STUDY ON MEMBRANE-MEMBRANE INTERACTIONS INDUCED BY HEAT STRESS AND APPLICATIONS TO PRODUCE CHITOSANASE AND ITS LIPOZYME FROM *STREPTOMYCES GRISEUS*

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Preface

This PhD dissertation work has been carried out under the supervision of Professor-Doctor Ryoichi Kuboi in Division of Chemical Engineering, Graduate School of Engineering Science, Osaka University, Japan from 2005 to 2009.

The objective of this study is to enhance production and secretion of chitosanase from *Streptomyces griseus* cells based on heat induced membrane-membrane interaction between liposomes and cell membrane. The membrane-membrane interaction induced by heat stress is effective to prepare new-type catalyst (chitosanase-LIPOzyme) that shows significantly higher in catalytic efficiency and stability than those of conventional chitosanase. The chitosanase-LIPOzyme could be prepared by the heat induced secretion of chitosanase harboring signal peptide (SP) associated-liposomes interacting cell membrane under stress conditions. The chitosanase-LIPOzyme could be effectively applied to produce functional biomaterial oligochitosans effectively even though under stress conditions. The author hopes that this research would contribute to the development of the effective bioprocess for production of proteins from microbial cells and the development of new type of biocatalyst LIPOzyme prepared from proteins harboring SP for biochemical engineering bioprocess.

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Contents

Summary

The production of biofunctional oligochitosan has widely attracted many researchers because of its various applications in food, pharmaceutical, cosmetics and so on. Chitosanase is known as a good enzyme that effectively hydrolyzes chitosan to small functional oligochitosans. Many studies have focused on the enhanced production of chitosanase from microbial cells and/or the effective ways to use chitosanase for such purposes. Recently, however, the importance of membrane-membrane interaction has been shown to play the crucial roles for the enhanced production and secretion of intracellular enzymes. In this study, the membrane-membrane interactions between liposomes and cell membrane induced by the heat stress are studied to prepare new-type catalysts (chitosanase-LIPOzymes) that show significantly higher in catalytic efficiency and stability than those of conventional chitosanase. The chitosanase-LIPOzyme prepared in this study represents a novel example of hydrolysis LIPOzyme that can be prepared from the enzyme harboring signal peptide associated-liposomes interacting onto cell membrane under stress conditions.

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In chapter I**,** the effective production and secretion of chitosanase from *S. griseus* cells were studied by the treatment of these cells with the neutral 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine (POPC) liposomes together with heat and/or oxidative stress. The effects of the liposomes and heat treatment on the cell growth and chitosanase production were first analyzed. The possible effects of the heat stress on the conformation and activity of chitosanase, as well as on the membrane fluidity of liposomes, were systematically investigated to elucidate the driving force of the heat induced liposome-chitosanase interaction. The interaction of liposomes with target chitosanase and cell lipid membrane play important roles for the enhanced production and secretion of chitosanase from these cells under heat stress condition. The fundamental investigations of the oxidative stress (hydrogen peroxide) affecting on the lipid and protein peroxidation of cell membrane of *S. griseus* pretreated with and without liposomes were further carried out to optimize the conditions for enhanced production and secretion of chitosanase. The interaction of liposomes with cell membrane could significantly prevent the oxidative damage on cell membrane; as a consequence, the growth and production of chitosanase from these cells pretreated with liposomes and heat stress at 41 ^{o}C were enhanced even under oxidative stress condition.

In chapter II, the surface properties of *S. griseus* cell treated with the heat and/or oxidative stress in the presence and absence of liposomes were quantitatively evaluated in order to optimize conditions for the interaction of liposomes with cell membrane and secreted chitosanase. The characterization of surface net charge and hydrophobicity (*HFS*) of *S. griseus* cells was carried out by using aqueous two-phase partition system (ATPS). The surface hydration of cell membrane and those of various kinds of liposomes together with their hydrogen bond stability under various stress conditions were systematically evaluated by using Fourier transform infrared spectrometer (FTIR). The results have shown that the heat treatment enhanced *HFS* values relating to the increasing membrane fluidity, and reducing surface hydration (water loss) of cell membrane under heat stress condition. Based on these fundamental investigations, the interactions of liposomes with the cell membrane such as the adsorption, fusion, and internalization could be effectively induced by the hydrophobic, electrostatic, and hydrogen bond interactions through the stresses-mediated variation of surface properties of both cell membrane and liposomes.

In chapter III, based on fundamental results obtained in the chapter I and II, the preparation method, the heat stress induced chitosanase secretion which resulted in the chitosanase-LIPOzymes (chitosanase bound-liposome membranes), was further investigated as a case study. The secreted chitosanase containing signal peptide (SP) associated with the lipid membrane of liposomes interacting onto cell membrane of *S. griseus* cells pretreated with inhibitor of signal peptidase (SPase) under heat stress condition. Characterization of chitosanase-LIPOzyme was made based on the protein molecular weight, chitosanase activity, and their catalytic efficiency and stability under stress conditions. The chitosanase-LIPOzymes prepared showed higher in catalytic efficiency and stability than those of conventional chitosanase under extreme heat and pH conditions. Finally, chitosanase-LIPOzymes was utilized to produce biofunctional oligochitosans from soluble chitosan.

General Introduction

The production of biofunctional oligochitosan has widely attracted many researchers because of its valuable applications in food, pharmaceutical, cosmetics and so on (Shahidi *et al*., 1999; Majeti *et al*., 2000; Dodane *et al*., 1998; Uchida *et al*., 1988; Suzuki *et al*., 1986; Hirano *et al*., 1991). Chitosanase is known as a good enzyme that effectively hydrolyzes chitosan to small biofunctional oligochitosans (Izume *et al*., 1987; Katsumi *et al*., 2005). The schematic illustration to obtain oligochitosan from chitin is shown in **Fig. 1**. Chitin that is contained in shells of fungy, crabs, shrimps, and so on can be hydrolyzed under alkaline condition to produce chitosan that is the best substrate of chitosanase enzyme to produce biofunctional oligochitosan with small molecular weight. Chitosan has been used as a substrate in many enzymatic studies, not only with chitosanase but also with chitinase and lysozymes, and several other classes of hydrolytic enzymes. It becomes immediately apparent that the various categories of enzymes have different references with respect to deacetylation of chitosan. Lysozymes and chitinase are more active against chitosan with high deacetylation, whereas chitosanase preferably attacks chitosan with low deacetylation (Tremblay *et al*., 2001). Many studies have focused on the enhanced production of chitosanase enzymes from microbial cells and/or the effective ways to use these enzymes for such purposes (Price *et al*., 1975; Tanabe *et al*., 2003; Jung *et al*., 1999; Ming *et al*., 2006; Kuroiwa *et al*., 2003; Jeon *et al*., 2000; Fukuda *et al*., 2007). Because of big potentials of chitosanase enzymes, many efforts have been dedicated to search the new sources of chitosanase enzymes as well as to study the new fundamental engineering and technologies for the effective production and recovery of this enzyme. To the best of my knowledge, the source of chitosanase enzymes is wide range from bacteria, molds, yeasts, insects, viruses, and plants. As summarized in **Table 1**, the chitosanase is produced by different organisms. The chitosanase produced by different organisms has different characteristics such as molecular weight, optimal conditions of

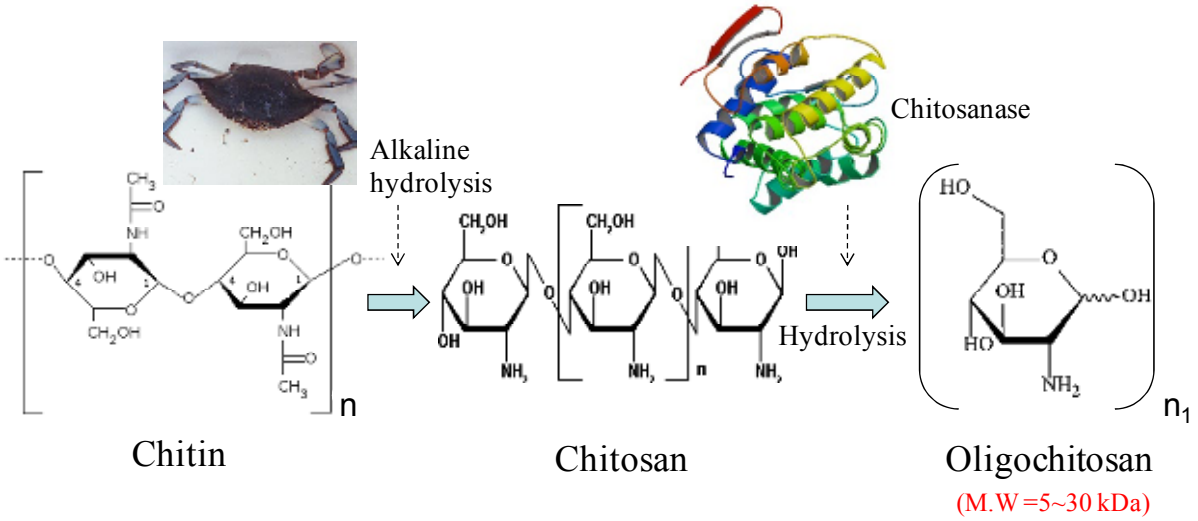


Fig. 1 Schematic illustration to obtain biofunctional oligochitosan using chitosanase enzyme

Table 1 Sources of chitosanase enzyme from different organisms

Organisms	Basic characteristics of chitosanase	Optimal conditions for hydrolysis activity	References
Streptomyces sp.	M.W. 29~30 kDa, hydrolysis of chitosan	pH 4.5, Temp. 40~55 ^{o}C	Price *et al*., 1975
Streptomyces grieus	M.W. 34 kDa, hydrolysis of chitosan and carboxymethyl -cellulose (CMC)	pH 5.7, Temp. 40~50 ^{o}C	Tanabe *et al*., 2003 and this study
Bacillus sp.	M.W. 32~36 kDa, hydrolysis of chitosan	pH 4.0, Temp. 45 ^{o}C	Chiang *et al*., 2003
Microbacterium sp.	M.W. 30 kDa, hydrolysis of chitosan	pH 6.6, Temp. 50~60^{o}C	Sune *et al*., 2006
Rhodotorula sp.	M.W. 36 kDa, hydrolysis of chitosan	pH 5, Temp. 45 ^{o}C	Somashekar *et al*., 1992
Sphingomonas sp.	M.W. 45 kDa, hydrolysis of chitosan	pH 6.5, Temp. 56 ^{o}C	Zhu *et al*., 2007
Aeromonas sp.	M.W. ~20 kDa, hydrolysis of chitosan	pH 4.5, Temp. 40 ^{o}C	Charles-Rodriguez *et al*., 2008
Aspergillus sp.	M.W. 109 kDa (hydrolyze chitosan to oligochitosan) and 29 kDa (hydrolyze chitosan to	pH 4.0, Temp. 50 ^{o}C	Chen *et al*., 2004; 2008

	glucosamine)		
Acinetobacter sp.	M.W. 35.4 kDa, hydrolysis of chitosan	pH 7.0, Temp. 36 ^{o}C	Zhu *et al*., 2003
Penicillium sp.	M.W. 28~29 kDa, hydrolysis of chitosan	pH 4.5, Temp. 45 ^{o}C	Fenton *et al*., 1981
Myxobacter sp.	M.W. 30~31 kD, hydrolysis of chitosan	pH 5.0, Temp. 70 ^{o}C	Hedges *et al*., 1974
Pseudomonas sp.	M.W. 35 kDa, hydrolysis of chitosan	pH 4, Temp. 40~50 ^{o}C	Yoshihara *et al*., 1992
Nocardia sp.	M.W. 26 kDa, hydrolysis of chitosan	pH 6, Temp. 40 ^{o}C	Sakai *et al*., 1991
Fusarium solani f. sp.	M.W. 36 kDa, hydrolysis of chitosan	pH 5.6, Temp. 40 ^{o}C	Shimosaka *et al*., 1993
*Citrus sinensis (*plant*)*	M.W. 37.4 kDa, hydrolysis of chitosan	pH 5, Temp. 60 ^{o}C	Osswald *et al*., 1994

temperature and pH as well as specific substrates for maximum hydrolysis activity. The catalytic potentials of those enzymes are also different between the organisms and are dependent on the engineering and technologies used to produce and to recover the chitosanase from such organisms. Chitosanase was first produced from *Streptomyces sp.* in 1975 (Price *et al*., 1975). Chitosanase of *Streptomyces sp*. is reported having molecular weight from 29 to 30 kDa and optimal conditions for its hydrolysis of chitosan at temperature from 40 to 55 ^{o}C and pH of 4.5 (Price *et al*., 1997). Recently, Tanabe *et al*. have reported that the chitosanase produced by *Streptomyces griseus* cell has molecular weight of 34 kDa and optimal conditions for its hydrolysis of chitosan at temperature from 40 to 50 ^{o}C and pH of 5.7.

Many efforts have been dedicated to the improvement of the production of chitosanase from microbial cells. The achievements can be clarified in terms as the following: (i) culture and fermentation engineering (Jung *et al*., 1999; Zhu *et al*., 2003; Chen *et al*., 2008); (ii) recombinant technology (Fink *et al*., 1991; Yamada *et al*., 1997; Seki *et al*., 2000; Yatsunami *et al*., 2002); (iii) mutation (Boucher *et al*., 1995; Chen *et al*., 2008); and (iv) cell surface

displaying chitosanase harboring vectors (Fukuda *et al*., 2007). Although these approaches have many advantages for the enhanced production of chitosanase, the obtained pure chitosanase is still costive to expand the production, recovery and applicable usage of this enzyme (Charles-Rodriguez *et al*., 2008). In addition, the conventional bioprocess for production of chitosanase from microbial cells is still problematic because of low productivity and restriction of microbial cell potentials as well as less understanding the properties and potential functions of cell membranes that play very important roles for cell growth, protein function, enzymatic activation, gene regulation, protein synthesis and transportation and so on. The production of chitosanase from *S. griseus* cells has been widely studied (Price *et al*., 1975; Tanabe *et al*., 2003; Jung *et al*., 1999; Katsumi *et al*., 2005). Undoubtedly, it is necessary to study new strategies to overcome the present disadvantages of conventional bioprocess for the effective production and secretion of chitosanase from *S. griseus* cells.

The secretion of proteins through cell membrane of *S. griseus* cell is predicted at least five different protein transport pathways. The protein transport pathways in *S. griseus* cell are schematically shown in **Fig. 2**, based on the predictions of signal peptides and various retention signals. Ribosomally synthesized proteins can be sorted to various destinations depending on the presence (+SP) or absence (-SP) of an amino-terminal signal peptide (SP) and specific retention signals such as lipid modification or cell wall-binding repeats. Proteins devoid of a signal peptide remain in the cytoplasm. Proteins with signal peptides are inserted into the membrane either spontaneously, via the Sec pathway or the Tat pathway (rich twin-arginine (RR)) or the ABC transporter, or the Com pathway or the cotranslation-translocation.

Basically, the components of the Sec-dependent secretion machinery can be divided into six groups: cytosolic chaperones, the translocation motor (SecA), components of the translocation channel (SecYEG and SecDF-YajC), SPases, SPPases, and, finally, folding factors that function at the trans-side of the membrane (Tjalsma *et al*., 2000). The secretion of

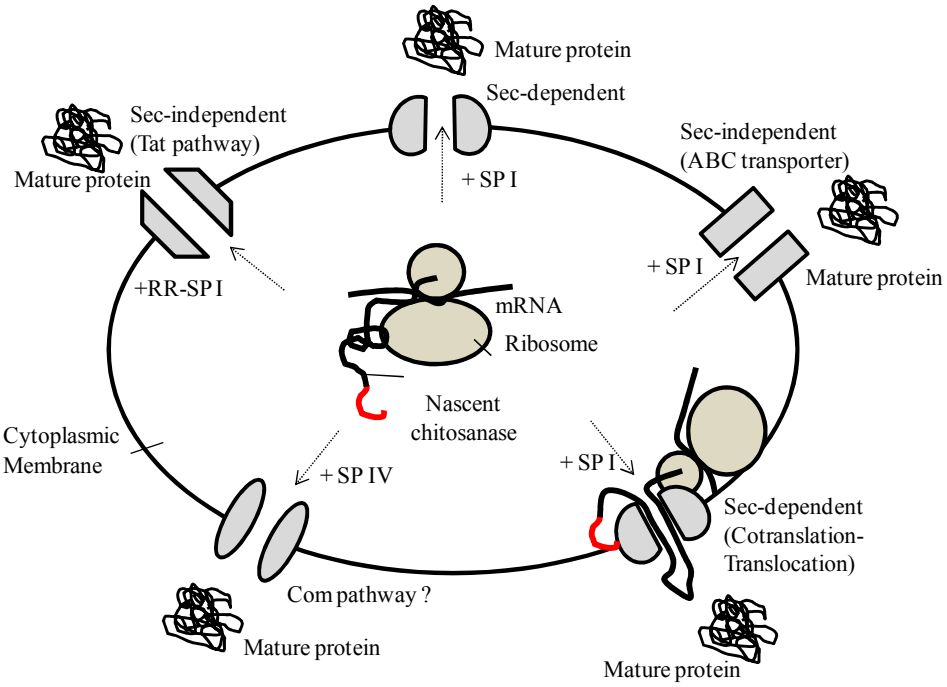


Fig. 2 Predicted pathways of protein transport in *Streptomyces griseus* cell

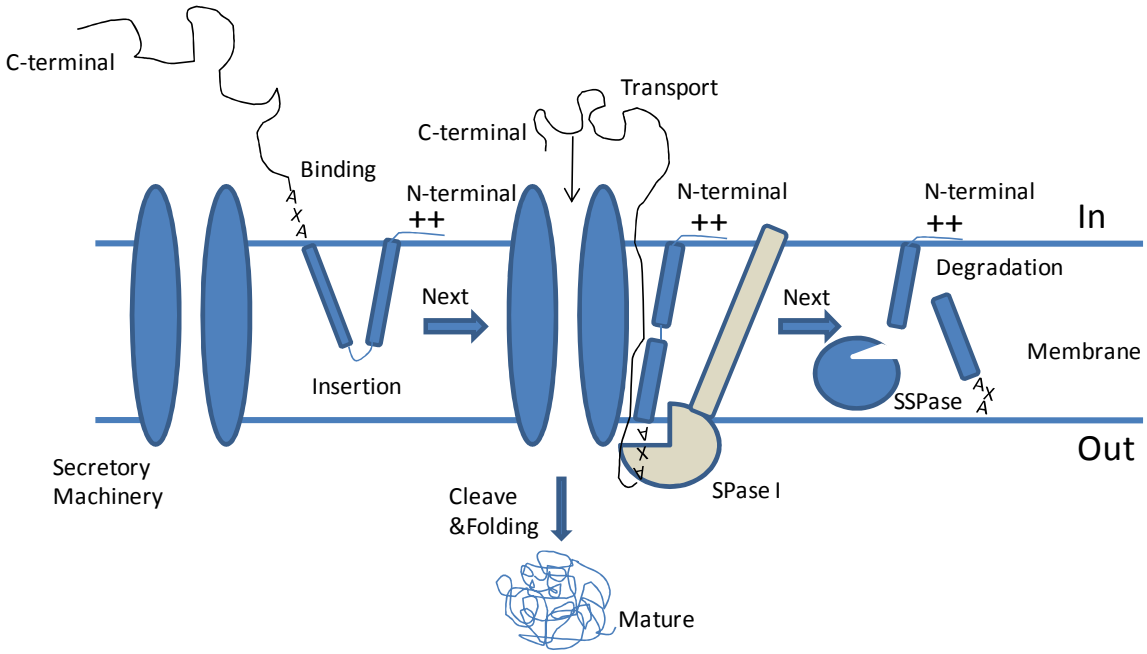


Fig. 3 Model for insertion of protein containing signal peptide (SP) into the cytoplasmic membrane. First, the positively charged N-domain interacts with negatively charged phospholipid in the membrane, after which the H-domain integrates loopwise into membrane. Next, the H-domain unloops, whereby the first of the mature protein is pulled through the membrane. During or shortly after the translocation across secretion machinery, the SP is first cleaved from mature protein by type I SPase and then, consequently, is degraded by the specific signal peptidase (SSPase). After its translocation, the mature protein folds into its native conformation (Tjalsma *et al*., 2000).

chitosanase through cell membrane of *S. griseus* has been clarified to be Sec-dependent secretion machinery dedicated by the type I signal peptidase (SPase I) that cleaves signal peptide (SP) of nascent chitosanase during or shortly after its translocation through secretion machinery (Tanabe *et al*., 2003). The model for SP binding to and its insertion into cytoplasmic membrane and its cleavage by type I SPase are shown in **Fig. 3**. The SP of chitosanase shares the common characteristics of SP type I, which is rich in positive charge, hydrophobic and α-helical conformation. Briefly, SP type I contains 3 distinct domains including N-domain, H-domain, and C-domain (Von-Heijne, 1990). The amino terminal N-domain of SP contains at least one arginine or lysine residue and this positively charged residue has been suggested to interact with translocation machinery (Akita *et al*., 1990) and negatively charged phospholipids in the lipid bilayer of the membrane during translocation (Deuerling *et al*., 1997). The H-domain, following the N-domain, is formed by a stretch of hydrophobic residues that seem to adopt α-helical conformation in the membrane (Briggs *et al*., 1986). Helix-breaking residues, found at the end of the H-domain, are thought to facilitate the degradation by specific signal peptidase (SSPase) (Dalbey *et al*., 1997). The C-domain, following the H-domain, contains the cleavage site for SPase I, which removes the SP from mature part of the secreted proteins during or shortly after translocation. The secreted proteins are then folded to native structure.

In addition, the major differences of protein secretion between five above pathways are dependent on the presence (+SP) or absence (-SP) of an amino-terminal signal peptide. In contrast to Sec-dependent pathway, proteins translocated via twin-arginine translocation (Tat) can be in a folded or unfolded conformation (Clark *et al*., 1997; Creighton *et al*., 1995; Hynds *et al*., 1998; Schaerlaekens *et al*., 2004). The Tat signal peptides have mean length and hydrophobic region similar to SP of Sec-dependent pathway but the difference is rich in twin-arginine (van Dijl *et al*., 2002). ABC transporters have a dual role in secretion, as they are responsible both for removal of the SP and for the translocation of mature proteins across

cytoplasmic membrane (den Blaanwen *et al*., 1996; Havarstein *et al*., 1995; Sahl *et al*., 1995). The signal peptide of proteins secreted through ABC transporters was found to be similar to that of Tat pathway (Zheng *et al*., 1999).

The export of proteins from *S. griseus* cell can also occur through both co-translation and post-translation. Full length of secreted polypeptides is still attached to a ribosome that can maintain the completed polypeptide in a loose, translocation competent conformation. Because cytoplasmic proteins lack such a segment and are synthesized on unattached ribosomes, the signal segment evidently directs the ribosome to the membrane (Walter *et al*., 1978). Recently, co-translational entry has also been demonstrated for membrane proteins by cleavage during synthesis (Chang *et al*., 1978). However, the selection between ribosome-dependent and ribosome-independent translocation is probably determined by the membrane system and by folding properties of each precursor protein (Verner *et al*., 1998; Kumamoto *et al*., 1998). Finally, it has also been reported the existence of one more pathway for protein translocation in Gram-positive bacteria, named Com pathway. The proteins are exported in a Sec-independent manner consisting of proteins encoded by comGC, comGD, comGE, and comGG genes. Although the SP shows certain similarities to SP of secretory proteins and lipoprotein of Tat- and Sec-dependent pathway, their translocation is dependent on a cleavage event at the cytoplasmic side of the membrane (Chung *et al*., 1998). The signal peptidase of Com pathway is probably related to type IV SPase (Lory *et al*., 1994). It has recently been reported that the heat and oxidative stresses can be utilized to activate many potential functions of bacterial cells.

The importance of heat and oxidative stresses is summarized in **Table 2**. It has been known that the cell lipid membrane is highly susceptible to the heat and oxidative stresses. The changes in membrane fluidity of bacterial cell sense to activate or inactivate many components such as the induction of chaperone molecules, changing in protein structures and functions that can be activated and/or inactivated in lipid membrane as well as the induced

Table 2 Importance of heat and oxidative stresses and cell stress responses

Stress	Efficiency	Cell Response	References
Cold heat shock (Heat shock at low temperature)	Membrane fluidity change	Increase in the unsaturated fatty acid content and/or decrease in fatty acid chain length to maintain membrane fluidity such as *Bacillus sp*., *Listeria sp*., *Vibrio sp*., *Pseudomonas sp*., *Brevibacterium ssp*.	Herbert *et al*., 1986, Russel *et al*., 1990
	Protein structure change	Weakening in hydrophobic bonds, denature of proteins, change amino acid composition in primary structure. Loss of enzymatic activity and protein functions	Jaenicke *et al*., 1990, Ochiai *et al*., 1979;1984, Schlatter *et al*., 1987
	Protein inactivation and enzyme reactivation	Change in configuration of protein; inhibition of cell growth and synthesis of DNA and RNA; activatation of oxidative enzymes, inhibition of the activity of fermentative enzymes	Inniss&Ingraham, 1978, Gounot *et al*., 1991
	Induction of chaperone:	Stimulate heat shock proteins (HSP) in yeast, activate heat shock genes such as GroES, GroEL	Berg *et al*., 1987
	Gene expression	Block protein translation at 5 ^{o}C in *E.coli*, induce polysomal run-off, accumulate 70S and ribosomal particles in *Vibrio sp*. Sense to ribosomal 30 S, activate genes to synthesize unsaturated fatty acids and so on at low temperature	Szer *et al*., 1970
Heat shock at high temperature	Membrane fluidity	Change in membrane fluidity influenced on the cell growth; change in ratio of unsaturated fatty acid and saturated fatty acid. Change in membrane fluidity that senses to various kinds of gene and activity and function of membrane proteins as well	Katsui *et al*., 1981;1982, Cronan *et al*., 1975, Kito *et al*., 1970, Sinensky *et al*., 1974, Cybulki *et al*., 2002, Los *et al*., 2004
	Cell membrane	Variation of surface net charge and hydrophobicity of cell membrane and local and surface net	Umakoshi *et al*., 1998a;1998b,

	surface properties	hydrophobicity of secreted proteins, enhancing translocation of protein through cell membrane by hydrophobic effects; enhancing transport of nascent protein across bacterial lipid cell membrane through the variation of lipid composition and fluidity of cell membrane; variation of surface hydration of cell membrane such as water loss that causes instability of hydrogen bonds	Yatvin *et al*., 1987, Ojeda *et al*., 2008
	Induction of chaperone	Induce/activate family of heat shock protein (Hsp) such as Hsp110, Hsp100, Hsp90, Hsp70 (DnaK), Hsp60 (GroEL), Hsp40, Hsp10 (GroES)	Gething *et al*. 1997, Hecker *et al*., 1990;1996, Baumann *et al*., 1997
	Protein structure change	Change in conformation of protein such as the hydrophobic, hydration and hydrogen bonds. Recruit the hydrophobic and hydrogen bond interactions of protein with chaperones, and/or lipid membrane	Ooi *et al*. 1987;1994, Kuboi *et al*. 2000, Wang *et al*., 2003
	Protein inactivation and enzymatic activation	At high temperature, some enzymes are lost activity but others are stable or enhanced. Specific heat accelerates enzymatic activity, cell metabolism, cell growth and synthesis of heat shock proteins	Nilsson *et al*. 1997, Chien *et al*. 1976
	Gene expression	Sensed by membrane fluidity changes, stimulate gene expression for heat shock genes such as HSP family producing Hsp110, Hsp90, Hsp100, Hsp40, GroEL, GroES, DnaK; genes are activated to synthesize saturated fatty acid at high temperature for maintaining the membrane fluidity .	Gething *et al*., 1997, Georgopoulos *et al*., 1993, Hecker *et al*., 1990;1996, Volker *et al*., 1994
Oxidative stress (Hydrogen peroxide)	Modulates protein phosphorylation through cystein-oxidation	Hydrogen peroxide is second messenger for signaling in the cell and is generated during aerobically growth of cell. Attendance in the redox process; activation of the cell metabolism under aerobic conditions; signaling at antigen receptor, enhancement of cell respiration and metabolism	Reth *et al*., 2000;2002, Mahadev *et al*., 2001, Droge *et al*., 1999, Finkel *et al*., 2001, Gamaley *et*

		al., 1999
	Induction of chaperones	Heat shock protein (Hsp33) is activated by disulfide bond formation under oxidative condition; the reduction of Hsp33 resulted in the dissociation of substrate binding to DnaK, DnaJ, GropE foldase system	Hoffmann *et al*., 2004
	Mediated tyrosine phosphorylation	Epidermal growth factor (EGF)-induced generation of hydrogen peroxide that mediated tyrosine phosphorylation	Bae *et al*., 1997
	Lymphocyte activation	Mimic as second messenger to activate lymphocyte	Reth *et al*., 2002

the synthesis of proteins, enzymes and/or fatty acids. The heat stress has also been reported to change the surface properties of bacterial cell such as changing in surface net charge and hydrophobicity and surface hydration of cell membrane resulting in the enhanced protein translocation across cell lipid membrane (Umakoshi *et al*., 1998[a] & 1998[b]; Yatvin *et al*., 1987, Ojda *et al*., 2008). Especially, many genes are activated under heat and oxidative stress conditions such as heat shock genes of HSP family, and genes corresponding for the synthesis of unsaturated and/or saturated fatty acids for stabilizing membrane fluidity under stress conditions, as shown in **Table 2**. Here, it is vividly recognized that the heat and oxidative stresses directly affect on the cell lipid membrane which in turn affects on the cell stress response. However, many potential functions of lipid membrane, especially under stress conditions, are still unknown. It is therefore necessary to explore the important roles of cell lipid membrane under stress conditions both *in vivo* and *in vitro* systems. For such words, the usage of liposome models that can mimic the lipid compositions of cell membrane to elucidate the profound aspects of membrane-membrane interaction, membrane-protein interaction, and membrane-gene interaction and so on is also favorable.

It has been well known that lipids fulfill three general functions (Meer *et al*., 2008). First, because of their relatively reduced state, lipids are used for energy storage in lipid droplets. Second, the matrix of cellular membranes is formed by polar lipids, which consist of a hydrophobic and a hydrophilic portion to form the membrane compartment to segregate the internal cellular components from environment. In addition to the barrier function, lipids provide membranes with the potential for budding, tubulation, fission and fusion, characteristics that are essential for cell division, biological reproduction and intracellular membrane trafficking. Lipids also allow particular proteins in membranes to aggregate, and others to disperse. Finally, lipids can act as first and second messengers in signal transduction and molecular recognition processes. Furthermore, some lipids function to define membrane domains, which recruit proteins from the cytosol that subsequently organize secondary signaling or effector complexes (Meer *et al*., 2008). The identification of new disease-related gene has revealed the involvement of lipid-related proteins, such as enzyme, transporter (ATP binding cassette (ABC) transporters) or lipid binding protein (Sud *et al*., 2007) such as the phosphoinositides (PI) identify endocytic membranes and allow them to recruit proteins from the cytosol that are involved in vesicle trafficking and other aspects of cellular homeostasis. Lipid mediators of signaling and recognition processes are numerous and work through specific protein–lipid interactions (Kobayashi *et al*., 2002). The dynamic of lipid translocation to the cytosolic leaflet causes a lipid quantity imbalance that can contribute to the membrane bending that is required for vesicle budding. Lipid asymmetry in membranes is a consequence of multiple factors, including the biophysical properties that dictate the ability of a lipid to cross the bilayer spontaneously, retentive mechanisms that trap lipids in one leaflet of the bilayer, and the presence of transporters that assist lipid translocation (Pomorski *et al*., 2006). Liposome, a closed phospholipid bilayer membrane, is spherical vesicle having inner aqueous phase and a nano-order interface (~5nm) harboring hydration and low hydrophobic layer on its surface. The interaction of liposomes with cell membrane is clarified into four ways:

Table 3 Liposomes-cell membranes interaction

Liposome	Cell	Mode of interaction	Applications	References
Egg-PC and PE neutral liposome	Erythocytes	Adsorption and fusion	Drug and gene delivery	Martin *et al*., 1976
Neutral PC liposome	Erythrocytes	Fusion	Induction of the budding of vesicles containing membrane proteins	Suzuki *et al*., 1999
Stearylamine-liposome	Erythrocyte ghost cells	Fusion	Drug and gene delivery	Nishiya *et al*., 1995
PEO-lipid modified- PC liposome	Erythrocytes	Internalization	Drug and gene delivery, gene therapy	Higashi *et al*., 1996
Cationic DOTAP, DODAP liposome	Human epidermoid carcinoma	Internalization	Drug and gene delivery, gene therapy	Almofti *et al*., 2002
Cationic DOTMP liposome	Human and murine Erythroleukemia	Internalization	Gene delivery, gene therapy	Friend *et al*., 1996
Cationic DOTAP liposome	Mammalian cell	Fusion and Internalization	Gene delivery	Wrobel *et al*., 1994
PEG modified liposome	Tumor cell	Adsorption, fusion and internalization	Drug delivery, disease treatment	Gabizon *et al*., 2004
Anionic liposome	Mammalian cell	Internalization	Gene delivery	Straubinger *et al*., 1988
Neutral PC and PE liposomes	Cholinese hamster fibrioblast	Lipid transfer from liposome to cytoplasmic membrane	Modify cell phospholipids membrane- great advance to study the metabolism of plasma membrane lipids	Sandra *et al*., 1979
Neutral PC liposome	Erythrocytes	Lipid transfer from liposome to cell	Induction of membrane protein release from less fluid membrane to high fluid membrane	Cook *et al*., 1980

adsorption, fusion, internalization, and lipid transfer (Ostro *et al*., 1989), as summarized in **Table 3**. The principle to control the interaction between liposomes and cell membrane has been dedicated and basically divided into three ways (Lian *et al*., 2001) such as (i) control the charge of liposomes (Scheule *et al*., 1997); (ii) control the size of liposomes (Senior *et al*., 1985; Devine *et al*., 1994); (iii) control the surface hydration of liposome by modification of liposomal surfaces using polymers and/or lipids (Torchilin *et al*., 1994[a]; 1994[b]). However, the controlling of the membrane fluidity, hydration, and hydrophobicity of both liposomes and bacterial cell membrane by the treatment of specific heat has not been dedicated to be studied. In this study, the heat and liposomes enhanced production and secretion of chitosanase from *S. griseus* cell treated with and without oxidative stress were first characterized on the basis of the interaction of liposomes with target chitosanase and cell membrane under stress conditions. The protein and lipid peroxidation of cell membrane in the presence and absence of liposomes under oxidative stress conditions was also evaluated to elucidate the importance of membrane-membrane interaction between liposomes and cell membrane of *S. griseus*. The interaction of liposomes with cell membrane under stress conditions was further characterized via the surface properties of cell membrane and various kinds of liposomes such as surface net charge and hydrophobicity, and membrane fluidity, and surface hydration. Based on these characterizations, the key factors to control the interaction between liposomes and cell membrane of *S. griseus* such as the adsorption, fusion and internalization can be relied on the hydrophobic, electrostatic and hydrogen bond interactions. The interaction between liposomes and cell membrane of *S. griseus* under the heat stress conditions is not only utilized for the enhanced production of chitosanase but this interaction also plays a key role to prepare the chitosanase-LIPOzymes based on membrane-membrane interaction induced secretion of chitosanase containing signal peptide (SP) associated-lipid membrane of liposomes interacting onto cell membrane of *S. griseus* under heat stress condition. The chitosanase-LIPOzymes show chitosanase activity with protein with molecular weight

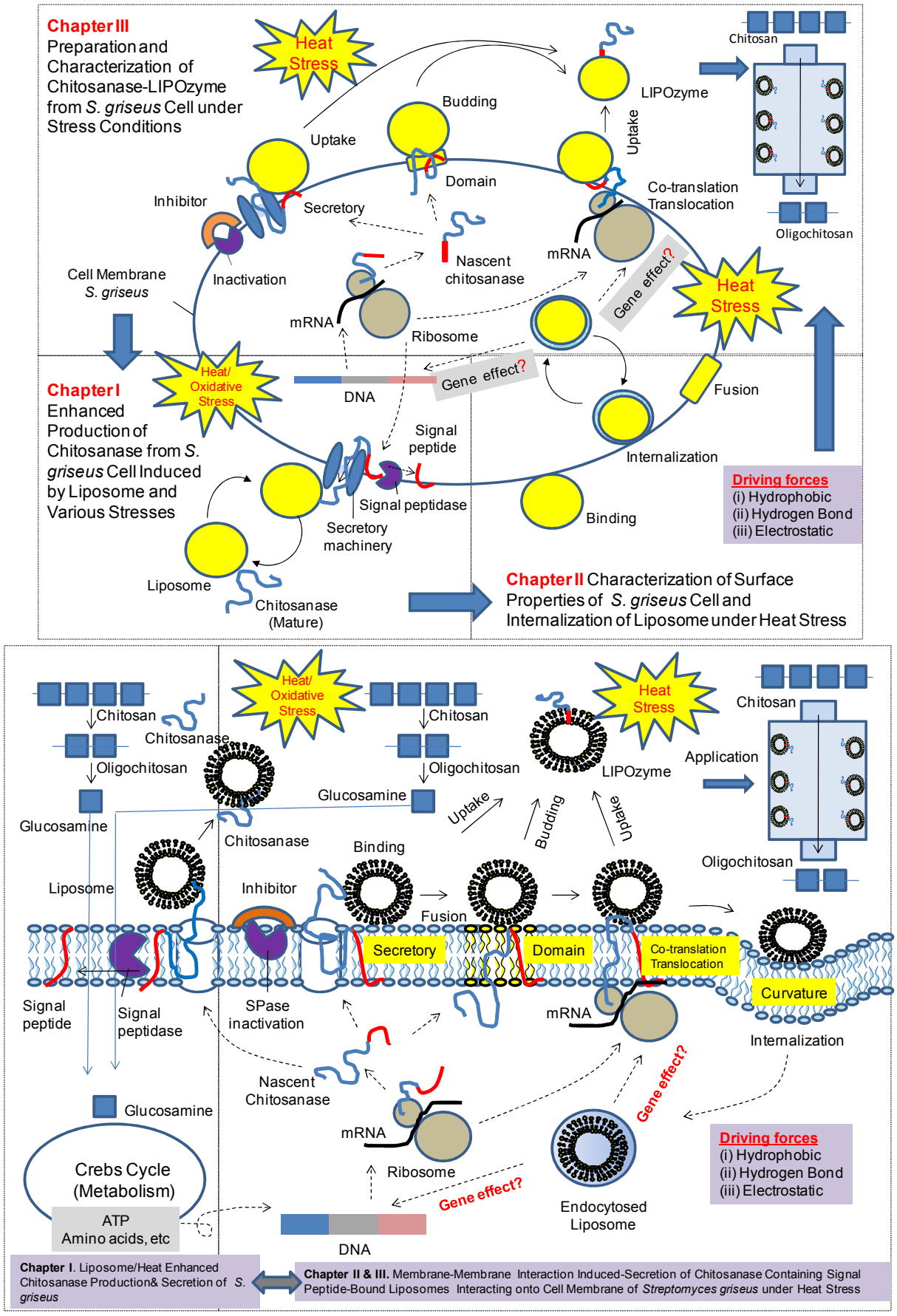


Fig. 4 Framework of present study

Chapter I

Enhanced Production of Chitosanase from *Streptomyces griseus* Cell Induced by Liposomes under Various Stress Conditions

Evaluate the cell growth and production/secretion of chitosanase of *S. griseus* cells treated by the heat, oxidative stress and liposomes

Study on kinetic of cell growth and production of chitosanase treated by the heat stress and liposomes by using Leudeking-Piret model

Evaluate the stress effects on the properties of chitosanase and liposomes such protein conformation, activity, membrane fluidity and so on

Characterize interactions of liposome with chitosanase and cell membrane under stress conditions

Chapter II

Characterization of Surface Properties of *Streptomyces griseus* Cell and Internalization of Liposome under Heat Stress Conditions

Characterize surface properties of *S. griseus* cell treated with without liposomes under various heat, pH and oxidative conditions

Evaluate stresses induced liposome-cell membrane interaction such as binding, fusion, internalization

Characterize the driving force of stresses induced liposome-cell membrane interaction

Chapter III

Preparation and Characterization of Chitosanase-LIPOzyme from *Streptomyces griseus* Cell under Heat Stress Conditions

Preparation of chitosanase-LIPOzymes based on membrane-membrane interaction induced secretion of chitosanase containing signal peptide bound-liposomes interacting onto cell membrane of *S. griseus*

Study on kinetics of enzymatic reaction of chitosanase-LIPOzymes using Michaelis-Menton and correlation between surface hydration of liposomes and activity of displayed chitosanase

Characterization of chitosanase-LIPOzymes by molecular weight, catalytic efficiency and stability under stress conditions

Possible applications of chitosanase-LIPOzyme to produce biofunctional oligochitosans

Fig. 5 Flowchart of this dissertation

equaling to the sum of mature chitosanase and SP and higher in catalytic efficiency and stability even under extreme pH and heat conditions. Finally, the efficiency of chitosanase-LIPOzymes can be applied to produce the biofunctional oligochitosans. The framework and flowchart of the present study are schematically shown in **Figs. 4** and **5**, respectively.

In chapter I, the effective production and secretion of chitosanase from *S. griseus* cells were studied by the treatment of these cells with the neutral 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine (POPC) liposomes together with heat and/or oxidative stress. The effects of the liposomes and heat treatment on the cell growth and chitosanase production were first analyzed. The possible effects of the heat stress on the conformation and activity of chitosanase, as well as on the membrane fluidity of liposomes, were systematically investigated to elucidate the driving force of the heat induced liposome-chitosanase interaction. The interaction of liposomes with target chitosanase and cell lipid membrane plays important roles for the enhanced production and secretion of chitosanase from these cells under heat stress condition. The fundamental investigations of the oxidative stress (hydrogen peroxide) affecting on the lipid and protein peroxidation of cell membrane of *S. griseus* pretreated with and without liposomes were further carried out to optimize the conditions for enhanced production and secretion of chitosanase. The interaction of liposomes with cell membrane could significantly prevent the oxidative damage on cell membrane; as a consequence, the growth and production of chitosanase from these cells pretreated with liposomes and heat stress at 41 ^{o}C were enhanced even under oxidative stress condition.

In chapter II, the surface properties of *S. griseus* cell treated with the heat and/or oxidative stress in the presence and absence of liposomes were quantitatively characterized in order to optimize the conditions for the effective interaction of liposomes with cell membrane and secreted chitosanase under such stress conditions. The characterization of surface net

charge and hydrophobicity (*HFS*) of *S. griseus* cells was carried out by using aqueous two-phase partition system (ATPS). The surface hydration of cell membrane and those of various kinds of liposomes together with their hydrogen bond stability under different stress conditions were systematically evaluated by using Fourier transform infrared spectroscopy (FTIR). The results have shown that the heat treatment enhanced *HFS* values and the increasing *HFS* values probably relate to the increasing membrane fluidity and the reducing surface hydration (water loss) of cell membrane under heat stress condition. Based on these fundamental investigations, the strategy to control the interactions of liposomes with the cell membrane such as the adsorption, fusion, and internalization can be considered by the adjustment of surface properties of both cell membrane and liposomes such as surface net charge and hydrophobicity, and surface hydration to the favorable states for their interaction.

In chapter III, based on the fundamental results obtained in the chapter I and II, the preparation method of chitosanase-LIPOzymes was further investigated as a case study. The membrane-membrane interaction induced secretion of chitosanase containing signal peptide (SP) bound liposomes interacting onto cell membrane pretreated with inhibitor of type I signal peptide (SPase) under specific heat conditions resulting in chitosanase-LIPOzymes formation. Characterization of these LIPOzymes was based on protein molecular weight, chitosanase activity, and the catalytic efficiency and stability under stress conditions. The stress-resistant chitosanase-LIPOzymes show higher catalytic efficiency and stability than those of conventional chitosanase under extreme pH and heat conditions. The correlation of activity of displayed chitosanase with surface hydration of various liposomes used to prepare LIPOzymes was also investigated to verify the successful displaying of chitosanase on liposomes. The effective production of biofunctional oligochitosans from chitosan catalyzed by various kinds of chitosanase-LIPOzymes was finally studied.

The results obtained in this work are summarized in General Conclusion. Suggestions for Future Work are described as extension of present dissertation.

