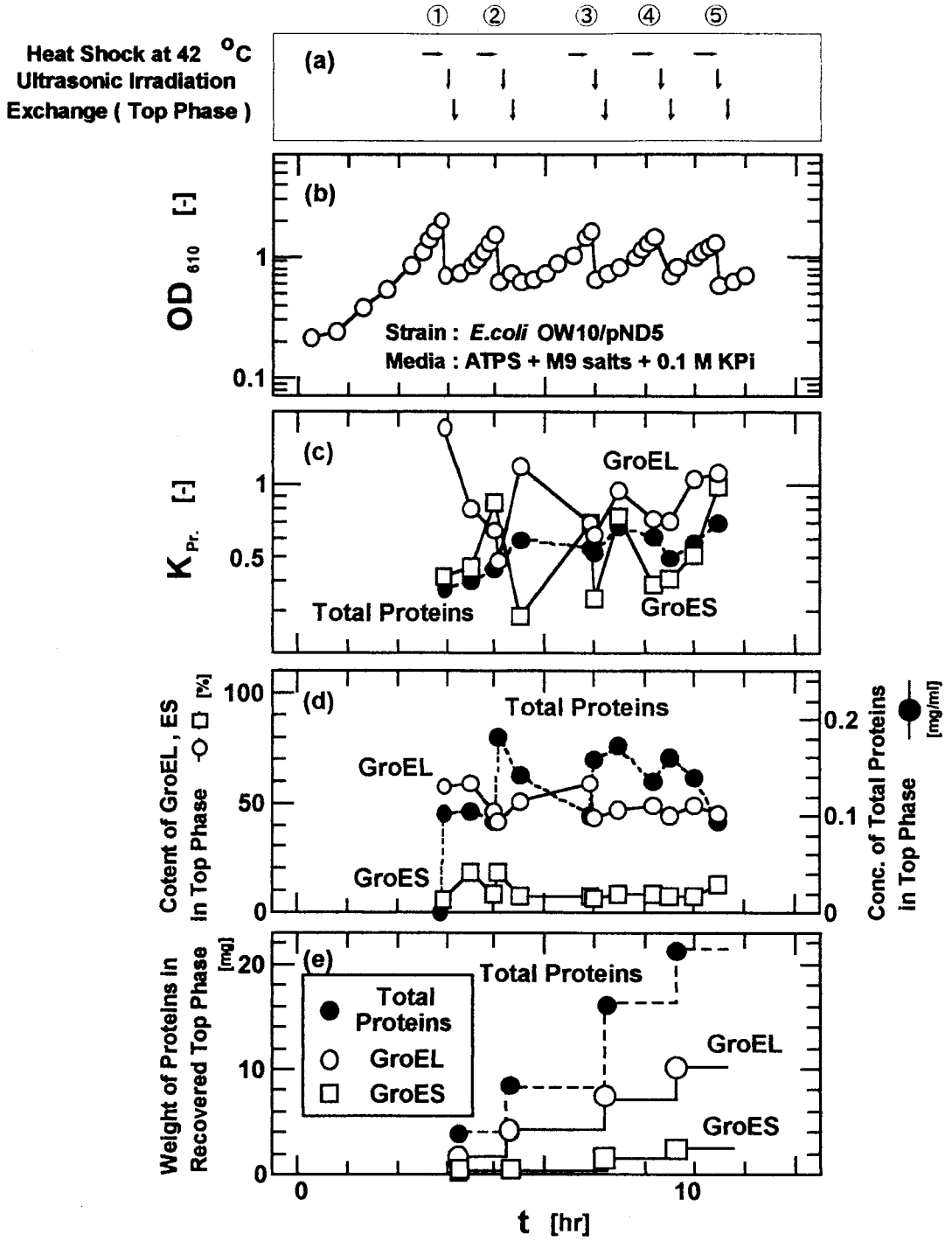


Fig. 6-17 The intermittent operation : the time course of (a)OD_{660}, (b) partition coefficient, K_{Pr}, (c)content of HSPs and concentration of total proteins in top phase (d), and integration of weight of total proteins and HSPs in recovered top phase (e). The arrows on this figure show the time of the treatment of heat shock (42°C, 45min) , ultrasonic irradiation (power; 100W, 8min), and exchange of top phase.

5. SUMMARY

Stress mediated extractive cultivation processes to produce and separate the cytoplasmic i) β-gal and ii) HSPs were successfully accomplished by integrating stress-response functions of cells and ATPS.

(1) The PEG/Dex two-phase systems, which were composed of two polymers, were suitable for the cultivation of recombinant *E.coli* cells because of their higher sensitivity against salts. Then, the addition of KPi salts had the minor effect to the growth rate of cells. While the normal *E.coli* cells were found to grow in the PEG/phosphate systems, of which salt concentration was relatively high.

