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Oxidative/heat stress enhanced production of chitosanase from *Streptomyces griseus* cells through its interaction with liposome

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The effective production and secretion of chitosanase from *S. griseus* cells, in the presence of hydrogen peroxide, were studied by the treatment of the cells with 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine (POPC) liposomes under heat condition. The variation of the conformation and activity of the chitosanase caused by the interaction of liposomes with target chitosanase under stress condition was systematically investigated by using circular dichroism (CD) spectra and dielectric dispersion analysis (DDA). The effect of the oxidative stress (hydrogen peroxide) on the lipid and protein peroxide of cell membrane of *S. griseus* pretreated with and without liposomes under heat stress at 41 °C was further carried out. The possible utilization of membrane–membrane interaction between liposomes and cell membrane induced by the heat treatment was further investigated to enhance the production of chitosanase from *S. griseus* under oxidative stress condition.

[**Key words:** Membrane stress biotechnology; Chitosanase; Liposome; Oxidative stress; *Streptomyces griseus*]

Chitosanase produced by *Streptomyces griseus* is secreting and non-membrane protein with 33.8 kDa in molecular weight (M.W). Chitosanase can be classified as glycosyl hydrolase that catalyzes the degradation of chitosan. The three-dimensional structure of chitosanase from *S. griseus* species has previously been analyzed (1). Chitosanase can be classified into three groups according to their specificity for the hydrolytic reaction of β-glucosidic linkage in partially N-acetylated chitosan molecules (2, 3).

"Stress" can often reveal the potential roles of bacterial cells. Heat is known to be sensitive and useful stimuli. A series of previous reports on heat shock protein (HSP), which is known as the molecular chaperone in bacteria, has described the important roles of HSPs for protein refolding (4, 5) and translocation (6, 7). Oxidative stress, induced by the reactive oxygen species (ROS), has been widely studied. It has been reported that the hydrogen peroxide (H_2O_2) could play important roles as second messenger in the initiation and amplification of signaling at the antigen receptor (8). A several excellent reviews about the functions of hydrogen peroxide as secondary messenger have already appeared (8–13). It has been also reported that the production rates of superoxide radical ($^{\cdot}O_2^-$) and hydrogen peroxide are linearly related to the number of the active respiration chains that reaches to the maximal values during the exponential growth and significantly decreased at the stationary phase (14).

Much effort to overcome the above restrictions has recently been conducted by several researchers. A stress-mediated bioprocess as another designed strategy enhances the productivity of the biological target (15, 16). The stress-mediated bioprocess utilizing the stress response function of bacterial cells is a preferable strategy to enhance the target production. It has been reported that the heat stress could enhance the periplasmic recovery of cytoplasmic β-galactosidase through the translocation of this enzyme across the inner membrane of *Escherichia coli* cells (17–19). In addition, under stress condition, the biological membrane and liposome induce so many sophisticated properties. The addition of the liposome into the cell culture has been reported to enhance the production and release of the chitosanase from *S. griseus* under heat stress condition at the specific temperature (20–22). On the basis of the recently reported concept of membrane stress biotechnology, a conventional bioprocess is further improved.

In this study, the effective production and secretion of chitosanase from *S. griseus* cells were studied by the treatment of these cells with the liposome prepared by 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine (POPC) under the heat and/or oxidative stresses. The interaction of the POPC liposomes with target chitosanase and the variation of the conformation and activity of chitosanase were systematically investigated by using circular dichroism (CD) spectra and dielectric dispersion analysis (DDA). The possible utilization of membrane–membrane interaction between liposomes and cell membrane induced by the heat treatment was further characterized under oxidative stress condition to enhance the production of chitosanase from *S. griseus*.

MATERIALS AND METHODS

Materials The 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine (POPC) was purchased from Avanti Polar Lipid (Alabaster City, AL, USA). Other lipids to prepare the cell-mimicing liposome, such as 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidic

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

Preparation of liposomes The POPC was 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 10 mM phosphate buffer containing 150 mM NaCl (pH 7) for overnight. The freezing–thawing cycle was repeated 5 times with hydrated POPC solution for the formation of unilamellar vesicles. 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 liposome containing lipids mimicking cell membrane (LMCM, DMPG/DOPE/POPC/POPA (56:22:20:2 in molar ratio)) was prepared by previously reported method (23).

Evaluation of secondary structure of chitosanase using CD spectra Conformational transition of chitosanase under oxidative stress conditions was analyzed in the presence and absence of liposomes 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 (21) 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 chitosanases was dissolved in 10 mM phosphate buffer containing 150 mM NaCl, pH 7.0. The effect of oxidative stress on the conformation of chitosanase with and without liposomes was also evaluated by co-incubation of 4.3 μM chitosanases 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 liposomes solution was well prepared to be transparent in the same buffer and their CD spectra were measured as the blank sample. The quartz 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.