Chapter I

Enhanced Production of Chitosanase from *Streptomyces griseus* Cell Induced by Liposomes under Various Stress Conditions

Introduction

Chitosanase produced by *Streptomyces griseus* is secreted non-membrane protein with 33.8 kDa in molecular weight (M.W). Chitosanase is glycosyl hydrolase that catalyzes the degradation of chitosan. The three-dementional structure of chitosanase from *S. griseus* species has been analyzed (Tanabe *et al*., 2003). On the other hand, chitosanase can be divided into three groups according to their specificity for the hydrolysis of β-glucosidic linkage in partially N-acetylated chitosan molecules. The class I chitosanases split the GlcNAc-GlcN and GlcN-GlcN bonds (Fukumizo *et al*., 1994, Mitsutomi *et al*., 1992). The class II chitosanases split only GlcN-GlcN bond (Izume *et al*., 1992). The class III chitosanases split GlcN-GlcN and GlcN-GlcNAc bonds (Mitsutomi *et al*., 1992). The family 46 chitosanases only degrade the chitosan but family 8 chitosanases hydrolyze both chitosan and carboxyl methyl-cellulose (CMC). However, no simple relationship between the structure and specificity of chitosanase has been found. It is therefore more knowledge about the structures and functions of chitosanase that may clarify the role of chitosanase in the degradation of chitinous compound in nature (Tanabe *et al*., 2003). In biological system, heat is known to be sensitive and useful stimuli. A series of paper in relation to heat shock protein (HSPs), known as the molecular chaperone in bacteria, have described the important roles of HSPs for protein refolding (Krzewska *et al*., 2001, Minami at al., 2001) and translocation (Jason *et al*., 2003, Yamamoto *et al*., 2005). The crucial roles of the heat on the functional properties of liposome are also focused on the heat induced increasing dynamic motion of

individual phospholipid molecules in the membrane. Such dynamic motion is so important to recruit the other molecules into the interactions in the membrane such as protein refolding (Yoshimoto *et al*., 1998;2000, Kuboi *et al*., 2000, Morita *et al*., 2000).

Oxidative stress, known as the effect of reactive oxygen species (ROS) on the biomolecules, has been widely studied. Hydrogen peroxide (H_2O_2) has also been found to play important roles as second messenger in the initiation and amplification of signaling at the antigen receptor (Reth *et al*., 2002). A several excellent reviews about the functions of H_2O_2 as secondary messenger have already appeared (Reth *et al*., 2002, Bae *et al*., 1997, Mahadev *et al*., 2001, Rhee *et al*., 2000, Gamaley *et al*., 1999, Finkel *et al*., 2001, Droge *et al*., 2002). It has been also reported that the production rates of superoxide radical ($^{\cdot}O_2^-$) and H_2O_2 are linearly related to the number of the active respiration chains that reaches to the maximal values during the exponential growth and significantly decreases at the stationary phase (Flecha *et al*., 1995). Under the oxidative conditions, the heat shock protein (Hsp33), a redox-regulated molecular chaperone, is activated by disulfide bond formation and subsequent dimerization and works as efficient chaperone holdase that binds to unfolding protein intermediates and maintains them in folding competent conformation. Reduction of Hsp33 is necessary but not sufficient for substrate protein release. The substrate dissociation from Hsp33 is linked to the presence of DnaK/DnaJ/GrpE foldase system, and then supports the refolding of the substrate protein. Upon the substrate release, the reduced Hsp33 dimers dissociate into inactive monomers. This regulated substrate transfer ultimately links substrate release and Hsp33 inactivation to the presence of available DnaK/DnaJ/GrpE and, therefore, to the return to non-stress condition (Hoffmann *et al*., 2004).

Liposome has many advantageous applications such as drug delivery system (DDS) (Taran *et al*., 1996, Koneracka *et al*., 2005). Liposome can covalently bind to functional ligands of activated gel beads under suitable condition; therefore immobilized liposome chromatography (ILC) is also very favorable method for bioseparation (Nagami *et al*., 2005,

Yoshimoto *et al*., 1998, 2000). Liposome can also assist the protein refolding by suitable hydrophobic interaction between them. The local and surface net hydrophobicity of phospholipid membrane and local hydrophobicity of protein has been considered as the key factor in liposome-assisted protein refolding (Yoshimoto *et al*., 1998; 2000).

In this chapter, the effective production and secretion of chitosanase from *S. griseus* cells were studied by the treatment of these cells with 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine (POPC) liposomes together with the heat and/or oxidative stresses. The efficiency of the liposomes and the heat treatment on the cell growth and chitosanase production was analyzed using Leudeking-Piret model. The interaction of liposomes with target chitonanase that could afftect on the conformation and activity of chitosanase was systematically investigated by using circular dichroism (CD) spectra, dielectric dispersion analysis (DDA). The interaction of neutral POPC liposomes with the cell membrane of *S. griseus* under various heat conditions was also evaluated through the variation of membrane fluidity of liposomes co-incubated with and without intact and spheroplast *S. griseus* cells. The membrane-membrane interaction was further verified through the fusion between POPC liposomes and LMCM liposomes using the fluorescence resonance energy transfer (FRET). The fundamental investigations of the oxidative stress (hydrogen peroxide) affecting on the lipid and protein peroxidation of cell membrane of *S. griseus* pretreated with and without liposomes under heat stress at 41 ^{o}C were further carried out. The interaction of liposomes with the target chitosanase and cell membrane under oxidative stress condition was characterized through (i) the conformational change of chitosanase using CD spectra with and without liposomes and (ii) the peroxidation of lipids and proteins in cell membrane in the presence and absence of liposomes. The possible utilization of membrane-membrane interaction between liposomes and cell membrane induced by the heat treatment to enhance production of chitosanase from *S. griseus* was further characterized under oxidative stress condition. On the basis of this evaluation, the

possible effect of the oxidative stress on the enhanced production of antibiotic antifugal iturin A from *Bacillus subtilis* cells treated by UV irradiation was finally discussed.

Materials and Methods

1. Materials

The lipids including 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine (POPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidic acid (POPA), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-rac-(1-glycerol)] (POPG) were purchased from Avanti Polar Lipid (Alabaster City, AL, USA). Chitosanase from *S. griseus* was purchased from Sigma Aldrich. Other chemical reagents of highly analytical grade were purchased from Wako Pure Chemical (Osaka, Japan).

2. Preparation of liposomes

The phospholipid molecules of POPC, POPA, and POPG were solubilized in chloroform. Using vacuum rotary evaporator, a phospholipid thin layer was prepared in the round bottom flask. Further dried process was operated with nitrogen flow for at least 5 h. In order to create the multi lamellar vesicles, the phospholipid thin dried layer was hydrated with 10mM phosphate buffer containing 150 mM NaCl (pH 7) for over night. The freezing-thawing cycle was repeated 5 times with hydrated POPC solution for lamellar vesicle formation. This vesicles solution was extruded through the polycarbonate membrane with pore size of 100 or 200 nm to form unilamellar vesicles prior to use.

The giant unilamellar vesicles (GUVs) containing lipids mimicking cell membrane were prepared by previous reported method (Moscho *et al*., 1996). Briefly, phospholipids (0.1 M) involving DMPG/ DOPE/POPC/POPA (56:22:20:2 in molar ratio) were dissolved in chloroform, and 20 μl of this solution was added to a 50 ml round-bottom flask containing

980 µl of chloroform and 100-200 µl of methanol. The aqueous phase (7 ml of distilled water or buffer) was then carefully added along the flask wall. It should be feasible to scale this procedure up or down, as desired. Liposomes were formed in distilled water, Hepes buffer (10 mM, pH adjusted to 7.4 with NaOH), sodium phosphate buffer (10 mM, with 11 mM $MgCl_2$, pH 7.4) and sodium borate buffer (50 mM, pH 9.2). The molecules could be entrapped in the vesicles or the enzymes to be incorporated into the liposome membrane were added to the buffer solution prior to evaporation of organic solvent. The organic solvent was removed in a rotary evaporator under reduced pressure (final pressure 1.3 kPa) at 40 ^{o}C and 40 rpm. As a consequence of the different boiling points of chloroform (61 ^{o}C at 100 kPa) and methanol (64 ^{o}C at 100 kPa), the two major boiling points could be observed. After evaporation for 2 min, an opalescent fluid was obtained with a volume approximately 6.5 ml. The resulting aqueous solution contained GUVs in high concentration.

3. Cultivation of *Streptomyces griseus* cells for chitosanase production

3.1 Production of chitosanase from *S. griseus* cell under heat stress in the presence of liposomes

Streptomyces griseus cells, which produced chitosanase and were stored in freeze-dried ampule at -80 ^{o}C, were grown in slant of seed culture (1 % manitol, 0.2 % peptone, 0.1 % meat extract, 0.1 % yeast extract, pH 6.8) at 37 ^{o}C during 24 h. Each slant of seed bacteria was aerobically cultivated with 100ml of nutrient broth including: KCl (0.05 %), KH_2PO_4 (0.1 %), $MgSO_4.7H_2O$ (0.05 %), $FeSO_4.7H_2O$ (0.001 %) and water-soluble chitosan (0.2 %), pH 6.8 at 37 ^{o}C for chitosanase production. In order to evaluate the effects of the heat stress and POPC liposome on the cell growth and chitosanase production, *S. griseus* cells were grown in 300 ml round flask containing 100 ml of chitosanase-production medium in the presence and absence of 1 mM POPC liposome with the diameter of 200 nm under heat stress condition. The cultivation process was kept in a water bath with a shaking speed of 150 rpm.

The first heating at 41 ^{o}C for 20 min was applied after 10 h of the incubation and such heat stress treatment was stopped and the cell incubation flasks were immediately transferred to another incubator setup at 37 ^{o}C, where the heating rate of water bath from 37 to 41 ^{o}C and its cooling rate from 41 to 37 ^{o}C was fixed at 1.7 K/min and 1.1 K/min, respectively. The parallel experiments were carried out with the addition of 1 mM POPC liposome into the culture broth of *S. griseus* in order to compare the cell growth and chitosanase production under heat stress condition with and without POPC liposome. The second and third heating at 41 ^{o}C were applied for 20 min after 30 and 50 h of the cultivation, respectively. The cells were incubated in the same nutrient broth at 37 ^{o}C as control experiment in order to compare their growth and chitosanase production with the heat and the heat/POPC condition.

3.2 Production of iturin A from *Bacillus subtilis* cells under UV irradiation

The wild type *Bacillus subtilis* cells were grown in the medium containing saccharose (30 g/l), soya bean powder (10 g/l), yeast extract (5 g/l), $(NH_4)SO_4$ (5 g/l), K_2HPO_4 (5 g/l), $MgSO_4$ (0.5 g/l), $MnCl_2$ (0.004 g/l), $CaCl_2$ (0.005), $FeSO_4.7H_2O$ (0.025 g/l), pH 7.0 for 10 h. These cells were recovered by centrifugation at 7000 x g and were dissolved by sterilized distilled water to concentration at the optical density at 550 nm (OD_{550}) of 1.0. The same volume of 10 μM caffeine buffer was also added before the irradiation of these cells by UV light at 254 nm for 1 min (Bok *et al*., 1992). The mutants of *B. subtilis* were selected based on their antibiotic antifungal activity (Kimura *et al*., 1990). The highest activity of iturin A producing mutants were chosen for further experiments. The mutants were fermented in 500 ml flask containing 150 ml above medium at 30 ^{o}C for 72 h in water bath shaker at 140 rpm (optimal conditions) for production of iturin A surfactant. The produced iturin A was obtained in supernatant after removing cell deposit by centrifugation. Iturin A was further purified by precipitation using cold ethanol and was charaterized by thin layer chromatography (Shoda *et al*., 1991).

3.3 Production of chitosanase from *S. griseus* cell pretreated with liposome and heat stress under oxidative stress condition

Briefly, the *S. griseus* cells were cultivated in the same medium and same other conditions, as described in section 3.1. The heat treatment at 41 ^{o}C on the *S. griseus* cells was carried out after 10 and 24 h of the incubation and the POPC liposomes (1 mM, 100 nm) was simultaneously added after incubation of 24 h. In order to evaluate the effect of oxidative stress on the cell growth and chitosanase production of *S. griseus* after treatment of heat stress at 41 ^{o}C and POPC liposomes, H_2O_2 (5 mM as final concentration) and POPC liposomes (1 mM, 100 nm in size) were simultaneously added to the culture broth of *S. griseus* cells. Two control experiments were used in this experiment to compare the cell growth and chitosanase production; (i) *S. griseus* cells were cultivated at 37 ^{o}C in the same medium with the addition of 5 mM H_2O_2 and (ii) *S. griseus* cells were cultivated at 37 ^{o}C in the same medium without any treatment.

4. Measurement of chitosanase activity

Chitosanase activity was measured with glycol chitosan as a substrate (Ohtakara *et al*., 1988). The reaction mixture containing 0.5 ml of 2 wt% glycol chitosan in 0.1 M phosphate buffer (pH 5.6) and 0.5 ml of enzyme solution was incubated at 37 ^{o}C for 10 min and then the enzymatic reaction was stopped by boiling for 4 min. This solution was cooled down and added to 1 ml of acetylacetone diluted in 0.5 N Na_2CO_3 (1:50 v/v) and 1 ml of distilled water and boiled for 20 min. An amount 5 ml of ethanol and 1 ml of enrich reagent (p-dimethylamino-benzaldehyde, DMAB) were then added and incubated at 65 ^{o}C for 10 min. The absorbance of the assay solution is measured at 530 nm using UV spectrophotometer (UV-1600A, Shimadzu, Japan). One unit of chitosanase is defined as the amount of enzyme that hydrolyzed glycol chitosan in order to release 1 μM glucosamine per min at given assay conditions. To measure the extracellular activity, the supernatant of cell culture was obtained

by centrifugation at 8000 rpm for 5 min to remove the cells and other materials. To measure the intracellular activity, the collected cells from centrifugation were resuspended on enzyme production medium and then using an ultrasonic device (UD-200 (Tomy Seiko Co., Ltd; 20 KHz) with micro-tip (TP-040)) disrupted the cells to release intracellular chitosanase. To evaluate the effect of liposomes on the intact recovery of target chitosanase, extracellular activity of chitosanase in the supernatant was measured with addition of 10 mM triton X-100 in order to liberate the encapsulated chitosanase in liposomes.

5. Kinetic of cell growth and its production based on the Leudeking-Piret models

Under optimal growth conditions, when the inhibitory effects of substrate and product are neglected, the rate of cell growth follows the well-known exponential relation. The microbial growth according to Leudeking-Piret is shown below:

$$\frac{dX}{dt} = \mu X \quad (1) \text{ or } \mu = \frac{1}{X}\frac{dX}{dt} \quad (2)$$

The rate of product formation is expressed by Leudeking-Piret

$$\frac{dP}{dt} = \propto \frac{dX}{dt} + \beta X \quad (3)$$

From Equation (3)

$$\frac{1}{X}\frac{dP}{dt} = \propto \frac{1}{X}\frac{dX}{dt} + \beta \quad (4)$$

$$\text{or} \quad \pi = \alpha\mu + \beta \quad (5)$$

Production yield could be defined as ratio of mass of formed product and mass of cell

$$\frac{dP}{dX} = \propto + \frac{\beta}{\mu X} \quad (6)$$

Biological meanings

X: biomass concentration (mg/l); μ: specific growth rate (1/h); π: specific production rate (1/h); dP/dX: production yield (mg/g); α: growth-associated product formation coefficient (g/g); β: non growth-associated product formation coefficient (g/g.h)

6. Evaluation of secondary structure of chitosanase using CD spectra

Conformational transition of chitosanase under heat and oxidative stress conditions in the presence and absence of liposomes was evaluated by using Jasco J-820 SFU spectropolarimeter. The molecular ellipticity of chitosanase as the function of the wavelength in range 250 to 190 nm was fitted based on the previous results (Chang *et al*., 1978) in order to get the information of the secondary structure of chitosanase. In the experiment shown the efficiency of the heat treatment, an amount 4.3 μM chitosanase was prepared in 10 mM phosphate buffer containing 150 mM NaCl, pH 7.0. In order to evaluate the interaction of POPC liposome with chitosanase under the heat stress condition at 41^{o}C for 60 min, the liposome solution at 1 mM POPC as final concentration was mixed with above chitosanase solution.

The effect of oxidative stress on the conformation of chitosanase with without liposomes was also evaluated by co-incubation of 4.3 μM chitosanase with 5 mM hydrogen peroxide in the presence and absence of 1 mM POPC liposomes. In order to minimize the noise on the CD spectra of chitosanase, 1 mM POPC liposome solution was well prepared to be transparent in the same buffer and their CD spectra was measured to compare with buffer solution. The quart cell with 1 mm in path length, the bandwidth of spectra at 0.1 nm and scanning rate at 10 nm/min were applied to measure CD spectra of chitosanase.

7. Evaluation of membrane fluidity by dielectric dispersion analysis

An impedance analyzer (RF impedance analyzer 4291B, Agilent Technology) was equipped with handmade brass electrode cell in order to measure the dielectric spectra of liposome in the frequency range between 1 MHz and 100 MHz.

The interaction of POPC liposome and chitosanase results in the change of surface properties of POPC liposome. The shift of relative permittivity (or dielectric dispersion) of POPC liposome as function of frequency may show the orientation of the solvent molecules

such as amplitude of the dielectric dispersion ($\Delta\varepsilon$), the lateral diffusion (f_{c1}) and the rotation (f_{c2}) of dipolar head groups of individual phospholipid molecules in the liposome (Shikata *et al*., 1998). Such dielectric parameters can be obtained based on the fitting analysis using Debye's equation as shown below (Schrader *et al*., 2001). The volume 125 μl of 30 mM POPC liposome was applied within hand-bath electrode in order to obtain capacitance and conductance values. The interaction between POPC liposome and chitosanase was evaluated as dielectric dispersion shift of the mixture 15 mM POPC and 4.3 μM chitosanase as the final concentration. The samples of pure POPC and POPC/chitosanase mixture were exposed to the heat stress at 31, 35, 37, 41, 43, 45, 47 and 49 ^{o}C for 20 min before being applied into an electrode cell. The heating time was set based on the previous findings on the conformational change of chitosanase under the heat stress condition (Jung *et al*., 2003). The normalized f_{c1} and f_{c2} frequencies are described as following formula as $(f_{c1,p} - f_{c1,0})/f_{c1,p}$ and $(f_{c2,p} - f_{c2,0})/f_{c2,p}$; where $f_{c1,p}$, $f_{c2,p}$ are characteristic frequencies of POPC/chitosanase mixture and $f_{c1,0}$, $f_{c2,0}$ are characteristic frequencies of pure POPC liposome.

Debye's equations:

$$\Delta\varepsilon' = \varepsilon' - \varepsilon'_w = \frac{\Delta\varepsilon_1}{1+(f/f_{c1})^2} + \frac{\Delta\varepsilon_2}{1+(f/f_{c2})^2} \quad (7)$$

$$\Delta\varepsilon'' = \varepsilon'' - \varepsilon''_w - \frac{G_{dc}}{2\pi f C_0} = \frac{\Delta\varepsilon_1(f/f_{c1})}{1+(f/f_{c1})^2} + \frac{\Delta\varepsilon_2(f/f_{c2})}{1+(f/f_{c2})^2} \quad (8)$$

$$\varepsilon' = C * C_0^{-1} \quad (9)$$

$$\varepsilon'' = \frac{G}{2\pi f C_0} \quad (10)$$

In these equations, the data were collected in the form of parallel connection of capacitance C (F) and conductance G (S) as the function of frequencies. The dielectric constant (ε') and its loss (ε'') were calculated based on equation (*Eq*. 9) and (*Eq*. 10). Similarly, the dielectric constant and loss of water (ε'_w, ε''_w) were calculated to evaluate the

increment of ε', $\varepsilon''$from the values of water ($\Delta\varepsilon'$, $\Delta\varepsilon''$) and the amplitude of dielectric dispersion ($\Delta\varepsilon_1, \Delta\varepsilon_2$) using equation (*Eq*. 7) and (*Eq*. 8); where the f, f_{c1}, f_{c2} are frequencies (MHz); C_0 is cell constant (F) and G_{dc} is direct current conductivity of sample.

8. Evaluation of interaction between liposome and *Streptomyces griseus* cell

The interaction between neutral POPC liposomes and cell membrane of *S. griseus* under a heat stress at 41°C was characterized by measuring the variation of the membrane fluidity of POPC liposomes in the presence and absence of the spheroplast or intact *S. griseus* cells. The 250 volumes of 1 mM POPC liposomes or spheroplast *S. griseus* cells (10^3/ml) were labeled by incubation with 1 volume of TMA-DPH dissolved in ethanol (2 mg/ml) at room temperature for 4 h prior to use. The TMA-DPH molecules favorably incorporate to hydrophobic environment based on its hydrophobic structure. These molecules insert the hydrophobic tails and expose hydrophilic head groups perpendicularly to the lipid bilayer of liposomes and cell membranes. The membrane fluidity of the POPC liposomes at 41 °C with and without the spheroplast and intact *S. griseus* cells was used to monitor the interaction of liposomes with the cell membranes. The degree of polarization (P) of the sample was recorded by exciting with vertically polarized light (360 nm) and the emission intensities (430 nm) of both parallel ($I_{//}$) and perpendicular ($I_{\pm}$) using polarizer (FP 2010, JASCO, Japan). The degree of polarization (P) was calculated as $P = (I_{//} - I_{\pm})/(I_{//} + I_{\pm})$ and membrane fluidity was defined as $1/P$.

9. Analysis of lipid and protein peroxidation

There are several spectrophotometric methods to evaluate the protein and lipid peroxides in vitro and in vivo such as the iodine libration method (Takagi *et al*., 1978, Buege *et al*., 1978), and measurement of protein and lipid hydroperoxides by the ferric-xylenol orange method (Gay *et al*., 2003) and measuring the lipid peroxides by thiobarbituric acid reaction (TBAR) (Ohkawa *et al*., 1979).

Brief description of the lipid peroxides by measuring TBARS (Ohkawa *et al*., 1979), the reaction mixture contains 0.1 ml of lipid sample, 0.2 ml of 8.1 % sodium dodecyl sulfate (SDS), 1.5 ml of 20 % acetic acid solution of various pH, and 1.5 ml of 0.8 % aqueous solution of thiobarbituric acid (TBA). The pH of 20 % acetic solution is adjusted with NaOH to above pH 3.0, and in the pH range of 1.0~3.0, 20 % acetic acid containing 0.26 M HCl is adjusted to the specific pH with NaOH. The mixture is finally made up to 4.0 ml with distilled water, and heated at 95 ^{o}C for 60 min. After cooling with tap water, 1.0 ml of distilled water and 5 ml of the mixture of *n*-butanol and pyridine (15:1, v/v) are added, and the mixture is shaken vigorously. After centrifugation at 4000 rpm for 10 min, the absorbance of organic layer (upper layer) is measured at 532 nm. The 1, 1, 3, 3-tetramethoxy-propane (TMP) is used as an external standard, and the level of lipid peroxides is expressed as nmol of molondialdehyde (MDA) per gram of wet weight of lipids, which is calculated from absorbance at 532 nm using TMP as an external standard.

The amount of protein and lipid hydroperoxides is measured by ferric-xylenol orange method (Gay *et al*., 2003). The lipid hydroperoxide is assayed by the M-PCA-FOX: the mix 200 µl of extracted lipid sample and 900 µl $ChCl_3$:MeOH (2:1) containing 4 mM BHT, votex, after 5 min, centrifuge at 6500 g for 6 min. Next chloroform layer containing target lipids is collected and dried by argon or oxygen-free nitrogen and then adding immediately in this order: 250 µl $ChCl_3$ + 4 mM butylated hydroxytoluen (BHT); 460 µl MeOH + 4 mM BHT; 30 µl xylenol orange (XO) and 20 µl Fe^{2+}. After incubation for 60 min in covered test tubes, measure absorbance at 560 nm. A blank sample containing the extract reduced with 1 mM triphenylphosphine subjected to identical protocol is measured the absorbance at 560 nm.

The protein hydroperoxide is assayed by G-PCA-FOX method. Briefly, 10 µl extract + 500 µl 0.2 M PCA; votex; ice 5 min; centrifuge at 6500g and then add 1100 µl of 6 M GuHCl to pellet; votex to dissolve pellet and wash the solution with 1100 µl chloroform containing 4 mM BHT; collect 850 µl of aqueous layer containing proteins; wash with 850 µl chloroform

containing 4 mM BHT resulting in getting 700 µl of aqueous layer. Finally, add to that aqueous solution in this order: 40 µl of 0.5 M perchloric acid (PCA); 25 µl of 5 mM XO; 25 µl of H_2O, and 10 µl of 5 mM Fe^{2+}; mix vigorously and incubate this sample for 60 min at room temperature in stopped containers, measure absorbance at 560 nm. A blank containing extract reduced with 1 mM sodium dithionite subjected to identical protocol is measured the absorbance at 560 nm.

10. The lipid mixing between neutral liposomes and LMCM liposomes

Labeled liposomes were prepared as the method described in our previous study (Ngo *et al*., 2005). Briefly, NBD-PE (1 mol%) and Rh-PE (1 mol%) together with 1 mM POPC phospholipids were dissolved in chloroform in round bottom-flask before evaporation under low pressure at 37 ^{o}C to create dried-lipid film layer. To create the large lamellar vesicles, the 10 mM phosphate buffer (pH 7) was added to dissolve lipid thin layers and was incubated at room temperature for 24 h. Finally, the freezing (-20 ^{o}C, 10 min) and thawing (37 ^{o}C, 10 min) were simultaneously applied 5 times. To obtain the unilamellar vesicles with size of 100 nm, the vesicle suspension was finally extruded through polycarbonate membrane with the pore size of 100 nm. The lipid mixing between liposomes was evaluated by co-incubation of the labeled POPC liposomes (0.1 mM lipid, 100 nm) with LMCM liposomes (0.1 mM lipid, 100 nm) or with LMCM giant unilamellar vesicles (0.1 mM lipid, 5~6 µm) at the same volume at 0, 20, 25, 30, 37, and 41 ^{o}C for 60 min. In principle, the fusion of labeled POPC liposomes with the LMCM liposomes causes a dilution of lipid probes and consequently reduces the efficiency of energy transfer between NBD-PE and Rh-PE lipid probes in liposome membrane, resulting in the enhanced emission intensity of NBD-PE probes. The fusion between the labeled POPC liposomes and LMCM liposomes was measured by following changes in fluorescence intensity at 530 nm using exciting the excited wavelength at 450 nm. The fluorescence of NBD-PE at 530 nm was recorded at 0 min (F_0) and at 60 min (F_{60}) with the

addition of triton X-100 (1 %) to disrupt the liposome membrane (F_{max}). The fusion efficiency is calculated as the following formula: $100x(F_{60} - F_0)/(F_{max} - F_0)$.

11. Secretion of encapsulated chitosanase through membrane of LMCM liposomes

Release of encapsulated chitosanase across the lipid mimicking cell membrane (LMCM) liposomes was characterized in the presence and absence of POPC liposomes under various heat conditions. A lipid mixture of 5 mM POPG/DOPE/POPC/POPA (56:20:22:2 in molar ratios) was utilized to fabricate 200 nm liposome entrapping chitosanase (0.16 mg/ml as final entrapped protein concentration). The release of chitosanase across the LMCM liposomes was studied after heating at 25 °C, 37 °C, 41 °C, and 45 °C for 20 min with and without POPC liposomes by co-incubation of one volume of 1 mM POPC liposomes with one volume of 1 mM LMCM liposome entrapping chitosanase. The amount of chitosanase released across the LMCM liposomes with and without the POPC liposomes was quantified by detecting the total protein concentration outside these liposomes before (C_{out}, 0_{min}) and after (C_{out}, 20_{min}) heat treatment at 41 °C for 20 min by using a BCA protein assay kit purchased from PIERCE (Rockland, IL, USA). A volume of 20 µl triton X-100 (20 %) was added to 50 µl of the above liposome mixture to detect the total protein concentration inside the LMCM liposome (C_{triton}). Yield of chitosanase release (Y%) is calculated by following formula $Y\%=(C_{out},20_{min} - C_{out},0_{min})/C_{triton\ X\text{-}100}$. The data and vertical bar represent mean values and standard deviation obtained from three independent experiments.

Results and Discussion

1. Enhanced production of chitosanase by liposome and heat treatment

The effect of the liposome addition on the growth of *S. griseus* cells and their production of the target chitosanase under heat stress condition was investigated. It was expected that the

treatment of heat stress and POPC liposomes could enhance the growth of *S. griseus* cells and their production of the target chitosanase. **Fig. 1-1a** shows the growth curve of *S. griseus* cells by measuring the time course of the cell optical density. The *S. griseus* cells were well adapted and grown in nutrient broth. Their growth rates during 10 h of initially cultivated stage were almost same. The observation also points out that *S. griseus* cells completed the logarithm phase and began reaching the stationary phase after 25 h of the cultivation.

The heat (41 ^{o}C, 20 min) and heat (41 ^{o}C, 20min)/ POPC treatments were applied into the cell culture broth after 10 h of the cultivation. The results show that both the heat and heat/POPC treatments enhanced the cell concentration in the logarithm phase in comparison with those of the control experiment. In this case, the relative estimation of the growth rates of *S. griseus* cells during logarithm phase shows that the cell growth rates under the heat and heat/POPC treatments are respectively 1.2 and 1.4 times higher than that at control experiment. Both the second and third heating in stationary phase did not cause the significant increase of cell concentration, as shown in **Fig. 1-1a**. This observation implies that the heat treatment at 41 ^{o}C enhanced the cell growth rate during the logarithm phase of the cultivation. Interestingly, the addition of POPC liposomes furthermore increased the growth of bacterial cells under heat stress condition at 41 ^{o}C. In this case, it seems that the heat stress activated the biological machinery of *S. griseus* cells and reinforced their nutrition consumption in order to synthesize cell materials for their fission. At this moment, the author can not explain sufficiently the roles of the heat stress and POPC liposomes for the enhanced growth of *S. griseus* cells. It may consider that the liposomes could act as the positive modulator of the protein or biological membrane, which activated the growth of *S. griseus* cells. The correlation of co-effect of heat and liposomes on the cell growth and chitosanase production from *S. griseus* would be explained in the same model. The heat and liposome treatments were thus shown to be effective for the cell growth although further investigation of the interaction between cell membrane and liposomes under specific heat condition is needed.

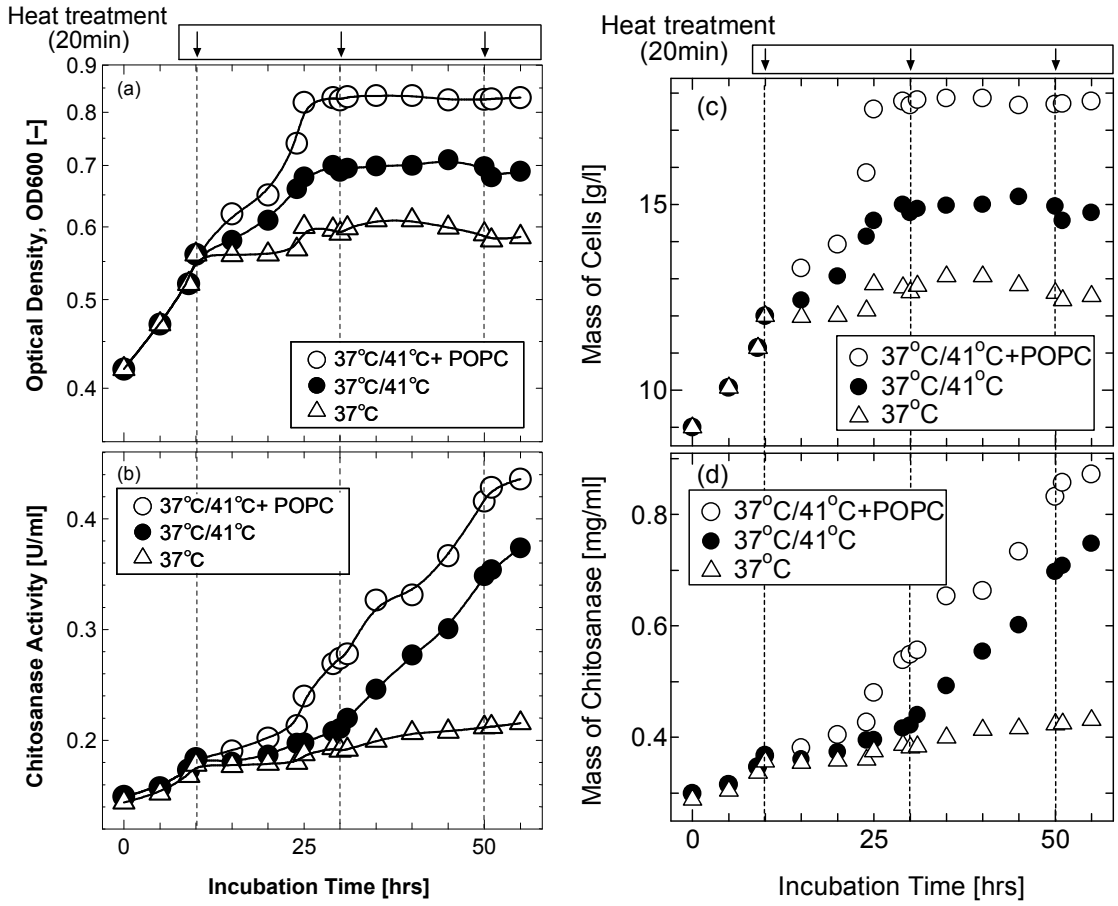


Fig. 1-1 Time course of the growth and chitosanase production of *S. griseus* cells under various heat conditions with and without liposomes; masses of cells and chitosanase is calculated based on the OD and specific activity of chitosanase from *S. griseus*

The target chitosanase production and release from *S. griseus* cells were also measured during the cultivation. **Figure 1-1b** shows the time course of chitosanase activity of *S. griseus* cells during their cultivation process. The total chitosanase activity was rapidly increased after 25 h of the cultivation when the *S. griseus* cells reached stationary phase. It was confirmed that almost chitosanases (more than 95%) were secreted to aqueous medium after that was synthesized in the cytosol of *S. griseus* cells. Relative estimation also points out that the chitosanase activities in the case of the heat (41 ^{o}C) and heat (41 ^{o}C)/POPC treatments are approximate as 1.8 and 2 times, respectively, higher than that in the control experiment. These results imply that the heat treatment at 41 ^{o}C enhanced the production and release of the target

chitosanase from *S. griseus* cells. Especially, the addition of POPC liposomes to the culture broth of *S. griseus* cells further increased the quantity of the target chitosanase. In this process, the heat treatment at 41 ^{o}C was applied three times for 20 min after 10, 30 and 50 h of the cultivation, respectively. The second and third heating resulted in more effective enhancement of the chitosanase activity. These results supported our expectation that the heat treatment could enhance the production and release of chitosanase. It is also considered that the heating point and heating period are important to create potential functional response of bacterial cells. Based on the effective conditions obtained by DDA and CD spectra analysis of the interaction between chitosanase and POPC liposomes, the optimal heating period at 41 ^{o}C is suggested to be 20 min for enhanced chitosanase production from *S. griseus* cells, as shown in **Fig. 1-1b**. The masses of cells and chitosanases calculated based on the OD values and specific activity of chitosanase are shown in **Figs. 1-1c** and **1-1d**. It is possible that heat stress activated the cell membrane and enhanced the internal tumor pressure of *S. griseus* resulted in enhancement of membrane fluidity. Thus in the logarithm phase, when the amount of chitosanase was slowly produced and released across the membrane, the proliferation of *S. griseus* cells was increased and was significantly higher than that of the control. In the presence of POPC liposomes, released chitosanase was bound to the liposome surfaces after the heat treatment. Therefore, the increase of chitosanase in the heat/POPC liposomes treatment could be observed in comparison with that of the heat treatment and the control in logarithm phase. The online monitoring of cell membrane state by DDA (data not shown) supports that the enhanced growth of *S. griseus* cells in the heat/POPC liposomes treatment may be a results of membrane fusion between POPC liposomes and cytoplasmic membranes and/or the POPC liposomes captured the chitosanase at the interface of cell membrane. In the stationary phase, the cell growth depended their biophysical states was saturated after 25 h of the cultivation for all cases although the second and third heat treatments were applied. However, the chitosanase activity from *S. griseus* cells still increased by second and third heat

treatments in comparison with that of the control. It is possible that heat treatment at 41 ^{o}C directly affected to the change in the membrane properties such as the increase of membrane fluidity to recruit the interaction between liposomes and cell membrane. As the consequence, the enhanced production and release of the target chitosanase from *S. griseus* could be obtained. It has also been reported that the heat stress enhanced the periplasmic recovery of cytoplasmic galactosidase by translocation across the inner membrane of *Escherichia coli* (Umakoshi *et al*., 1998) and liposome that has chaperone-like function for protein refolding (Yoshimoto *et al*., 1998 & 2000) and the heat activates the molecular chaperones in bacteria, which also exhibits important roles for protein refolding (Krzewska *et al*., 2001; Minami *et al*., 2001) and enhanced translocation (Jason *et al*., 2003; Yamamoto *et al*., 2005). In this work, heat stress (41 ^{o}C) and POPC liposomes were successfully applied for enhancement of the production of the target chitosanase from *S. griseus*. The heat stress and liposome treatments could therefore be utilized in order to enhance the target production in stress-mediated bioprocess.

2. Specificity of substrate for production of chitosanase by *Streptomyces griseus*

In order to verify the substrate specificity for the production of chitosanase by *S. griseus* cell, the glucose and chitosan media were used to cultivate the *S. griseus* cells. As shown in **Fig. 1-2**, the growth of these cells in the glucose and chitosan media was quite good and almost similar. The total protein concentration produced by *S. griseus* cells cultivated in glucose medium was significantly higher than that in chitosan medium. In addition, the target production of chitosanase by these cells cultivated in those media was compared to elucidate the substrate specificity for chitosanase production. The production of chitosanase by these cells cultivated in glucose medium was not significantly observed, whereas the production of chitosanase was effective only in the case that cells were cultivated in the chitosan medium. This observation implies that the chitosan is specific substrate for the production of

chitosanase by *S. griseus* cell rather than glucose. It is because of that chitosanase is extracellular enzyme, which can hydrolyze large molecular weight chitosan to smaller molecular weight oligochitosans and glucosamine that can be adsorbed directly by the *S. griseus* cells. Indeed, *S. griseus* cells need to secrete chitosanase to the aqueous medium to digest chitosan to glucosamine and/or small oligochitosans for their surviving. Once these cells were cultivated in the glucose medium, the cells could directly utilize this substrate for their growth and production of many kinds of proteins but not including chitosanase, as shown in **Fig. 1-2**.

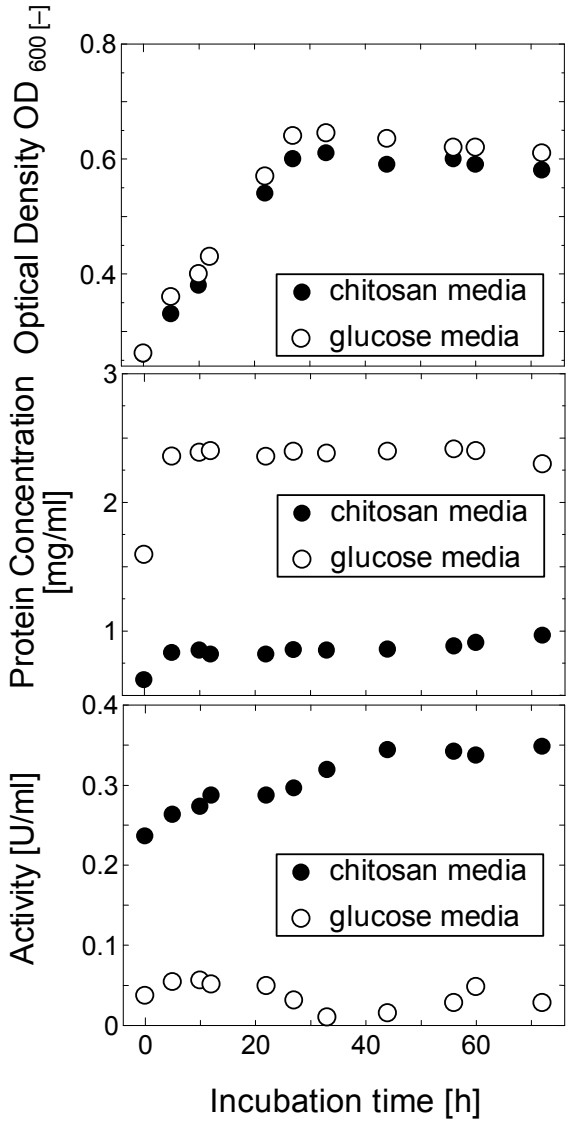


Fig. 1-2 Effect of nutritive compositions on the growth of *Streptomyces griseus* cells and their production of chitosanase

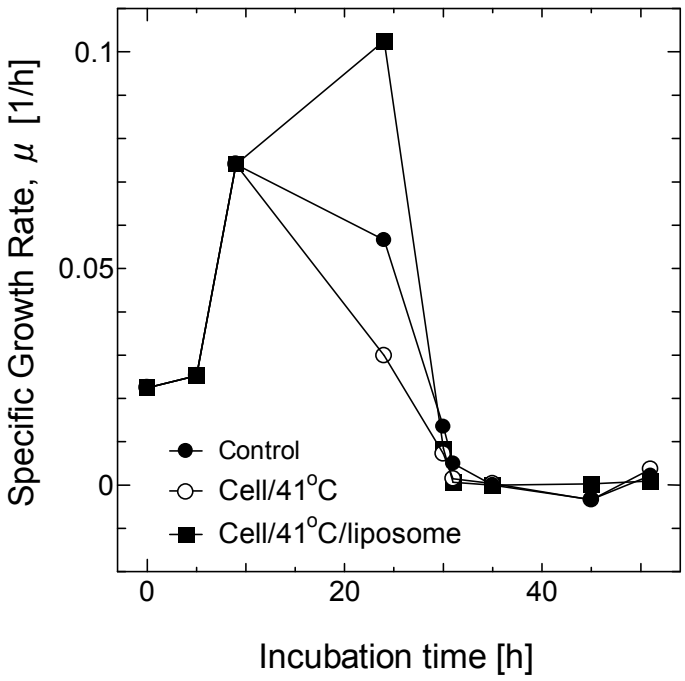


Fig. 1-3 Kinetic of specific growth rate of *S. griseus* cell under heat stress with and without liposome; condition of experiment is the same as that shown in **Fig. 1-1**; and mass of cells was estimated based on the correlation between OD value and wet weight of cells

3. Kinetic of cell growth and production of chitosanase by *Streptomyces griseus* by Leudeking-Piret models

3.1 Kinetic of cell growth rate

Figure 1-3 shows the experimental data of specific growth rate of *S. griseus* cell treated with the heat stress at 41 ^{o}C in the presence and absence of POPC liposomes versus incubation time in batch culture mode. This analysis verifies the effects of the heat and liposomes on the growth rate of *S. griseus* cell. It is shown that the treatment of heat stress at 41 ^{o}C did not increase the specific growth rate of this cell in comparison with that of the control. However, the co-treatment of the heat stress at 41 ^{o}C and POPC liposomes increased the specific growth rate of this cell in comparison with that of the control. The maximal specific growth rate (μ_{max}) of *S. griseus* cell at 37 ^{o}C treated with and without the heat stress at 41 ^{o}C was almost 0.073 h^{-1} after incubation of 10 h, while μ_{max} was increased to 0.102 h^{-1}

once this cell was treated with the heat stress at 41 ^{o}C and POPC liposomes simultaneously, after incubation of 24 h. This observation implies that the enhanced specific growth rate of *S. griseus* cell was mainly contributed by liposome under heat stress condition. The reduction of specific growth rate of this cell after incubation of 30 h could relate to the restriction of nutrients for the cell growth in the batch culture mode.

3.2 Kinetic of production rate

Figure 1-4 verifies the effects of the heat stress at 41 ^{o}C and POPC liposome on the specific production rate of chitosanase by *S. griseus* cells. The result shows that although the maximum specific production rate of chitosanase by *S. griseus* cell treated with the heat stress at 41 ^{o}C was almost same as the control at 0.06 h^{-1} after incubation of 10 h, the specific production rate of chitosanase by *S. griseus* cell under the heat stress at 41 ^{o}C was still kept

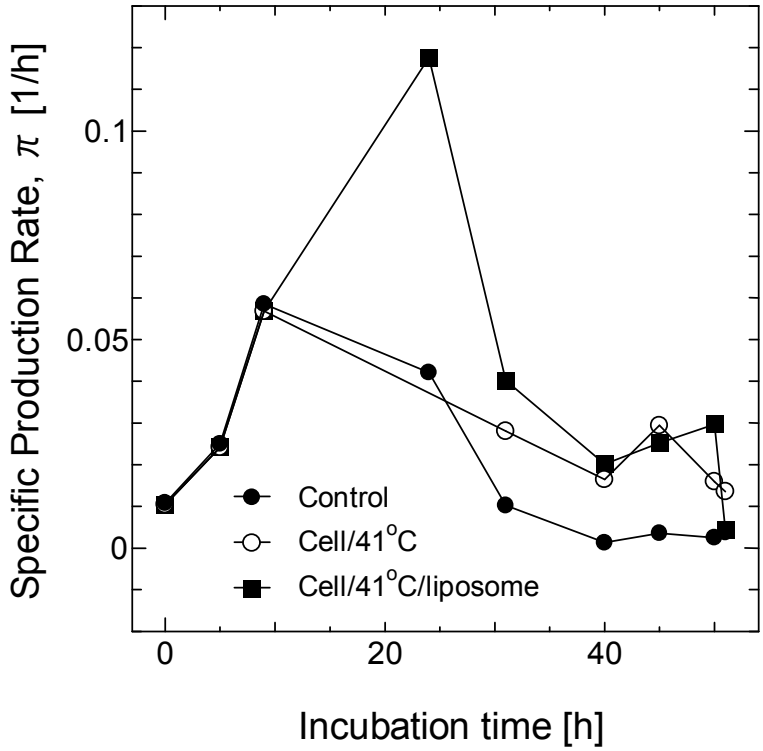


Fig. 1-4 Specific production rate of chitosanase by *S. griseus* cell under heat stress with and without liposome. The experimental condition is the same as that shown in **Fig. 1-1**. The mass of chitosanase in supernatant is estimated based on its specific activity as compared with that of the standard chitosanase from *S. griseus* cell purchased from Sigma Aldrich

higher than that of the control during the fermentation process. Interestingly, the addition of POPC liposomes to the fermented broth of *S. griseus* cells pretreated with the heat stress at 41 ^{o}C further increased the specific production rate of chitosanase with its maximal value almost 0.118 h^{-1} after incubation of 24 h although after that it was gradually reduced during the fermentation process. This observation implies that the *S. griseus* cells treated with heat stress at 41 ^{o}C even in the presence or absence of POPC liposomes enhanced the specific production rate of chitosanase by *S. griseus* cells.