(2) The heat induced translocation of β-gal and the productivity of HSPs and total proteins were improved by the addition of salts, especially KPi salt into the growth media.

(3) The partition coefficient of β-gal was dramatically increased when the Na_2SO_4 were added to the PEG / phosphate media. For the partitioning of HSPs in the PEG / Dex two-phase systems, the partition coefficients of GroEL were increased to the 100 times greater values, when KPi was added to these systems. Those of GroES were increased when Na_2SO_4 was added.

(4) Although the ultrasonic irradiation was applied repeatedly, both recombinant and normal *E.coli* cells were able to be grown at the same growth rate as before.

(5) Based on the above results and those in chapter 4, the extractive cultivation process was developed and extended to a semi-continuous operating mode, where the top phase containing target β-gal or HSPs was recovered following intermittent ultrasonic irradiation with /without heat stress and the bottom phase containing the cells was recycled together with new top phase solution to repeat production and recovery of the target proteins.

GENERAL CONCLUSIONS

A systematic approach using aqueous two-phase systems (ATPS) to quantify and to integrate various stress-response functions of bacterial cells was successfully carried out for the design and development of the effective production and selective recovery process of intracellular proteins, exploiting the stress mediated enhancement of intra- and inter-molecular hydrophobic interaction. The evaluation method of the surface properties of cells, especially those under stressed conditions, was firstly established to obtain the quantitative relationship between the stress factor and the stress responded change in surface properties by using the aqueous two-phase partitioning method. Secondly, the kinetic/physical properties of bacterial cells and their membranes were evaluated through the kinetic analysis of cell disruption and enzyme release processes as a cell response against the mechanical stress. The enhancement of stress response functions of cells by using hydrophobic interaction between cells and hydrophobic synthetic polymer was clarified. Thirdly, the method to manipulate the lytic process, cellular stress response against chemical stress, was investigated by controlling the hydrophobic interaction between cell membrane and the detergent through the kinetic analysis of cell lysis process using nonionic detergent, Triton X-100. Fourthly, the bacterial cell function induced by sublethal stress was clarified based on the hydrophobic interaction between intracellular biopolymers including heat shock proteins (HSPs), which were induced by various stresses, especially concerning with i) heat-induced translocation and ii) heat-enhanced production and renaturation of the intracellular protein mediated by HSPs. The observed heat-induced translocation of β-gal was verified by using the liposomes as a model

systems. Finally, the bioprocesses integrating the stress-response functions of cells and aqueous two-phase systems was developed in relation to i) the selective recovery of periplasmic β-galactosidase and ii) the effective production of intracellular heat shock proteins (HSPs; GroEL and GroES).

The physicochemical properties of bacterial cell surfaces (isoelectric point, surface net and local hydrophobicity) have been systematically characterized by using the partitioning method in ATPS. The isoelectric point (pI) can be determined by the cross partition method. At the pI, the surface net hydrophobicity (HFS_{Cell}) and local hydrophobicity (LH_{Cell}) of bacterial cells were determined from the partitioning behaviors in ATPS changing the mode of partitioning. By using parameters, such as pI, HFS_{Cell}, and LH_{Cell}, the surface properties of bacterial cells exposed to various stresses were successfully characterized, so that, especially, the cells exposed to heat treatment were found to change not their charges but their surface net and local hydrophobicities.

The cell response against the physical stresses induces the following transient behaviors such as i) the destruction of cell structure and ii) the release of intracellular enzymes at different rates. Through the kinetic analysis of cell disruption process, the generalized operational parameters were firstly presented as a specific energy supplied into the disruption media. The generalized values of the disruption rate constant K_C and the release rate constant for each enzyme K_i (i=1,2,..) were well correlated with the cell strength against the disruptive stress and also the intracellular location of each enzyme, respectively. The values evaluated from the release selectivity against the total proteins (K_i/K_T) were found to show the intracellular location of the enzyme (Location Factor, LF_i), which indicated that

intracellular enzymes were transported to the individual compartments across the membrane depending on stressed conditions. The hydrophobic interaction between the membrane surface and the polymer molecules was also found to be characterized and mediated by the LF_i values. The selective release of periplasmic enzyme was successfully achieved at the optimal condition determined by LF_i values.

The cell response against the chemical stress are mainly categolized by i) the solubilization of outer membrane of *E.coli* cells and ii) the release of periplasmic enzymes. The release process of various enzymes induced by the disruption of the outer membrane was characterized based on the first-order kinetics. The enzyme release process was systematically analysed by using the quantitative parameters such as i) operational parameters of nonionic detergent (Triton concentration; C_{Triton}, molecular weight; M_W) and ii) evaluated properties of cells (surface hydrophobicity of cells; HFS_{Cell}, the local hydrophobicity; LH_{Cell}, location factor of intracellular enzymes; LF_i). From the generalized correlation, the importance of the hydrophobic interaction between the detergent and the membrane surface was studied in detail. As a case study, the operational condition to give both the higher yield and the higher selectivity of cytoplasmic β-gal was obtained based on the generalized relationship.