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 liposomes in the frequency range between 1 MHz and 100 MHz. The shift of relative permittivity (or dielectric dispersion) of POPC liposomes as function of frequency may show the orientation of the 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 liposomes. Such dielectric parameters can be obtained based on the fitting analysis using Debye's equation as shown below (24). 30 mM POPC liposome sample (125 μl) was applied to the electrode in order to obtain capacitance and conductance values. The interaction between POPC liposomes and chitosanase was evaluated as a shift in the above parameters of the dielectric dispersion (15 mM POPC and 4.3 μM chitosanase as the final concentration). The samples of pure POPC and POPC/chitosanase mixture were exposed to oxidative stress condition (5 mM hydrogen peroxide) for 20 min before being applied into a brass electrode cell. 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 liposomes.

Debye's equations:

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

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

$$\varepsilon' = C^* C_0^{-1} \quad (3)$$

$$\varepsilon'' = \frac{G}{2\pi f C_0} \quad (4)$$

In these equations, the data were corrected 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 Eqs. (3) and (4). Similarly, the dielectric constant and loss of water (ε'_w, ε''_w, respectively) were calculated to evaluate the increment of ε', ε'' from the values of water ($\Delta\varepsilon'$, $\Delta\varepsilon''$) and the amplitudes of dielectric dispersion ($\Delta\varepsilon_1$, $\Delta\varepsilon_2$) using Eqs. (1) and (2); 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.

Analysis of lipid and protein peroxides The amount of protein and lipid peroxides was measured by ferric-xylenol orange method (25). After the mixture was vigorously mixed for 5 min, the sample was centrifuged at 6500 ×*g* for 6 min. Chloroform layer containing target lipids was collected and dried by oxygen-free nitrogen and then the following reagents were added immediately; 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, the sample was measured absorbance at 560 nm. A blank sample containing the extracted lipid, reduced with 1 mM triphenylphosphine was subjected to identical protocol and measured the absorbance at 560 nm.

The protein peroxide was assayed by G-PCA-FOX method. Briefly, 10 μl sample of extracted protein and 500 μl 0.2 M PCA were mixed vigorously in ice, and then centrifuged at 6500 ×*g* for 5 min. After 1100 μl of 6 M GuHCl solution was added to pellet, the sample was vortexed to dissolve pellet and washed the solution with 1100 μl chloroform containing 4 mM BHT. Finally, the obtained sample was added to the aqueous solution in the following 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+}. After the incubation of this sample for 60 min at room temperature, the absorbance at 560 nm was measured. A blank containing extracted protein, reduced with 1 mM sodium dithionite, was subjected to identical protocol and measured the absorbance at 560 nm.

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 (20). 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 °C for 60 min in the presence and absence of 5 mM hydrogen peroxide 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 subtracted to that of water or buffer. The accuracy of the frequency reading is better than ± 0.1 cm^{-1}.

Measurement of chitosanase activity Chitosanase activity was measured with glycol chitosan as a substrate (26). 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 °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

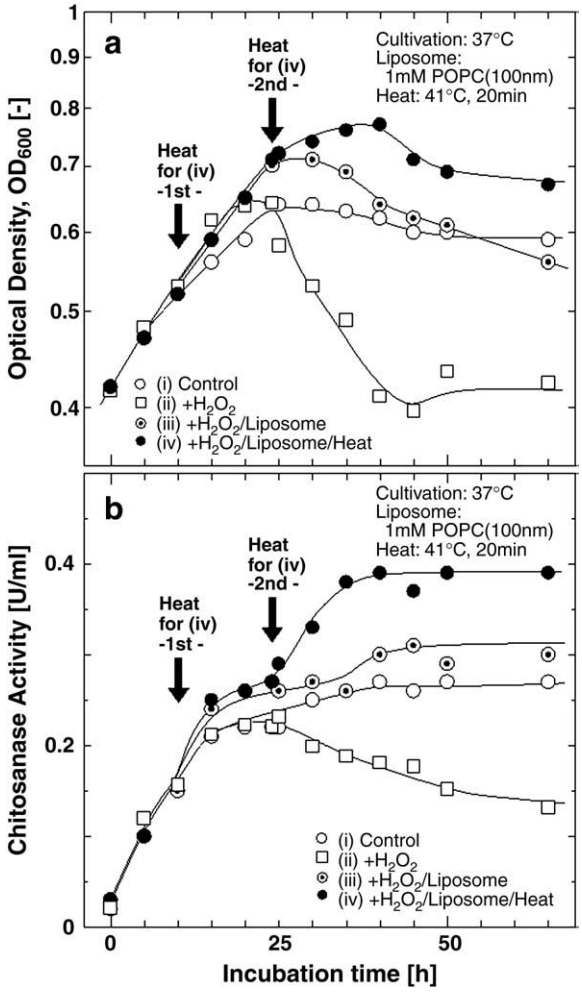

FIG. 1. Production of chitosanase by *S. griseus* cells treated with heat stress and liposomes under the oxidative stress condition. The cultivation was carried out at 37 °C and the heat stress at 41 °C for 20 min was applied to cell broth twice after incubation of 10 and 24 h. The oxidative of 5 mM H_2O_2 was added to above cell culture together with and without 1 mM POPC liposomes. The cultivation was continuous at 37 °C after above treatment. Symbols: "open circle": Control, "open square"; with H_2O_2, "double open circle"; with H_2O_2/Liposome, "closed circle"; with H_2O_2/Liposome under heat stress.