3.4 Production yield of chitosanase by *Streptomyces griseus* cell

The production yield of chitosanase by *S. griseus* cell was calculated based on the correlation between the specific growth rate and specific production rate shown in **Fig. 1-5**. The production yield reflects the potential of *S. griseus* cell for the production of chitosanase under certain condition. The results are shown in **Fig. 1-6**, the production yield of chitosanase by *S. griseus* cell treated with the heat stress at 41 ^{o}C was approximately twice higher than that of the control after incubation of 31 h. Interestingly, the addition of POPC liposomes to fermented broth of *S. griseus* cell under the heat stress at 41 ^{o}C further increased the production yield of chitosanase to approximate 4.5 times higher than that of the control. These results once more confirm that the heat stress and POPC liposomes can play important roles for the enhanced production of chitosanase by *S. griseus* cells.

The efficiency of the heat stress and liposomes on the growth of *S. griseus* cell and its chitosanase production are summarized in **Table 1-1**. Although the treatment of the heat stress at 41 ^{o}C and liposomes effectively enhanced the growth and production of chitosanase from *S. griseus* cell, the mechanism behind this phenomenon has not been elucidated yet.

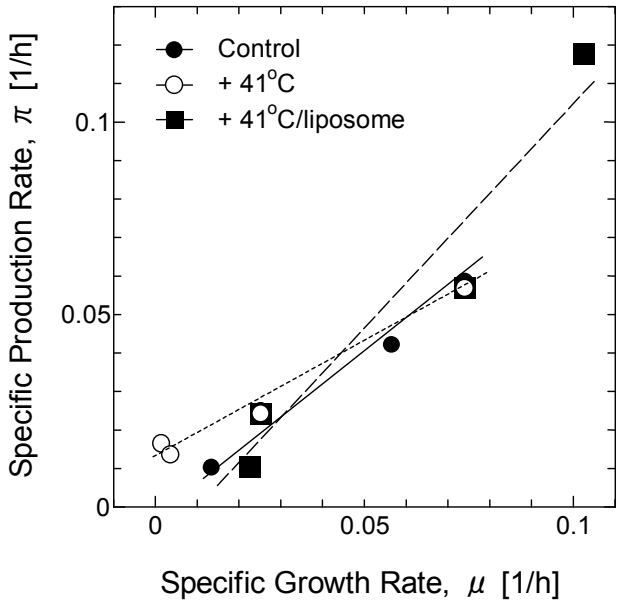


Fig. 1-5 Correlation between the specific growth rate and specific production rate of chitosanase of *Streptomyces griseus* cell. The data were plotted based on **Fig. 1-3** and **Fig. 1-4**. On the basis of this plotting, α and β values of each experimental condition could be obtained

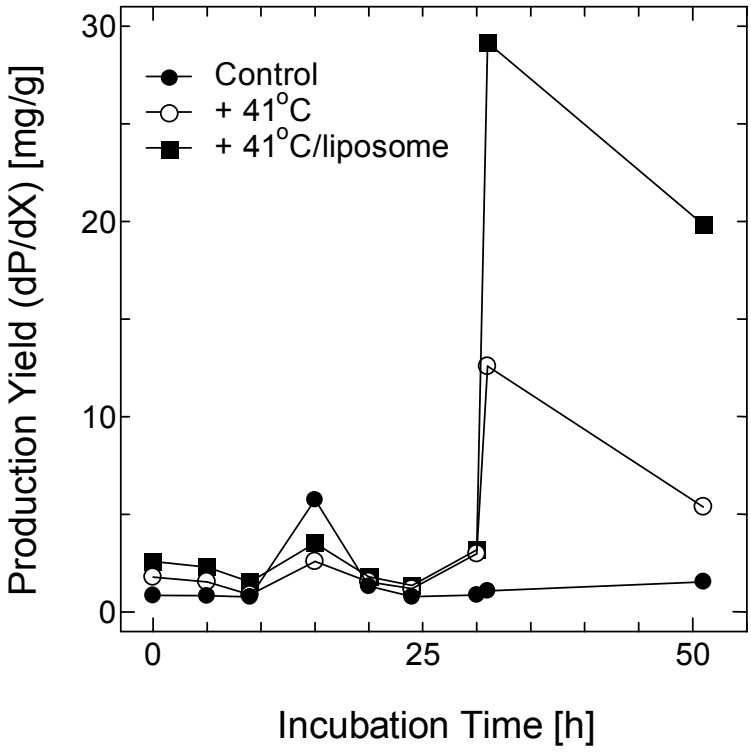


Fig. 1-6 Production yield of chitosanase by *Streptomyces griseus* cell under heat stress with and without liposome. Data were calculated based on α, β obtained by plotting in **Fig. 1-5** as the description shown in experimental section

Table 1-1 Effeciency of the heat stress and the liposome on the growth of *Streptomyces griseus* cell and its production of chitosanase

Term	Control	Control (+ 41 ^{o}C)	Control (+ 41 ^{o}C/liposome)
Maximal specific growth rate, μ_{max} (h^{-1})	0.073	0.073	0.102
Maximal specific production rate, π_{max} (h^{-1})	0.061	0.061	0.118
Maximal production yield, dP/dX (mg/g)	5.8	12.5	28.1

4. Enhanced secretion of chitosanase from *S. griseus* cell by liposome and heat treatment

Figure 1-7 shows the effects of the addition of POPC liposomes on the chitosanase release by intact and spheroplast *S. griseus* cells cultivated at 37 ^{o}C with and without the treatment of heat stress at 41 ^{o}C. In both intact and spheroplast cells at normal conditions (37 ^{o}C), a significant change in the chitosanase activity was not observed (0.18-2.0 U/ml). In the case of the intact cells, the chitosanase release was increased under the heat treatment at 41 ^{o}C and was 1.8 times higher than that at 37 ^{o}C. The addition of POPC liposome at 41 ^{o}C further enhanced the chitosanase release, resulting in 2.2 times higher activity of chitosanase (0.43 U/ml). It has been reported that the addition of POPC liposome at 41 ^{o}C increased the release of chitosanase from the intact *S. griseus* cells, implying the possible interaction of chitosanase with the heat-stressed cell membrane (Ngo *et al*., 2005). The former results on the interaction of cell membrane-chitosanase are corresponding with the previous findings. However, in the case of spheroplast cell without peptidoglycan layers, the chitosanase activity was not increased only under the heat stress at 41 ^{o}C although the value was increased by adding

POPC liposomes at 41 °C. These results imply that the enhanced release of chitosanase could be related not only to the interaction of chitosanase itself with the cell membrane (Ngo *et al*., 2005) but also to a possibly important role of liposome membrane itself.

As shown in **Fig. 1-7**, the total proteins released from both cells are also shown. In contrast to the behaviors of chitosanase release, no significant change of the release of the total proteins was observed in three independent experiments in the case of intact cells regardless of the heating treatment or liposome.

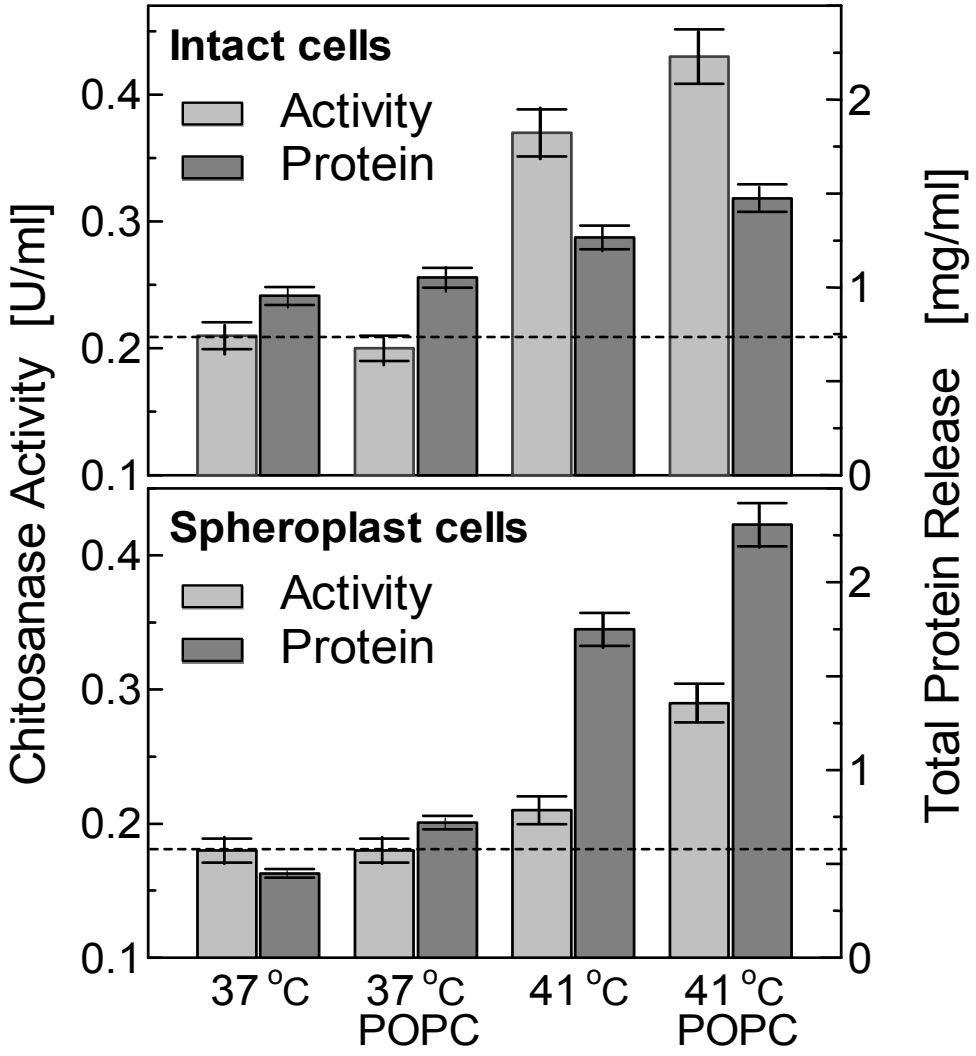


Fig. 1-7 Release of chitosanase by intact and spheroplast *Streptomyces griseus* cells under various heat conditions with and without liposomes

The release of total proteins from the spheroplast cells was significantly higher than that from the intact cells. Under the heat stress at 41 ^{o}C, the total proteins released from the spheroplast cell with and without the POPC liposome were almost 1.4 times higher than those from the intact cells, implying that the proteins associating with membrane could be released under the heating condition owning to their conformational change. The analysis of proteins released from these cells by SDS-PAGE showed that the amount of the chitosanase released from the spheroplast cells was smaller than that from the intact cells under above conditions similarly in the case of the chitosanase activity tested above. This observation implies that the POPC liposome itself affects to the release of proteins including chitosanase rather than the folding of nascent chitosanase to native conformation in the case of spheroplast cells.

The difference of specific activity of chitosanase released in total extracellular proteins between intact and spheroplast *S. griseus* cells shows an additional effect of direct interaction of liposome with cell membrane such as roles of peptidoglycan layers on the release and activity of chitosanase. The bacterial peptidoglycan layers have been reported to play important roles on the secretion and activity of the periplasmic enzymes involved in the folding of the secreted nascent enzymes into their native conformation ones (Arnoud *et al*., 1996), implying that the support of peptidoglycans and periplasmic enzymes could fold the nascent chitosanase to the native one in the case of intact cell once the nascent chitosanase was released across cell membrane to periplasmic space. Moreover, the binding and/or the fusion of the POPC liposomes with the membrane of *S. griseus* cells induced by the heat stress might also create the lipid domains, which support for the release of chitosanase through membrane bound-protein secretory pathway. These assumptions are strongly supported by the enhanced release of chitosanase from spheroplast cell at 41 ^{o}C with POPC liposome together with previous findings to show the significant roles of (i) interaction between cell membrane and liposomes in the protein transfer from cells to liposomes (Enoch *et al*., 1977) and (ii) the lipid mixing between liposomes and cell membrane induced the

release of the shed-vesicles containing membrane proteins from erythrocyte cells (Suzuki *et al*., 1999). It is therefore seems that the "direct" interaction between the lipid membranes plays a key role in protein release and transfer.

5. Efficiency of liposomes and heat treatments on the enhanced production and secretion of chitosanase from *S. griseus* cells

The efficiency of the heat and liposomes treatment on the enhanced production and secretion of chitosanase was elucidated by considering the interaction of the liposome with chitosanase and cell membrane under the heat stress condition. Such interactions play important roles to enhance the chitosanase production and secretion from *S. griseus* cells. The interactions between liposomes and chitosanase were evaluated by DDA and CD spectra. The interaction of liposomes with cell membrane was evaluated through the variation of liposomal membrane fluidity in the presence and absence of intact and spheroplast *S. grieus* cells and that membrane-membrane interaction under heat stress condition was further elucidated by the lipid mixing between POPC liposomes and lipid mimicking cell membrane (LMCM) liposomes. The efficiency of lipid mixing (membrane fusion) on the secretion of encapsulated chitosanase through protein bound lipid membrane mode was finally studied under various heat conditions.

5.1 Interaction of liposome with chitosanase under heat stress evaluated by DDA

The dielectric dispersion analysis (DDA) of POPC liposome solution in the presence and absence of chitosanase was carried out under various heat conditions in the frequency range from 1 to 100 MHz in order to detect heat stress-mediated interaction between protein and phospholipid membrane. Under heat specific heat condition, the interaction of protein with liposome leads to the variation of surface properties of liposome. The fluctuations on hydrocarbon phase of membrane strongly depend on temperature and can be correlated with

molecular order and microdynamic of the dipolar head groups, located on the membrane surface (Schrader *et al*., 2001). On the other hand, the variation of electrostatic interactions at the bilayer surface may exercise an influence on the lateral area per lipid molecule and may be mediated by the rotational isomerization of the acyl chain, on the domain structure of membrane (Schrader *et al*., 2001). Dielectric dispersion analysis is a powerful technique to investigate the reorientation of the solvent molecules on the surface of phospholipid membrane (Morita *et al*., 2000; Shikata *et al*., 1998; Schrader *et al*., 2001; Hianik *et al*., 1997; Smith *et al*., 1996). Dielectric dispersion analysis was also applied to evaluate the interaction of cell membrane and protein (Morita *et al*., 2000).

Dielectric parameters of liposome, based on the Debye's equation, can be obtained such as the amplitude of dielectric dispersion ($\Delta\varepsilon$), the lateral diffusion and the rotation of dipolar head groups on the membrane surface (as characteristic frequencies f_{c1}, f_{c2}, respectively). **Figure 1-8** shows the f_{c1} and f_{c2} values of dipolar head groups in pure POPC liposome and

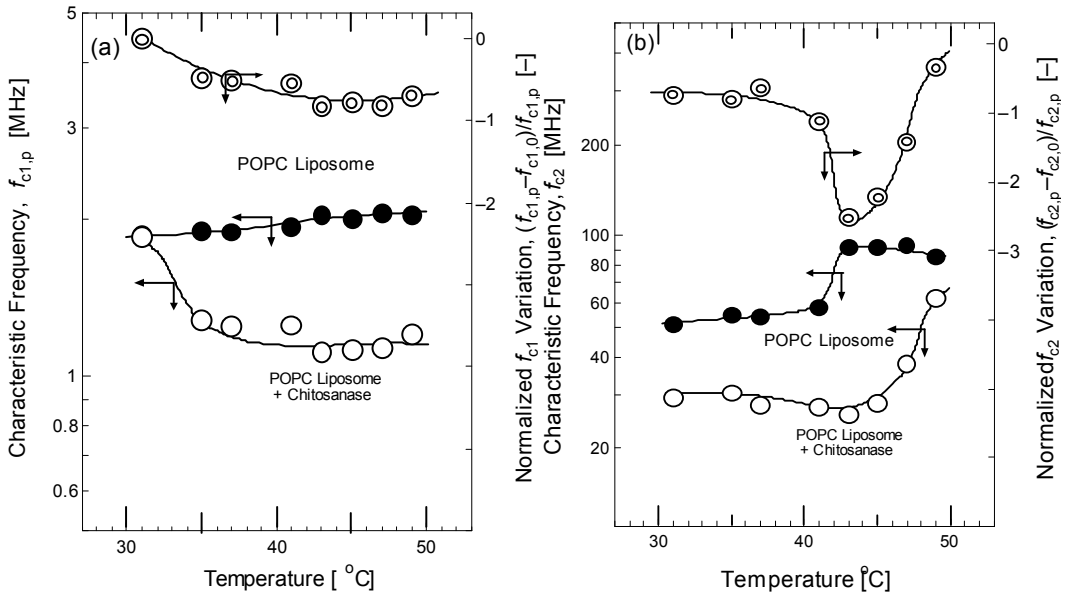


Fig. 1-8 Temperature dependence of dielectric parameters ((a) f_{c1} and (b) f_{c2}) of the POPC liposome with and without chitosanase, the f_{c1} and f_{c2} can herewith be corresponding with the lateral and rotational mobility of phospholipid molecules

POPC/chitosanase complex under various heat condition at 31, 35, 37, 41, 43, 45, 47 and 49 ^{o}C. In the case of pure POPC liposome, the observation shows that the f_{c1} values were thermally stabilized with increasing temperature as shown in **Fig. 1-8a**. However, the f_{c2} values were increased with increasing temperature as shown in **Fig. 1-8b**. The observation also shows that the addition of pure chitosanase to POPC liposome solution caused a significant decrease of the f_{c1} and f_{c2} values. It had been reported that the frequency of thermal fluctuation of dipolar head group is estimated by kT/h (where: k is Bolzmann constant, h is Planck constant and T is absolute temperature) (Smith *et al*., 1996). The above observation implies that the chitosanase may interact with POPC liposome resulted in restriction of lateral area per lipid molecules. Therefore, the decrease of both lateral diffusion and rotation of dipolar head groups on phospholipid membrane surface could be observed. The role of temperature inducing the interaction of chitosanase and POPC liposome was relatively estimated as the function of normalized variation of the f_{c1} and f_{c2} values of POPC in the presence and absence of chitosanase. The calculation, plotted in the **Figs. 1-8a** and **1-8b**, shows that the normalized variation of f_{c1} and f_{c2} values was decreased and minimized at the temperature range of 40-45 ^{o}C. This integration implies that the strongest interaction of chitosanase with POPC liposomes could be obtained at temperature range 40-45 ^{o}C. It has also been reported that the membrane fluidity reflecting the lateral diffusion and rotation of phospholipid molecules strongly depends on the contribution of temperature (Morita *et al*., 2000; Smith *et al*., 1996). The heat contributes to the configurational entropy and the activation energy, which associates with the transition from unpolarized to polarized state of phosphocholine head groups and breaks the intermolecular bonds of PO^{-}_{4} …$CH_3{}^{+}N$ that are restricting the reorientation of the dipoles between molecular phospholipids (Smith *et al*., 1996). Therefore, the increasing of the interaction between dipolar head groups in the POPC liposome with the other molecules such a chitosanase is expected by increasing temperature.

It is also suggested that heat treatment at 40-45 ^{o}C caused the conformational transition of chitosanase, resulting in the variation of it surface properties, including of both local and surface net hydrophobicity (Jung *et al*., 2003). Therefore, the favorable forces inducing the interaction of chitosanase with POPC liposome under specific heat condition at 40-45 ^{o}C could be considered as hydrophobic and electrostatic interactions. Here, it is concluded that the specific heat enhanced the interaction of chitosanase with POPC liposome resulting in the prevention of the heat damage on chitosanase structure. The effective conditions for their strongest interaction could be obtained at temperature range 40-45 ^{o}C. Therefore, the optimal temperature at 41 ^{o}C and POPC liposomes were utilized in the following experiments in order to enhance the target chitosanase production and recovery from *S. griseus* cells.

5.2 Interaction of chitosanase with liposomes evaluated by CD spectra

The conformational transition of chitosanase in the presence and absence of POPC liposome was investigated under heat stress condition. The typical CD spectra of the

Table 1-2 Summary of secondary structure of chitosanase from *S. griseus* after the treatment of heat stress at 41 ^{o}C in the presence and absence of POPC liposomes

Experiments	Secondary structure of chitosanase			
	α-helix (%)	β-sheet (%)	β-turn (%)	Random (%)
25 ^{o}C	39	6	36	19
25 ^{o}C+POPC	39	6	37	18
41^{o}C	12	25	18	45
41^{o}C + POPC	28	9	18	45

chitosanase at above conditions are shown in **Fig. 1-9**. The variation of molecular ellipticity values at the wavelengths of 208 nm, 222 nm and 218 nm could be obtained at above conditions.

The results are summarized in **Table 1-2** as the description in the experiments and methods section. At 25 ^{o}C, the secondary structure of chitosanase with and without POPC liposome exhibited as the α-helix content of 39 % and the β-sheet content of 6 %. In the case of heat condition at 41 ^{o}C, there was drastic decrease of the α-helix content (remained only 12 %) and significant increase of the β-sheet content (25 %) of chitosanase. It was so interesting that, in the presence of POPC liposome at 41 ^{o}C, the α-helix content was maintained (28 %) and slight increase of the β-sheet (9 %) was observed. These results may imply that the heat treatment could easily change the native structure of chitosanase and POPC liposome could hydrophobically and electrically interact with chitosanase, resulting in the slightly conformational change of the chitosanase structure in liposome to protect it from heat damage. It is possible that there was the hydrogen bond transition between phospholipid acyl chains in liposome vesicle and chitosanase and the transition between α-helix, β-sheet, β-turn and random in chitosanase under the heat stress. At the non-heat stress conditions (25-30 ^{o}C), the native conformation of chitosanase was not favorable enough to recruit the interaction with POPC liposome as the observation of CD and DDA shown in **Fig. 1-9**. It is also suggested that the specific heat at 40-45 ^{o}C caused the conformational transition of chitosanase, resulting in the variation of their surface properties including of both local and surface net hydrophobicity (Jung *et al*., 2003). The membrane fluidity, reflecting the dynamic motion of individual phospholipid molecules and local hydrophobicity of membrane surface, was also increased with the increase of temperature. Consequently, liposome recruited the other molecules into the interactions in their interior and interface (Morita *et al*., 2000). It has also been reported that small unilamellar POPC liposomes were utilized as artificial chaperone, which can refold the denatured protein resulting in enhancement of native protein

recovery (Yoshimoto *et al*., 1998 & 2000).

The target of this evaluation is to elucidate the interaction of chitosanase with POPC liposome under heat stress condition. In this case, the interaction of chitosanase with POPC liposome, a model of biological membrane may also contribute to the explanation that heat and POPC liposome enhanced the chitosanase production of *S. griseus* in the stress-mediated bioprocess. It is possible that POPC liposome interacted and protected the target chitosanase in the culture broth of *S. griseus* under heat stress condition. The driving force, which induced the interaction between chitosanase and POPC liposome under heat stress condition, can be discussed as mainly hydrophobic force. However, as shown in **Fig. 1-8a**, partially electrostatic force may also contributed to bring chitosanase to interface of POPC liposome.

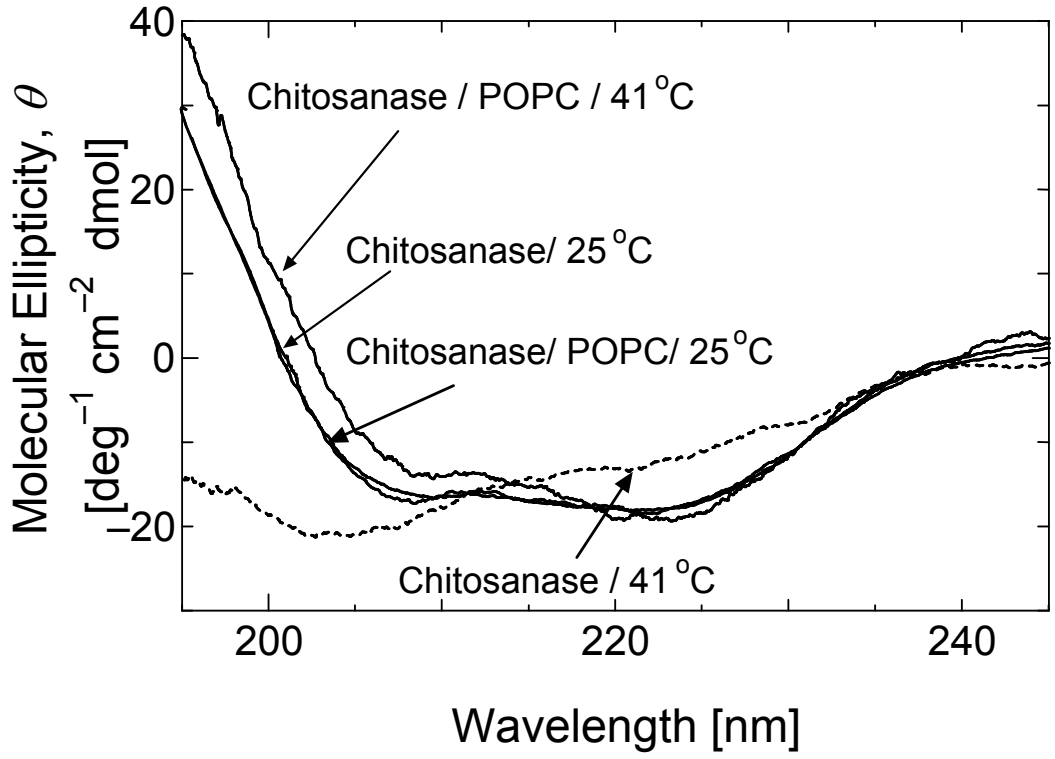


Fig. 1-9 Conformational transition of chitosanase from *S. griseus* cell under various heat conditions with and without liposome

5.3 Interaction of neutral liposome with cell membrane of *S. griseus* cell through the variation of liposomal membrane fluidity

The interaction of the POPC liposomes with cell membrane under heat stress conditions was investigated. **Figure 1-10** shows the time course of the membrane fluidity (1/*P*) of pure POPC liposomes with and without the co-incubation of spheroplast or intact *S. griseus* cells. The results indicate that the 1/*P* values under the heat stress at 41 ^{o}C were almost unchanged (1/*P* = 4.8) throughout incubation of 2 h. On the contrary, 1/*P* values of the POPC liposomes in the presence of spheroplast cells were drastically decreased from 4.8 to 3.7 and those in the presence of intact cells were decreased from 4.9 to 4.3 throughout incubation of 2 h. These results imply that the direct interaction of the POPC liposomes with spheroplast and intact *S. griseus* cells could occur in a manner to the reduction of the membrane fluidity of the POPC liposomes. The above phenomena might support the hypothesis that the POPC liposomes could pass through the peptidoglycan layer and directly interact with the lipid membranes of *S. griseus* cells under the heat stress condition.

Several possibilities of such interaction could be considered as the follows (i) POPC liposomes containing TMA-DPH could fuse to the cell membrane; (ii) intact POPC liposome could interact with cell proteins and released chitosanase at the cell surface under a heat stress at 41 ^{o}C. In both cases, the membrane fluidity of POPC liposome could be reduced. The results also show that the membrane fluidity of the spheroplast *S. griseus* cells decreased with the increasing temperature and that the direct interaction of POPC liposomes with the cell membranes occurred effectively under a heat stress at 41 ^{o}C. The interaction of the POPC liposomes with the cell membrane plays crucial roles for the enhanced release of chitosanase from *S. griseus* cells under the heat stress condition.

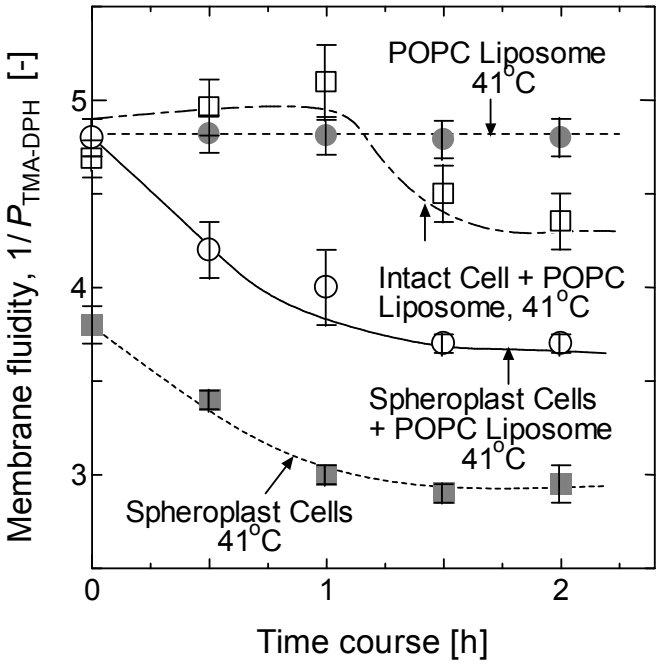


Fig. 1-10 Variation of membrane fluidity of POPC liposomes in the presence and absence of *S. griseus* cells under heat stress condition

5.4 Lipid mixing between neutral liposome and LMCM liposomes

The lipid mixing between neutral POPC liposomes and lipid mimicking cell membrane (LMCM) liposomes at different temperatures is shown in **Fig. 1-11**. The typical signal of fluorescence energy transfer between NBD-PE and Rh-PE labeled in POPC liposomes is shown before and after mixing with LMCM liposomes at 41 °C for 60 min in **Fig. 1-11a**. In principle, the mixing between labeled liposomes and non-labeled liposomes reduces the efficiency of fluorescence energy transfer from NBD-PE to Rh-PE, resulting in the increased fluorescence intensity of NBD-PE in time dependence. The addition of triton X-100 to this mixture disrupted all liposomes, resulting in the deletion of fluorescence energy transfer between NBD-PE and Rh-PE. The calculated amount of lipid mixing is described in the experimental section and is shown in **Fig. 1-11b**. The results show that the amount of lipid mixing between neutral POPC liposomes and LMCM liposomes was increased by increasing temperature. Among tested temperatures, the highest amount of lipid mixing between them

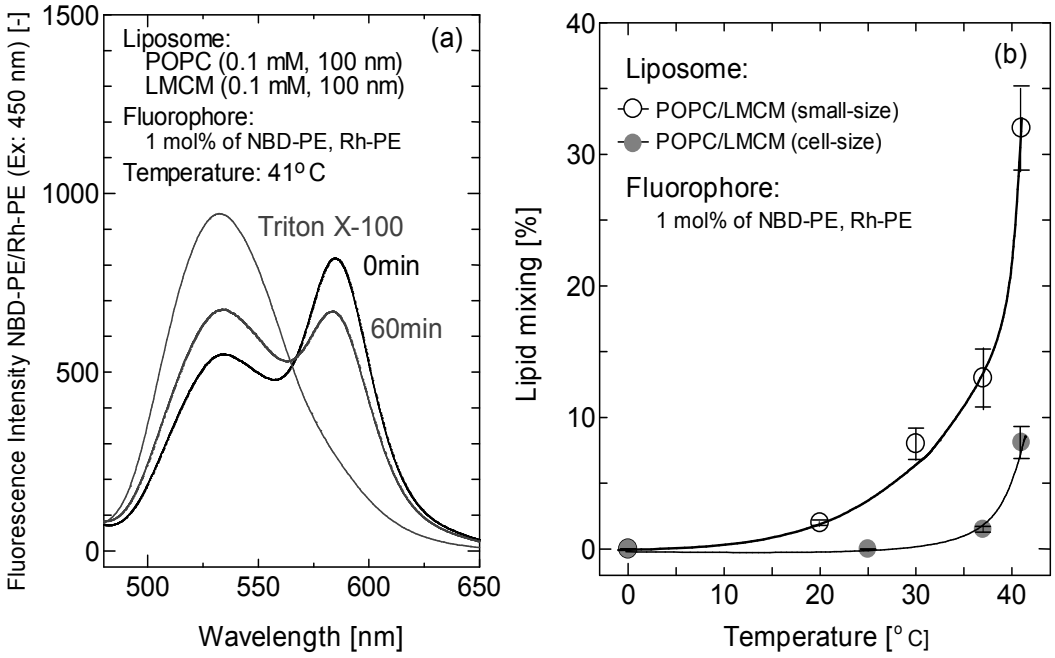


Fig. 1-11 Effect of temperature on lipid mixing of POPC liposomes with small and cell-size LMCM liposome vesicles by using fluorescence resonance energy transfer (FRET)

was 33 % at 41 ^{o}C. In addition, the lipid mixing between neutral POPC liposomes and LMCM giant vesicle (GUV) also showed the importance of temperature on the fusion between the lipid membranes. These observations imply that heat induced the interaction and fusion of neutral POPC liposomes with LMCM liposomes.

6. Secretion of chitosanase through lipid mimicking cell membrane (LMCM) liposome under heat stress in the presence of neutral liposome

In order to verify the possible secretion of chitosanase through the induction of membrane interaction between the membrane of *S. griseus* cell and the POPC liposome, the release of encapsulated chitosanase across lipid mimicking cell membrane (LMCM) liposomes was further invesitigated with and without the POPC liposomes under various heat conditions. As shown in **Fig. 1-12**, the release of chitosanase across the LMCM liposomes without the POPC liposome was not significant at every tested temperature, whereas an

addition of the POPC liposomes to the solution of LMCM liposomes encapsulating chitosanases significantly increased the release of these enzymes across LMCM liposomes to 11, 17, and 14 % at 37, 41 and 45 ^{o}C, respectively. The above phenomena imply that the POPC liposomes could be effective modulators for the release of chitosanase through the lipid membrane of LMCM liposomes with maximum output at 41 ^{o}C (**Fig. 1-12**). The activity of the released chitosanase across the LMCM liposomes with and without the treatment of POPC liposomes was also detected in all experimental temperatures. The results show that the activity of released chitosanase with the treatment of the POPC liposomes well corresponded to the activity of native chitosanase from *S. griseus* cell although there was almost no activity of the released chitosanase detected without the treatment of POPC liposomes at every tested temperature.

There are several important membrane properties those affect the release of encapsulated chitosanase across the LMCM liposomes treated with the POPC liposomes: (i) lipid mixing between membranes (Enoch *et al*., 1997; Suzuki *et al*., 1999), (ii) membrane fluidity (Cook *et al*., 1980), (iii) lipid composition (Yatvin *et al*., 1987) and so on. Under the heat stress at 41 ^{o}C, the POPC liposomes could directly fuse with LMCM liposomes as shown in **Fig. 1-11**. It is possible that the fusion of two membranes induced the release of chitosanase across LMCM liposomes. The lipid mixing of the membranes has been reported to play a role for the transfer of several intrinsic membrane proteins between artificial phospholipid membranes (Enoch *et al*., 1997) and the transfer of membrane proteins from less fluid membrane to high fluid membrane (Cook *et al*., 1980). The fusion of liposomes to the membrane of erythrocyte cell induced the release of shed-vesicles containing intrinsic membrane proteins (Suzuki *et al*., 1999). In addition, Yatvin *et al*. have also reported that the membrane lipid composition played an important role for the translocation of the nascent proteins in the heated *Escherichia coli* cell (Yatvin *et al*., 1997). On the other hand, the conformation of chitosanase under specific heat condition may also play a role for its interaction and release across lipid

membrane. Jung *et al*. have reported that the conformation of chitosanase was varied by the heat treatment, resulting in the maximal values in both local and surface net hydrophobicity of chitosanase in range of 40~45 ^{o}C (Jung *et al*., 2003). These factors involving high fluidity of the lipid membrane and the favorable conformation of chitosanase strongly recruited the hydrophobic interaction between chitosanase (intermediate state of protein folding or molten globule states) and the POPC liposomes (Ngo *et al*., 2005). It is therefore explained that the release of chitosanase across lipid membranes, as shown in **Fig. 1-12**, was maximized at 41 ^{o}C in comparison with that at 37 ^{o}C and 45 ^{o}C.

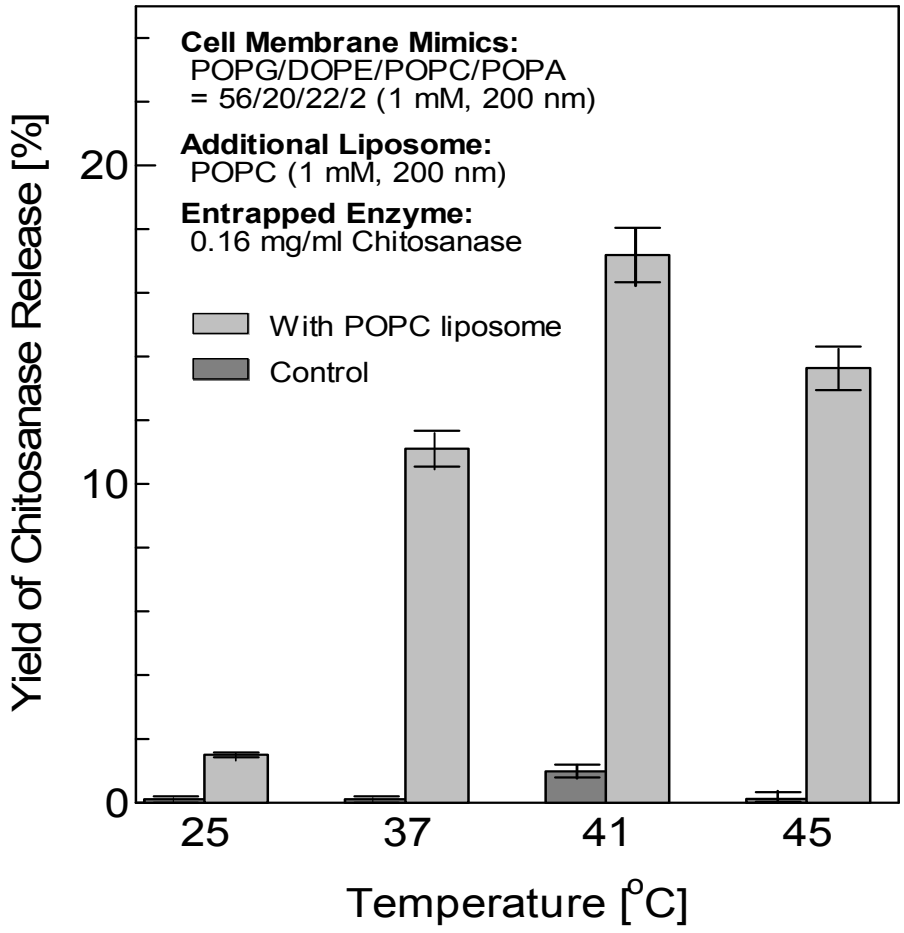


Fig. 1-12 The release of chitosanase across the lipid mimicking cell membrane (LMCM) liposomes in the presence and absence of POPC liposomes under various heat conditions

7. Characterization of chitosanase production under oxidative stress conditions

7.1 Enhanced production of iturin A from *Bacillus subtilis* by using UV irradiation

In order to characterize the possible effects of oxidative stress on the growth and production of target proteins from bacterial cells, the production of iturin A from *Bacillus subtilis* treated by UV irradiation in my previous study may be compared and shown here. Iturin A is cyclic lipopeptide containing seven amino acids L-Asp-D-Tyr-D-Asp-L-Gln-L-Pro-D-Asp-L-Ser and one β-amino acid. This peptide can specifically bind to cell membrane of various kinds of toxic fungus such as *Aspergillus flavus*, *A. parasiticus* to create the pore in the membrane, resulting in the leakage of cytosolic solution and cell death. This efficiency of iturin A is considered as antibiotic antifungal surfactant that has attracted many

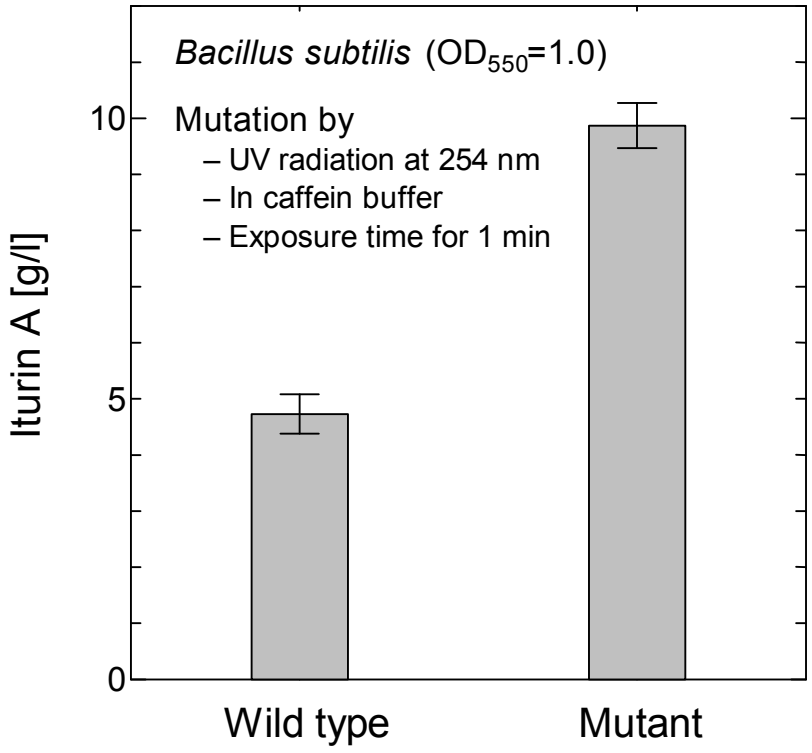


Fig. 1-13 Enhanced production of antibiotic antifungal iturin A from *Bacillus subtilis* by treatment of UV radiation. The iturin A was purified and quantified from fermented broth of *B. subtilis* mutant as the description in the experimental section.

researchers studying the effective methods for the enhanced iturin A production (Kimura *et al*., 1990; Ohno *et al*., 1993; Shoda *et al*., 1991; Bok *et al*., 1992).

As shown in **Fig. 1-13**, the production of iturin A by mutants was almost twice higher than that of wild-type *B. subtilis* cells. This observation implies the effects of UV light treatment on the *B. subtilis* cells. It has been reported that UV light treatment at 254 nm directly affected on the DNA of *B. subtilis* cells , consequently, resulted in the mutation of these cells. Under lethal condition of UV light, almost all bacteria were died but the mutants those could survive might have high potential for the production of iturin A in specific medium (Bok *et al*., 1992). In this study, although the production of iturin A by the mutant could enhance to twice higher than that by wild-type *B. subtilis*, the stability of high activity iturin A producing mutants was still problematic such as the loss of iturin A activity of the next generation of *B. subtilis* mutants. In addition, the mutation by UV light irradiation not only specifically affected on the DNA but during and/or after UV radiation the unsaturated phospholipids in bacterial cell membrane were also susceptible to UV light at the wavelength of 254 nm, which can activate oxygen molecules to generate various kinds of reactive oxygen species (ROS) (Girotti *et al*., 2001). The photosensitivity of lipids could be enhanced under UV light irradiation to form lipid peroxidation such as LOOH and LOH those could significantly affect on the cell membrane and other molecules (Girotti *et al*., 2001). Probably, the production of iturin A and its activity were also dependent on the state of cell lipid membrane under oxidative stress condition produced by UV irradiation. In order to compare with it, the effects of ROS on the lipid peroxidation of cell membrane, the cell growth and production of target enzyme such as the growth and production of chitosanase from *S. griseus* cells under oxidative stress (H_2O_2) were further studied in the presence of liposomes. It was expected that the interaction of liposomes with bacterial cell membranes induced by the specific heat treatment could prevent oxidative stress damage on the bacterial cell membrane to protect cell growth and to induce the enhanced production of target enzymes.