Based on the evaluated properties of bacterial cells, the biological response of bacterial cells was analysed as targetted the cytoplasmic β-galactosidase in *E. coli* cells. The heat stress was found to induce i) reduction of the amounts of intracellular soluble proteins, ii) the formation of their inactive aggregates, iii) degradation of them, and iv) translocation of cytoplasmic β-galactosidase. Especially, the phenomena of heat-induced

translocation of β-gal across the inner membrane were analysed by using LF_G values. The K_G/K_T values were found to be maximal when cells were stressed at the temperature of 45~47°C and further enhanced by the addition of salts. From the result on the surface properties of both β-gal and cell membrane under the heat stress, it is suggested that (1) the conformational change of cytoplasmic proteins to the partially unfolded state with higher local hydrophobicity, (2) the increase in membrane fluidity of inner membrane, (3) the enhancement of hydrophobic interaction between lipid and protein, and (4) the inhibition of translocation by GroEL restabilizing the heat-damaged proteins, all phenomena could drive the heat-induced translocation of β-gal across the inner membrane.

The mechanism of heat-induced translocation of *in vitro* β-gal was verified by using the model-cell systems prepared by phospholipid. Exposing the liposomes entrapping β-gal to heat treatment (40~50 °C, 1~60min) was also found to induce its translocation across the liposome membrane. The translocated activity of β-gal to the outer aqueous phase indicated the maximal value when the liposomes entrapping β-gal were heated at 45°C for 30min. The gel permeation profiles of the liposomes before and after heat treatment (45°C, 30min) also supported the translocation of β-gal across the liposome membrane. The surface hydrophobicity of liposome membrane, $HFS_{Liposome}$, was increased with increasing temperature and the local hydrophobicity of β-gal, LH_G, which was maximized at the temperature of 40~50 °C. In this way, the *in vitro* mechanisms of β-gal translocation have found to be triggered by the enhancement of hydrophobic interaction between the liposome surface and β-gal molecules. These results were well corresponding to the results

obtained *in vivo*. From the results *in vivo* and *in vitro*, the simple and plausible scheme on the heat-induced translocation of β-gal was finally presented on the basis of the hydrophobic interaction between the liposome and the proteins. This is applied for the heat-induced translocation of other cytoplasmic proteins.

Based on these results, the bioprocess using the stress-response function of cells and aqueous two-phase systems for the production and separation of intracellular proteins was developed in relation to the following two functions such as i) heat-induced translocation enhanced by salts and ii) refolding (renaturation) of intracellular proteins. In both phenomena, the conditions were optimized on the basis of the results on stress-response against mechanical, chemical, and mainly against heat stress. In the former case, the cytoplasmic β-gal was selected as a target enzyme. The cultivation condition was selected at moderately high temperature and high salt concentration to give the high translocation of target and the optimal growth of cells. In the latter case, the efficient recovery of the heat shock proteins (HSPs) such as GroEL and GroES was accomplished by combining the heat and osmotic stress. At the optimal condition, the extractive cultivation process was carried out, where the top phase containing the target was recovered and the interface and the bottom phase containing the surviving cells was recycled for further cultivation with a new top phase. This operation was repeated several times. In both cases of the intermittent extractive cultivation process, the β-gal and HSPs were successfully recovered with high selectivity and with high recovery yield.

SUGGESTIONS FOR THE FUTURE WORK

(1) Evaluation of Other Stress-Response Functions of Bacterial Cells. In this study, two functions of bacterial cells, *i.e.* (1) reactivation or refolding of the proteins partly denatured under sublethal stresses and (2) their specific translocation across inner membrane induced by heat and/or osmotic stresses (the latter one or combined one should be studied further), were mainly investigated among their various stress response functions. Among other important functions, especially (3) the aggregates formation and (4) the degradation of proteins, both mediated by stress-induced other HSPs and protease, should be further studied by using the same methodology presented in this work, *i.e.* quantitative evaluation of the surface properties of outer and inner cell membranes, intracellular location of enzymes, and their release rate constants. In practical process level, the control of (3) aggregates formation may contribute both to solve the serious problem in relation to the formation of inclusion body of recombinant proteins and to develop new bioprecipitation processes. And by the control of (4) the degradation by the protease, selectivity and recovery yield of the target protein may also be improved. In all of these processes mediated by some stress proteins / HSPs, the important issue may be evaluation and control of the 'hydrophobic interaction' in the target proteins, the functional stress proteins such as molecular chaperon and HSPs, and/or the cell membrane.

(2) Stress Mediated Translocation of Other Proteins and Those Fused with β-Galactosidase. It has been shown that the surface properties of proteins and phospholipid membrane were varied depending on the variation of conformation and the stress conditions. In chapter 4, the change in

surface hydrophobicity of cytoplasmic β-galactosidase induced by heat stress were shown and, especially, the dependence of the biological function (transmembrane) on their properties were presented, where the surface properties, especially, local hydrophobicity of proteins played an important role. It is interesting to study whether this stress mediated translocation is general stress response function of cells applicable to other proteins and also those fused with β-galactosidase. The quantitative characterization of surface properties of those proteins and cell membranes and their hydrophobic interactions may give the sound base for the rational design and development of novel and advanced bioprocesses and materials which would recognize and respond to environmental stresses.

(3) *in vitro* Reconstitution of Intracellular Materials Relating to *in vivo* Functions. Based on the quantitative characterization of the properties and functions of proteins at various stress conditions, the functions of stress proteins such as HSPs and molecular chaperons should be verified in the *in vitro* reconstituted systems using the phospholipid membrane (liposomes), which is used as a cell model. The biological role of the stress proteins can also be characterized by applying the presented method to the reconstituted model cells.

(4) Model Simulation of Biological Functions based on the Hydrophobic Interaction. A possible model for the translocation of β-gal was presented in this work. Several possible pathways were herewith neglected for the simplification of the model. The improved model should be investigated by considering the effect of the presence of HSPs and salts, the aggregation of proteins, the re-translocation of translocated proteins. Further, the possible application of the improved model for the *in vivo* results has to

be shown by comparing the results *in vitro* and *in vivo*.

(5) Application of Model Cells As a Functional Biomaterials. The liposomes trapping β-galactosidase and HSP (GroEL) were used as model systems of *in vivo* heat-induced transmembrane of proteins. They can also be used as a functional biomaterials by clarifying the effect of composition of host membrane, the effect of the types of trapping protein, and in details quantitative relationships between 'stress factor' and 'response of the materials'.

The above approaches both *in vivo* and *in vitro* seem to be corresponding well each other, suggesting that the common phenomena in relation to the role of hydrophobic interaction may underlie in both cases. The breakthrough of the approaches based on the fusion or integration of microscopic to macroscopic views is needed for the design and development of new-conceptual biomaterials and bioprocess. These may be accomplished by the characterization of surface properties, interaction, and stress response functions of biomaterials, biomembranes, and cells as a common parameter.

NOMENCLATURE

AP = fractions of aggregate proteins

ATPS = aqueous two-phase systems

C, C_0 = cell concentration [mg-(dry)cell/ml]

$[D_{4,in}]$, $[D_{4,out}]$ = tetrameric β-gal of inactive state

D_C, D_i = the remained fraction of cell disruption and enzyme and protein release [-]

DF_C, DF_S = 1-D_C, 1-D_S [-]

$DRF_i = RF_i - RF_T$ [-]

d_p = average particle size of bacterial cells [nm]

HFS = surface hydrophobicity of proteins, liposomes, and cells

I = intermediate states of β-gal inserted to lipid membrane

K = partition coefficient of biopolymers, liposomes and cells

K_C, K_i = (V/P)k_C, (V/P)k_T [m^3 J^{-1}]

k_C, k_i = rate constant of cell disruption, cell lysis or enzyme release [min^{-1}] (chapter 2 and 3)

k_1, k_2 , k_d = kinetic parameters of model simulation of heat-induced translocation of protein [min^{-1}] (chapter 5)

LF_i = location factor of intracellular enzyme i (=K_i / K_T) [-]

LH = local hydrophobicity of proteins or cells

MP = fraction of membrane proteins

$[N_{4,in}]$, $[N_{4,out}]$= concentration of tetrameric β-gal of active state

PP = fraction of periplasmic proteins

OD = optical density

pI = isoelectric point of proteins or cells [-]

P = acoustic power of ultrasonic wave [W]

q = specific energy supplied into the disruption media [J m^{-3}]

q_{opt} = optimal specific energy of target enzyme [J m^{-3}]

R_i = released amounts of enzymes or total soluble proteins per weight of packed cells [U/mg-cell or mg-pr/mg-cell]

$RF_i = 1\text{-}D_i$ [-]

SP = fraction of soluble proteins

$SRF_i = RF_i/RF_T$ [-]

t = stressing time [min]

V = working volume of disruption media [ml]

<Subscript>

C = cell concentration determined by optical density

G = β-galactosidase

i = kinds of enzymes (i = 1, 2, and 3 are AcP, G6PDH, Fum, respectively)

Lysis = treatment with Triton X-100

S = average cell size

T = total soluble proteins

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