7.2 Enhanced production of chitosanase from *S. griseus* cells treated by liposomes and specific heat under oxidative stress condition

The efficiency of UV irradiation on the cell growth and production of iturin A from *B. subtilis* mutant could relate to the effects of oxidative stress. Here, the growth and production of chitosanase from *S. griseus* cells pretreated with heat stress at 41 ^{o}C and the neutral POPC liposomes were investigated under oxidative stress condition. In **Fig. 1-14a**, the growth of *S. griseus* cells treated with 5 mM H_2O_2 at 37 ^{o}C after incubation of 24 h was significantly reduced as compared to that of the control (cells were incubated at 37 ^{o}C without any treatment). This result implies that the oxidative stress was severe to the cell growth of *S. griseus* cells. This effect was probably similar to that of UV irradiation (produced ROS) on the growth of *B. subtilis* producing iturin A, as shown in previous discussion. In another experiment, the oxidative stress (5 mM H_2O_2) was added to the suspension of *S. griseus* cells pretreated with the heat stress at 41 ^{o}C in the presence and absence of liposomes. The result shows that the treatment of heat stress at 41 ^{o}C on *S. griseus* cell after incubation of 10 and 24 h increased the cell growth even in under oxidative stress as compared with that of the control. The addition of POPC liposomes further enhanced the growth of *S. griseus* cells pretreated with the heat stress even under oxidative stress condition. This observation shows the importance of the heat stress and POPC liposomes for the cell growth even under oxidative stress conditions.

Figure 1-14b shows the chitosanase production by *S. griseus* pretreated heat stress at 41 ^{o}C with and without liposomes under oxidative stress condition of H_2O_2. The production of chitosanase was evaluated based on the chitosanase activity in fermented broth. The results show that the chitosanase activity obtained in the case *S. griseus* cells grown at 37 ^{o}C under oxidative stress without liposome was significantly reduced, being 40 % lower than that of the control. Interestingly, the addition of POPC liposomes to the *S. griseus* cells pretreated heat stress at 41 ^{o}C increased the chitosanase production even under oxidative stress condition and

its chitosanase production was 1.5 times higher that that of the control. Similar to our previous paper (Ngo *et al*., 2005), it was confirmed that almost all chitosanase (more than 95%) synthesized by *S. griseus* cell was released to aqueous medium. The above result implies that the heat stress and liposomes play important role for the enhanced production of chitosanase from *Streptomyces griseus* cells even under oxidative stress conditions.

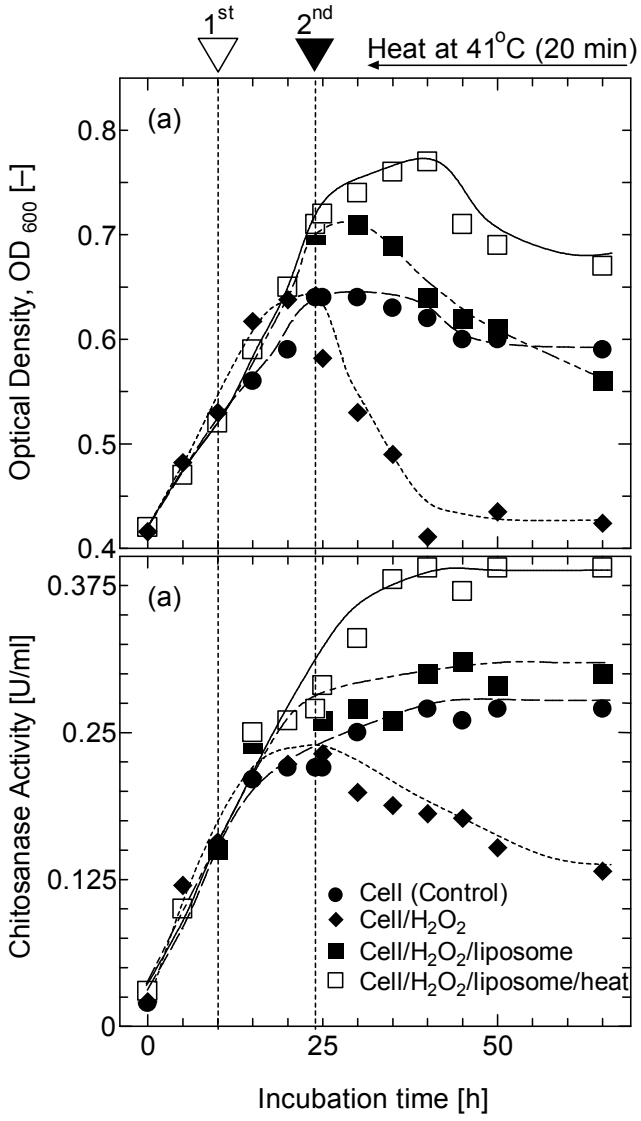


Fig. 1-14 Production of chitosanase by *S. griseus* cells treated with heat stress and liposomes under the oxidative stress condition. The cultivation was carried out at 37 ^{o}C and the heat stress at 41 ^{o}C for 20 min was applied to cell broth twice after incubation of 10 and 24 h. The oxidative of 5 mM H_2O_2 was added to above cell culture together with and without 1 mM POPC liposomes. The cultivation was continuous at 37 ^{o}C after above treatment.

As previously reported (Ngo *et al*., 2005), the treatment of the heat stress (41 ^{o}C) and POPC liposomes enhanced the growth and production of chitosanase of these cells to 1.4 and 2 times, respectively, higher than those of the cells cultivated at 37 ^{o}C. In the case of oxidative stress effect, the treatment of *B. subtilis* by UV irradiation caused the mutation of these cells as well as the cell death caused by the effect of ROS produced by UV irradiation (Ngo *et al*., 2002). Here, although the production of chitosanase by *S. griseus* cells could be carried out through the pretreatment of liposomes and heat stress at 41 ^{o}C under the oxidative stress condition, the reduced growth and lower production of chitosanase imply the severe effects of oxidative stress. However, this observation also implies the importance of liposomes, which could protect the cell growth and its chitosanase production of *S. griseus* under the oxidative stress condition. It is therefore possible that the addition of liposomes into the culture broth of *B. subtilis* cells under UV irradiation could protect the cell growth and finally enhanced production of iturin A by its mutants. The possible utilization of oxidative stress to inhibit the signal peptidase to not cleave signal peptide (SP) of secreted chitosanase for the preparation of chitosanase harboring SP bound liposomes interacting onto cell membrane of *S. griseus* would also be considered.

8. Interaction of liposomes with cell membranes and chitosanase can significantly prevent the severe damage of oxidative stress on cell membrane

In order to verify the efficiency of liposomes for the enhanced production of chitosanase even under oxidative stress condition, the conformational change of chitosanase under strong oxidative stress condition with and without liposomes was investigated. The results show that the conformation of chitosanase under strong oxidative stress at 5 mM H_2O_2 was completely destroyed and it could not be detected by CD spectra as shown in **Fig. 1-15**. However, the addition of POPC liposomes could significantly prevent the damage of the oxidative stress on conformation of chitosanase. The secondary structure of chitosanase under oxidative stress in

the presence and absence of POPC liposomes was also analyzed as shown in **Table 1-3**. The secondary structures of chitosanase with and without liposomes non-stressed conditions were almost same such as α-helix (39 %), β-sheet (6 %), turn (36 %) and random (19 %). The addition of POPC liposomes significantly maintained α-helix content (23 %) and mildly increased the β-sheet content (14 %) of chitosanase. The result in **Table 1-3** also shows that the oxidative stress also damaged on structure of chitosanase, resulting in the increasing in β-turn and random conformation of these enzymes even in the presence of POPC liposomes. The efficiency of POPC liposomes on the chitosanase under the oxidative stress condition could be clarified into two possibilities (i) interaction between liposomes and chitosanase occurs effectively under oxidative stress condition, and (ii) POPC liposomes have scavenger-like functions under oxidative stress condition. The interaction between liposomes and chitosanase could stabilize the configuration of chitosanase under oxidative stress condition (**Fig. 1-15**). In relation to scavenger function of POPC liposomes, it has been reported that phosphatidylcholine vesicles could decompose the hydrogen peroxide to water and oxygen (Yoshimoto *et al*., 2007).

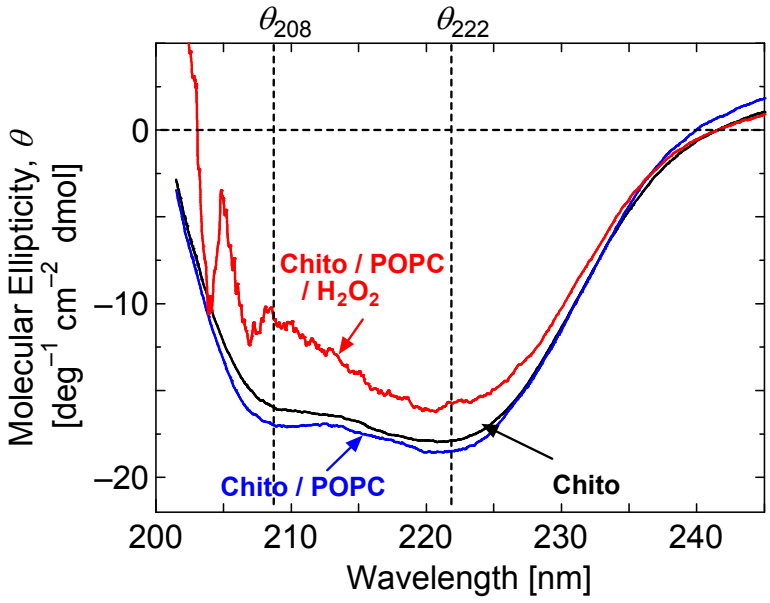


Fig. 1-15 Conformational transition of chitosanase under oxidative stress condition in the presence and absence of POPC liposomes; chitosanase (4.3 μM) was incubated with 5 mM H_2O_2 in the presence and absence of 1 mM POPC liposomes

Table 1-3 Summary of secondary structure of chitosanase from *S. griseus* after the treatment of oxidative stress in the presence and absence of POPC liposomes. Chitosanase (4.3 μM) was incubated with 5 mM H_2O_2 in the presence and absence of 1 mM POPC liposomes. The secondary structure contents were obtained by fitting the molecular ellipticity of chitosanase as the function of wavelength in range from 250 to 190 nm (Chang *et al*., 1978)

Experiments	Secondary structure of chitosanase			
	α-helix (%)	β-sheet (%)	β-turn (%)	Random (%)
Chitosanase	39	6	36	19
Chitosanase/ POPC	39	6	37	18
Chitosanase + oxidative	ND	ND	ND	ND
Chitosanase + POPC + oxidative	23	14	27	36

ND: not determined

9. Liposome can prevent the lipid and protein peroxidation

The important roles of POPC liposomes for the prevention of oxidative stress damage on the chitosanase and cell lipid membrane of *S. griseus* was further studied. **Figure 1-16** shows the protein peroxidation (ProOOH) of chitosanase under oxidative stress condition with and without POPC liposomes. The results showed that the formation of the ProOOH was increased in time dependence. After an incubation of 18 h, the amount of ProOOH was approximate $9x10^{-6}$ M although this value was reduced to $6.1x10^{-6}$ M after an incubation of 24 h. The addition of POPC liposomes to the above oxidative condition of chitosanase significantly prevented the peroxidation of these enzymes and the amount of ProOOH formation was significantly lower than those under oxidative stress without liposomes. After an incubation of 18 h, the amount of ProOOH was $4.2x10^{-6}$ M but it was further reduced to $1.7x10^{-6}$ M after 24 h. These results clearly showed the roles of POPC liposomes for the prevention of protein peroxidation induced by the oxidative stress.

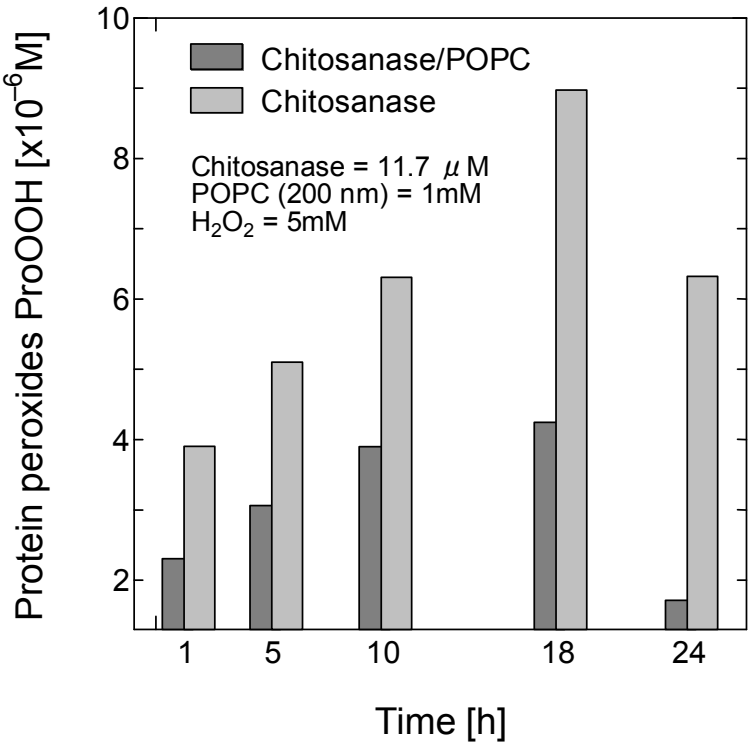


Fig. 1-16 Peroxidation of chitosanase catalyzed by hydrogen peroxide in the presence and absence of liposomes

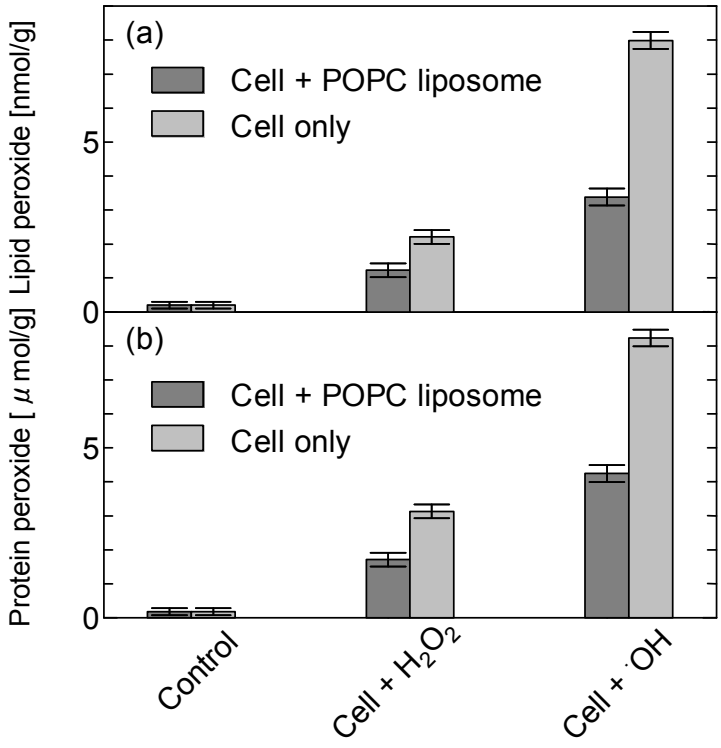


Fig. 1-17 Lipid and protein peroxides of *S. griseus* cell under various oxidative stresses with and without liposomes

The efficiency of POPC liposomes on the prevention of protein peroxide and lipid peroxide of cell membrane under oxidative stress condition was also investigated. Here, two kinds of ROS such as hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\cdot}OH$) were used to oxidize *S. griseus* cells in the presence and absence of POPC liposomes. **Figure 1-17A** shows the lipid peroxide of cell membrane mediated by various kinds of oxidative stresses. The results show that the lipid peroxide formation per gram of lipid of cell membrane of *S. griseus* cells treated with H_2O_2 and $^{\cdot}OH$ was 2.2 and 8.0 nmol/g, respectively. However, the addition of POPC liposomes significantly prevented the lipid peroxidation of cell membrane under such oxidative stress conditions. The amount of lipid peroxide of cell membrane in the presence of liposomes was 1.2 and 3.4 nmol/g, respectively. These observations imply that the liposomes could also play a role for the protection of lipid cell membrane of *S. griseus* under oxidative stress conditions.

The efficiency of POPC liposomes on the prevention of protein peroxide of cell membrane under oxidative stress was also investigated, as shown in **Fig. 1-17B**. The results show that the amount of protein peroxide formation of cell membrane caused by H_2O_2 and $^{\cdot}OH$ was 3.1 and 8.2 μmol/g, respectively. However, the addition of POPC liposomes significantly prevented the protein peroxides formation under oxidative stress conditions. This observation implies that the POPC liposomes could play very important roles to prevent the damage of oxidative stress on lipids and proteins of cell membranes of *S. griseus* cells.

Summary

The heat treatment enhances the growth and production of chitosanase of *S. griseus* cells treated without and with POPC liposomes to 1.4 and 2.0 times higher than those of the control. The specificity of substrate for the effective production of chitosanase by *S. griseus* cells was suggested to use water soluble chitosan. The heat treatment on *S. griseus* cells mainly enhanced the specific production rate of chitosanase rather than specific growth rate but

simultaneous treatment of these cells by liposomes and heat stress enhanced both specific growth rate and specific production rate of chitosanase. The maximum specific growth and production rates of *S. griseus* treated with only heat stress at 41 ^{o}C were almost same as those of the control at 0.073 and 0.061 h^{-1}, respectively. However, these values of *S. griseus* cells treated with heat stress at 41 ^{o}C and POPC liposomes simultaneously were 0.102 and 0.118 h^{-1}, respectively. The secretion of chitosanase through the cell membrane of *S. griseus* cells cultivated under the heat stress at 41 ^{o}C without and with POPC liposomes was 1.8 and 2.2 times higher those of the control.

In attempt to elucidate the efficiency of the heat treatment and liposomes on the enhanced production and secretion of chitosanase by *S. griseus* cells, the interaction of liposomes with target chitosanase and cell membrane was evaluated. Although the interaction of the neutral POPC liposomes with secreted chitosanase could play a role for the enhanced secretion of chitosanase by *S. griseus* cell, the membrane-membrane interaction of the neutral POPC liposomes with LMCM liposomes (in vitro) under heat stress at 41 ^{o}C induced the secretion of entrapped chitosanase through protein bound-lipid membrane of LMCM liposomes to 17 % of initial entrapped chitosanase. The interaction of POPC liposomes with cell membrane (in vivo) might show the similar effect of membrane-membrane interactions induced and/or enhanced the secretion of chitosanase through the cell membrane of *S. griseus* cells under heat stress condition.

The membrane-membrane interaction between liposomes and cell membranes is considered as a key to solve our previous problems for the enhanced production of antibiotic antifungal iturin A from *Bacillus subtilis* by using UV irradiation. Although the production of iturin A from *B. subtilis* mutants was twice higher than that of the wild-type *B. subtilis*, the stability of high iturin A activity producing mutants was problematic. It is supposed that the UV irradiation produced reductive oxygen species (ROS) that directly attacked bacterial DNA, proteins and lipids of cell membranes. It is therefore important to study the production of

chitosanase from *S. griseus* cells pretreated with the heat stress and liposomes under oxidative stress condition. The cell growth and its production of chitosanase were significantly reduced immediately after the addition of 5 mM H_2O_2 as compared with the control. However, these cells pretreated simultaneously with the heat stress at 41 ^{o}C and POPC liposomes enhanced the cell growth and production of chitosanase to higher values than that of the control.

Several effects of liposomes on the preventing of the oxidative stress damage on the cell growth and the target chitosanase were systematically investigated. The CD spectra measurement shows that the conformation of chitosanase was stabilized by the interaction with the POPC liposomes under the oxidative stress condition. The susceptibility of chitosanase, lipids and proteins of cell membrane under oxidative stress condition in the presence and absence of POPC liposomes was also characterized. The results clearly show that POPC liposomes could significantly prevent the lipid and protein peroxidation of cell membrane of *S. griseus* under oxidative stress condition. This effect of liposomes once more confirms the importance of membrane-membrane interaction between liposomes and cell membrane under heat and/or oxidative stress conditions for the production and secretion of chitosanase by *S. griseus* cells as well as possible bridge of this fundamental phenomenon to understand the enhanced production of iturin A by *B. subtilis* mutants.

Chapter II

Characterization of Surface Properties of *Streptomyces griseus* Cell and Internalization of Liposome under Heat Stress Conditions

Introduction

Liposome, a closed phospholipid bilayer membrane, is spherical vesicle having inner aqueous phase and a nano-order interface (~5nm) harboring hydration and low hydrophobic layer on its surface. Because of non-toxic and highly biocompatible properties, liposomes are often used to encapsulate water-soluble drugs or genes in their aqueous space and/or the lipid-soluble materials within the membrane itself. Liposomes release their contents by interacting with cells in one of four ways: adsorption, endocytosis, lipid exchange, or fusion (Ostro *et al*., 1989). The current applications of liposomes are mostly related to the phamarceutical preparation of drug-liposomes for the delivery of drugs and/or genes (Allen *et al*., 1991; Lee *et al*., 1995; Lian *et al*., 2001). However, rapid clearance of liposome containing target materials in the biological system limited its usage. Many efforts have been dedicated to the inhibition of the rapid clearance of liposomes and that can be clarified into 3 principles such as (i) control of charge, (ii) surface hydration and (iii) size of liposomes (Lian *et al*., 2001). However, many disadvantages in the usage of charged liposomes, and surface modified-liposomes as the carriers are still remained. For example, the positively- and negatively-charged liposomes are recognized by receptors found on a variety of cells, including macrophages as well as the effects of the cationic liposomes on DNA instability, immune-mediated clearance, and inflammatory response (Scheule *et al*., 1997). The surface modification is often done by incorporating gangliosides, poly(ethyleneglycol), and poly(acrylamide) that are chemically conjugated to hydrophilic polymers (Torchilin *et al*.,

1994[a]). However, the slow renal clearance of extremely large PEG polymers may be concerns (Lian *et al*., 2001) as well as their safety in humans is less well understood (Torchilin *et al*., 1994[b]). In relation to size control, most recent investigations have used unilamellar vesicles, 50-100 nm in size, for systemic drug delivery applications (Senior *et al*., 1985; Devine *et al*., 1994).

The controlling of the membrane fluidity, hydrophobicity, and surface hydration of both liposome and bacterial cell membrane by the treatment of specific heat has not been dedicated to be studied. Although the heat treatment on the enhanced endocytosis of liposomes modified with poly(ethylene oxide) bearing-lipid has been reported, the mechanism of such effect has not been well clarified though the authors implied that the heat effect might relate to the cell metabolism (Higashi *et al*., 1995). The stability of the neutral POPC liposomes at near or higher physiological temperature is dependent on their fluidity, and according to the previous findings, their stability was high under extreme temperature (Jorgensen *et al*., 1993; Oberholzer *et al*., 1995; Zuidam *et al*., 1993). In addition, the specific heat treatment also showed to be effective on the translocation of protein across cell membrane by increasing hydrophobic interaction between translocated protein and cell lipid membrane (Umakoshi *et al*., 1998[a]; 1998[b]).

In most previous studies, the strategy to control the internalization of liposomes into cell is the controlling of charge, hydration, and size of liposomes. Here, a possible way to control the surface properties of both neutral liposomes and cell membranes was presented, focusing on membrane fluidity, hydrophobicity and hydration by the treatment of specific temperature. Previously, the interaction of the neutral POPC liposomes with cell membranes of *S. griseus* effectively occurred by the heat treatment at 41 ^{o}C. Such heat treatment and/or heat induced the interaction of the neutral liposomes and cell membranes could vary cell surface properties such as surface net charge and surface net hydrophobicity (*HFS*) (Ngo *et al*., 2008[a]), resulting in the enhancement of the cell growth of *S. griseus* bacterial cells and their production and

secretion of chitosanase effectively (Ngo *et al*., 2005; 2008[b]). Furthermore, the enhanced production of chitosanase was probably affected by the internalized POPC liposomes at gene expression level of chitosanase. This assumption is based on the previous study showing that the POPC liposomes could enhance gene expression level of green fluorescence protein (GFP) (Bui *et al*., 2008). The neutral liposomes prepared by phosphatidylcholine lipid vesicles are often used for the enzymatic reactions inside liposomes because of their safety and biocompatibility for entrapped biomaterials (Oberholzer *et al*., 1995; Yoshimoto *et al*., 2004; Walde *et al*., 2001; Fisher *et al*., 2002).

The content of this chapter is to elucidate the heat induced/enhanced interaction of neutral liposomes with cell membranes, focusing on the internalization, although the adsorption and the fusion of these liposomes with cell membranes were also dependent on the heat treatment. First, the surface properties of cell membranes such as surface net charge (*Z*) and hydrophobicity (*HFS*), and surface hydration of *S. griseus* cells under various conditions of pH, oxidative, and heat were characterized. Then the internalization and the cellular uptake of these liposomes by *S. griseus* cells under various heat and/or pH conditions were investigated by using the liposomes labeled by the fluorescent probes integrated lipid membrane. In the attempt to clarify the endocytotic pathway of these liposomes, various inhibitors of endocytotic pathway were used to treat the *S. griseus* cells. Next, the internalization of these liposomes was characterized by measuring the amount of intracellular liposomes and their interaction with cell membrane was observed under various temperatures by fluorescent microscopy. The driving forces of the heat induced such interactions were characterized by the variation of surface net hydrophobicity of *S. griseus* cells pretreated with and without liposomes. Finally, the surface hydration of cell membrane and liposomes prepared by lipids mimicking cell membrane (LMCM) was also characterized under different heat, oxidative and pH conditions. The strategy to control the membrane-membrane interaction between liposomes and cell membrane of *S. griseus* cells was finally discussed based on the effects of

electrostatic, hydrophobic and hydrogen bond interactions that could be controlled by changing of heat and pH conditions.

Materials and Methods

1. Materials

The lipids including 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine (POPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidic acid (POPA), 1,2-dioleyl-*sn*-glycero-3-phosphoethanolamine (DOPE) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-rac-(1-glycerol)] (POPG) were purchased from Avanti Polar Lipid (Alabaster City, AL, USA). The inhibitors of endocytotic pathways such as o-phenanthroline, cytochalasin B&D, trypsin were purchased from Sigma (St. Louis, MO, USA). Poly (ethylene glycol) (PEG 1540, 4000, 6000; Mw 1.5 kD, 3 kD and 7 kD, respectively) and dextran (Dex) (60~90k, 100~200k; Mw 60-90 kD and 100-200 kD, respectively) were purchased from Wako Pure Chemicals Ltd. (Osaka, Japan). Rhodamine-PE (Rh-PE) and NBD-PE fluorescent lipid probes were and other chemical reagents of highly analytical grade were purchased from Wako Pure Chemicals (Osaka, Japan).

2. Preparation of liposomes

Fluorescent labeled liposomes were similarly prepared as the method described in our previous study (Ngo *et al*., 2005). Briefly, 1 mM POPC or 1 mM POPG phospholipids were dissolved with NBD-PE (1 mol% of lipid) and/or Rh-PE (1 mol% of lipid) in chloroform in round bottom-flask before evaporation under low pressure at 37 ^{o}C to create dried-lipid film layer. The large lamellar vesicles were created from the dried-lipid thin layers in 10 mM phosphate buffer (pH 7) at room temperature for 24 h. Finally, the freezing (-20 ^{o}C, 10 min) and thawing (37 ^{o}C, 10 min) were simultaneously applied with total 5 times. To obtain the

unilamellar vesicles, the above vesicle suspension was finally extruded through polycarbonate membrane with the pore size of 100 nm prior to use.

The giant vesicles containing lipids mimicking cell membrane (LMCM) were prepared by previous reported method (Moscho *et al*., 1996). Briefly, phospholipids (0.1 M) involving DMPG/ DOPE/POPC/POPA (56:20:22:2 in molar ratio) were dissolved in chloroform, and 20 µl of this solution was added to a 50 ml round-bottom flask containing 980 µl of chloroform and 100-200 µl of methanol. The aqueous phase (7 ml of distilled water or buffer) was then carefully added along the flask walls. It should be feasible to scale this procedure up or down, as desired. Liposomes were formed in distilled water, Hepes buffer (10 mM, pH adjusted to 7.4 with NaOH), sodium phosphate buffer (10 mM, with 11 mM $MgCl_2$, pH 7.4) and sodium borate buffer (50 mM, pH 9.2). The molecules could be entrapped in the vesicles or the enzymes to be incorporated into the liposome membranes those were added to the buffer solution prior to evaporation of organic solvent. The organic solvent was removed in a rotary evaporator under reduced pressure (final pressure 1.3 kPa) at 40 ^{o}C and 40 rpm. As a consequence of the different boiling points of chloroform (61 ^{o}C at 100 kPa) and methanol (64 ^{o}C at 100 kPa), the two major boiling points could be observed. After evaporation for 2 min, an opalescent fluid was obtained with a volume approximately 6.5 ml. The resulting aqueous solution contained GUVs in high concentration.

3. Evaluate interaction of neutral liposome with cell membrane of *S. griseus*

3.1 Internalization of neutral POPC liposomes into *S. griseus* cells

The sample of 0.025 mM POPC liposomes (100 nm) labeled with Rh-PE (1 mol% of lipid) was co-incubated with *S. griseus* cell suspension (10^3/ml) at 4, 37, and 41 ^{o}C for 60 min. The emission intensities of labeled liposomes in the mixture of cells/liposomes were measured at 0 min (F_0) and 60 min (F_{60}) by exciting to the wavelength at 530 nm using fluorescence polarizer (FP 6500, JASSCO, Japan). The triton X-100 (1 % as the final

Table 2-1 Effect of inhibitors on different endocytotic pathways

Inhibitor	Corresponding functions	Inhibition of endocytotic pathways	References
O-phenathroline	Specific inhibition of phosphoinositol- phospholipase C (PI-PLC)	Curvature of cell membrane	(Sluss *et al*., 1990[a])
Cytochalasin B&D	Inhibit the ATPase activity, and block actin filament elongation or disrupt microfilament/microtubule associated-membrane	Phagocytosis	(Flanagan *et al*., 1980; Miyake *et al*., 1978)
Trypsin	Destroy cell receptors on the cell surface	Clathrin dependent adsorptive pinocytosis/ phagocytosis	(Chyung *et al*., 2003; Drin *et al*., 2003)

concentration) was added to disrupt the lipid cell membrane of *S. griseus* and the emission intensity of rhodamine labeled-liposomes was recorded as the maximum value (F_{max}). The efficiency of liposome internalization was calculated according to the following fomula: 100 x$(F_0 - F_{60})/F_{max}$ (1)

In order to clarify the endocytotic pathway of the neutral liposomes, the *S. griseus* cells (10^3/ml) were pretreated with various kinds of inhibitors to inhibit the endocytotic pathways of these cells. The inhibition effect of inhibitors is shown in **Table 2-1**. The inhibitors (o-phenanthroline (5 mM), cytochalasin B (2.5 μg/ml), cytochalasin D (1μg/ml), and trypsin (0.01 wt%) were used to treat *S. griseus* cells (10^3/ml) to inhibit the different pathways of endocytosis. The neutral POPC liposomes (0.025 mM) were co-incubated with the above treated cells and their fluorescent emission intensities at 0 min (F_0) and 60 min (F_{60}) and with addition of 1 % triton X-100 (F_{max}) were recorded and calculated similarly to the case of the rhodamine-labeled POPC liposomes those internalized into *S. griseus* cells without pretreatment of inhibitors.

3.2 Fusion of neutral liposomes with *S. griseus* cells

The fusion was evaluated by using fluorescence resonance energy transfer (FRET) based on previous finding (Struck *et al*., 1981). The sample (1 mol% of liposomal lipid) of POPC (0.025 mM, 100 nm in size) containing NBD-PE (1 mol% of lipid) and Rh-PE (1 mol% of lipid) was co-incubated with *S. griseus* cells (10^3/ml) at different temperatures of 4, 37, and 41 ^oC for 60 min. In principle, the fusion of labeled POPC liposomes with the cell membrane reduces the efficiency of energy transfer between NBD-PE and Rh-PE fluorescent probes incorporating in liposome membrane, consequently, the enhanced emission intensity of NBD-PE as a function of time. The fusion between the labeled liposomes and cell membranes was measured by following changes in fluorescence intensity at 530 nm by exciting wavelength at 450 nm. The fluorescence intensities of NBD-PE were recorded at 0 min (F_0) and at 60 min (F_{60}) with the addition of triton X-100 (1 %) to disrupt the lipid membranes of liposomes (F_{max}). The fusion efficiency was calculated by the following formula: $100x(F_{60} - F_0)/(F_{max} - F_0)$ (2).

Finally, the adsorption amount of the neutral liposomes onto cell membranes of *S. griseus* was fitted to the mass balance of the total neutral liposomes that could βken up by *S. griseus* cells under different temperatures.

3.3 Cellular uptake of liposomes under various pH conditions

The effect of pH on the cellular uptake of POPC and POPG liposomes was similarly evaluated using rhodamine labeled-liposomes incubated with *S. griseus* cells (10^3/ml) at pH 2.2, 2.8, 6.8, and 8.5 at 4 ^oC for 60 min. The cells after such incubation were washed by cold PBS buffer 5 to 6 times and the fluorescence intensity of labeled liposomes bound cell membranes and that freely existed in the buffer were recorded by exciting the sample at 530 nm to calculate amount of liposomes taken up by cells.

4. Fluorescence microscopy for observation of liposomes-cell interactions

The sample of 0.125 mM POPC liposomes labeled Rh-PE was co-incubated with 10^3/ml *S. griseus* cells pretreated with without 5 mM o-phenanthroline at 4, 37, and 41 ^oC for 60 min. The cells were washed 5 to 6 times by cold PBS buffer. The fluorescence images of these cells are a consequence of the interaction between the labeled liposomes and cell membranes induced by the specific heat treatment. The fluorescence images of these cells were observed using fluorescence microscopy (Olympus IX 51, Japan), setup with the light filters for rhodamine fluorescence.

5. Characterization of surface net hydrophobicity of *S. griseus* using ATPS

The basic compositions of the systems (the total weight is 5 g) for the partitioning of the cells were 7~13 wt% PEG 1540, 4000, 6000 and 7~13 wt% Dex 60-90k, 100-200k. The aqueous two-phase partitioning systems (ATPS) were prepared by mixing the stock solutions of 30 wt% PEG and 30 wt% Dex with the cell suspension. The pH values of these systems were adjusted by the addition of high concentration HCl and/or NaOH solutions and for the determination of surface net hydrophobicity (*HFS*) of these cells, all ATPSs were adjusted to pH at isoelectric point (pI) of *S. griseus* cell at 3.2 as previous finding (Ngo *et al*., 2008[a]).

The surface net hydrophobicity (*HFS*) of the *S. griseus* cells pretreated with and without liposomes was analyzed by ATPS (Kuboi *et al*., 1994), in a similar manner to that for amino acids, proteins and bacterial cells (Albersson *et al*., 1986). The partition coefficient of *S. griseus* cells in ATPS without salt and/or ligand is dependent on electrostatic, hydrophobic properties of both cell surface and ATPS medium. The partition coefficient of these cells (ln K_{cell}) can be performed as the following formula.

$$\ln K_{cell} = \ln K_{el.} + \ln K_{hphob.} \quad (3)$$

The $K_{el.}$ and $K_{hphob.}$ are the contributors to solute partitioning due to electrostatic and hydrophobic effects, respectively. $K_{el.}$ is dependent on the surface charge of the solute and the

difference in electrostatic potentials between two phases. At isoelectric point (pI) of the solute, ln $K_{el.}$ can also be negligible. The partition coefficient, therefore, corresponds to the product of hydrophobic factor of two-phase system (*HF*) and the hydrophobic factor of solutes (*HFS*) such as *S. griseus* cells in this case (Kuboi *et al*., 1994). It is therefore the partition coefficient of cells based on the hydrophobic effect that can be performed as follow.

$$\ln K_{cell} = \ln K_{hphob.} = HF \text{ x } HFS \qquad (4)$$

When the solutes are amino acids, *HFS* corresponds to the hydrophobicity scale (relative hydrophobicity, *RH*) of amino acid, which Nozaki and Tanford defined in water/dioxane and water/ethanol systems (Nozaki *et al*., 1971). By measuring partition coefficient of various amino acids which have defined *RH* value, *HF* can be quantitatively determined (Tanaka *et al*., 1991).

Streptomyces griseus cells were obtained by growing in seed culture medium as previous report (Ngo *et al*., 2005). After centrifugation, the cells were washed several times with the distilled water. The final concentration of these cells was adjusted to approximate 10^3/ml prior to use. This cell suspension was treated at 25, 30, 37, and 41 ^oC with and without 0.025 mM POPC liposomes (100 nm in size) for 60 min. The treated cells were washed several times with cold PBS buffer and distilled water to remove free-binding liposomes before applying into ATPS setup with experimental temperatures. The partition of above cells can be calculated by measuring cell concentration in the top (C_{top}) and bottom phase (C_{bottom}) of ATPS by UV adsorption at 600 nm (OD_{600}) and shown as follow.

$$(K_{cell} = C_{top} / C_{bottom}) \qquad (5).$$

The calculation of the surface net charge (*Z*) of the *S. griseus* cells was performed as follows (Albertsson *et al*., 1986):

$$\ln K_1 = \ln K_{cell,NaCl} = \ln K_0 + \gamma_1 Z \quad (6)$$

$$\ln K_2 = \ln K_{cell,Na_2SO_4} = \ln K_0 + \gamma_2 Z \quad (7)$$

$$\Delta \ln K = \ln K_1 - \ln K_2 = (\gamma_1 - \gamma_2) Z = \Delta\gamma Z \quad (8)$$

where γ_1, γ_2 are the electrostatic potentials between the two phases with NaCl and Na_2SO_4, respectively, and $\Delta\gamma$, an increment of the electrostatic potential, was determined based on the partitioning of amino acids ($\Delta\gamma = 0.056$).

6. Infrared spectroscopy of cell surface under various heat and pH conditions

The spheroplast *S. griseus* cells were prepared by lysozyme hydrolysis (1 mg/ml as final concentration) to remove outer peptidoglycan layer of intact *S. griseus* cells as our previous method (Ngo *et al*., 2008[b]). The concentration of these cells was adjusted at 10^6/ml in distilled water prior to use. The above spheroplast suspension was treated with the temperature at 25, 37, and 41 ^oC for 60 min similar to the case that these cells were treated at the different pH of 1.3, 5.6, 7.7, and 10.2 for 60 min and were treated with various concentrations of hydrogen peroxide (H_2O_2) at 1, 2, 3, 4, and 5 mM for 1 h before applying to FTIR to observe the surface hydration of cell membrane through the infrared spectra of phosphate group of lipid membrane. The sample of 30 µl spheroplast *S. griseus* cells suspension at each condition of temperature and pH was applied in 50 µm thick-cell with CaF_2 window. The infrared spectra were measured with a FTIR 4100 spectrometer (JASSCO, Japan) equipped with an Hg-Cd-Te detector. The resolution was set up at 4 cm^{-1}; the frequency range from 1700 to 1000 cm^{-1} was collected for each sample. The infrared spectra of samples were substracted to that of water or buffer. The accuracy of the frequency reading is better than ±0.1 cm^{-1}.

Results and Discussion

1. Characterization of surface characteristics of *Streptomyces griseus* cell

1.1 Characterization of surface net charge of *S. griseus* cells

The surface net charges of the *S. griseus* cells and liposomes treated cells were evaluated at various pH conditions by using ATPS method. **Figure 2-1a** shows a typical example of the

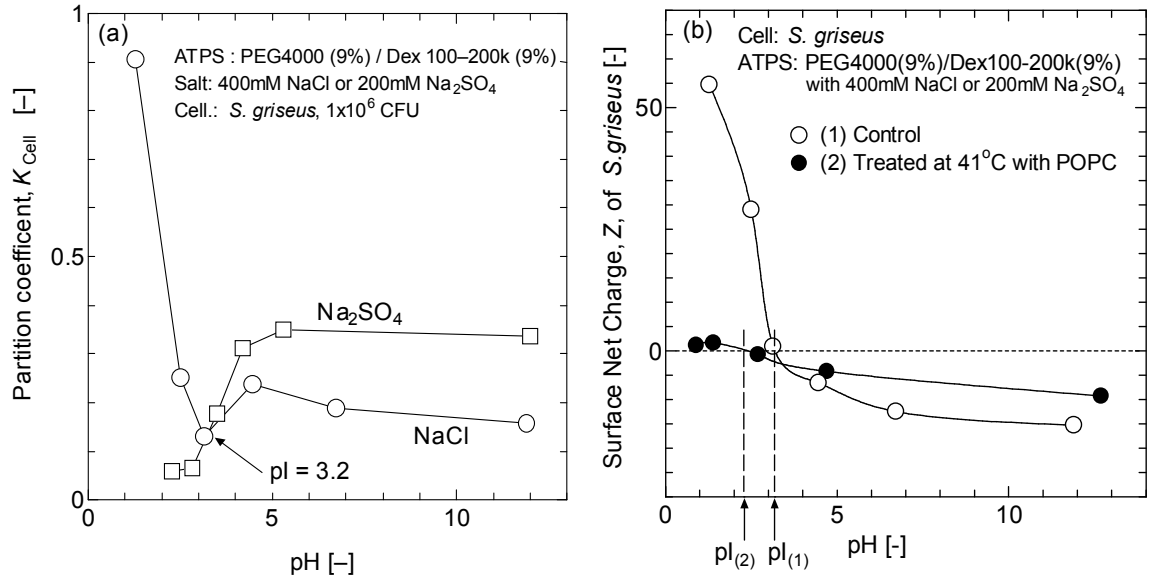
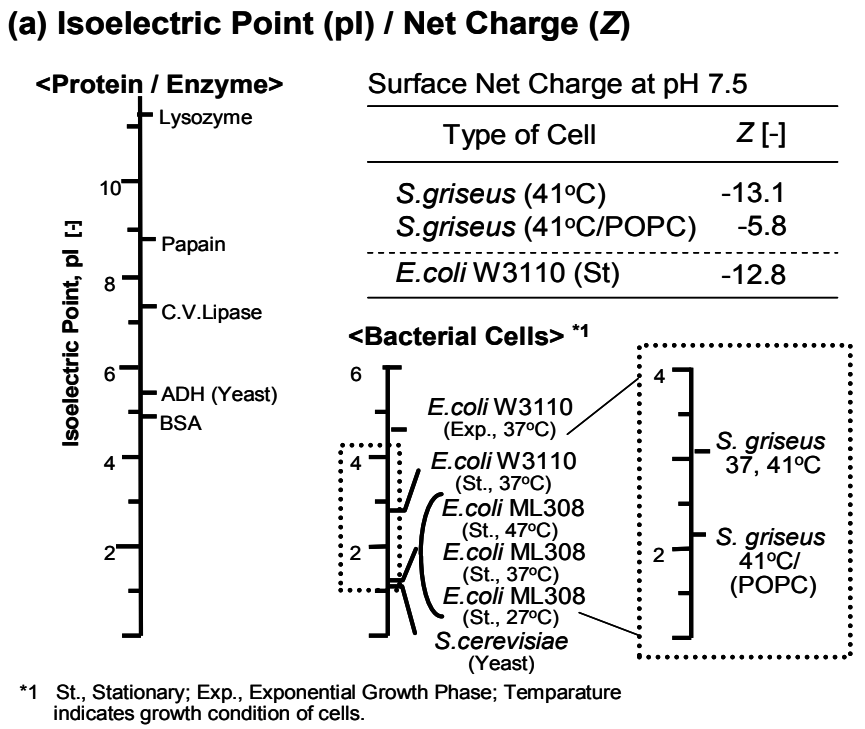


Fig. 2-1 Dependence of the partition coefficient (a) and surface net charge (b) of *S. griseus* cells and liposomes treated cells evaluated by the ATPS method on the pH

pH dependence of cell partitioning in the PEG 4000 (9 %)/Dex 100-200k (9 %) system with NaCl or Na_2SO_4 under heat stress condition at 41 ^oC. The partition coefficient of the *S. griseus* cells was dependent on the pH in the different ATPS conditions. In the case of the ATPS containing 400 mM NaCl, the partition coefficient of the cells decreased with increasing pH. On the contrary, the partition coefficient of these cells increased with increasing pH in the ATPS containing 200 mM Na_2SO_4. It has been reported that the surface net charge of the biomolecules can be calculated from the partitioning behavior in ATPS having positive and negative electrostatic potentials between the two phases. The electrostatic potential of the above two types of ATPS was first evaluated based on the partition behavior of charged amino acids (arginine and glutamine), where their surface net charge could be calculated. Based on the electrostatic potential of the ATPS, the surface net charge of the *S. griseus* cells was calculated and plotted in **Fig. 2-1b**. From similar calculations, the surface net charge of liposomes treated *S. griseus* cells under heat stress is also shown in **Fig. 2-1b**. The values of the isoelectric point (pI) of the *S. griseus* cells and the liposome-treated cells, determined as the crossing-point (Albertsson *et al*., 1986), are shown as 3.2 and 2.3,

respectively. **Figure 2-2a** shows the summary of the electrostatic properties of the two types of cells. The surface net charge (*Z*) and the isoelectric point (pI) values of the *S. griseus* cells were similar to that of *E. coli* W3110 strains in a stationary phase cultivated at 37 ^{o}C. However, the values were varied after heating the cell at 41 ^{o}C with POPC liposomes, resulting in a significant reduction of the surface net charge of the *S. griseus* cells (*Z* reduced from -13.1 to -5.8 at pH 7.5). This observation implies that the neutral POPC liposomes might be aggregated on the surface of the *S. griseus* cell to reduce its surface net charge and pI value.

The surface net hydrophobicity (*HFS*) of *S. griseus* cells pretreated with and without POPC liposomes at 41 ^{o}C was further evaluated by using the aqueous two-phase partitioning method at their determined isoelectric point (pI) value according to the previously reported method (Albertsson *et al*., 1986; Kuboi *et al*., 1994; Umakoshi *et al*., 1996). The obtained *HFS* values are shown in **Fig. 2-2b**. The *HFS* values of *S. griseus* cells at 37 and 41 ^{o}C were -358 and -122 kJ/mol, respectively. These were relatively high among various types of bacterial cells shown here, showing that the surface of the *S. griseus* cells is more hydrophobic. In the case of *S. griseus* cells pretreated liposomes at 41 ^{o}C, the HFS value was reduced to -510 kJ/mol, showing that the cell surface of the *S. griseus* cells became more hydrophilic through the interaction with POPC liposomes at 41 ^{o}C. However, the *HFS* value of these cells cultivated at 37 ^{o}C was not significantly changed even in the presence and absence of POPC liposomes. These results also imply that the heat stress at 41 ^{o}C induced the direct interaction of neutral POPC liposomes with the membrane of the *S. griseus* cells. Because the POPC liposomes have hydrophilic surfaces (*HFS* ~ 410 kJ/mol at 41 ^{o}C), as consequence of liposomes binding onto cell surface of *S. griseus* cells, the *HFS* of these cells was decreased. The driving force, which induced interaction between the neutral POPC liposomes and cell membranes of *S. griseus* cells under heat stress condition, mostly relied on the hydrophobic interaction.



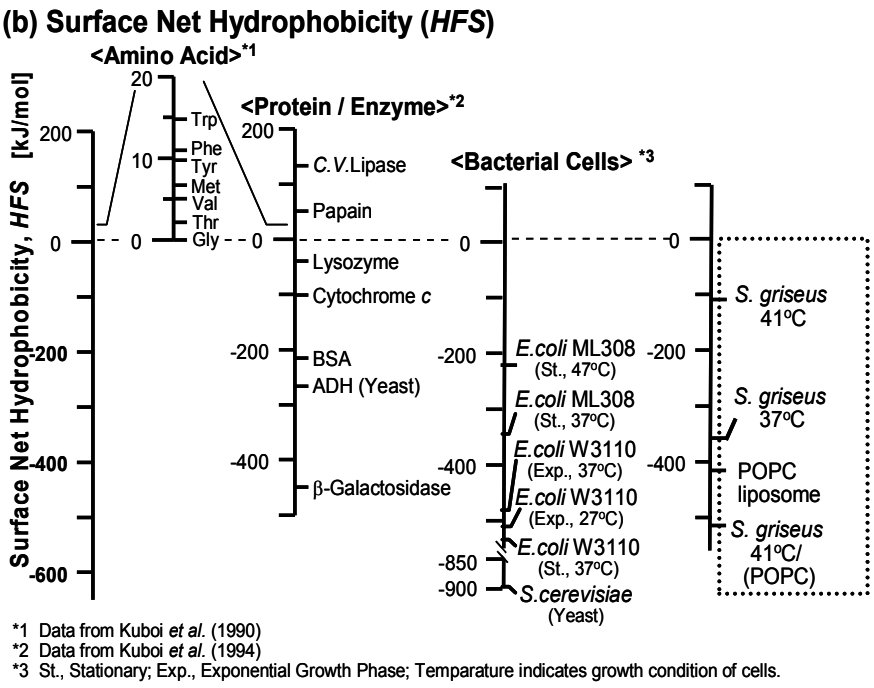


Fig. 2-2 Comparison of pI and *HFS* values of *S. griseus* cells and liposome-treated cells under various heat and pH conditions

1.2 Characterization of surface net hydrophobicity of *S. griseus* cell at different temperatures in the presence and absence of neutral POPC liposomes

The possible effect of surface net hydrophobicity (*HFS*) of both cell membrane and liposomes on their interaction was characterized under various temperatures by using ATPS. The heat treatment often affects on the fluidity of liposomes and surface net hydrophobicity (*HFS*), and surface hydration of cell membranes (Umakoshi *et al*., 1998[a]; 1998[b]; Ngo *et al*., 2008).

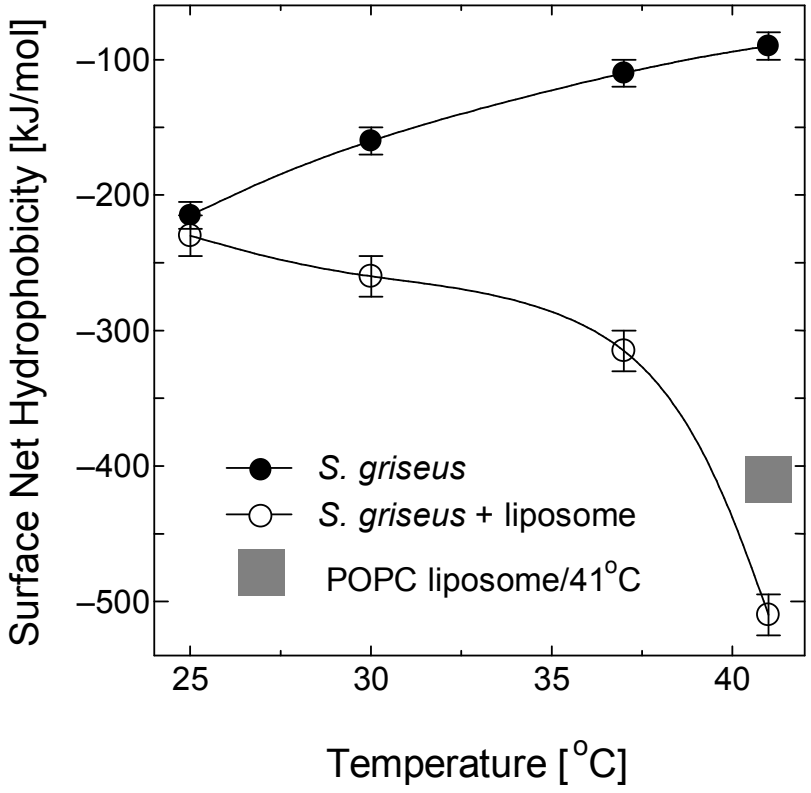


Fig. 2-3 Effect of different temperatures on the surface net hydrophobicity of *Streptomyces griseus* cells pretreated with without POPC liposomes. The cells (10^3/ml) were coincubated with neutral POPC liposomes (0.025 mM) at desired temperature for 60 min. Free binding of liposomes was removed by washing the cells using cold PBS buffer at 4 ^oC. These cells were dissolved again in the distilled water at same concentration of 10^3/ml prior to operate in the ATPS as described in the experimental section. The pH of ATPS was adjusted at 3.2, the pI value of *S. griseus* cell (Ngo *et al*., 2008). The data and vertical bar represent mean values and standard deviation obtained from three independent experiments.

Here, the *HFS* of *S. griseus* cells pretreated without neutral liposomes is first presented under different temperatures. As shown in **Fig. 2-3**, the *HFS* values were increased by the increasing temperature. The *HFS* values of these cells at 25, 30, 37, and 41 ^{o}C were, respectively, -215, -160, -110, and -90 kJ/mol. This observation well corresponds to the previous findings that the heat treatment at specific temperature could enhance the *HFS* of bacterial cells, including *S. griseus* (Umakoshi *et al*., 1998[a]; 1998[b]; Ngo *et al*., 2008[a]).

In addition, the surface of neutral POPC liposome is quite hydrophilic (*HFS* is approximate -410 kJ/mol). It is therefore predicted that the interaction of these liposomes with *S.* gri*seus* cells could vary cell surface properties such as surface net charge and hydrophobicity. As shown in **Fig. 2-3**, the *HFS* values of *S. griseus* cells pretreated with the neutral POPC liposomes were reduced once the mixture of cells and neutral POPC liposomes those were exposed to higher temperature. Those *HFS* values of cell surface at 25, 30, 37, and 41 ^{o}C were respectively -230, -260, -315, and -510 kJ/mol. This observation implies that the increasing temperature in specific range enhanced the interaction of the neutral liposomes with cell membranes, resulting that the surface of *S. griseus* cells became more hydrophilic. In addition, the interaction of neutral POPC liposomes with cell membranes at 41 ^{o}C also showed the reduction of surface net charge of *S. griseus* cells, as characterized in our previous study (Ngo *et al*., 2008[a]).

The effect of the heat treatment on the enhanced surface net hydrophobicity of *S. griseus* cell as well as its surface net charge and hydrophobicity were reduced by the interaction of neutral POPC liposomes with cell membrane under specific heat conditions strongly suggested that their interactions were significantly relied on the hydrophobic interaction by the changing temperatures.

2. Characterization of surface hydration of *S. griseus* cells

2.1 Characterization of cell surface under different heat and pH conditions

The effects of the temperature and pH on the surface hydration of spheroplast *S. griseus* cells were also studied using Fourier transform infrared spectroscopy (FTIR). The infrared (IR) spectra of bacterial functional groups in the range of frequency from 1300-1085 cm^{-1} are summarized in **Table 2-2**. The IR bands and the corresponding frequencies for *S. griseus* cells are based on the vibration patterns reported for bacteria (Ojeda *et al*., 2008; Helm *et al*., 2006; Dittritch *et al*., 2005; Jiang *et al*., 2004).

Figure 2-4A shows the IR frequencies of the PO_2^- antisymmetric stretching modes of PC, PA and PG in small unilamellar vesicles (SUV). In PA-SUV, the antisymmetric stretching mode has the maximum frequency at 1189.9 cm^{-1} while those values of PC-SUV and PG-SUV are 1232.3 cm^{-1} and 1205.3 cm^{-1}, respectively. It has been reported that antisymmetric stretching modes of dimyristoylphosphatidic acid (DMPA) and egg yolk phosphatidic acid (EPA) vesicles in the gel phase have maximum frequency at 1173.1 cm^{-1} and 1187.2 cm^{-1}, respectively (Nabet *et al*., 1994). The dipalmitoylphosphatidylcholine (PC) vesicle in liquid-crytalline state has maximum frequency at 1231.7 cm^{-1} (Yamamoto *et al*., 1995), and phosphatidylglycerol (PG) is between 1250 and 1205 cm^{-1} (Fragata *et al*., 1997). Here, the obtained results are well corresponding with the previous findings. The higher IR frequencies of the PO_2^- antisymmetric stretching modes the lower surface hydration of non-esterified phosphate group in liposomes is (Yamamoto *et al*., 1995). It is therefore concluded that the surface hydration of POPC liposomes is less than that of POPA and POPG liposomes.

Figure 2-4B shows the IR spectra of spheroplast *S. griseus* cell at various temperatures in the aqueous spectra of P=O group (1300-1150 cm^{-1}). The IR spectra were shown at 25 ^oC (1236.4 cm^{-1}) and at 37 ^oC (1232.3 cm^{-1}), and at 41 ^oC (1251.6 and 1236.4 cm^{-1}). The IR adsorption band at 1251.6 cm^{-1} is attributed to the double bond stretching of P=O of general phosphoryl group and phosphodiester of nucleic

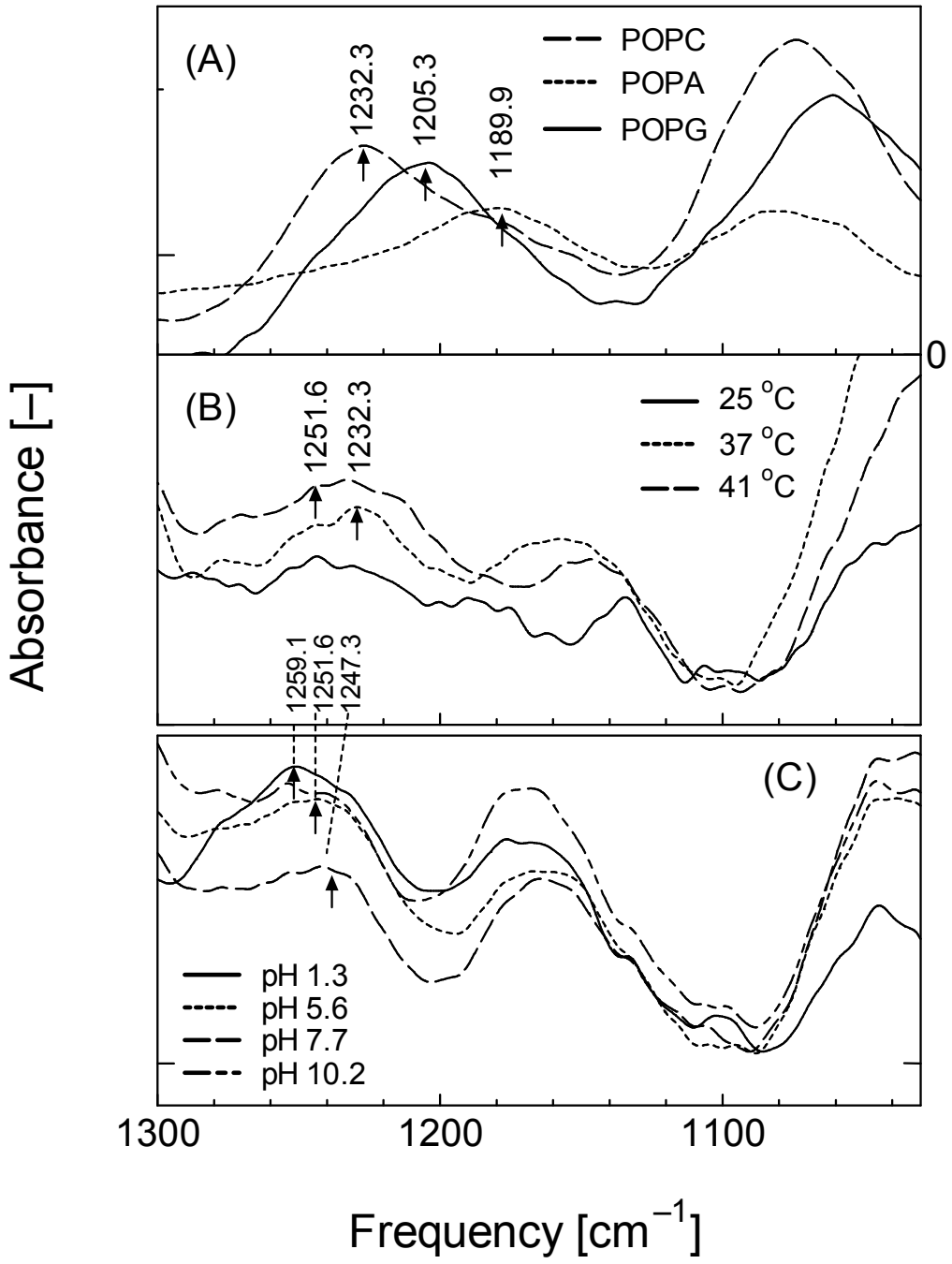


Fig. 2-4 Infrared spectra of PO_2^- group of liposomes (A) and that of PO_2^- groups of spheroplast of *Streptomyces griseus* cell (10^3/ml) in distilled water at different temperatures (B) and at various pH conditions (C). The approximate band positions are also indicated in the figure as the arrow. The spheroplast of *S. griseus* cells were exposed to the heat conditions at 25, 37, and 41 ^oC for 60 min and to the pH at 1.3, 5.6, 7.7, and 10.2 for 60 min at room temperature before applying to FTIR. The samples of POPC, POPG, and POPA liposomes (100 nm in size, 10 mM) were also prepared in distilled water to record the infrared spectra of PO_2^- group in aqueous region.

Table 2-2 Summary of the infrared absorption bands of bacterial functional groups (Ojeda *et al*., 2008)

Frequency (cm^{-1})	Functional group assignment	References
≈1300-1250	Vibration of C-O from esters or carboxylic acids	Helm *et al*., 2006
≈1262	Vibration of –COOH and C-O-H; double bond stretching of >P=O of general phosphoryl groups and phosphodiester of nucleic acids	Dittritch *et al*., 2005
≈1225	Stretching of P=O in phosphates	Jiang *et al*., 2004
≈1200-950	Asymmetric and symmetric stretching of PO_2^- and $P(OH)_2$ in phosphates; vibration of C-OH, C-O-C, and C-C of polysaccharides	Dittritch *et al*., 2005
≈1085	Stretching of P=O of phosphodiester, phosphorylated proteins, or polyphosphate products	Dittritch *et al*., 2005

acids (Dittritch *et al*., 2005). The appearance of IR bands at 1232.3 cm^{-1} corresponds to the stretching of P=O in phosphates (Jiang *et al*., 2004). The above results show that the increasing temperature brought the frequency shift of P=O group to higher value. As a consequence, the loss of water bound P=O groups in aqueous spectra region occurred on the cell membrane surface. This observation suggests that the surface hydration of cell membrane was decreased by the increasing temperature that might contribute to the enhancement of *HFS* (Ngo *et al*., 2008[a]) and/or the destabilization of hydrogen bonds mediated water molecules surrounding cell membrane.

Figure 2-4C shows the IR spectra of spheroplast *S. griseus* cells at the different pH conditions in the aqueous spectra of P=O groups (1300-1150 cm^{-1}). The IR adsorption bands were appeared frequency at approximate 1259.1 cm^{-1} at pH 1.3 and 10.2. However, at the pH near physiological conditions of 5.6 and 7.7, the frequencies are shifted to lower at 1251.6

and 1247.3 cm^{-1}, respectively. This observation implies that the surface hydration of spheroplast *S. griseus* cells was highly susceptible with the variation of pH and the loss of water on the cell surface of *S. griseus* under extreme pH conditions. The loss of water on the surfaces of cell membrane probably caused the destabilization of the hydrogen bonds on cell surface under extreme pH conditions, consequently, recruiting hydrogen bond interactions between the neutral liposomes and cell membranes.

2.2 Characterization of cell surface of *S. griseus* under oxidative stress conditions

The infrared (IR) spectra of lipid mimicking cell membrane (LMCM) liposomes are shown in **Fig. 2-4A**. The IR spectra of these liposomes were almost similar to those of cell membrane of *S. griseus*. This observation implies the similarity of lipid compositions between LMCM liposomes and cell lipid membranes. The spheroplast *S. griseus* cells pretreated with POPC liposomes under oxidative stress at 5 mM of H_2O_2 after incubation of 1 h showed infrared spectra similar to that of cells of the control, as shown in **Figs. 2-4B** and **2-4C**. Whereas, the infrared spectra of spheroplast cells non-treated with POPC liposomes was significantly changed under oxidative stress condition, as shown in **Fig. 2-4D**. These observations imply that the oxidative stress damaged on the lipid cell membrane, resulting in the disappearance of IR spectrum at the frequency 1231 cm^{-1} that corresponds to IR bands of phosphatidylcholine head group in the aqueous region of spheroplast cells. As a consequence, the surface properties of spheroplast *S. griseus* cells might significantly change such as surface net charge and hydrophobicity, and surface hydration although the quantity of these variations should be furthermore characterized. Here, undoubtedly the POPC liposomes interacted with cell membranes of *S. grieus* prevented the oxidative damage on the lipid cell membranes. These results together with the results obtained by measuring lipid and protein peroxidation of cell membrane in the presence and absence of neutral POPC liposomes, described in chapter I, support the possibility that the interaction between liposomes and cell

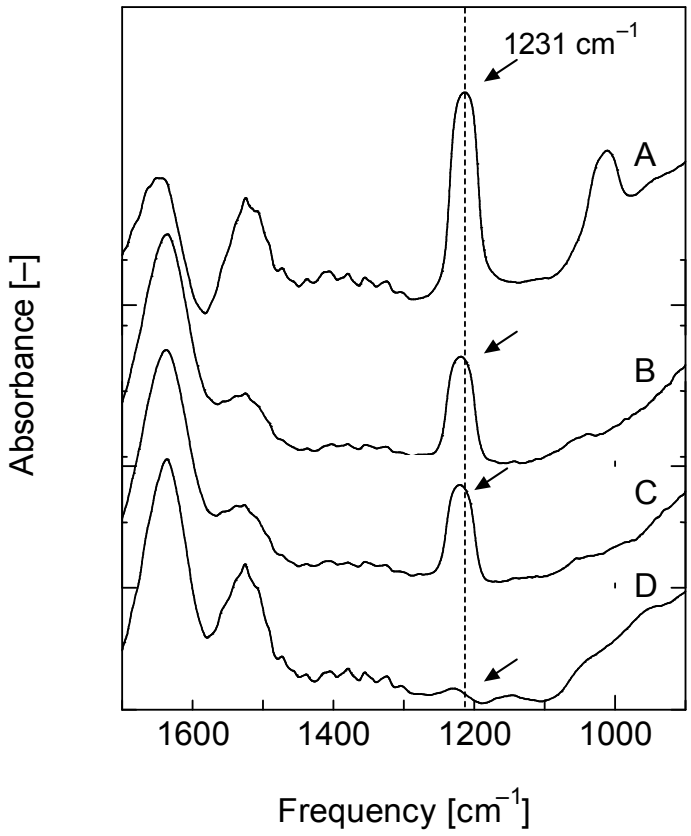


Fig. 2-4 Infrared (IR) spectra of lipid mimicking cell membrane giant vesicle (LMCM-GUV) and spheroplast *Streptomyces griseus* cells under oxidative stress (hydrogen peroxide) with and without POPC liposomes**.** IR spectra of LMCM-GUV (A); spheroplast *S. griseus* (B); spheroplast *S. griseus*/H_2O_2/POPC liposomes (C); and spheroplast *S. griseus*/H_2O_2 (D). The lipid mimicking cell membrane giant unilamellar vesicles (LMCM-GUVs) were prepared by lipid composition as following: DMPG/DOPE/POPC/POPA (56:20:22:2 in mol%). The LMCM-GUVs (10 mM) and *S. griseus* spheroplast cells (10^6/ml) were exposed to 5 mM H_2O_2 for 1 h at room temperature.

lipid membranes plays very important roles for the growth and chitosanase production of *S. griseus* cells under oxidative stress conditions.

3. Heat induces interaction of neutral liposomes with cell membrane

3.1 Heat stress induces internalization of neutral liposomes into *S. griseus* cells

In order to verify the effect of the heat treatment on the interaction of the neutral liposomes with the membrane of *S. griseus* cells, the internalization of POPC liposomes into

these cells pretreated with and without inhibitors of endocytotic pathway was first investigated at different temperatures, as shown in **Fig. 2-5**. **Figure 2-5A** shows the typical emission signals of rhodamine labeled-liposomes co-incubated with *S. griseus* cells at temperature of 41 ^{o}C. The decrease of emission intensity of the labeled liposomes after incubation of 60 min with the above cells implies the internalization of these liposomes, consequently, caused the reduction of rhodamine fluorescence of internalized liposomes, as previously reported (Simoes *et al*., 2001). The addition of triton X-100 disrupted cell lipid membranes resulting in the recovery of the initial fluorescence of such labeled-liposomes. The amount of the neutral liposomes internalized into *S. griseus* cells was also estimated after the treatment of these cells with and without an inhibitor (o-phenanthroline) at 37 and 41 ^{o}C for 60 min. **Figure 2-5B** shows the amount of internalized liposomes into cells in the absence of inhibitor (control) that was 27 % at 37 ^{o}C while that value was further increased to 37 % at 41 ^{o}C, of the initial lipid concentration of POPC liposomes (0.025 mM). This observation implies that the increasing temperature in specific range could enhance the internalization of neutral POPC liposomes into *S. griseus* cells. To elucidate the efficiency of the heat-induced the internalization of the neutral liposomes, the *S. griseus* cells were pretreated with inhibitor of the endocytotic pathway. Then, the internalization of POPC liposomes into these cells was observed at different temperatures. The internalization of POPC liposomes was reduced from 27 % to 7 % at 37 ^{o}C, and was decreased from 37 % to 18 % at 41 ^{o}C, as the consequence of cells pretreated with inhibitor. However, even in the presence of inhibitor the internalization of POPC liposomes into these cells was increased from 7 to 18 % by increasing temperature from 37 to 41 ^{o}C. The above results imply that the heat treatment at specific temperature plays a role to induce and enhance the internalization of the neutral liposomes into *S. griseus* cells. The mode of their internalization was probably relied on the endocytotic pathway.

The kinetics of the neutral liposomes internalized into *S. griseus* cells at different temperatures are also shown in **Fig. 2-6**. The increasing temperature increased the rate and the

amount of neutral liposomes internalized into these cells. The amount of the neutral liposomes internalized into these cells at 37 and 41 ^{o}C was reached maximum within 20 and 15 min of incubation, respectively. The rates of the internalization of the neutral liposomes within such period at 37 and 41 ^{o}C were 33.75 x 10^{-14} and 61.67 x10^{-14} M/min/cell, respectively. The amount of the neutral liposomes internalized into *S. griseus* cells at 37 and 41 ^{o}C was therefore relatively estimated as 469 x 10^{5} liposomes/cell and 643 x 10^{5} liposomes/cell, respectively. This result shows the importance of internalized liposomes, which might affect the growth of cell and the production of chitosanase as previously reported (Ngo *et al*., 2005; Bui *et al*., 2008).

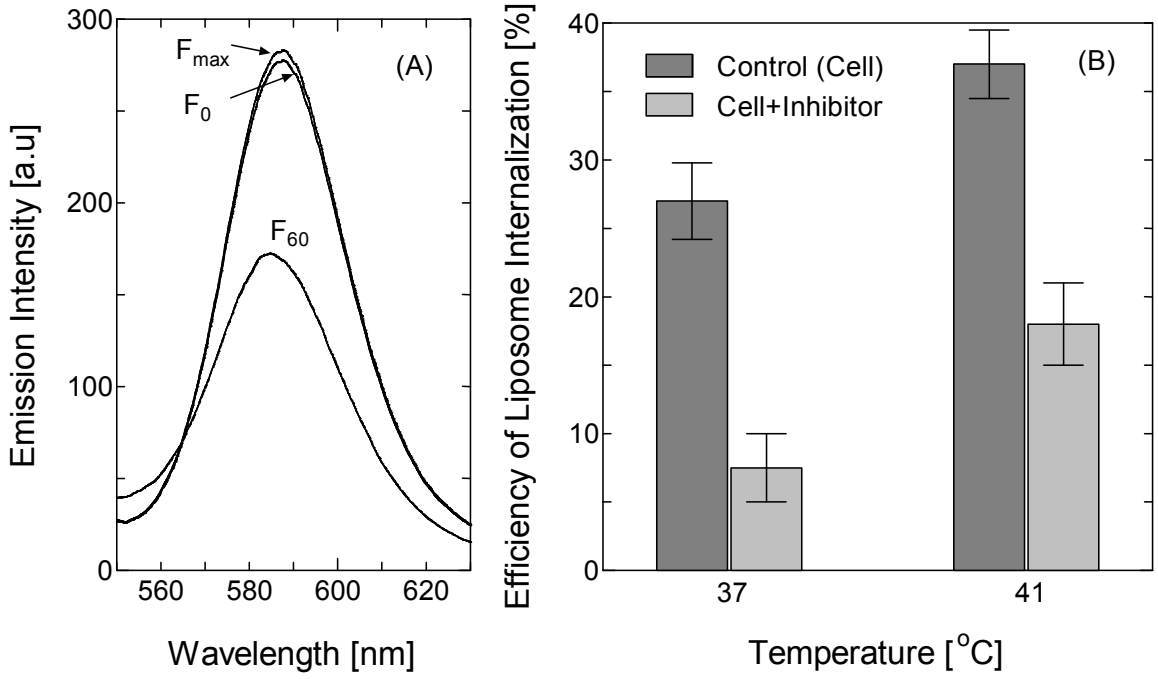


Fig. 2-5 Effect of heat treatment on the internalization of the neutral POPC liposomes into *Streptomyces griseus* cells pretreated with without inhibitor of endocytotic pathway. POPC liposome (0.025 mM as the final lipid concentration, 100 nm in size) labeled 1 mol% Rh-PE were incubated with *S. griseus* cells (10^{3}/ml) pretreated with o-phenanthroline (5 mM as final concentration) as an inhibitor of endocytotic pathway. The mixture of cells and labeled liposomes was coincubated at 37 and 41 ^{o}C for 60 min. Typical data is shown for rhodamine-PE labeled liposomes incubating with *S. griseus* cell without inhibitor at 41 ^{o}C after incubation of 60 min with without triton X-100 (A) and efficiency of liposome internalization calculated as shown in experimental section (B). The data and vertical bar represent mean values and standard deviation obtained from three independent experiments.

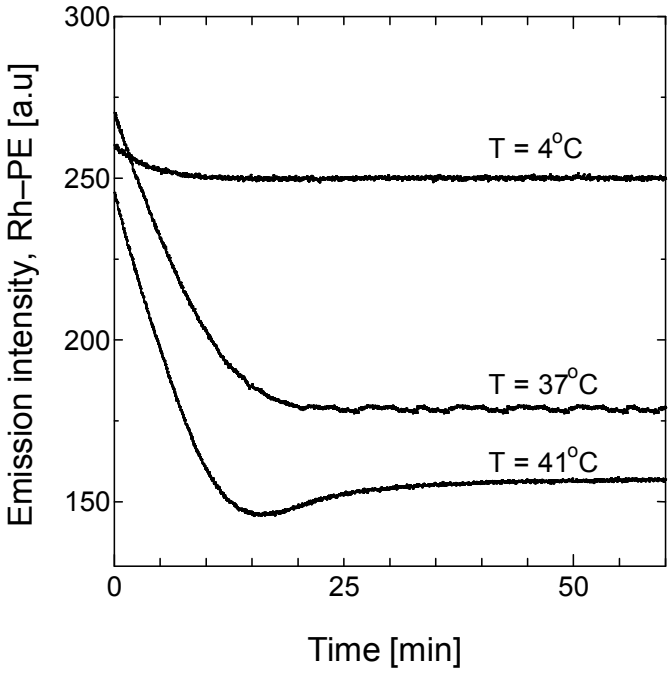


Fig. 2-6 Kinetics of neutral liposomes internalized into *S. griseus* cells at different temperatures. The conditions of this experiment are same as that shown in **Fig. 2-5**. The amount of neutral POPC liposomes internalized into *S. griseus* cells (10^3/ml) was calculated by measuring the maximum lipid concentration of these liposomes inside these cells at 37 ^{o}C/20 min (675 x 10^{-11} M phospholipid) and at 41 ^{o}C/15 min (925 x 10^{-11} M phospholipid), of initial liposome concentration of 0.025 mM. The phospholipid concentration of one POPC liposome with size of 100 nm is approximate 1.44 x 10^{-19} M by using some parameters such as surface areas of outer and inner PC headgroup are 0.74 and 0.61 nm^2, respectively and the thickness of phospholipid bilayer is 3.7 nm (Huang *et al*., 1978).

Table 2-3 Fusion, adsorption and internalization of neutral liposomes into *Streptomyces griseus* cells pretreated with and without inhibitor of endocytotic pathways under specific heat at 41 ^{o}C. The data and vertical bar represent mean values and standard deviation obtained from three independent experiments.

Term	Control (Cell)	Cell + Inhibitor
Fusion (%)	8±1.5	8.5±1.0
Adsorption (%)	55±5.0	73.5±6.5
Internalization (%)	37±2.5	18±3.0

3.2 Characterization of endocytotic pathway of neutral liposomes into *S. griseus* cell

In order to clarify the most related endocytotic pathway of the neutral liposomes into *S. griseus* cells induced by the heat treatments, the *S. griseus* cells were first treated with various kinds of inhibitors, which specifically inhibited the endocytotic pathways of these cells as summarized in **Table 2-1**. The internalization of these liposomes into *S. griseus* cells at different temperatures was comparatively investigated. As shown in **Table 2-4**, the amount of internalized liposomes into *S. griseus* cells pretreated with o-phenanthroline at 37 and 41 ^{o}C was significantly reduced as compared with that of the control. However, the internalization of POPC liposomes into *S. griseus* cells pretreated with cytochalasin B&D or trypsin was mildly inhibited as compared with that of the control at the temperatures of 37 and 41 ^{o}C. This observation implies that the internalization of the neutral POPC liposomes into *S. griseus* cells was due to the bacterial membrane curvature catalyzed by phosphoinositol phospholipase C (PI-PLC). The bacterial membrane curvature is an important step of the endocytosis of solutes through the cell membrane.

Table 2-4 Effect of inhibitors on the endocytosis of the neutral POPC liposomes into *S. griseus* cells under various temperatures. The *S. griseus* cells (10^3/ml) were pretreated with o-phenanthroline (5 mM), cytochalasin B (2.5 μg/ml), cytochalasin D (1 μg/ml), trypsin (0.01 wt%) to inhibit different endocytotic pathways. The internalization of the neutral POPC liposomes into these cells was carried out similar to the operation shown in **Fig. 2-5**.

Temperature (^{o}C)	Efficiency of Liposome Endocytosis (%)			
	Control	O-phenanthroline	Cytochalasin B&D	Trypsin
37	27.0±2.8	7.5±2.5	24.8±2.7	25.5±2.7
41	37±3.0	18±2.5	30.1±2.8	30.3±3.0

Previous studies have reported that o-phenanthroline specifically inhibits the activity of PI-PLC (Sluss *et al*., 1990[a]). The bacterial PI-PLC is a small, water-soluble enzyme that cleaves the natural membrane lipids such as phosphatidylinositol (PI), lyso-PI, and glycosyl-PI (Sluss *et al*., 1990[b]; Grieth *et al*., 1999; Heinz *et al*., 1998). PI-PLC-catalyzes the cleavage of interfacial substrate PI (which is an anionic lipid) in bacterial membrane to water-soluble cIP. The binding of PI-PLC enzymes to the surface of phosphatidylcholine (PC) interface at monodisperse, micelle and vesicle form by electrostatic and hydrophobic interaction significantly enhanced the hydrolysis activtiy of these enzymes (Berg *et al*., 2004; Zhang *et al*., 2004; Feng *et al*., 2003; Zhou *et al*., 1997; Qian *et al*., 1998). Recently, two distinct phosphatidylinositol-specific phospholipase C enzymes from genus *Streptomyces* have been found and both enzymes do not hydrolyze phosphatidylcholine (Qian *et al*., 1998). These findings might support the hypothesis that the PI-PLC enzymes of *S. griseus* could effectively interact with POPC liposomes interacting cell membrane under the specific temperatures, resulting in the enhanced hydrolysis activity of these enzymes to cleave the natural lipid PI. The cleaving of PI headgroup of phosphoinositol lipids in the bacterial lipid membrane caused the membrane curvature that is a possible pathway for the internalization of the neutral POPC liposomes into *S. griseus* cells.

3.3 Fluorescence images of rhodamine-labeled liposomes bound to cell membrane

The interactions of the neutral liposomes with cell membrane of *S. griseus* induced by the specific heat were further clarified by using fluorescence microscopy. **Figures 2-7A**, **2-7B**, and **2-7C** show the fluorescence images of the labeled-liposomes interacting onto cell membranes of *S. griseus* at 37 and 41 ^{o}C. The results indicate that there was no fluorescence image of *S. griseus* cells incubated with the labeled-liposomes at 4 ^{o}C, whereas the fluorescence images of these cells incubated with the labeled-liposomes were clearly observed

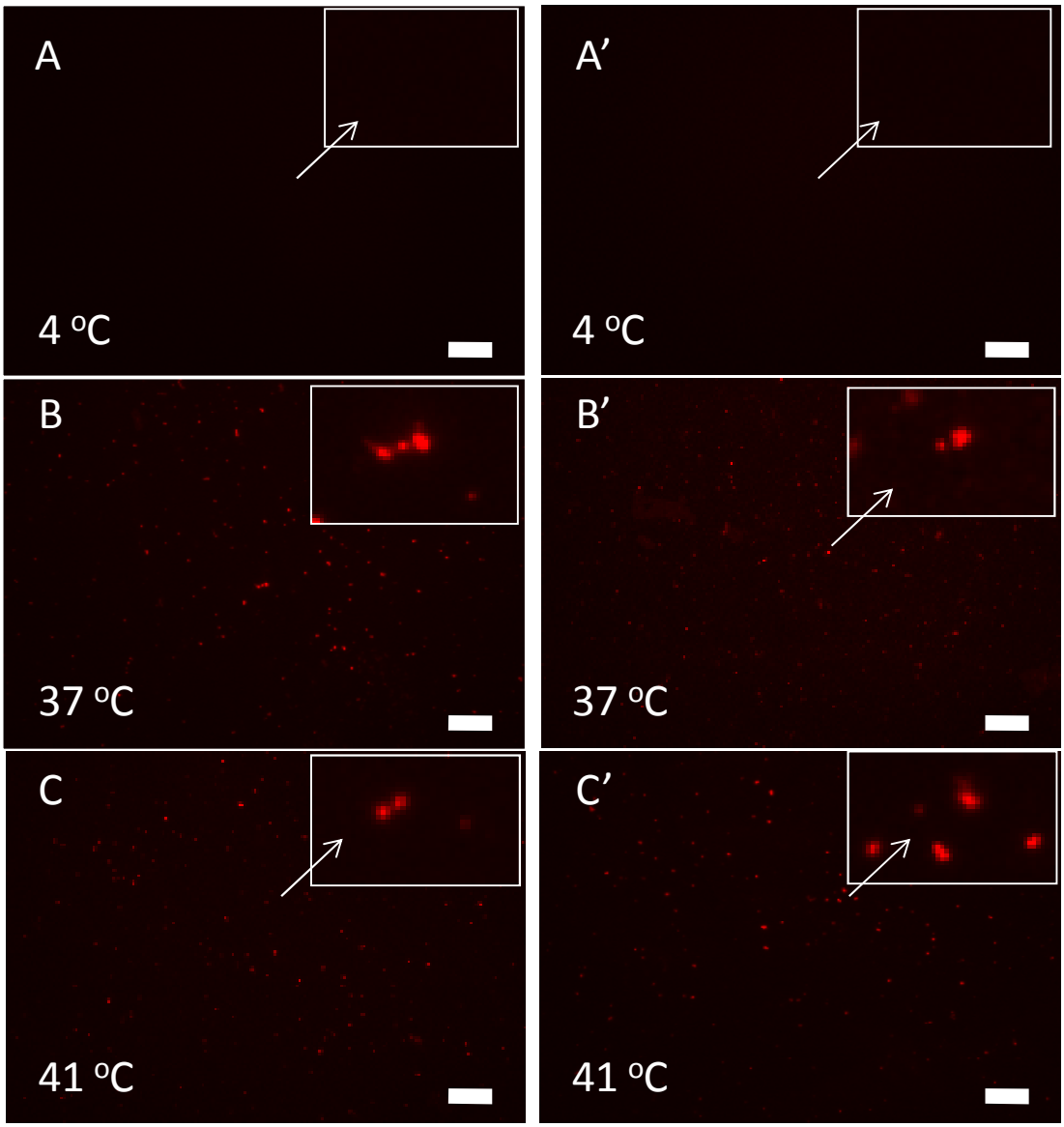


Fig. 2-7 Fluorescence micrographs of *Streptomyces griseus* cells pretreated with without inhibitor of endocytotic pathway, incubated with rhodamine-PE labeled POPC liposomes at different temperatures. POPC liposomes (0.125 mM as final lipid concentration, 100 nm in size) containing 1 mol% Rh-PE were incubated with *S. griseus* cells (10^3/ml) pretreated with without o-phenanthroline (5 mM as final concentration), an inhibitor of endocytotic pathway. The mixture of cells and labeled-neutral liposomes was heated at 37 and 41 °C for 60 min. Free binding labeled-liposomes were removed by washing the cells using PBS buffer 5 to 6 times at 4°C. The A, B and C panels show the images obtained by the interaction of rhodamine labeled-liposomes to the cell surface of *S. griseus* at 4, 37 and 41 °C; the A', B' and C' panels are images obtained from the interaction of the labeled-liposomes to the cells pretreated with inhibitor at 4, 37 and 41 °C. The white boxes show the zoom-up images of *S. griseus* cells interacting rhodamine labeled-liposomes and bar shows scale of 100 μm.

at 37 and 41 ^{o}C. This observation implies that the interaction of the neutral POPC liposomes with the cell membrane of *S. griseus* did not occur at low temperature but the heat treatment at high temperature of 37 and 41 ^{o}C induced and enhanced their interaction effectively. These results confirmed that the interactions between neutral POPC liposomes and cell membranes involved the adsorption, fusion of labeled-liposomes onto the surface of cell membrane; and their internalization into *S. griseus* cells induced by the heat treatment at specific temperatures.

The effect of inhibitors on the interactions of the neutral liposomes with the cell membranes of *S. griseus* was also characterized under various heat conditions. **Figures 2-7A'**, **2-7B'**, and **2-7C'** show the fluorescence images of the labeled liposomes those interacted with cell membranes of *S. griseus* pretreated with o-phenanthroline, an inhibitor of endocytotic pathway, at temperature of 4, 37, and 41 ^{o}C. There was no significant difference in the interaction of neutral POPC liposomes with cell membrane of *S. griseus* cells pretreated with and without this inhibitor at 4, 37 and 41 ^{o}C, as shown in **Fig. 2-7**. This observation confirms that the interaction between the neutral liposomes and the cell membranes was significantly induced by the heat treatment at specific temperatures and such interactions were involving the adsorption, fusion and internalization.

Indeed, together with the above results measuring the fluorescence of the internalized labeled liposomes coincubated with *S. griseus* cells under the specific heat conditions, the hypothesis of liposome internalized into *S. griseus* cells is proposed that the specific heat could induce the adsorption and fusion of POPC liposomes onto cell membrane. As the consequence, the PI-PLC enzymes of *S. griseus* cells were activated to cleave the bacterial PI headgroups specifically to induce the membrane curvature that plays important roles for the internalization of neutral POPC liposomes. The driving forces of heat induced the interactions of the neutral POPC liposomes with cell membranes of *S. griseus* are mainly hydrophobic and hydration effects.

4. Effect of pH on uptake of neutral liposome by *S. griseus* cells

In order to elucidate whether the electrostatic interaction could also play a role for the interactions between the neutral POPC liposomes and *S. griseus* cells, the cellular uptake of these liposomes under various pH conditions was also studied. The previous study has shown that the isoelectric point (pI) of *S. griseus* cell was approximate 3.2 (Ngo *et al*., 2008[a]). It is therefore realized that the surface net charge of these cells can be controlled by changing the pH condition around pI value. In addition, it is also reminded that the low temperature (4 ^{o}C) is strongly required in this experiment in order to minimize the possible effect of the high temperature on the interaction of the neutral liposomes with cell membranes. As shown in **Fig. 2-8**, the cellular uptake of the neutral POPC liposomes was not significantly observed at 4 ^{o}C under various experimental pH conditions. In the control experiment, the cellular uptake of negatively charged POPG liposomes by *S. griseus* cells at the same conditions of pH and temperature was comparatively investigated. The results show that the cellular uptake of the negatively charged liposomes was almost 100 % at pH 2.2 and 2.8 whereas these values were almost 0% at pH 6.8 and 8.5. The above results imply that there was almost no interaction between the neutral POPC liposomes and the cell membranes of *S. griseus* cells by the electrostatic interaction at neutral pH at low temperature. The interaction between the negatively charged liposomes and cell membranes was based on the variation of surface net charge of cell membrane under different pH conditions. The electrostatic interaction between negatively charged liposomes and cell membrane at pH smaller than pI (3.2) is because of that the cell membrane became positively charged surface at pH 2.2 and 2.8 but it became negatively charged surface at pH 6.8 and higher. In addition, surface net charge of POPC liposomes is not changed by changed pH. Indeed, the interaction between the neutral POPC liposomes and cell membranes was not dependent on the electrostatic interaction by variation of surface net charge of cell membrane under various pH conditions.

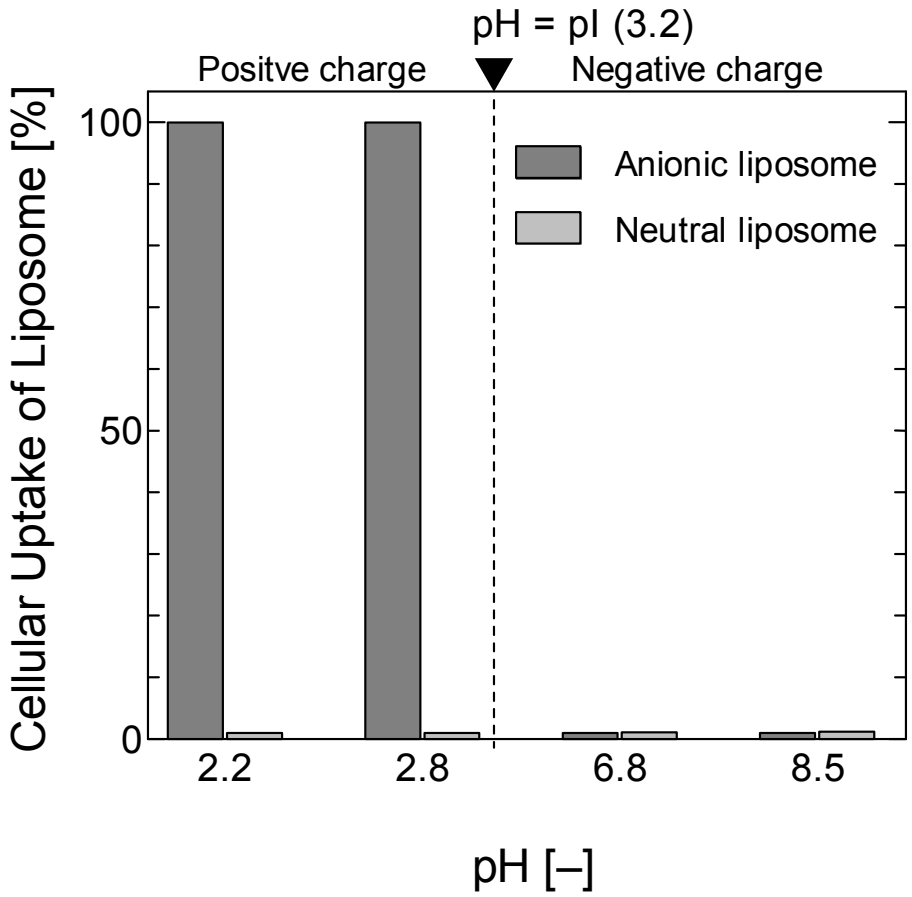


Fig. 2-8 Effects of pH on the cellular uptake of the neutral liposomes by *Streptomyces griseus* cells. The cells (10^3/ml) were coincubated with neutral POPC liposomes (0.025 mM) at different pH conditions. The low temperature at 4 ^oC was kept during such incubation to minimize the hydrophobic effect on the interaction of neutral liposomes with cell membrane. The control experiment using anionic POPG liposomes (0.025 mM) coincubating with above cells at same pH and temperature conditions was also performed to observe the cellular uptake of anionic liposomes by these cells by electrostatic effect. Previously, the pI of *S. griseus* cells is determined at 3.2 (Ngo *et al*., 2008[a])

Summary

The surface properties of *S. griseus* cell were characterized based on the variation of surface net charge and hydrophobicity and surface hydration of cell membranes under various conditions of heat, pH, and oxidative, as summarized in **Table 2-5**.

Table 2-5 Efficiency of heat, oxidative and pH treatment on the variation of cell surface properties of *Streptomyces griseus* evaluaed by ATPS and FTIR

Treatments	Variation of cell surface properties of *S. griseus*		
	Surface net charge	Surface net hydrophobicity	Surface hydration
Heat	Not significant change	Significant change	Change
Oxidative	Change	Change	Significant change
pH	Significant change	Not significant change	Change

The surface net charge of *S. griseus* cells is varied by the treatment of cells by pH and oxidative stress rather than the heat. The isoelectric point (pI) of this cell is 3.2, as evaluated by ATPS. The pH change to lower value of pI (3.2) varies the cell membrane to positively charged surface (ex. Z = +28 at pH 2.8). However, the cell membrane becomes negatively charged surface once pH shifts to higher 3.2 (ex. Z = -13.1 at pH 7.5). The result also shows that the interaction of the neutral POPC liposomes with cell membranes induced by the heat treatment causes the decrease in the surface net charge of cell membrane and the pI of these cells that shifts from 3.2 to 2.2. The interaction of liposomes with cell membrane of *S. griseus* significantly reduced the surface net charge of cell membranes at pH smaller than 2.2. For example, negatively charged surface (Z) of this cell pretreated POPC liposomes under the heat stress at 41 ^{o}C was reduced from -13.1 to -5.8 at pH 7.5.

The surface net hydrophobicity (*HFS*) of this cell is significantly varied, depending on the treatment of heat and oxidative rather than pH. The *HFS* of this cell increases by increasing temperatures. For example, the *HFS* values at 25 and 41 ^{o}C are -220 and -90 kJ/mol, respectively. The *HFS* values of *S. griseus* cells pretreated with the neutral POPC

liposomes at different temperatures are also characterized. The reduction of *HFS* values of *S. griseus* cells co-incubated with POPC liposomes at higher temperature shows the importance of heat treatment on the interaction between liposomes and cell membranes. For example, the *HFS* values of these cells after treatment with POPC liposomes (*HFS*~410 kJ/mol at 41 ^{o}C) at 25 and 41 ^{o}C are -231 and -510 kJ/mol, respectively. The reduction of *HFS* of *S. griseus* cells in the presence of liposomes is dependent on the temperature, implying that the heat treatment at specific temperature plays important role for the interaction of neutral liposomes with cell membranes. The interaction of the neutral POPC liposomes with cell membranes occurs more effectively at higher temperatures in specific heat range.

The surface hydration of *S. griseus* cells is dependent on the heat, pH and oxidative treatments. The treatment of heat at 41 ^{o}C and extreme pH (strong acidic and alkaline) causes the loss of water on cell membrane surface. As the consequence, the surface hydration of cell membrane is reduced under the extreme heat and pH conditions. This reduction is possible to contribute mildly to the increased *HFS*, as well as to the destabilization of hydrogen bonds, which are mediated by the water molecules of cell surface hydration. The cell lipid membrane is very susceptible with the oxidative stress. The oxidative stress severely damages the lipids of cell membranes; however, the membrane-membrane interaction between POPC liposomes and cell membranes is able to prevent the oxidative damage on cell membranes, as the results shown by FTIR for both spheroplast *S. griseus* cell and model of cell membrane by using LMCM-GUV.

Finally, based on the stresses, which induce the variation of cell surface properties of *S. griseus* such as surface net charge and hydrophobicity, and surface hydration, the strategies to control the interaction between liposomes and cell membranes are considered to rely on the electrostatic, hydrophobic and hydrogen bond interactions. As a case study, the interaction of the neutral liposomes (POPC) and negatively charged POPG liposomes with cell membranes is studied. The interaction of the neutral liposomes with cell membranes induced by the heat

treatment can be controlled based on the *HFS* and surface hydration of both cell membranes and liposomes. The internalization of these liposomes is significantly dependent on the temperature. The amount of POPC liposomes internalized into *S. griseus* cells at 37 and 41 ^{o}C is approximate 469x10^5 and 643x10^5 liposomes/cell, respectively. The internalization of the neutral POPC liposomes into these cells is based on endocytotic pathway (membrane curvature) under specific heat condition. Finally, the interaction between negatively charged liposomes and cell membranes of *S. griseus* under various pH conditions shows a conventional way to control the interaction between charged liposomes and cell membrane based on electrostatic interaction.

Chapter III

Preparation and Characterization of Chitosanase-LIPOzyme from *Streptomyces griseus* Cells under Heat Stress Conditions

Introduction

The production of the functional biomaterial oligochitosan has been attractive widely because of its valuable functions and properties. It has been reported that oligochitosan possesses many good functions for food (Shahidi *et al*., 1999), pharmaceutical (Majeti *et al*., 2000) and cosmetic (Dodane *et al*., 1998) usages. The pentameric and hexameric chitosans have been also reported to express antibacterial activity (Uchida *et al*., 1988), antitumor activity (Suzuki *et al*., 1986), and immune-enhancing effects (Hirano *et al*., 1991). Conventionally, oligochitosan is produced by acid hydrolysis of chitosan (Horowitz *et al*., 1957). However, the enzymatic hydrolysis has more advantages to produce oligochitosan because of milder and easier operation to control the enzymatic reaction to produce oligochitosan and to restrict the production of glucosamine (Izume *et al*., 1987). Several approaches have then been studied to enhance enzymatic hydrolysis potential for the production of oligochitosan such as the direct immobilization of chitosanase on the agar gel (Ming *et al*., 2006; Kuroiwa *et al*., 2003) and the circulation of chitosanase in the ultrafiltration membrane reactor (Jeon *et al*., 2000) to continuously produce the oligochitosan. Although these approaches have been proved as prospective methods, some disadvantages for commercial utilization are still remained due to the low production yield such as the low activity and stability of enzyme, high cost and limited availability.

Liposome, a closed phospholipid bilayer membrane, has inner aqueous phase and a nano-order interface (~5nm) harboring hydration and low hydrophobic layer on its surface. The enzymatic reactions inside small and giant liposome vesicles have been dedicated to be

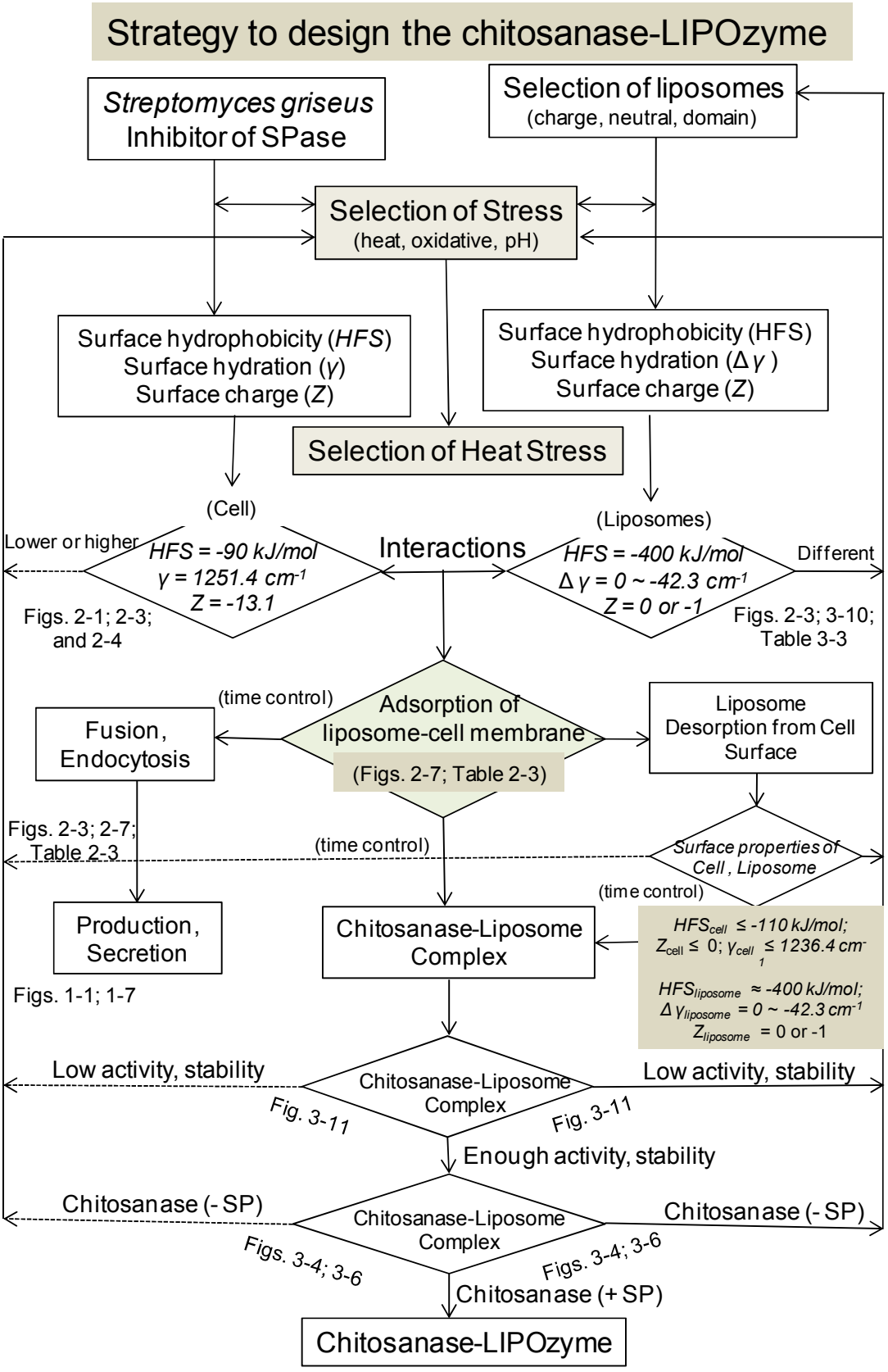
studied by many researchers (Fischer *et al*., 2002; Yoshimoto *et al*., 2004; Oberholzer *et al*., 1995 & 1999; Treyer *et al*., 2002) because of its merits for the applications in medical and biomedical treatments (Walde *et al*., 2001). Furthermore, the enzymes entrapped inside liposomes have been proved to be more stable under high temperature (Wang *et al*., 2003; Han *et al*., 1998) and higher selectivity (Yoshimoto *et al*., 2004). However, the permeability of outside substrates through the lipid membrane is certainly main problem for enzymatic reaction inside the liposomes. In the efforts to enhance the permeability, most of these methods required organic solvents, which could give a rise to considerable solvent residues that are unacceptable in food products, and the low yield of entrapped enzymes inside liposomes as well as the large-scale application would be taken into account difficulty.

In other approaches utilizing the merits of liposome surface such as surface charge, hydrophobicity, surface hydration and hydrogen bond network, the enzyme immobilization onto liposomes has been proved to be more effective and stable in enzymatic reaction (Durour *et al*., 1996). It has also been reported that the liposomes could recognize the molecule through the combined interactions such as electrostatic, hydrophobic and hydrogen bond interactions (Yoshimoto *et al*., 2000; Kuboi *et al*., 2004). Liposome itself could recruit the specific peptide of the oxidized and fragmented superoxide dismutase (SOD) onto its lipid membrane by the electrostatic, hydrophobic and hydrogen bond interactions to create active SOD-LIPOzyme (Tuan *et al*., 2008), and effects of liposome surface on the regulation of gene expression (Bui *et al*., 2008). Liposome modified with Mn−porphyrin complex could simultaneously induce antioxidative-LIPOzyme like activity of both superoxide dismutase and peroxidase (Umakoshi *et al*., 2008).

As previously reported, the interaction of liposomes with cell membranes of *S. griseus* could effectively occur under the specific heat condition. Such interaction could vary the surface properties of cell membranes such as surface net charge and surface net hydrophobicity (Ngo *et al*., 2008[a]), resulting in the enhanced production of chitosanase by

these cells (Ngo *et al*., 2005) and the enhanced secretion of this enzyme probably because of the protein bound-lipid cell membrane mode (Ngo *et al*., 2008[b]). Conventionally, chitosanase of *S. griseus* cell is secreted based on the regulation of signal peptide (SP) bound to the lipid cell membrane and secretory machinery and during or shortly after its secretion through cell membrane the SP is cleaved by signal peptidase type I (Tanabe *et al*., 2003). It is therefore important to inhibit the signal peptidase to not cleave the SP bound chitosanase secreted by these cells and the treatment of the liposomes at specific temperatures were applied simultaneously to induce the secretion of chitosanase containing signal peptide associated-lipid membrane of liposomes (chitosanase-LIPOzymes). It is expected that the chitosanase-LIPOzymes are highly effective and stable under extreme heat and pH conditions to digest natural chitosan (only solubilized at low pH). Moreover, they can be reutilized with low cost and high compatibility to design the continuous production of the functional biomaterial oligochitosan.

The purpose in this chapter is to design the effective chitosanase-LIPOzymes based on the specific heat induced secretion of chitosanase containing SP associated-lipid membrane of the liposomes as the first attempt (**Fig. 3-1**). The strategy to design the effective chitosanase-LIPOzymes is based on the membrane-membrane interactions between liposomes and cell membrane of *S. griseus* induced by the heat stress, based on the fundamental investigation shown in chapter I and II. The chitosanase-LIPOzymes were prepared from the fermentation broth of *S. griseus* cells pretreated with inhibitor of signal peptidase together with the treatment of liposomes under specific heat. The prepared chitosanase-LIPOzymes were characterized focusing on the molecular weight of chitosanase (with without SP) and its enzymatic activity displayed onto liposome surface under the extreme heat and pH conditions. The chitosanase-LIPOzymes prepared by various kinds of liposomes with highly catalytic efficiency and stability were further utilized to study their applicable possibilities to produce biofunctional oligochitosans.



Scheme 1 Design of chitosanase-LIPOzymes based on heat induced secretion of chitosanase containing SP bound liposomes interacting cell membrane of *S. griseus*

The importance of membrane-membrane interactions between liposomes and cell membranes of *S. griseus* induced by heat stress has shown important roles to enhance the cell growth and its production and secretion of chitosanase enzyme, as shown in chapter I. In chapter II, the interaction between liposomes and cell membranes has been also fundamentally investigated via stresses controlling the surface properties of both cell membrane and liposomes such as surface net charge and hydrophobicity, and surface hydration. These factors of surface properties of *S. griseus* cell were quantified at each condition of heat, oxidative and pH treatment. Such quantitative data are very important to design the optimal process for preparation of chitosanase-LIPOzymes, as shown in **Scheme 1**. The investigation also showed that the heat treatment significantly varied the surface net hydrophobicity (*HFS*) and surface hydration (γ) of cell surface as well as that of liposomes; as the consequence, the interaction between liposomes and cell membrane could be effectively controlled by heating-up and/or heating-down to induce the adsorption, fusion, and internalization of liposomes in time dependency. For the preparation of chitosanase-LIPOzymes, the adsorption and desorption of liposomes onto cell membranes could be effectively controlled by the heat induced the variation of *HFS* and surface hydration of both cell membranes and liposomes. The neutral, negative and neutral-domain liposomes were chosen to prepare chitosanase-LIPOzymes instead of cationic liposomes because cell membrane that is negatively charged surface at neutral pH refuses the fusion and internalization of the above liposomes more effectively. Indeed, the heat treatment could effectively induce the adsorption of these liposomes onto cell membranes through the hydrophobic and hydrogen bond interactions. For example, the *HFS* and IR spectra (γ) of cell PO_2^- group at 37 ^oC are -110 kJ/mol and 1233.2 cm^{-1}, respectively. However, these values at 41 ^oC are -90 kJ/mol and 1251.6 cm^{-1}, respectively, and zeta potential (Z) of these cells is not significantly affected by the temperature shift. The interaction between the neutral liposomes and cell membranes occurred most effectively at 41 ^oC through the adsorption, fusion, and

internalization, depending on the period of heat treatment. The heating-up of cell at 41 ^{o}C for 20 min and heating-down at 37 ^{o}C for 20 min were chosen to induce secretion of chitosanase containing SP through the cell lipid membranes of *S. griseus* pretreated inhibitor of signal peptidase (SPase) and these chitosanases bound liposomes interacting cell membrane, as previously reported (Ngo *et al*., 2005; 2008[b]). The liposomes used to prepare chitosanase-LIPOzymes should be neutral (Z = 0) or negatively charged surface (Z = -1), and increment of infrared spectra ($\Delta\gamma$) range between 0 and -42.3 cm^{-1} (POPC, POPG, POPA and POPC/Ch liposomes), and their *HFS* is approximate -410 kJ/mol. The most important factor to prepare chitosanase-LIPOzyme is based on SP bound mature chitosanase that is not cleaved by the SPase I during and/or shortly after the secretion of nascent chitosanase through translocation machinery of *S. griseus* cells pretreated with inhibitor of SPase I, as the description in later section. Here, the SP bound mature chitosanase can mimic its biological function to promote the association of the chitosanase with liposomes interacting onto cell membranes to form its LIPOzymes. The chitosanase-LIPOzymes were characterized based on the existence of SP through protein molecular weight and their catalytic efficiency and stability under various heat and pH conditions.

Materials and Methods

1. Materials

Chitosanase from *Streptomyces griseus* was purchased from Sigma (St. Louis, MO, USA). The lipids including 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine (POPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidic acid (POPA), Cholesterol (Ch), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-rac-(1-glycerol)] (POPG) were purchased from Avanti Polar Lipid (Alabaster City, AL, USA). The β-lactam inhibitor (amoxicillin) was purchased from LKT

Laboratories Inc. (St. Paul, USA). PhastGel^{TM} Homogeneous 12.5, Silver Staining kit, Protein PlusOne^{TM} were purchased from GE Healthcare Bioscience AB (Uppsala, Sweden). Other chemical reagents of highly analytical grade were purchased from Wako Pure Chemicals (Osaka, Japan).

2. Preparation and characterization of chitosanase-LIPOzyme

The *Streptomyces griseus* cells (initial contrentration of 10^6/ml) were grown in 300 ml round flask containing 100 ml chitosanase production medium including KCl (0.05 %), KH_2PO_4 (0.1 %), $MgSO_4.7H_2O$ (0.05 %), $FeSO_4.7H_2O$ (0.001 %) and water-soluble chitosan (0.2 %). The cultivation was carried out in water bath shaker at 140 rpm and 37 ^oC for 24 h. The cultivation was stopped and amoxicillin that is β-lactam class of antibiotic at optimal concentration (12 mg/l as final concentration) was added to inhibit the activity of signal peptidase. It has been reported that bacterial signal peptidase was effectively inhibited by β-lactam class of antibiotics (Black *et al*., 1998; Paetzel *et al*., 1998) such as the inhibition effects of amoxicillin on the growth of bacteria (Andes *et al*., 1998; Thorburn *et al*., 1996). Simultaneously, the various kinds of liposomes with 100 nm in size such as POPA, POPC, POPG, and POPC/Ch liposomes were prepared hygienically as our previous paper (Ngo *et al*., 2005) and were asceptically added to the cell broth at final concentration of 0.4 mM lipid. The treatment of the heat at 37 ^oC (20 min) and 41 ^oC (20 min) as one cycle was continuously applied to such cell broth and such heat treatment was 15 to 20 cycles. The cultivation was stopped and the separation of supernatant from cells was done by centrifugation at 7000 rpm at 5 ^oC for 10 min. The supernatant containing mature chitosanase, chitosanase with SP bound-liposome (chitosanase-LIPOzymes) and other proteins were characterized by measuring of chitosanase activity, protein molecular weight, protein concentration, and lipid concentration of liposomes. The sample was further purified to remove mature chitosanase and other proteins, which could not stably bind liposomes. For this purpose, the module of

dialyzer membrane column (TORAY, Japan) was chosen. The chitosanase-LIPOzyme sample was loaded and circulated inside this membrane module for 30 min to wash out the mature chitosanase and other proteins. Finally, the chitosanase-LIPOzymes could be separated effectively to characterize the existence of chitosanase containing SP on the basis of chitosanse activity, protein molecular weight, protein concentration as well as the lipid concentration of liposome.

3. Analysis

The activities of chitosanase-LIPOzymes and free chitosanase were similarly measured using glycol chitosan as the substrate dissolved in 10 mM phosphate buffer at pH 5.6 at 37 ^{o}C (Ohtaka, 1988) and the description shown in experimental section of chapter I. One unit of chitosanase is defined as the amount of enzyme that hydrolyzes glycol chitosan to release 1 μM glucosamine per min under given assay conditions. Protein concentration was measured by using BCA protein assay kit purchased from PIERCE (Rockland, IL, USA). The lipid concentration of POPC and POPC/Ch liposomes was measured by using PL-IC lipid assay kit (Wako, Japan). Other lipids such as POPG and POPA were measured by using lipid probe (rhodamine-PE) labeled liposomes. For the characterization of molecular weight of chitosanase harboring SP, SDS-PAGE was used. A 10 μl sample of chitosanase-LIPOzymes after disrupting liposomes by triton X-100 (1 % as final concentration) was dissolved with 17 μl of SDS-PAGE sample buffer and 5 μl of mercaptoethanol and was heated for 4 min at 95 ^{o}C for denaturation of protein. The 1-4 μl samples were applied onto a ready-made gel (Homologous 12.5, GE Healthcare, Chicago, IL) designed for the electrophoresis system (PhastSystem, GE Healthcare, Chicago, IL). The gel was finally stained with silver staining method and chitosanases with and without signal peptide were detected as a major staining band at 38.8 kDa and 33.5 kDa, respectively. In another experiment, the surface hydration of POPA, POPC, POPC/Ch and POPG liposomes was characterized through the antisymmetric

infrared spectra of PO_2^- in their lipid headgroups using FTIR (JASCO, Japan). Liposomes were prepared with size (100 nm) and lipid concentration (10 mM) in distilled water, described in experimental section and in previous study (Ngo *et al*., 2005). The sample of 30 µl liposome suspensions at room temperature and neutral pH for the infrared spectroscopic analysis was applied in 50 µm thick-cell with CaF_2 window. The infrared (IR) spectra of liposomes were measured with a FT/IR 4100 spectrometer (JASSCO, Japan) equipped with an Hg-Cd-Te detector. The resolution was 4 cm^{-1} and the frequency in range from 1700 to 950 cm^{-1} was collected for each sample. The subtraction of spectra in buffer was carried out to remove the contribution from water band with the accuracy of the frequency reading better than ±0.1 cm^{-1}.

4. Kinetic of enzymatic reaction using Michaelis-Menton models

The apparent activities of chitosanase-LIPOzymes and free chitosanases were measured by using glycol chitosan (82.084 kDa) as the substrate. In these enzymatic reactions, the concentration of chitosanase enzymes was fixed at 1.76 x 10^{-10} M for both sample of chitosanase-LIPOzymes and free chitosanase in order to utilize most enzymes in the reaction with the substrate that varied from 1.10 x 10^{-9} M to 3.65 x 10^{-8} M. The lipid concentrations of POPA, POPC, and POPC/Ch liposomes used to prepare chitosanase-LIPOzymes were 0.34 x 10^{-5} M, 0.32 x 10^{-5} M, and 0.39 x 10^{-5} M, respectively.

For the steady-state approach to the analysis of an enzymatic reaction, we assume the conversion of single substrate, S, to a single product, P, by an enzyme, E. Another assumption of equilibrium-state, the concentration of enzyme and substrate complex, ES, is in thermodynamic equilibrium with the free forms of enzyme and substrate and that the ES complex is held together by noncovalent force. In other words, the rate constants for the formation of ES complex (k_1, units of $M^{-1}s^{-1}$) and its breakdown to the free components (k_{-1}, units of s^{-1}) are considerably faster than the catalytic step (k_{cat}, units of s^{-1}). In addition to the

steady-state, we assume that the breakdown of the ES complex following catalytic conversion into product and enzyme is so fast that we may neglect its formation. Thus the slow step is the chemical step and we denote the rate constant as k_{cat}. (Gabi *et al*., 2007).

$$E + S \underset{k_{-1}}{\overset{k_1}{\leftrightarrows}} ES \xrightarrow{k_{cat}} E + P \qquad \text{(scheme 1)}$$

Assume ES complex is in a 'steady-state', then $k_{cat}/K_m \geqq 10^9$ and the ES complex is in 'equilibrium-state', kcat/Km < 10^9. Then steady-state assumption can be performed as the following:

$$k_1 [E] [S] = k_{-1} [ES] + k_{cat} [ES] \qquad (1)$$

The velocity of product formation in this enzymatic reaction is therefore performed by Michaelis-Menton model to estimate some parameters such as Michaelis constant (K_m), maximum velocity (V_{max}) and catalytic rate constant (k_{cat}).

$$v = \frac{V_{max}[S]}{K_m + [S]} \quad (2)$$

$$k_{cat} = \frac{V_{max}}{[E]} \quad (3)$$

5. Stability of chitosanase-LIPOzyme under stress condition

The stability of chitosanase-LIPOzymes and mature chitosanase with and without liposome under the extreme heat and pH conditions was assayed by measuring chitosanase activity, as the description in experimental section in chapter I. The stability of these enzymes at different temperatures was assayed by the exposure of these enzyme samples to 25, 30, 37,

41, 45, 50, 55, and 65 ^{o}C for 10 min. Then 150 µl enzyme sample was reacted with 150 µl of 2 % glycol chitosan for 10 min at given assay temperatures at pH 5.5 to measure the activity of chitosanase (Ohtaka, 1988). The stability of these enzymes under different pH conditions at 1.3, 2.3, 4.5, 5, 5.9, 7.6, and 10.2 was assayed by incubation of these enzyme samples at given pH values for 24 h. Then 150 µl enzyme sample was reacted with 150 µl of 2 % glycol chitosan (dissolved in water at desired pH value) for 10 min at 45 ^{o}C to measure the activity of chitosanase (Ohtaka, 1988).

6. Physical stability and lifetime of chitosanase-LIPOzymes at stored condition

After preparation of chitosanase-LIPOzymes, their physical structure stability was checked by using quart crystal microbalance (QCM). Briefly, the solution of chitosanase-LIPOzymes (0.32 mM as final lipid concentration and liposomal size of 100 nm) was continuously pumped onto the surface of gold electrode. The velocity of liposomal influx was setup at 5 rpm using mini rotary pump. The frequency shift was recorded in time dependence. The adsorption modes of liposomes on the surface of gold electrode are distincted as the intact immobilization and/or the rupture states. The stability of chitosanase activity stored at 4 ^{o}C was also investigated by measuring its activity in time dependence. The measurement of chitosanase activity was described well in experimental section of chapter I.

7. RP-HPLC analysis of oligochitosans produced by various chitosanase-LIPOzymes

The composition of the oligochitosans was analyzed by HPLC on TSK gel NH_2-60 column (4:6 x 250 mm, TOSOH Co. in Japan), as the previous report (Jeon *et al*., 2000). The enzymatic reaction was carried out at 45 ^{o}C and pH 5.5 for 1 h. After incubation of chitosanase-LIPOzymes or mature chitosanase with glycol chitosan at the E/S ratio of 5 % and 7 %, respectively, one mililiter of reaction mixture was mixed with 1 ml of acetonitrile. Then, 200 µl of this mixture was chromatographed using elution buffer (60 % acetonitrile,

40 % distilled water) at a flow rate of 1.0 ml/min and temperature of column at 45 °C. The oligosaccharides were detected by monitoring the refractive index.

Results and Discussion

1. Preparation of chitosanase harboring signal peptide by *Streptomyces griseus* cells

1.1 Strategies to prepare of chitosanase-LIPOzymes

It has been reported that the extracellular chitosanase is released through the secretory machinery of *S. griseus* cell by the hydrolysis of its SP catalyzed by the signal peptidase (Tanabe *et al*., 2003). **Figure 3-1** shows the schematic illustration for the preparation of chitosanase-LIPOzymes from *S. griseus* cells by treatment of these cells with liposomes and specific heat condition. This operation has been described in experimental section.

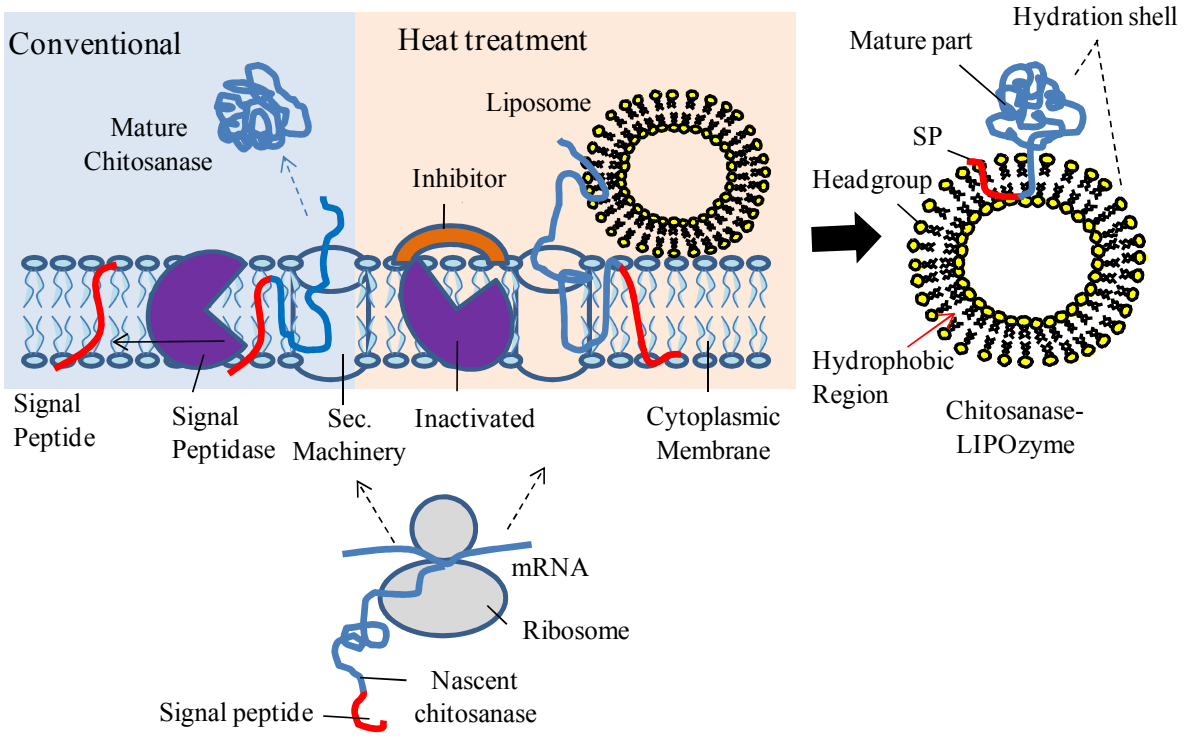


Fig. 3-1 Schematic illustration for the preparation of chitosanase-LIPOzyme by *S. griseus* cell on the basis of the heat induced the secretion of chitosanase containing SP associated-lipid membrane of liposome.

The chitosanase containing SP could interact effectively with the lipid membrane of liposomes through the lipid association of the SP by electrostatic and hydrophobic interactions. These interactions play important roles to display chitosanase stably onto liposome surfaces together with the stabilization of the hydrogen bond interactions between chitosanase and the surface hydration of liposome membranes mediated by water molecules.

1.2 Optimal conditions for treatment of inhibitor

Type I signal peptidases are not inhibited by the standard protease inhibitors (Zwizinski *et al*., 1981; Talarico *et al*., 1991; Black *et al*., 1992; Kuo *et al*., 1993; Kim *et al*.,1995). For example, Kim *et al*. have found that *E.coli* signal peptidase is not inhibited by diisopropyl fluorophosphates (DFP), phenylmethylsulphonyl fluoride (PMSF), soyabean trypsin inhibitor, APMSF, elastinal, benzamindine, leupeptin, tosyl-lysine chloromethyl ketone, *n*-tosylphenyalanine chloromethyl ketone, chymostatin, *p*-chloromercuribenzoate, EDTA, o-phenanthroline, phosphoramidon, and bestatin. However, β-lactam compounds can inhibit *E. coli* signal peptidase in a pH and time dependent manner. SmithKline Beecham Pharmaceuticals (Harlow, Essex, UK) has studied extensively the potential of β-lactam (or penem)-type inhibitors against bacterial type I signal peptidase (Allsop *et al*., 1995; 1996, Perry *et al*., 1995). The most effective penem compounds are the 5*S* stereoisomers, which are capable to inhibit both the gram-negative *E. coli* and the gram-positive bacterial signal peptidases. The 5*S* stereoisomer is opposite stereochemically to that of the 5*R* *β*lactams that are required for β-lactamases and penicillin-binding proteins. The crystal structure of a bacterial signal peptidase in complex with a β-lactam group inhibitor was also reported (Paetzel *et al*., 1998).

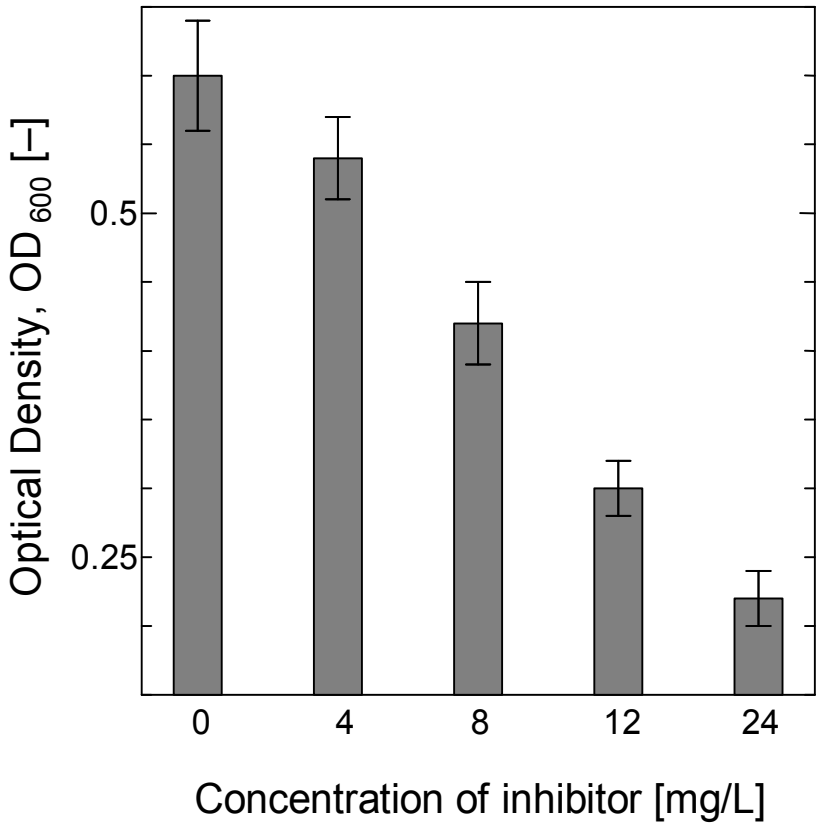


Fig. 3-2 Effect of concentration of amoxicillin on the growth of *Streptomyces griseus* cells after an incubation of 24 h at 37 ^{o}C

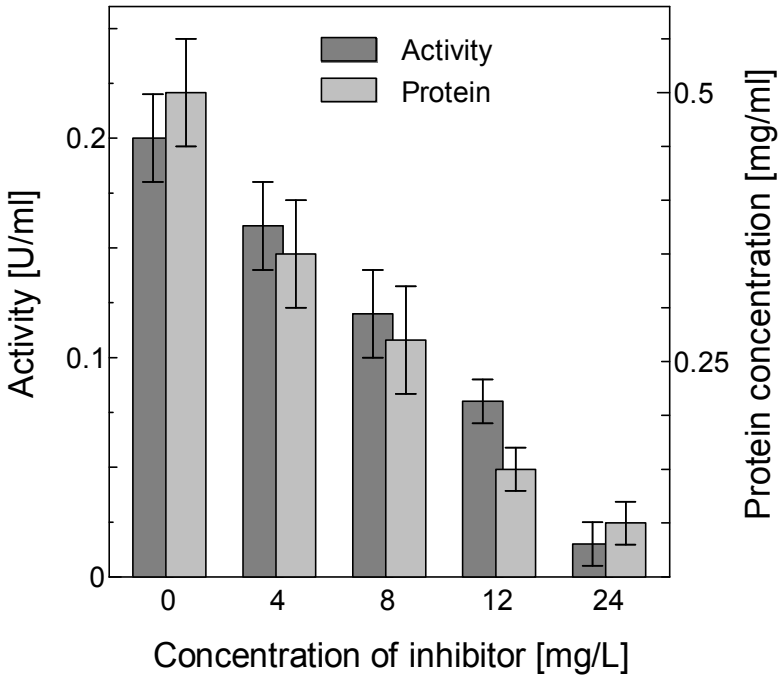


Fig. 3-3 Effect of concentration of amoxicillin on the secretion of chitosanase and total proteins from *Streptomyces griseus* cells after an incubation of 24 h at 37 ^{o}C

In this study, *S. griseus* cells were prepared with amoxicillin (β*l*actam group) to inhibit the activity of type I signal peptidase to not cleave SP of nascent chitosanase during or shortly after the secretion of these enzymes across secretion machinery of these cells. It has also been reported the *in vivo* activities of amoxicillin and amoxicillin-clavulanate against 17 strains of *Staphylococus pneumonia* with minimum inhibitory concentrations (MIC) of 0.12–8.0 mg/liter (M. Paetzel *et al*. 1998).

In the preliminary experiment, the inhibition effect of amoxicillin on the growth of *S. griseus* cells as well as their production of chitosanase and release of proteins were first optimized the concentration for the inhibition of the secretion of protein. As shown in **Fig. 3-2**, the growth of *S. griseus* cells was reduced with increasing concentration of inhibitor. At the concentration of 12 mg/l of inhibitor, the growth of *S. griseus* cell is inhibited almost 50% in comparison with that of the control after incubation of 24 h at 37 ^{o}C. The secretion of chitosanase and total proteins by *S. griseus* cells were also decreased by increasing concentration of inhibitor, as shown in **Fig. 3-3**. Interestingly, after the treatment of *S. griseus* cells with amoxicillin (12 mg/l as final concentration), the secretion of total proteins and chitosanase was significantly blocked almost 70% and 60%, respectively, in comparison with that of the cells cultivated at 37 ^{o}C without any treatment. Although amoxicillin caused the negative effects on the growth and secretion of chitosanase of *S. griseus* cells cultivated at 37 ^{o}C, the dose of this inhibitor was chosen at 12 mg/l to inhibit the type I signal peptidase of *S. griseus* cells. The chitosanase-LIPOzymes were prepared according to the description in experimental section.

2. Characterization of chitosanase-LIPOzyme

2.1 Characterization of chitosanase and its signal peptide

The SP of chitosanase from *Streptomyces griseus* can herewith be classified as the signal peptide type I (leader peptide) (Tanabe *et al*., 2003). The primary structure analysis of this SP

as well as its associated-mature chitosanase using http://us.expasy.org/tools/protscale.html was shown in **Fig. 3-4**. The SP contains 53 amino acids in length with molecular weight approximately 5.3 kDa and isoelectric point (pI) at 13 as shown in **Fig. 3-4A**. The SP of chitosanase shares the common characteristics of SP type I, which is rich in positive charge, hydrophobic and α-helical conformation.

Basically, SP type I contains 3 distinct domains including N-domain, H-domain, and C-domain (Von-Heijne, 1990). The amino terminal N-domain of SP contains at least one arginine or lysine residue and this positively charged residue has been suggested to interact with translocation machinery (Akita *et al*., 1990) and negatively charged phospholipids in the lipid bilayer of the membrane during translocation (Deuerling *et al*., 1997). The H-domain,

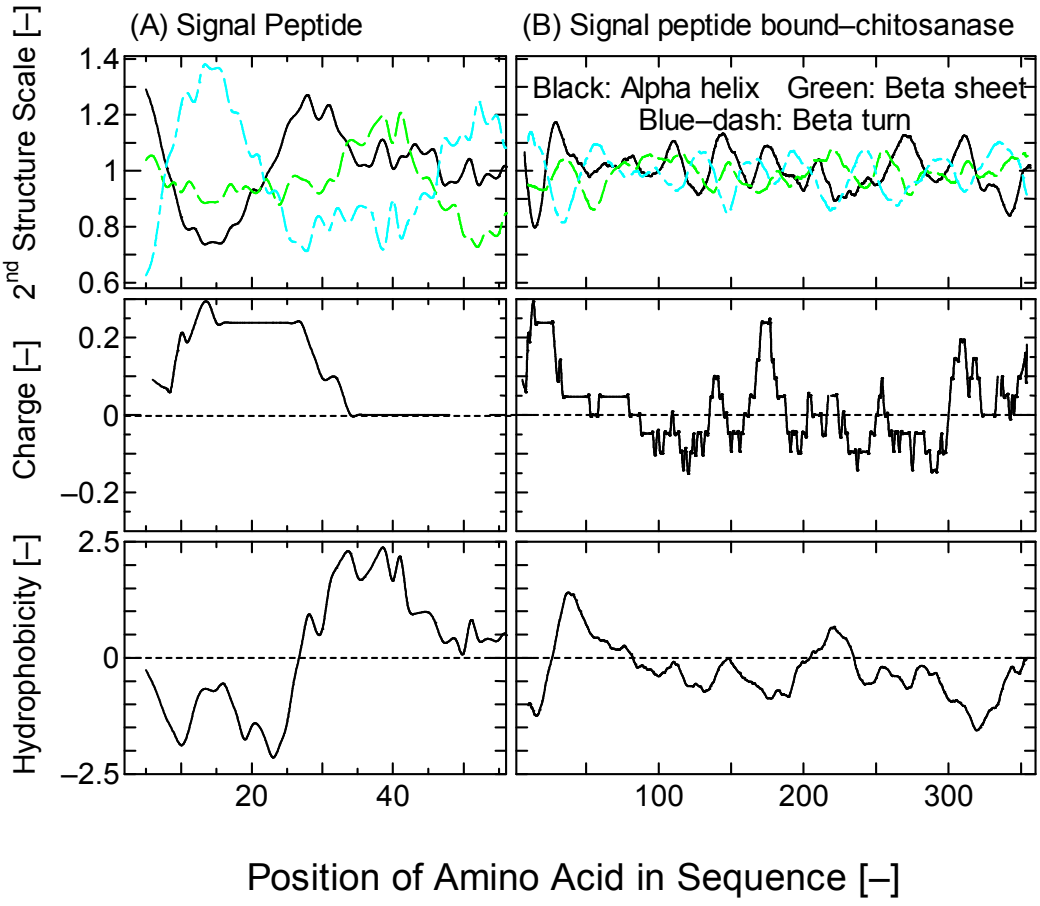


Fig. 3-4 Characteristics of chitosanase from *S. griseus* and its signal peptide analyzed on the basis of its amino acid sequence

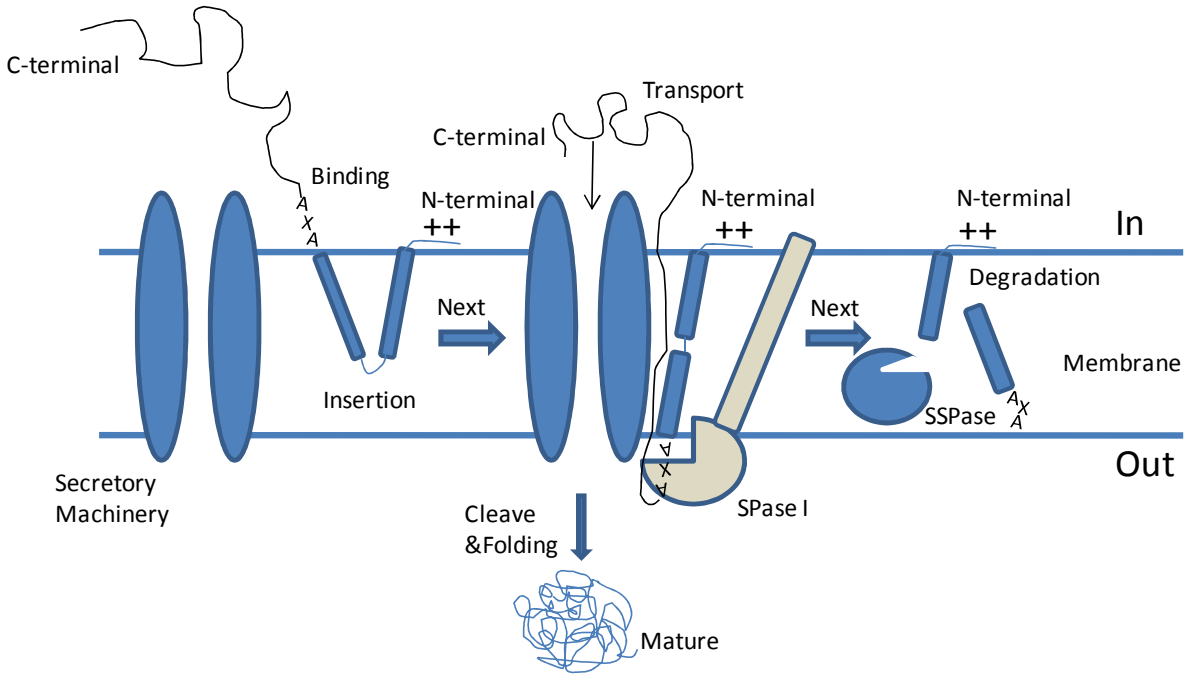


Fig. 3-5 Model for insertion of protein containing signal peptide (SP) into the cytoplasmic membrane. First, the positively charged N-domain interacts with negatively charged phospholipid in the membrane, after which the H-domain integrates loopwise into membrane. Next, the H-domain unloops, whereby the first of the mature protein is pulled through the membrane. During or shortly after the translocation across secretion machinery, the SP is cleaved from mature protein and consequently degraded by the specific signal peptidase (SSPase). After its translocation, the mature protein folds into its native conformation (Tjalsma *et al*., 2000).

following the N-domain, is formed by a stretch of hydrophobic residues that seem to adopt α-helical conformation in the membrane (Briggs *et al*., 1986). Helix-breaking residues found at the end of the H-domain, are thought to facilitate cleavage by specific signal peptidase (SSPase) (Dalbey *et al*., 1997). The C-domain, following the H-domain, contains the cleavage site for SPase, which removes the SP from mature part of the secreted protein during or shortly after translocation. The mature part of the protein is thereby released from the membrane and can fold into its native conformation as schematially shown in **Fig. 3-5**.

Undoubtedly, the signal peptide plays very important role to immobilize chitosanase stably in liposome membrane. The strategy is the treatment of *S. griseus* cells with the

inhibitor to inhibit the activity of type I signal peptidase (SPase I). Then the heat stress and various kinds of liposomes are applied together to cell cultivation. The interaction of liposome with cell membrane was controlled by using heat stress at 41 and 37 ^{o}C for 20 min (15~20 times) to induce the secretion of nascent chitosanase through membrane-bound pathway to directly display this chitosanase with signal peptide on the liposome (LIPOzyme).

2.2 Analysis of signal peptide associated-chitosanase

The chitosanase-LIPOzymes produced by *S. griseus* cell were described in the experimental section. The initial *S. griseus* cells with concentration (10^6/ml) were fermented in a 300 ml round-bottom flask containing 100 ml chitosanase production medium in the presence of amoxicillin as the β-lactam class inhibitor of signal peptidase (12mg/l as final concentration). The cultivation was carried out at 37 ^{o}C in a water bath at a shaking 140 rpm for 24 h. Liposomes (100 nm in size) such as POPA, POPC, and POPC/Ch (0.4 mM as final concentration) were added to cell culture broth. This cell-incubating flask was transferred between the water bath incubators kept at 41 ^{o}C for 20 min and 37 ^{o}C for 20 min. The heat fluctuation at 41 ^{o}C (20 min)/ 37 ^{o}C (20 min) was repeated 15 to 20 times. During the treatment, the rates of heating up of cell culture broth from 37 ^{o}C to 41 ^{o}C and its cooling down from 41 ^{o}C to 37 ^{o}C in water bath incubator were fixed at 1.7 K/min and 1.1 K/min, respectively. Based on previous study, the heat treatment could control the association and/or dissociation of liposomes with cell membranes by the hydrophobic and hydrogen bond interactions. These interactions controlled by the heat are possible to prepare the liposomes chitosanase harboring SP bound lipid membranes of liposomes (chitosanase-LIPOzymes).

A possibility of the recovered chitosanase harboring SP was characterized by measuring its activity and molecular weight using SDS-PAGE. The expression of chitosanase activity in the purified chitosanase-LIPOzyme solution confirmed the abundance of chitosanase enzyme.

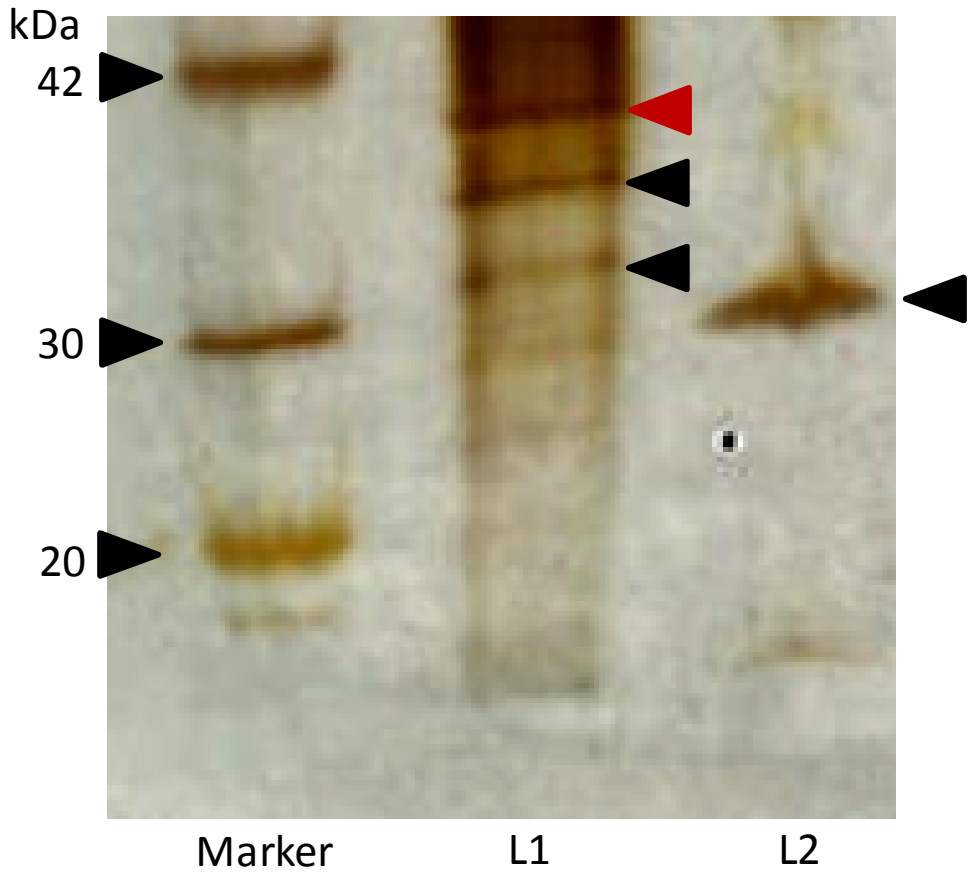


Fig. 3-6 SDS-PAGE analysis of chitosanase with signal peptide produced by *Streptomyces griseus* using Phastgel homologue 12.5 with silver staining for observation of protein. The cells were treated with inhibitor and liposome as well as the heat stress at 41 ^{o}C. L1: Protein components produced by *S. griseus* cells; L2: standard chitosanase; M: Marker.

These results are well corresponding with previous findings (Ngo *et al*., 2005, 2008[a], 2008[b]). The existence of SP associated-chitosanase in this sample was further characterized by using the SDS-PAGE, as shown in **Fig. 3-6**. The molecular weight of the protein shown as red arrow mark in L1 was estimated approximately 39 kDa that was higher than molecular weight of mature chitosanase marked by arrow in L2 (33.5 kDa). The result also shows that there was no band of chitosanase (without SP) existed in L1 after purification of chitosanase-LIPOzyme sample although some impure proteins were remained with distinct molecular weights. The protein expressing chitosanase activity and with molecular weight of 39 kDa, which is well

corresponding with the sum of the molecular weight of mature chitosanase and SP (38.8 kDa) shows the evidence of chitosanase harboring SP in LIPOzyme samples. It is therefore found that the chitosanase-liposome complex was formed based on its SP associated-lipid membrane of liposomes interacting onto cell membranes of *S. griseus* under the specific heat condition.

Table 3-1 Summarize some basic parameters of phospholipids of liposomes

Factor	POPC (Huang *et al.*, 1978)	POPA (Huang *et al.*, 1978)	POPC/Ch (70:30 mol%) (Falck *et al.*, 2004)	
			POPC	Cholesterol
Surface area of outer head group ($Å^2$)	74	60	44	38
Surface area of inner head group ($Å^2$)	61	60	44	38
Thickness of bilayer (Å)	37	37	34	

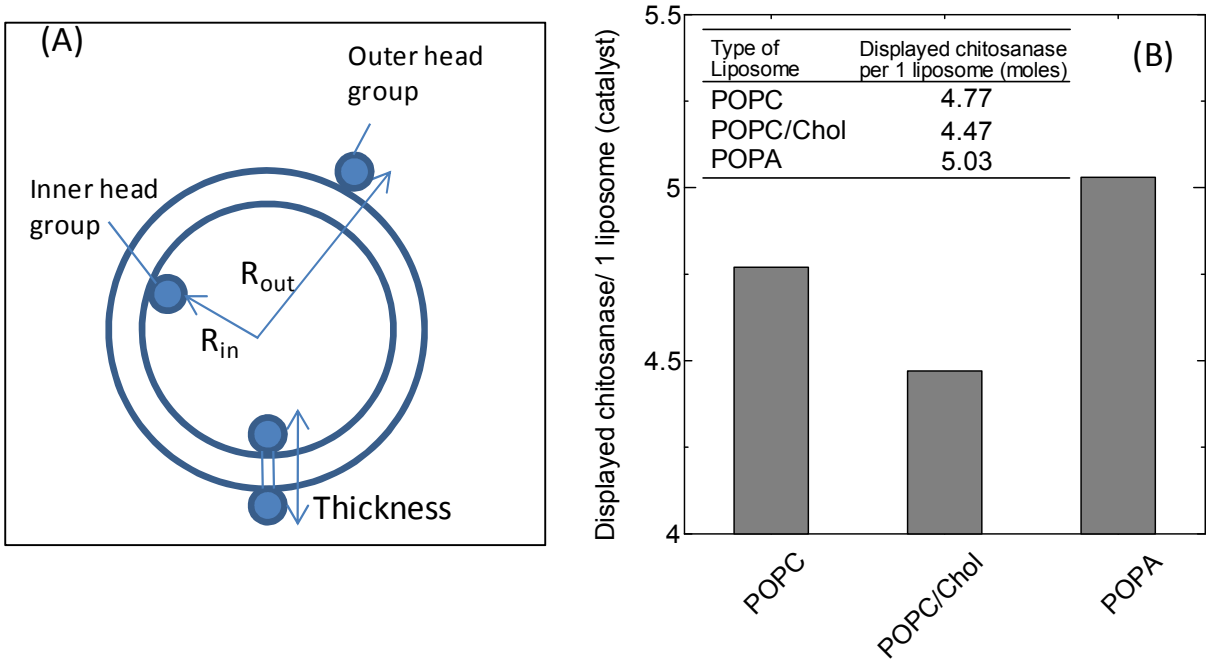


Fig. 3-7 The model of spherical liposomal vesicle prepared by extruding method (A) and calculated number of chitosanase molecules harboring SP displayed onto one liposome (B)

Table 3-2 Input parameters for different chitosanase-LIPOzymes

Type of liposome of LIPOzymes	Estimated size (nm)	Protein concentration (M)	Lipid concentration (M)
POPC	100	$1.76x10^{-10}$	$0.32x10^{-5}$
POPC/Ch (70:30 mol%)	100	$1.76x10^{-10}$	$0.39x10^{-5}$
POPA	100	$1.76x10^{-10}$	$0.34x10^{-5}$

2.3 Calculation for chitosanase-LIPOzyme

The model of liposome is shown in **Fig. 3-7A**. The number of chitosanase molecules harboring SP displayed on one liposome was approximate 5 molecules for the case of POPA and POPC liposomes while it was approximate 4 molecules for the case of the POPC/Ch liposome (**Fig. 3-7B**). This result means that there is no significant difference between negatively charged, neutral, and/or domain-formed liposomes (POPA, POPC, and POPC/Ch) in the interaction with chitosanase containing the SP secreted by *S. griseus* cells under the heat stress condition at 41 ^{o}C. Under the heat stress at 41 ^{o}C, the interactions of these liposomes with cell membrane of *S. griseus* cells could play important roles to display chitosanase containing SP-associated lipid membranes of liposomes (chitosanase-LIPOzymes). Probably, the inhibition effects of amoxicillin inhibited the activity of signal peptidase, which could not cleave the SP of nascent chitosanase during and/or shortly after the translocation of this enzyme through cell membrane of *S. griseus*.

3. Characterization of enzymatic activity of chitosanase-LIPOzymes

The enzymatic activity of chitosanase-LIPOzymes prepared in the previous section was further characterized through the kinetic analysis of the glucosamine production using glycol

chitosan as the substrate. **Figure 3-8A** shows the rate of product (glucosamine) formation versus the substrate (glycol chitosan) concentration. All chitosanase-LIPOzymes prepared by POPA, POPC, and POPC/Ch liposomes significantly accelerated the rate of product formation twice to four times higher than the rate of product formation of the control. The maximum velocity (V_{max}) of enzymatic reaction of each LIPOzymes was measured and compared. Among three kinds of chitosanase-LIPOzyme assayed here, the V_{max} of chitosanase-LIPOzyme prepared by POPA liposome showed the highest activity at 12.4 x 10^{-10} M/s. The chitosanase-LIPOzymes prepared by neutral (POPC) and domain formed (POPC/Ch) liposomes showed the slower rate at 7.1 x 10^{-10} M/s and 5.8 x 10^{-10} M/s, respectively.

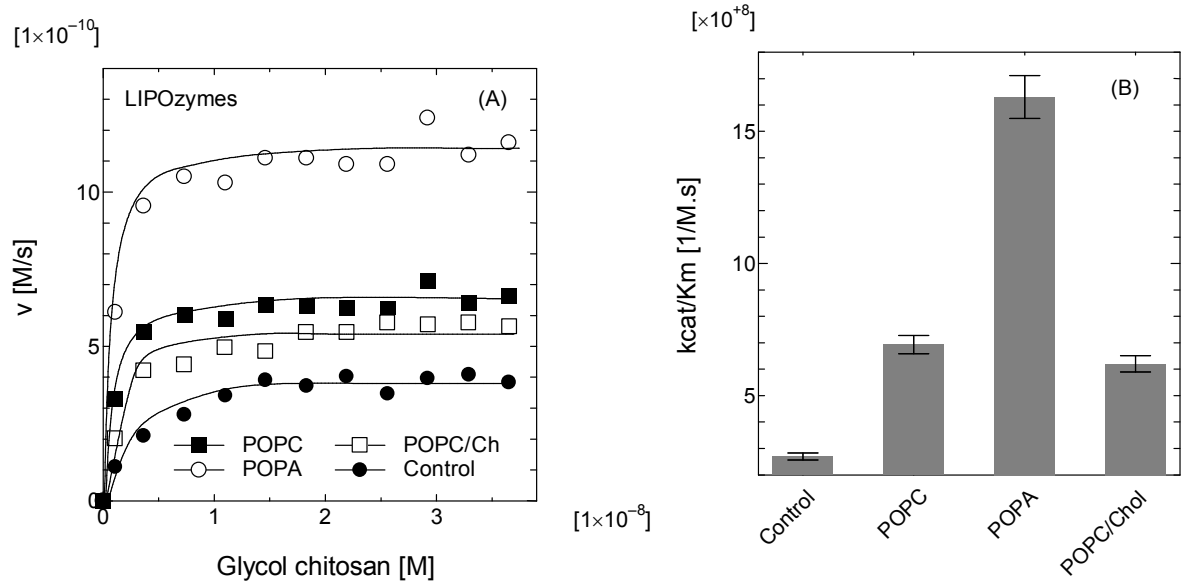


Fig. 3-8 Rate of product (glucosamine) formation versus concentration of substrate (glycol chitosan) (A) and efficiency of catalytic rate k_{cat}/K_m (B) catalyzed by mature chitosanase (control) and various kinds of chitosanase LIPOzymes. The enzymatic reaction was carried out by the coincubation of 150 µl chitosanase-LIPOzyme or mature chitosanase (protein concentration is fixed at 1.76 x 10^{-10} M) with 150 µl glycol chitosanase (its concentration was varied from 1.10 x 10^{-9} M to 3.65 x 10^{-8} M) at 45 ^{o}C for 10 min. The enzymatic reaction was stopped by boiling at 95 ^{o}C for 4 min and glucosamine concentration was quantified as the previous method (Ohtaka, 1988)

In order to verify the applicability of chitosanase-LIPOzymes, which reach the steady-state ($k_{cat}/K_m \geq 10^9$) or equilibrium mechanism ($k_{cat}/K_m < 10^9$), the efficiency of catalytic rate (k_{cat}/K_m) of various kinds of chitosanase-LIPOzymes are performed in **Fig. 3-8B**. The highest k_{cat}/K_m value could be obtained with the chitosanase-LIPOzyme prepared by POPA liposomes (1.6×10^9 (1/M.s)) corresponding to the steady state, in which the rate of chitosanase-LIPOzyme-substrate complex formation equals to the rate of its breakdown into chitosanase-LIPOzyme and its product. In the neutral and domain formed chitosanase-LIPOzyme, the k_{cat}/K_m values were lower at 6.9×10^8 and 6.2×10^8 (1/M.s), respectively, corresponding to the equilibrium state, in which the rate of chitosanase-LIPOzyme-substrate complex formation and its equilibrium that is more slowly affected by the breakdown into chitosanase-LIPOzyme and its product.

There are several effects of liposomes on the hydrolysis potential of chitosanase containing SP displayed on liposome surfaces such as electrostatic, hydrophobicity, and surface hydration. As above hypothesis (**Fig. 3-5**), the SP first bound to surfaces of liposomes by electrostatic interaction and then its hydrophobic region could penetrate into membrane bilayer of liposomes based on the hydrophobic interaction. The importance of hydrophobic interaction between signal peptide and liposome membrane could be realized between the neutral and charged liposomes. Although the electrostatic interaction of chitosanase containing SP with the negatively charged POPA liposomes was more effective than that interaction with the neutral (POPC) and domain formed (POPC/Ch) liposomes, the hydrophobic interaction of SP of chitosanase with lipid membrane of liposomes could be a key for the efficiency and stability of this enzyme on liposome surfaces. In addition, the negatively charged surfaces of POPA liposomes played a role to recruit the electrostatic interaction with positively charged substrate (glycol chitosan) resulting in the increasing diffusion rate of substrate bound to the surface of chitosanase-LIPOzyme. Here, some characteristics of liposome should be clarified to give a deeper insight of its efficiency of the

catalytic potential: (i) The liposome enhanced diffusion rate of substrate bound to chitosanase-LIPOzyme resulted in the enhanced production rate. (ii) The liposome as solid surface supported for the formation of chitosanase-LIPOzyme-substrate complex. (iii) The charge, fluidity, hydration and hydrogen bond stability of liposome membrane were able to affect on the catalytic potential and the stability of chitosanase.

The hypothesis of liposomes effects on the surface displayed chitosanase activity is shown in **Fig. 3-9**. In this model, the SP (rich in positive charge and hydrophobic) could mimic its biological functions to interact with the lipid membranes of liposomes by the electrostatic and hydrophobic interactions. The chitosanase containing SP was therefore displayed stably on the liposome surfaces to carry out its enzymatic reaction. Those characteristics are very important to prepare stable chitosanase-LIPOzymes rather than coventional adsorption of mature chitosanase on liposomes. The stability of hydrogen bond interaction between chitosanase and liposomes mediated by water molecules was proposed to play very important roles for the catalytic efficiency and stability of displayed chitosanase on liposomes.

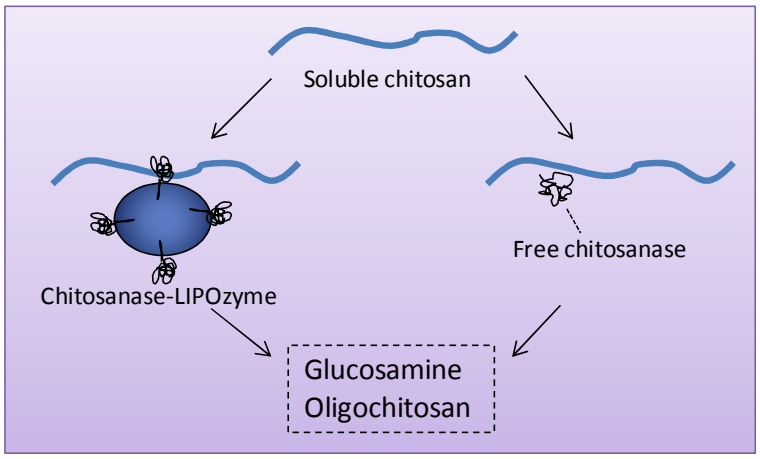


Fig. 3-9 The hypothesis of chitosanase-LIPOzyme in this study and free chitosanase catalyzing an enzymatic reaction of glycol chitosan to glucosamine and/or oligochitosans (Katsumi *et al*., 2005; Fukamizo *et al*., 1995)

4. Effect of surface hydration of liposomes on activity of their displayed chitosanase

A possible mechanism on the chitosanase-LIPOzyme formation was further investigated focusing on the role of surface hydration of the liposomes. It is found that the SP could mimic its biological functions to interact with the lipid membrane of liposomes by the electrostatic and hydrophobic interactions. These interactions were able to display chitosanases harboring SP on the liposome surfaces, consequently, stabilized the hydrogen bond interactions between chitosanases and liposome membranes mediated by water molecules.

Figure 3-10A shows the infrared frequencies of the PO_2^- antisymmetric stretching modes of PC, PA and PC/Ch in small unilamellar vesicles (SUV). In PA-SUV, the antisymmetric stretching mode had the maximum frequency at 1189.9 cm^{-1} while that value of PC-SUV is 1232.3 cm^{-1}. Incorporation of 30 mol % of Cholesterol (Ch) into PC-SUV brought about 1.9 cm^{-1} shift to lower frequency. It has been reported that antisymmetric stretching modes of dimyristoylphosphatidic acid (DMPA) and egg yolk phosphatidic acid (EPA) vesicles in the gel phase had the maximum frequency at 1173.1 cm^{-1} and 1187.2 cm^{-1}, respectively (Nabet *et al*., 1994). In the dipalmitoylphosphatidylcholine vesicle in liquid-crytalline state, this value was 1231.7 cm^{-1} and the incorporation of more than 26 mol % Ch into PC-SUV brought about 3.1 cm^{-1} shift to lower frequency (Yamamoto *et al*., 1995) because Ch increased the space around P-O bond and enhanced the hydration of PO_2^-. In fact, the infrared spectra of PO_2^- in PC, PA, and PC/Ch vesicles show the hydration of these functional groups in the different headgroups (Casal *et al*., 1987) and the lower frequency the higher hydration of PO_2^- is. Among these liposome vesicles, the hydration of PO_2^- group in PA headgroup shows the highest value. The incorporation of Ch into POPC liposome vesicles slightly increased the hydration of PO_2^- group as compared with that of pure POPC liposome. In the phosphotidylcholine bilayer the P^----N^+ dipoles align parallel to the plane of membrane surface and unesterified phosphate oxygen forming hydrogen bonds with adjacent molecules mediated by water molecules (Pearson, 1979). The influence of the surface hydration of the

lipid headgroup on the activity of hydrolysis enzymes associated-lipid membrane has also been proved (Yamamoto *et al*., 1995). In our study, the relationship between the surface hydration of various liposomal membranes and the activity of displayed chitosanases on such liposomes is shown in **Fig. 3-10B**. It seems that the activity of chitosanase-LIPOzymes was proportional to the hydration of the liposomal headgroup of PO_2^-. The highest activity was obtained in the case of chitosanase-LIPOzyme prepared by POPA liposome as 1.53 U/mg although there was not significant difference in the activity of that prepared by POPC (1.39 U/mg) and POPC/Ch (1.38 U/mg) liposomes. This observation implies the effects of surface hydration of liposomal membranes on the enhanced activity of chitosanase harboring SP bound liposomes although the membrane fluidity and surface charge of liposomes could also be playing the role affecting on the activity of the displayed chitosanase, as summarized and shown in **Table 3.2**.

Previously, the hydrophobic and electrostatic interactions of bovine myelin basic protein (MBP) with egg yolk phosphatidic acid (EPA) decreased the conformational disorder of the lipid acyl chain, resulting in the hydrogen bond network that was replaced by the penetration of MBP into EPA membrane (Nabet *et al*., 1994). The chitosanase harboring SP could mimic its biological functions in the complex with liposome by the penetration of the SP into the lipid membranes of liposomes based on electrostatic and hydrophobic interactions, resulting in the formation of hydrogen bonds between PO_2^- and water molecules that was replaced by electrostatic and hydrophobic interactions with the SP. The mature part of chitosanase was therefore immobilized stably on liposome surface to display its active center on liposomal membrane. The stability of hydrogen bond interaction between chitosanase and liposome membrane mediated by water molecules-bound PO_2^- group might reflect the effects of the hydration of lipid membrane on the enhanced hydrolysis potential of displayed chitosanase, especially, the stability of chitosanase-LIPOzymes under stress conditions such as the heat stress and acidic and/or alkaline conditions.

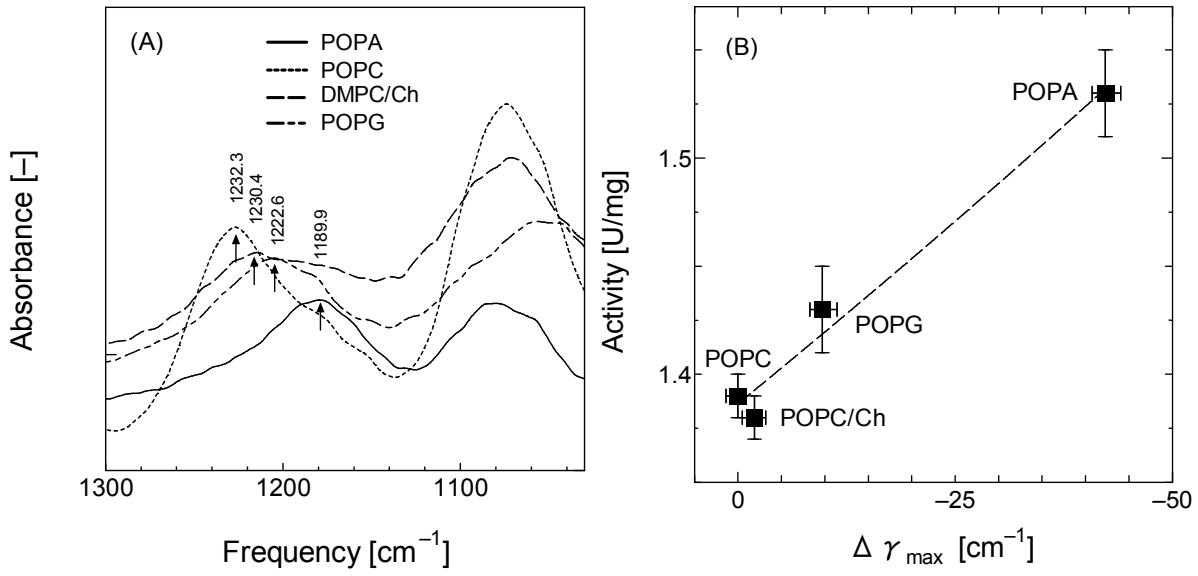


Fig. 3-10 Infrared spectra of various liposomes for the PO_2^- antisymetric stretching bands at room temperature (A) and the relationship between activity of chitosanase-LIPOzymes and the $\Delta\gamma_{max}$ (B). The antisymmetric stretching band shift ($\Delta\gamma_{max}$) is normalized by the subtraction of typical infrared frequency of PO_2^- of each liposome to that of POPC liposome. The enzymatic reaction and activity measurement were carried out similarly to the conditions described in **Fig. 3-9** and in the experimental section

Table 3-3 Summarize the effects of surface hydration, membrane fluidity and surface charge of liposomes on the activity of chitosanase-LIPOzymes

Type of liposome	Hydration ($\triangle\gamma_{max}$) [cm^{-1}]	Membrane Fluidity ($1/P^{TMA\text{-}DPH}$) [-]	Charge (*Z* potential) [-]	Chitosanase Activity [U/mg]
POPA	-42.32±1.7	5.70±0.7	-1	1.53±0.01
POPG	-9.73±1.7	5.10±0.4	-1	1.43±0.01
POPC	0.00±0.05	4.80±0.4	0	1.39±0.02
POPC/Chol	-1.91±1.3	2.50±0.4	0	1.38±0.02

+ Fluidity of liposome ($1/P$) was measured by using TMA-DPH lipid probe as the description in our previous study (Ngo *et al*., 2008[a])

+ Charge of liposome: liposome unit was defined its *Z* potential at negative (Z = -1), neutral (Z = 0), and positive (Z = +1)

5. Stability of chitosanase-LIPOzyme under stress conditions

In order to clarify the role of signal peptide (SP) on the stability of chitosanase-LIPOzymes under different biophysical conditions such as pH and temperatures, the activities of various kinds of chitosanase-LIPOzyme and that of mature chitosanase (control) coincubated with without liposomes were comparatively investigated.

The activities of various kinds of chitosanase-LIPOzymes and that of mature chitosanase under different heat and pH conditions are shown in **Fig. 3-11**. As shown in **Fig. 3-11A**, the activities of all kinds of chitosanase-LIPOzymes and that of the control were dependent on the temperature shifts. The activities of these chitosanase-LIPOzymes were significantly higher than that of the control at all temperatures. The activities of chitosanase-LIPOzymes at optimal temperature (45 ^{o}C) were more than twice higher than that of the control. Especially, under the heat stress (55~65 ^{o}C), the activities of these chitosanase-LIPOzymes were highly maintained, whereas the activity of the control enzyme was significantly lost its activity. As shown in **Fig. 3-11B**, the activities of various kinds of chitosanase-LIPOzymes and that of the control under various pH conditions were comparatively performed. The activity of the control was significantly dependent on the pH in the range of 2.3 to 10.2 while that of chitosanase-LIPOzymes was much more stable. The activities of all kinds of chitosanase-LIPOzymes at pH conditions (2.3~10.2) were significantly higher than that of the control. Especially, under acidic (pH 2.3) and alkaline (pH 10.2) conditions, the activities of chitosanase-LIPOzymes prepared by POPA, POPC and POPC/Ch liposomes were almost maintained their activities that were significantly higher than that of mature chitosanase, which were almost lost the activity under such extreme pH conditions. These observations imply that chitosanase containing SP associated-lipid membrane of POPA, POPC and POPC/Ch liposomes shows higher in the efficiency and stability of hydrolysis potential in comparison with that of the mature chitosanase, especially, under the extreme heat and pH conditions.

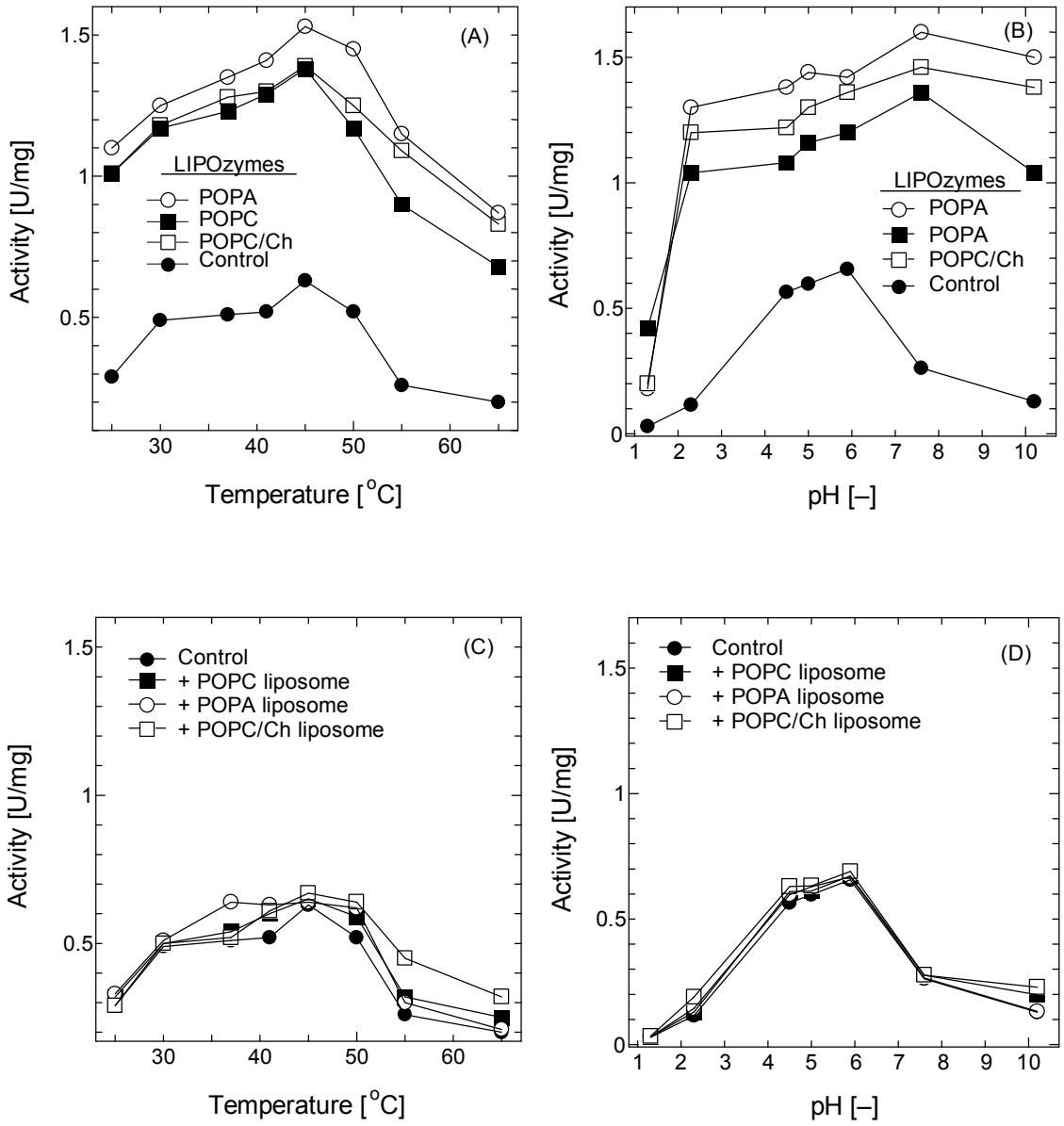


Fig. 3-11 Effect of different heat and pH conditions on the activity of chitosanase-LIPOzymes (A, B) and that effect on the activity of mature chitosanase coincubated with without liposomes (C, D). The enzymatic reaction and activity measurement were carried out similarly to the conditions described in **Fig. 3-9** and in the experimental section

The above results might show the roles of SP in the complex of chitosanase with liposome membrane. To further verify the role of the SP, the activities of the mature chitosanase (without SP) coincubated with without liposomes were comparatively investigated under various heat and pH conditions. As shown in **Fig. 3-11C** and **Fig. 3-11D**,

there was not significant difference in the activities of the mature chitosanase coincubated with without POPA, POPC and POPC/Ch liposomes under given heat and pH conditions although slight effects of liposomes on the activity of this enzyme could be observed under the heat stress condition (**Fig. 3-11C**). These observations confirm that the protein/lipid complex between mature chitosanase and liposomes was not stable just based on the conventional absorption of the liposomes to stabilize its activity and conformation under the heat and extreme pH conditions. Thus, the association of SP with lipid membranes of liposomes by electrostatic, hydrophobic and hydrogen bond interactions was playing crucial roles for the enhanced activity and the stability of chitosanase on liposome surfaces.

The key factor to stabilize the conformation of protein is hydrophobic interaction between hydrophobic residues inside protein. The quantitative analysis of the hydrophobic interaction has been divided into two terms (i) the changes in the hydration free energy and (ii) the conformational free energy; both are strongly dependent on the types of amino acid residues (Ooi, 1994). The hydration is essential feature in the native conformation of a protein in aqueous solution. The hydrophilic residues located at the molecular surface and the hydrophobic residues located inside to stabilize the conformation of protein. The loss in hydration free energy; and the gain in conformational free energy were produced by non-bonded interaction between dehydrated atomic groups. In one model, a hydration shell is formed based on the interaction of protein with water molecules in the first hydration layer surrounding the protein (Ooi *et al*., 1987). The interactions of protein with water molecules in the first hydrated layer interfere with the water structure of the second and further remote layers, resulting in the changes in free energy (Ooi *et al*., 1987). It has also been found that charge-charge interactions are attractive at all pH values where the protein unfolds. The temperature and pH dependences in these interactions contribute to acid denaturation of protein but the other effects such as hydrogen bonding and solvation of protein are important as well (Yang *et al*., 1993). Based on their suggestions, it is possible that the stability of

chitosanase on the surface of liposomes under stress conditions is dependent on the stability of the hydration shell surrounding chitosanase on liposome surface mediated by hydrogen bond interaction of such water layer with the PO_2^- headgroup of liposomes. These hydrogen bond interactions were strengthened by the association of the SP of chitosanase with lipid membrane of liposome through the electrostatic and hydrophobic interactions but it was not available between mature chitosanase (without SP) and liposomes. Furthermore, the electrostatic contributes in the unfolding of protein are estimated to be relatively small with several kcal/mol near neutral pH. The electrostatic energy contributes to the enthalpy term in folding or unfolding free energy; and its contributions to the entropy term may occur indirectly through a change in hydration on ionization (Ooi, 1994). The thermal stability of enzymes was remarkably increased after being entrapped inside liposomes prepared by phosphatidylcholine (Wang *et al*., 2003; Han *et al*., 1998). The immobilization efficiency of chymotripsin on phosphatidylcholine liposomes was thermally dependent on the hydrophobic interactions between hydrophobic parts of enzymes and hydrophobic tails of phospholipids in liposomes (Durour *et al*., 1996). These may show the importance of hydrophobic, electrostatic, and hydrogen bond interactions on the stability of the protein/lipid complex.

6. Possible model of chitosanase containing signal peptide interacting lipid membrane of liposomes

A possible hypothesis of chitosanase-LIPOzymes is shown in **Fig. 3-12**. The key factor promoting the association of chitosanase harboring SP with lipid membranes of liposomes is SP because it can mimic its biological function to interact with liposomes effectively by electrostatic and hydrophobic interactions. This interaction of chitosanase harboring SP with liposomes is important to display chitosanase on surface of liposomes. The displayed chitosanase exposes its active center on liposome surface to catalyze the enzymatic reaction. The active center of chitosanase involves two amino acid residues of aspartic acid (Asp40)

and glutamic acid (Glu22) (Fukamizo *et al*., 1997; 2000, Katsumi *et al*., 2005, Yoon *et al*., 2001, Honda *et al*., 1999, Tremblay *et al*., 2001). The tryptophan and arginine residues have been reported to play important roles for catalytic activity and thermal stability of chitosanase molecules of *Streptomyces* sp. (Fukamizo *et al*., 1997; 2000, Honda *et al*., 1999). It is also possible that the positively charged arginine residue can interact with lipid membrane of liposomes, especially, with anionic liposomes by the electrostatic interaction.

It is well known that the fluorescence behavior of tryptophan residues is dependent on the dispersed dielectric constant environment of tryptophan. The fluorescence behavior of tryptophan in various dielectric constant environments prepared by dioxane/water mixture is shown in **Table 3-4**. The dielectric constant of water is approximate 78 and that of liposomal interface and hydrophobic region of lipid bilayer of liposomes is approximate 40 and 2~4, respectively. The intrinsic fluorescence of tryptophan in chitosanase of *S. griseus*

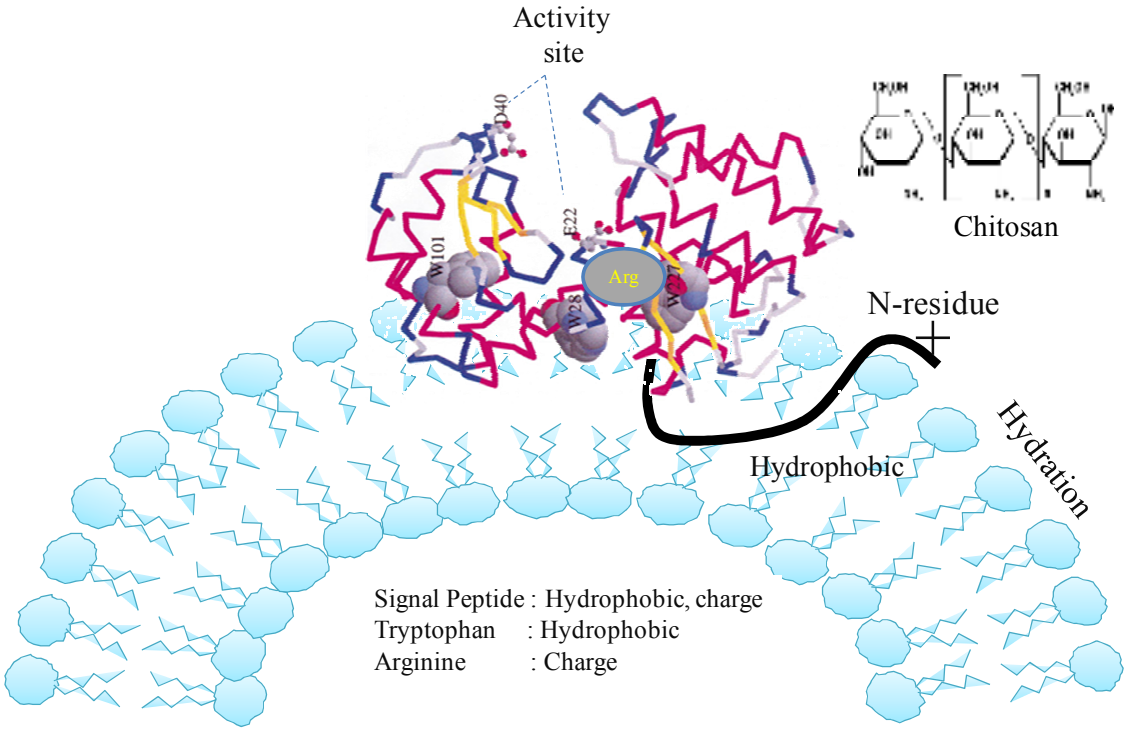


Fig. 3-12 Hypothesis of chitosanase harboring SP displayed on surface of liposomes for enzymatic activity of chitosanase-LIPOzymes

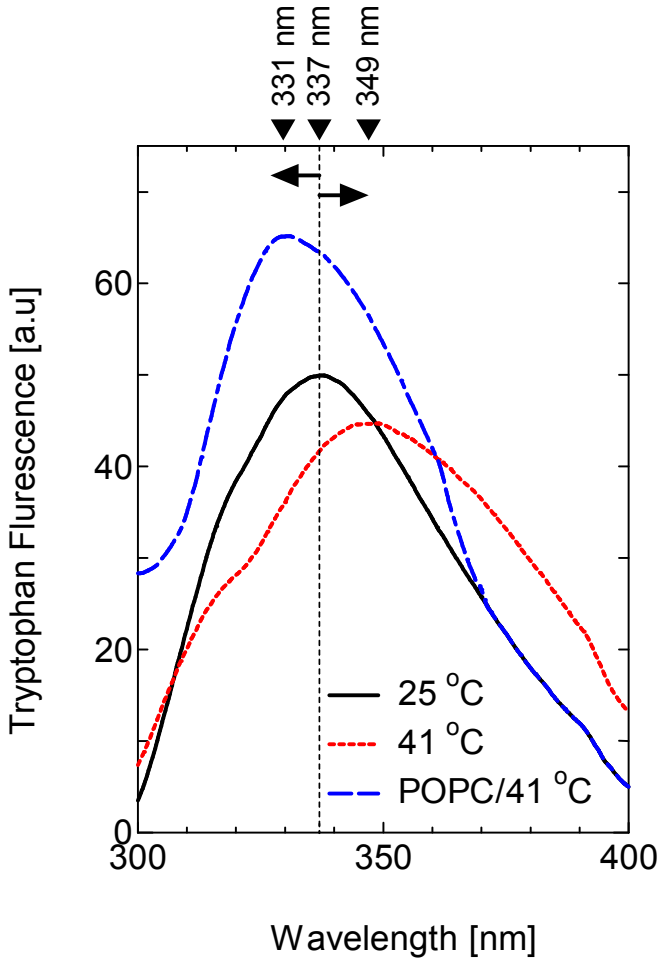


Fig. 3-13 Fluorescence of tryptophan residues in chitosanase of *S. griseus* under various heat conditions in the presence and absence of POPC liposomes

Table 3-4 Fluorescence behavior of tryptophan residues in various dielectric constant media

Dielectric constant (ε) Dioxane/water medium	Maximum wavelength (nm) of tryptophan fluorescence
2	334
10	343
18	346
31	347
40	349
78	352

co-incubated with and without POPC liposomes is shown under various temperatures in **Fig. 3-13**. The result shows that the maximal wavelength of tryptophan fluorescence (λ_{max}) in

chitosanase is 337 nm at 25 ^{o}C. The heat treatment at 41 ^{o}C increased λ_{max} to 349 nm in the absence of liposomes; whereas this value is 331 nm at 41 ^{o}C in the presence of POPC liposomes. This observation shows that the heat treatment at 41 ^{o}C changed the conformation of chitosanase, resulting in tryptophan residues that are exposed to the aqueous phase, consequently, the enhanced λ_{max} of tryptophan. In the presence of POPC liposomes under such heat condition, the interaction between chitosanase and liposomes, based on the hydrophobic interaction, resulted in burying the tryptophan residues in hydrophobic region of liposome membranes to cause the reduction of λ_{max} of tryptophan (331 nm) that is corresponding to λ_{max} of tryptophan in environment with dielectric constant (ε=2~4). The above results support the hypothesis shown in **Fig. 3-12**.

7. Physcial stability and lifetime of chitosanase-LIPOzymes at stored conditions

After the preparation of chitosanase-LIPOzymes, the physical structure of liposomes used to prepare such LIPOzymes was also assayed by using quart crystal microbalance (QCM). As shown in **Fig. 3-14**, the adsorption of intact POPC vesicles used to fabricate chitosanase-LIPOzyme on gold electrode surface caused the reduction of the frequency to -150 Hz, which well corresponds to the increasing of adsorbed mass of intact vesicles (monolayer) on gold electrode. The stability of these vesicles on surface of gold electrode implies that liposomes were still intact structure after the interaction with chitosanase containing SP. In order to confirm whether these vesicles are really intact form on gold electrode, the triton X-100 (1% as final concentration) was added to disrupt the intact vesicle. The result shows in **Fig. 3-14** that the lipid bilayer adsorbed on surface of gold electrode reduced the frequency to approximate -50 Hz, which well corresponds to absorbed mass of lipid bilayer. These observations confirm that the prepared chitosanase-LIPOzyme maintained their intact structure after the interaction with chitosanase containing SP. The association of SP with the lipid membrane of liposomes based on the electrostatic and hydrophobic

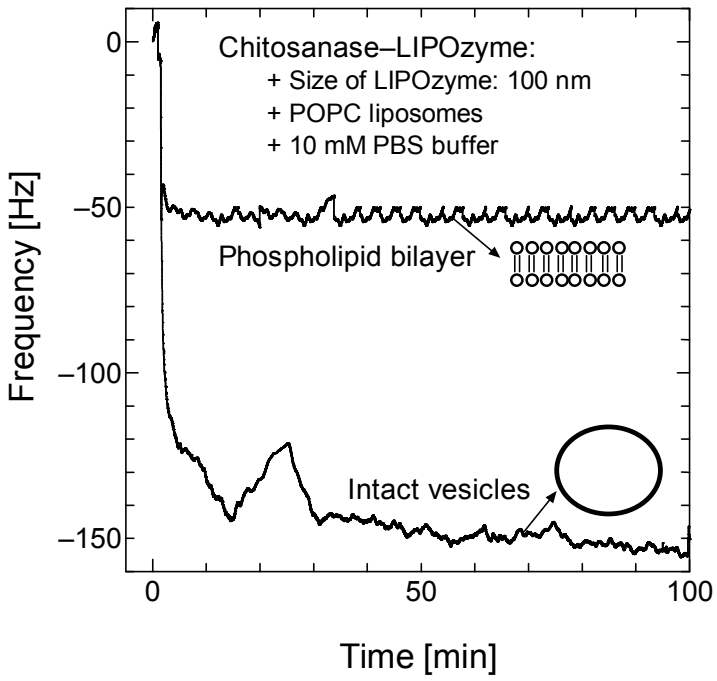


Fig. 3-14 Physical stability of chitosanase-LIPOzyme prepared by POPC liposome using quart crystal microbalance (QCM) technique

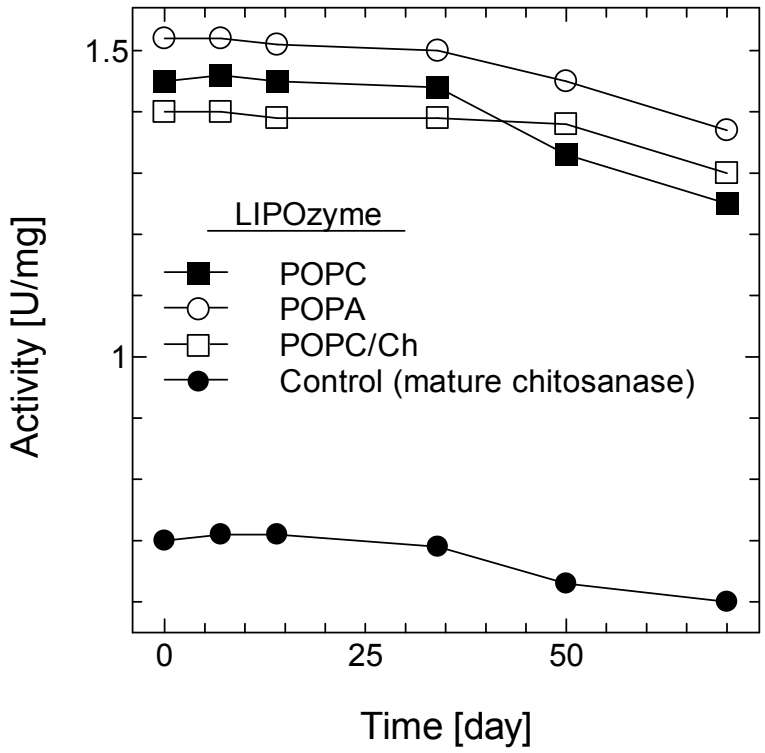


Fig. 3-15 Time dependency of activity of chitosanase-LIPOzymes prepared by *Streptomyces griseus* and various kindes of liposomes stored at 4 ^{o}C. Chitosanase and its LIPOzymes were kept in 10 mM PBS buffer at 4 ^{o}C in refrigorator

interactions did not cause rupture of liposomes.

The frequency shift (ΔF) using QCM is dependent on the absorbed mass (Δm) onto surface of gold electrode. According to Sauerbrey's equation $\Delta F = - \Delta m/c$; where c is sensitivity of frequency shift constant (1.77 ng/cm^2). The frequency shift is herewith used to evaluate the mode of liposome adsorption onto gold electrode.

The stability of chitosanase-LIPOzymes stored at 4 ^oC was also investigated by measuring their chitosanase activities in the time dependence. As shown in **Fig. 3-15**, chiosanase-LIPOzymes prepared by various kinds of liposomes such as POPC, POPA, and POPC/Ch liposomes dissolved in PBS buffer showed higher activity than that of the control. The chitosanase activity of all LIPOzymes was highly maintained within 75 days kept in PBS buffer at 4 ^oC although their activity was mildly reduced after storage of 34 days. This tendency of chitosanase-LIPOzymes is similar to that of the control (mature chitosanase). This investigation is meaningful to understand the stability of chitosanase activity of the stored chitosanase-LIPOzymes at low temperature.

8. Application of chitosanase-LIPOzymes to produce oligochitosan

The optimal conditions for the highest activity of mature chitosanase and various kinds of chitosanase-LIPOzymes are summarized in **Table 3-5** such as the temperature, pH and ratio of enzyme-substrate (E/S). The merits of all kinds of chitosanase-LIPOzymes show the wider range of temperature and pH and lower ratio E/S (5 %) required to optimize enzymatic activity, compared to restricted conditions of temperature, pH and higher ratio of E/S (7 %) that were required for the optimal conditions of mature chitosanase.

The enzymatic reaction using various kinds of chitosanase-LIPOzymes to digest glycol chitosan to oligochitosans was carried out as the description in experimental section. Then its products were analyzed by RP-HPLC (Jeon *et al*., 2000). As shown in **Fig. 3-16**, the molecular weight distribution of oligochitosans (P_1, P_2, P_3, P_4, and P_5) after enzymatic

Table 3-5 Summary the optimal conditions for the activity of chitosanase-LIPOzymes

Type of LIPOzyme	Temperature [^{o}C]	pH [-]	Ratio (E/S) [w/w]
POPC	45±5	2.3—10.2	0.05
POPC/Ch	45±5	2.3—10.2	0.05
POPA	45±5	2.3—10.2	0.05
Control	45±2	5.5 ±1	0.07

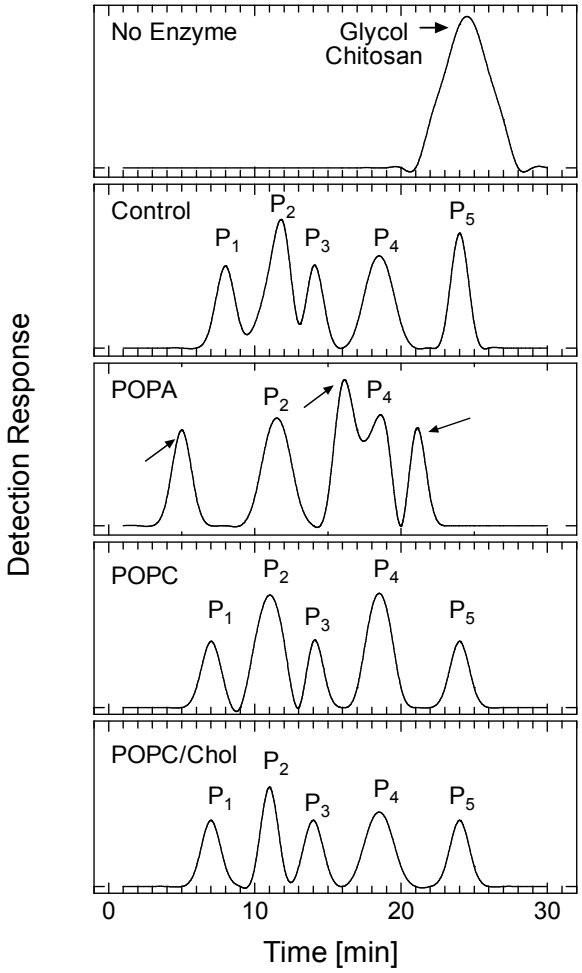
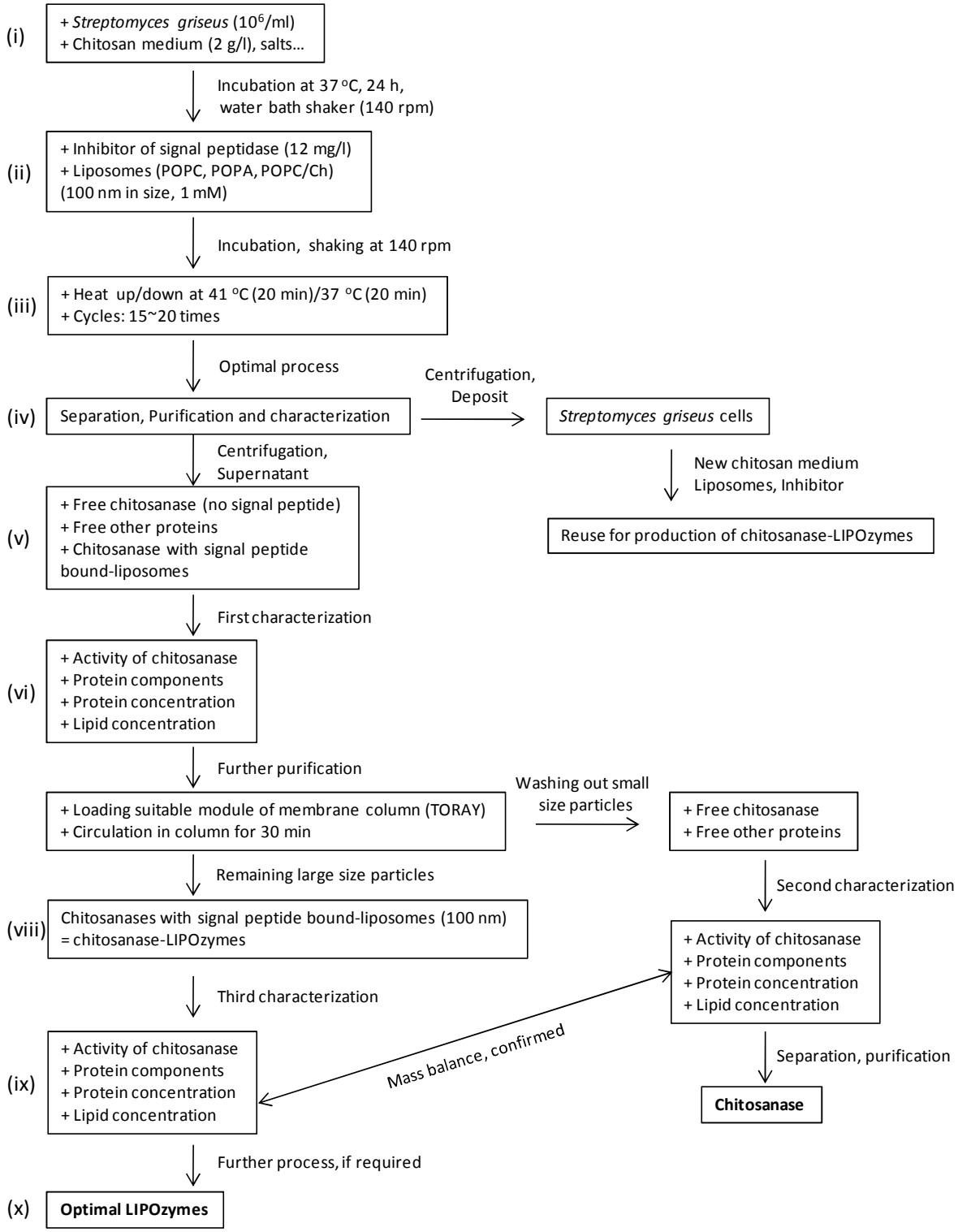


Fig. 3-16 P-HPLC analysis of oligochitosan formation from glycol chitosan catalyzed by mature chitosanase (control) and chitosanase-LIPOzymes. The enzymatic reaction for production of oligochitosan was described in experimental section. The operation of RP-HPLC was performed on the TSK gel NH_2-60 column (4:6 x 250 mm) with mobile phase (acetonitrile 60 %, distilled water 40 %), temperature of column at 45 ^{o}C, flow rate at 1 ml/min.

reaction of chitosanase-LIPOzymes prepared by POPC, POPC/Ch liposomes was almost similar to that obtained by mature chitosanase (control). However, the oligochitosans formed in an enzymatic reaction by chitosanase-LIPOzyme prepared by POPA liposomes showed three different oligochitosans (arrow marks) and two similar ones (P_2 and P_4). The conversion efficiency of glycol chitosan to oligochitosans was 100 % in case of chitosanase-LIPOzyme prepared by POPA liposome although that was 75 % and 73 % in the case of chitosanase-LIPOzymes prepared by POPC and POPC/Ch liposomes, respectively, after 1 h of enzymatic reaction, as compared to that of the control (55 %). These results imply that the conversion efficiency of chitosanase-LIPOzymes could be dependent on the types of liposomes used to prepare chitosanase-LIPOzymes. Reasonably, the characteristic of different liposomes such as charge, fluidity, hydrophobicity and hydrogen bond stability would βken into account to design chitosanase-LIPOzymes for the production of target oligochitosans. In this study, three kinds of liposomes such as negatively charged (POPA), neutral (POPC) and domain formed (POPC/Ch) liposomes were utilized to design chitosanase-LIPOzymes. The difference in charge, membrane fluidity and surface hydration of these liposomes (**Table 3-3**) might show their influences on the activity of displayed chitosanase although the specific production of the target oligochitosans using different chitosanase-LIPOzymes should be dedicated to be studied on the different liposomes.

Summary

Chitosanase-LIPOzymes could be prepared based on the membrane-membrane interaction specific heat induced secretion of chitosanase containing SP associated-lipid membrane of the liposomes interacting onto cell membranes of *S. griseus* cell under specific heat condition, as summarized in **Scheme 2**. The optimal conditions for the treatment of *S. griseus* cells were first established such as concentration of inhibitor of type I SPase, optimal



Scheme 2 Preparation and characterization of chitosanase-LIPOzymes by membrane-membrane interaction induced the secretion of chitosanase containing SP associated lipid membrane of liposomes interacting onto cell membranes of *S. griseus* cells pretreated with inhibitor of type I SPase under the specific heat condition

conditions of heat and liposomes treatment. The optimal concentration of inhibitor (amoxicillin) was chosen at 12 mg/l to inhibit the activity of bacterial signal peptidase type I of *S. griseus* cells after an incubation of 24 h at 37 ^{o}C. The heat treatments at (41 ^{o}C, 20 min) and (37 ^{o}C, 20 min) were used to treat *S. griseus* cells after treatment with inhibitor and various kinds of liposomes. These heat treatments were continuously applied to such *S. griseus* cells 15~20 times for each temperature at 37 or 41 ^{o}C to induce the membrane-membrane interaction of liposomes with cell membranes resulting in the secretion of chitosanase containing SP associated lipid membrane of liposomes interacting onto cell membrane for preparation of chitosanase-LIPOzymes.

Characterization of chitosanase containing SP displayed on the surface of liposomes was based on the protein molecular weight, chitosanase activity, together with its catalytic efficiency and stability on liposome surface under extreme heat and pH conditions. The dependence of the activity of displayed chitosanase on the surface hydration of liposomes is also subjected to be investigated.

SP of chitosanase belongs to type I SP, which contains 53 amino acids and three distinct domains (i) the H-domain (rich in positive charge) can electrostatically bind to negatively charged lipid membrane or translocation machinery; (ii) the N-domain (rich in hydrophobicity) can insert into hydrophobic region of cell lipid membrane and has cleaving site for the degradation by specific signal peptidase (SSPase); (iii) C-domain (hydrophilic domain) contains the cleaving site for signal peptidase type I (SPase I) to cleave SP from mature chitosanase.

The characterization of protein molecular weight of chitosanase-LIPOzymes shows the existence of protein with approximate 39 kDa, which has chitosanase activity and its molecular weight corresponds to the sum of SP and mature chitosanase (38.8 kDa). It is therefore confirmed that the chitosanase-LIPOzymes could be prepared by the secretion of chitosanase containing SP associated-lipid membrane of liposomes interacting onto cell

membranes of *S. griseus* cells under the heat stress condition. The number of chitosanase molecules displayed per one liposome was relatively estimated as 4~5 molecules and this value was almost similar among LIPOzymes prepared by POPC, POPA, and POPC/Ch liposomes.

The SP plays important roles to stably display its bound-chitosanase on liposome surfaces by mimicking its biological function in the association with the lipid membranes of liposomes by electrostatic and hydrophobic interactions. The catalytic efficiency of chitosanase-LIPOzymes is significantly higher than that of the conventional chitosanase. The maximum conversion velocity (V_{max}) of chitosanase-LIPOzymes prepared by POPA, POPC, and POPC/Ch liposomes is $12.4x10^{-10}$, $7.1x10^{-10}$, and $5.8x10^{-10}$ M/s, respectively, as compared with that of conventional chitosanase ($3.9x10^{-10}$ M/s). The efficiency of catalytic rate (k_{cat}/K_m) of all chitosanase-LIPOzymes is almost 2 to 5 times higher than that of the conventional chitosanase. The k_{cat}/K_m of chitosanase-LIPOzymes prepared by POPA liposomes reaches steady state of enzyme-substrate complex formation and the breaking down into enzyme and product at $1.6x10^{9}$ 1/M.s while those values prepared by POPC and POPC/Ch liposomes are $6.9x10^{8}$, and $6.2x10^{8}$ 1/M.s, respecitively, corresponding to the equilibrium state of enzyme-substrate complex formation. The k_{cat}/K_m value of conventional chitosanase is $2.9x10^{8}$ 1/M.s.

The catalytic efficiency of chitosanase containing SP displayed on liposomes is significantly dependent on the surface hydration, membrane fluidity and surface charge of liposomes. The catalytic activity of displayed chitosanase is higher on the liposomes, which have higher surface hydration, higher membrane fluidity and negative charge. For the neutral liposomes, the activity of displayed chitosanase seems to be dependent on the membrane fluidity and surface hydration although it is not clearly different in this study.

The stability of chitosanase-LIPOzymes prepared by POPA, POPC, and POPC/Ch liposomes under extreme heat and pH conditions is significantly higher than that of the

conventional chitosanase even with or without liposomes. This result shows the effects of SP that can associate with liposomal membranes by the electrostatic and hydrophobic interactions to stabilize the surface hydration of displayed chitosanase through hydrogen bond interaction mediated by water molecules between chitosanase and liposome surfaces.

Furthermore, chitosanase-LIPOzymes show higher in catalytic efficiency and stability as compared with that of the conventional chitosanase under extreme pH and heat conditions. The conversion of chitosan to specifically targeted oligochitosans by chitosanase-LIPOzymes can be regulated by modification of surface properties of liposomes such as surface charge, domain and surface hydration. Based on the findings in this study as well as those in the previous studies (Fukamizo *et al*., 1997; 2000, Katsumi *et al*., 2005, Yoon *et al*., 2001, Honda *et al*., 1999, Tremblay *et al*., 2001), the possible molecular mechanism of chitosanase harboring SP displayed on the surface of liposome is hypothesized. In this hypothesis, the SP, tryptophan (W28, W110, W227) and arginine (Arg205) are proposed to play important roles to interact with liposomal membranes for the stability and efficiency of chitosanase under extreme pH and heat conditions.

General Conclusions

The membrane-membrane interaction between liposomes and cell membranes of *S. griseus* cell induced by heat stress plays important role for the enhanced production and secretion of chitosanase from *S. griseus* cells. The interaction between liposomes and cell membrane can be effectively controlled by stress induced the variation of the surface properties of cell membranes and liposomes such as surface net charge and hydrophobicity, and surface hydration, resulting in the eleactrostatic, hydrophobic and hydrogen bond interactions between them. The interactions between liposomes and cell membrane under heat stress condition not only enhance the production and secretion of chitosanase from *Streptomyces griseus* cells but can also be utilized to induce the secretion of chitosanase containing SP associated-liposomes to produce chitosanase-LIPOzymes, which express higher activity and stability under stress conditions as compared with that of the conventional chitosanase.

In chapter I, the interaction of liposomes with target chitosanase and cell membrane under heat, and oxidative stress condition plays a key role for the enhanced cell growth, production and secretion of chitosanase from *S. griseus* cells. In *in vivo* experiments, it has been found that the heat treatment enhances the growth and production of chitosanase of *S. griseus* treated without or with POPC liposomes to 1.4 and 2.0 times higher than those of the control. The heat treatment on *S. griseus* cells mainly enhances the production rate of chitosanase but co-treatment of these cells by liposomes and heat stress enhances both cell growth rate and production rate of chitosanase. The secretion of chitosanase through the cell membrane of *S. griseus* cells cultivated under the heat stress at 41 ^{o}C without and with POPC liposomes is 1.8 and 2.2 times higher those of the control. In *in vitro* experiment, the membrane-membrane interaction of the neutral POPC liposomes with lipid mimicking cell membrane (LMCM) liposomes under the heat stress such as 41 ^{o}C induces the secretion of entrapped chitosanase

through protein bound-lipid membrane of LMCM liposomes to 17% of initial entrapped chitosanase, showing a possible way of protein secretion through protein bound-lipid membrane.

The membrane-membrane interaction between liposomes and cell membranes is considered as a key for the cell growth and production of target chitosanase under oxidative stress condition. Although cell growth and production of chitosanase are significantly reduced immediately after the addition of 5 mM H_2O_2 as compared with the control, these cells pretreated with the heat stress at 41 ^oC and POPC liposomes enhance the growth and production of chitosanase higher than that of the cells cultivated at only 37 ^oC (control). Several effects of liposomes to prevent the damage of oxidative stress on the cell growth as well as the target chitosanase were systematically investigated. The CD spectra measurement shows that the conformation of chitosanase is stabilized by interacting with POPC liposomes under oxidative stress condition. The susceptibility of chitosanase, cell lipid membrane and its proteins under oxidative stress condition in the presence and absence of POPC liposomes was also characterized. The results clearly show that POPC liposomes can significantly prevent the lipid and protein peroxidation of cell membrane of *S. griseus* under oxidative stress condition. This effect of liposomes once more confirms the importance of membrane-membrane interaction for the enhanced production and secretion of chitosanase by *Streptomyces griseus* cells under heat and/or oxidative stress conditions as well as possible bridge of this fundamental phenomenon to the understanding of UV irradiation of the enhanced production of iturin A by *Bacillus subtilis* mutants. The addition of liposomes into the fermented broth of these mutants is expected to enhance the cell growth and production of iturin A after or during UV irradiation treatment on wild-type *B. subtilis*.

In chapter II, the interaction between liposomes and cell membrane under stress conditions can be controlled by electrostatic, hydrophobic, and hydrogen bond interactions. The strategy to control these interactions is based on the characteristics of the surface

properties of *S. griseus* cell and those of liposomes that can be varied by the treatment of specific heat, pH, and oxidative stress. The results show that the surface net charge of *S. griseus* cell is varied depending on the treatment of cell by pH and oxidative stress rather than the heat. The surface net hydrophobicity (*HFS*) of this cell is significantly varied depending on the treatment of heat and oxidative rather than pH. The *HFS* of this cell is increased by increasing temperatures. For example the *HFS* values at 25 and 41 ^{o}C are -220 and -90 kJ/mol, respectively. The *HFS* of *S. griseus* cells pretreated with the neutral POPC liposomes at different temperatures was also characterized. The *HFS* of these cells at 25 and 41 ^{o}C in the presence of POPC liposomes are -231 and -510 kJ/mol. This result shows the interaction of POPC liposomes (*HFS*~410 kJ/mol) with cell membranes resulted in the variation of the cell surface from hydrophobic surface to more hydrophilic surface. The reduction of *HFS* of these cells treated with liposomes by increasing temperature implies that the heat plays important role to induce the interaction of neutral POPC liposomes with cell membrane.

The surface hydration of *S. griseus* cell is significantly dependent on the treatment of the heat, pH and oxidative condition. The treatment of heat stress (41 ^{o}C) and extreme pH (strong acidic and alkaline) causes the loss of water of cell membrane surface. As the consequence, the loss of water of the cell membrane might contribute to the mild increase of hydrophobicity as well the destabilization of hydrogen bonds network mediated by water molecules on the cell membrane. The cell lipid membrane is very susceptible with the treatment of oxidative stress. The oxidative stress significantly damages the lipids of cell membrane but the interaction between POPC liposomes and cell membrane can prevent the oxidative stress damage on cell membrane as the evaluation by FTIR using both spheroplast *S. griseus* cell and LMCM-GUV.

It is therefore considered that the interaction between liposomes and cell membrane can be controlled based on the surface characteristics of both liposomes and cell membrane by considering the driving force for their interaction. As a case study, the interaction between the

neutral POPC liposomes and cell membrane is shown to be effective under the specific heat conditions with specific *HFS*, and hydration of cell membrane and liposomes surface. The internalization of neutral POPC lioposomes can be controlled by the treatment of specific heat resulting in the interaction of these liposomes such as the adsorption, fusion with the cell membrane to induce the membrane curvature of *S. griseus* for the endocytosis of these liposomes. The heat effects on this process is also elucidated by variation of cell surface properties such as the enhanced surface net hydrophobicity and destabilization of hydrogen bond caused and the driving force induced such interactions is based on the heat induced the loss of water on the cell membrane. As a consequence, the interaction between neutral liposomes and cell membrane can occur at various modes such as adsorption, fusion, and internalization by hydrophobic and hydrogen bond interactions rather than the electrostatic interaction that is conventionally considered to control membrane-membrane interaction. The quantitative factors of surface characteristics of both cell membrane and liposomes such as surface net hydrophobicity (*HFS*), surface hydration, and charge (*Z*) are very important to design an effective process for preparation of chitosanase-LIPOzymes shown in next chapter.

In chapter III, the importance of membrane-membrane interaction induced by the heat stress can also be utilized to design a process for production of chitosanase-LIPOzymes based on the specific heat induced secretion of chitosanase containing SP associated-lipid membrane of the liposomes interacting onto cell membrane of *S. griseus*. The most effective conditions based on the fundamental investigation in chapter I and II can be strategically applied to produce these LIPOzymes. The interaction between liposomes shows in three modes such as the adsorption, fusion, and internalization of neutral liposomes. For the effective production of chitosanase-LIPOzymes from *S. griseus* cells, the controlling the adsorption of neutral liposomes with cell membranes by the treatment of specific heat is considered to be a key factor although the fusion and internalization of these liposomes also contribute to the enhanced production and secretion of chitosanase from *S. grieus* cell, as shown in chapter I,

and II. The interaction of neutral liposomes with cell membrane depends on the variation of *HFS* and surface hydration of cell membrane and liposomes rather than surface net charge under heat stress conditions. It is therefore shown that the controlling of membrane-membrane interaction for the preparation of chitosanase-LIPOzymes is mainly based on *HFS*, and surface hydration of cell membrane and liposomes under different temperatures.

In addition, the secretion of entrapped chitosanase through the lipid membrane of LMCM liposomes induced by the membrane-membrane interaction under specific heat conditions mentioned in the chapter I might show a possible way of chitosanase secretion through protein-bound lipid membrane pathway. Here, the strategy to prepare the effective chitosanase-LIPOzymes is based on the interaction of liposomes with chitosanase containing SP because the SP can mimic its biological functions to associate with the lipid membrane of liposomes via the electrostatic and hydrophobic interactions.

The chitosanase-LIPOzymes prepared by various kinds of liposomes including negatively charged, neutral, and domain formed liposomes show significantly higher in catalytic efficiency and stability under extreme heat and pH conditions. For example, the maximum conversion velocity (V_{max}) of chitosanase-LIPOzymes prepared by POPA, POPC, and POPC/Ch liposomes is 12.4×10^{-10}, 7.1×10^{-10}, and 5.8×10^{-10} M/s, respectively, as compared with that of conventional chitosanase (3.9×10^{-10} M/s). The efficiency of catalytic rate (k_{cat}/K_m) of all chitosanase-LIPOzymes was almost 2 to 5 times higher than that of conventional chitosanase. The k_{cat}/K_m of chitosanase-LIPOzyme prepared by POPA liposomes reaches steady state of enzyme-substrate complex formation at 1.6×10^{9} 1/M.s while those values prepared by POPC and POPC/Ch liposomes are 6.9×10^{8}, and 6.2×10^{8} 1/M.s, respectively, corresponding to the equilibrium state of enzyme-substrate complex formation. The k_{cat}/K_m value of conventional chitosanase is 2.9×10^{8} 1/M.s.

The catalytic efficiency of chitosanase containing SP displayed on liposome surface

shows to be significantly depended on the surface hydration, membrane fluidity and surface charge of liposomes. The catalytic of activity chitosanase-LIPOzymes is higher with the liposomes those have higher surface hydration, higher membrane fluidity and negative charge. For the neutral liposomes, the activity of displayed chitosanase seems to be dependent on the membrane fluidity and surface hydration although it was not clearly different in this study.

The stability of chitosanase-LIPOzyme prepared by POPA, POPC, and POPC/Ch liposomes under extreme heat and pH conditions is significantly higher than that of conventional chitosanase even with or without liposomes. This result shows the effects of SP that can associate with liposomal membrane by electrostatic and hydrophobic interactions to stabilize the surface hydration of chitosanase through hydrogen bond interaction, mediated by water molecules between chitosanases and liposome surfaces.

The molecular mechanism of chitosanase harboring SP displayed on liposomes is also hypothesized. The catalytic center of chitosanase-involved aspartic acid (Asp40) and glutamic acid (Glu22) and Glu22 plays a role for proton donor and Asp40 activates water to attack C1 carbon of the substrate sugar chitosan. The most important factor to display this chitosanase on surface of liposome is SP that can mimic its biological function to associate with liposome membrane based on the hydrophobic and electrostatic interaction. Other characteristics of chitosanase might also be considered to design the effective chitosanase-LIPOzymes such as tryptophan and arginine residues those can also play a role in hydrophobic and electrostatic interaction with liposomes, especially, under extreme pH and heat conditions.

Finally, chitosanase-LIPOzymes show higher in catalytic efficiency for the conversion of chitosan to biofunctional oligochisans as compared with that using conventional chitosanase. The specific conversion of chitosanase-LIPOzymes can be regulated by modification of liposomes used to prepare LIPOzymes. It is therefore that the chitosanase-LIPOzymes have very big potentials to produce biofunctional oligochitosans for applications for foods, medicines, cosmetics and so on.

Suggestions for Future Works

To extend the finding obtained in this work, the following studies are suggested.

(1) Further characterization of chitosanase harboring signal peptide (SP)

As I have shown some evidences for the existence of chitosanase containing SP bound liposomes to form chitosanase-LIPOzymes in chapter III. However, because of their merits, it is necessary to verify whether the secreted chitosanase bound liposomes contain SP. In this study, the chitosanase-LIPOzymes prepared by several kinds of liposomes such as POPA, POPC, and POPC/Ch liposomes will be characterized based on three steps:

(i) Utilization of hollow dialyzer membrane column (TORAY, Japan) to purify the chitosanase-LIPOzymes from supernatant. Briefly, the prepared chitosanase-LIPOzymes will be circulated inside and/or loaded from outside membrane module. The liposomes (100 nm) harboring chitosanase containing SP will be stucked at the membrane interface. The impure proteins will be washed out from target chitosanase-LIPOzymes by continuous buffer influx through membrane module. The chitosanase containing SP will associate lipid membrane of liposomes stably by electrostatic and hydrophobic interactions of SP in chitosanase rather than other hydrophilic impure proteins. As a consequence, the pure chitosanase-LIPOzymes will be obtained after this operation.

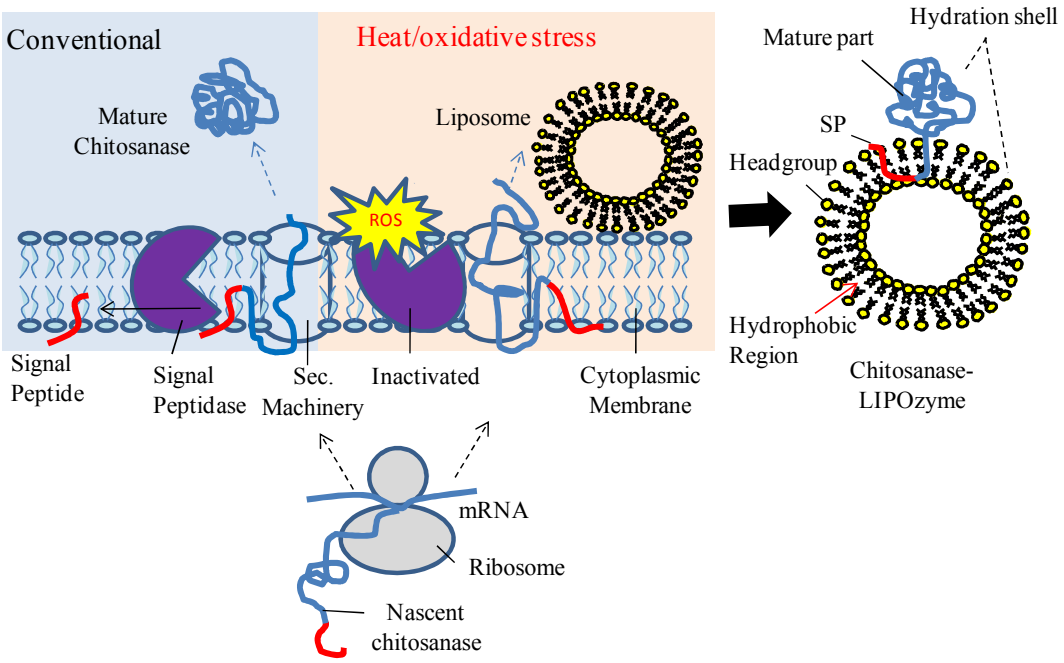
(ii) Purification of type I signal peptidase (SPase I) from *Streptomyces griseus*: The purpose to purify the SPase I is because the SPase specifically cleaves the SP from mature part of chitosanase that is proposed to be displayed on the liposome surface. The mature part of chitosanase will be removed from the liposomes but the SP of chitosanase is still remained in the liposome membrane. The triton X-100 that can disrupt the liposome membrane will be used to recover the SP from liposomes.

(iii) Characterization of SP will be based on the molecular weight using SDS-PAGE and mass spectroscopy, and amino acid sequence of SP to compare with its known sequence (Tanabe

et al., 2003).

(2) Study on the production of chitosanase-LIPOzymes from *Streptomyces griseus* cells treated with the heat and oxidative stress in the presence of liposomes

As the results shown in chapter I and II, the effect of the oxidative stress on the cell growth and production of chitosanase of *S. griseus* is severe. However, once these cells are treated with the heat stress and liposomes, the growth of cell and the production of chitosanase are enhanced. The analysis of protein and lipid peroxidation caused by the oxidative stress shows the possibility that the oxidative stress can be utilized to inhibit the type I signal peptidase (SPase I) in the cell membrane. For these words, the oxidative stress can mimic the function of inhibitor to inactivate the activity of SPase I. Then the heat and liposomes are applied to induce the secretion of chitosanase containing SP bound liposomes to produce chitosanase-LIPOzymes. The schematic illustration is shown in the scheme 1.



Scheme 1 The schematic illustration to prepare chitosanase-LIPOzyme by treatment of cell to oxidative and heat stress in the presence of liposomes

(3) Elucidation of novel pathway for secretion of protein harboring SP across lipid membrane

In this dissertation, the membrane-membrane interaction between liposomes and cell membranes induced by heat stress plays important role for the enhanced secretion of chitosanase containing SP associated liposomes. The evidence of SP bound chitosanase might support a possible hypothesis of chitosanase secretion through protein bound lipid membrane pathway. The strategy in this work is utilization of liposomes as model of cell lipid membrane that can encapsulate the chitosanase harboring SP. The secretion of encapsulated chitosanase harboring SP across the lipid membrane will be investigated under various stress conditions in the presence of different liposomes as the strategy shown in chapter I.

(4) Applications of chitosanase-LIPOzymes to produce target oligochitosans

Because of the limitation of time, the productions of oligochitosans by using chitosanase-LIPOzymes have not been dedicated such as how to control the selectivity of enzymatic activity of chitosanase-LIPOzymes to produce specific oligochitosan. Throughout the dissertation, it can be recognized that the efficiency of chitosanase-LIPOzymes catalyzing chitosan to smaller molecular weight oligochitosans is significantly higher than that using conventional chitosanase, especially, under extreme pH, heat conditions. However, it is also important to find out the key factors of chitosanase-LIPOzymes, which can hydrolyze the chitosan to specific oligochitosans with desired molecular weights. The modification of the surface of liposomes, used to prepared chitosanase-LIPOzymes, as well as the efficiency of various kinds of stress on surface properties of LIPOzymes such as surface hydration, membrane fluidity and hydrogen bond stability will be strongly considered to enhance the specificity of enzymatic reaction. Then the continuous production of oligochitosans by using chitosanase-LIPOzymes immobilized on gel chromatography and/or membrane module reactors will be dedicated.

(5) Molecular mechanisms of chitosanase harboring SP displayed on liposomes to form effective chitosanase-LIPOzymes

The purposes are to elucidate the molecular mechanisms of chitosanase harboring signal peptide (SP) displayed on the liposome surface that catalyzes an enzymatic reaction to hydrolyse the soluble chitosans to smaller molecular weight oligochitosans and/or glucosamine. The interaction of SP and various kinds of amino acid residues in chitosanase with liposomes will be focused at molecular level to elucidate the catalytic efficiency and stability of chitosanase-LIPOzymes under extreme pH and heat conditions. The surface properties of chitosanase harboring SP and its LIPOzymes will be characterized under various heat and pH conditions such as surface net charge and hydrophobicity, and surface hydration and hydrogen bond stability, using aqueous two-phase system (ATPS), FTIR, CD spectra and dielectric dispersion analysis, immobilized liposomes chromatography (ILC). In addition, the basic characteristics of soluble chitosan polymer under various heat and pH conditions will be also taken into account of investigations to elucidate how it is interacting with surface of chitosanase-LIPOzymes. Finally, the hydrolysis of natural chitosan polymer (low water solubility) will be also assayed to elucidate the effect of surface hydration of liposomes and the catalytic activity of chitosanase-LIPOzymes under extreme pH and heat conditions. Because the natural chitosan can be highly soluble at low pH condition so that it can be digested by hydrolysis of chitosanase-LIPOzymes but not conventional chitosanase.

Nomenclatures

ε	Dielectric constant	[-]
f, f_{c1}, f_{c2}	Characteristic frequencies	[MHz]
C	Capacitance	[F]
C_0	Cell constant	[F]
G, G_{dc}	Conductivity	[S]
ε', ε''	Dielectric constant and dielectric constant loss	[-]
ε'_w, ε''_w	Dielectric constant and dielectric constant loss of water	[-]
$\Delta\varepsilon'$, $\Delta\varepsilon''$	Increment of dielectric constant from water	[-]
$\Delta\varepsilon_1$, $\Delta\varepsilon_2$	First and second amplitude of dielectric dispersion	[-]
Z	Zeta potential	[-]
θ	Molecular ellipticity	[mdeg/mol]
$1/P$	Membrane fluidity	[-]
μ	Specific growth rate	[h^{-1}]
π	Specific production rate	[h^{-1}]
α	Growth associated product formation coefficient	[g/g]
β	Non-growth associated product formation coefficient	[g/g.h]
dP/dX	Production yield of enzyme	[mg/g]
HFS	Surface net hydrophobicity	kJ/mol
Z	Zeta potential	[-]
RH	Hydrophobic scale of amino acid which is defined in water/dioxane and water/ethanol by Nozaki and Tanford (1971)	[mol/kJ]
HFS	Surface net hydrophobicity	[kJ/mol]
HF	Hydrophobic factor	[mol/kJ]
k_{cat}	Rate constant	[1/s]
Km	Michaelis constant	[-]
k_{cat}/Km	Efficiency of catalytic rate	[1/M.s]
v	Catalytic velocity	M/s
V_{max}	Maximum catalytic velocity	M/s
γ	Frequency of IR spectra of PO_2^- of liposome and/or cell membrane	cm^{-1}

List of Abbreviation

ROS	Reductive oxygen species
MA-ILC	Metal affinity-immobilized liposome chromatography
ILC	Immobilized liposome chromatography
Hsp	Heat shock protein
DnaJ/DnaK/GrpE	Molecular chaperone system in *E.coli* synthesized by corresponded genes
GroEL/GroES	Molecular chaperone system in *E.coli*
GM1	Ganglioside
MG	Molten globule
CD	Circular Dichroism
DDA	Dielectric dispersion analysis
POPC	1-palmitoyl-2-*sn*-oleoyl-3-phosphatidylcholine
OD	Optical density
UV	Ultra violet
F	Faraday (standard international unit of capacitance)
S	Siemens (standard international unit of electric conductivity)
ATPS	Aqueous two-phase partition system
Dex, PEG	Dextran and Polyethylene glycol
FRET	Fluorescence resonance energy transfer
Z	Zeta potential
[ES]	Complex enzyme-substrate
LMCM-GUV	Lipid mimicking cell membrane-giant unilamellar vesicle
ProOOH, LOOH	Protein peroxides and lipid peroxides
ROS	Reductive oxygen species
pI	Isoelectric point
QCM	Quart crystal microbalance
TMA-DPH	trimethylammonio-diphenylhexatriene
Rh-PE	Lissamine rhodamine B-phosphatidylethanolamine,
NBD-PE	N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine
POPA	1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidic acid
POPG	1-palmitoyl-2-oleoyl-*sn*- glycero-3-[phospho-rac-(1-glycerol)]
DOPE	1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine
Ch	Cholesterol

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