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Selective separation process of proteins based on the heat stress-induced translocation across phospholipid membranes

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Abstract

Heating of several protein solutions at 40–47°C for 5–60 min in the presence of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) liposomes induced the translocation of β-galactosidase (β-gal), α-glucosidase (α-glu) and bovine carbonic anhydrase (CAB) from outer to inner aqueous phase across the liposome membrane. The translocated amounts of β-gal at various temperatures were maximized under suitable heating conditions (45°C, 30 min). Those of α-glu and CAB were maximized at 40–45 and 60°C, respectively. Each maximum value could be correlated with the corresponding local hydrophobicity of each protein evaluated by the aqueous two-phase partitioning method. The possibility to apply these heat-induced translocation phenomena to the bioseparation of proteins was successfully demonstrated for the model mixture solution of β-gal, α-glu and CAB.

Keywords: Heat stress-induced translocation; Phospholipid membranes; Proteins; β-Galactosidase; α-Glucosidase; Bovine carbonic anhydrase

1. Introduction

Since translocation phenomena of proteins across the phospholipid and/or biological membrane are important among a variety of biological functions, these mechanisms have been widely studied, including those of membrane, periplasmic, and/or secretory proteins, which have hydrophobic signal peptide sequence in their molecules [1,2]. Recently, some cytoplasmic proteins having no signal sequence and no ability of secretion have been reported to translocate to periplasm of *Escherichia coli* cells or surrounding media across the biological membranes by exposing the cells to the heat stress condition [3–5]. The role of molecular chaperone (e.g. SecB, SecY, GroEL, DnaJ, DnaK) on the translocation phenomena has been also studied [6–8].

Such translocation phenomena may be utilized for the bioprocess to produce and separate recombinant proteins from their host cells. From the point of view of in vivo application, however, the effective utilization of such phenomena has been restricted to the specific proteins, having signal sequences [9], or the modified N-terminal with its sequence [10]. Especially in the case of in vitro application, the novel bioseparation process can be developed by exploiting such translocation phenomena. The partly unfolded (molten globule) state of the protein [11], or the existence of local hydrophobic binding site on the surface [12], have been reported to be required for the translocation across the membrane [13,14].

In the previous study, the heat-induced translocation of the cytoplasmic β-galactosidase (β-gal) from

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inner to outer aqueous phase across the liposome membranes [13] and from cytoplasm to periplasm across the inner membrane of *Escherichia coli* cells [14] have been investigated based on the surface properties, which have been evaluated by the aqueous two-phase partitioning method of the phospholipid and biological membranes and that of the target β-gal. In this study, the heat-induced translocation phenomena of β-gal from the outer aqueous phase of the liposome to the inner phase were firstly investigated in order to apply the previous findings to the separation of target proteins. The role of the variation of local hydrophobicity of the β-gal surface was then studied. The possibility of the stress-mediated separation process of the proteins is discussed based on the responsive behavior of three enzymes, α-glucosidase (α-glu), β-gal, and carbonic anhydrase from bovine (CAB) under heat stress conditions.

2. Experimental

2.1. Materials

1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) was purchased from Avanti Polar Lipids (Birmingham, UK). Tetrameric enzyme β-galactosidase (β-gal) and *o*-nitrophenyl-β-D-galactopyranoside (ONPG) as the substrate were purchased from Sigma (St. Louis, MO, USA). Monomeric proteins, α-glucosidase (α-glu) and carbonic anhydrase from bovine (CAB), were purchased from Sigma. Poly(ethylene glycol) (PEG 4000, M_r=3 kDa) and dextran 100–200 kDa (Dex; M_r=100–200 kDa) were obtained from Wako Pure Chemicals (Osaka, Japan). Nonionic detergent, Triton X-100 (M_r=0.65 kDa), for solublizing the liposome membrane, was purchased from Sigma. The salts and other chemicals were of analytical grade.

2.2. Preparation of liposomes

Multilamellar vesicles (MLVs) were firstly prepared by hydrating a POPC thin film with a 20 m*M* Tris–HCl buffer (pH 7.5) followed by a freeze–thaw method (five times) with dry ice–ethanol. Large unilamellar vesicles (LUVs) composed of POPC were prepared by extrusion (15 times) of MLVs through polycarbonate filters with a pore size of 200 nm using an extrusion device (Liposofast; Avestin, Ottawa, Canada).

2.3. Heat treatment of liposome–protein solution

The above liposome solution was mixed with the solution of β-gal, α-glu, or CAB (protein concentration, 6.0 mg/ml). The mixed solution was heated at various temperatures (25–60°C) for various periods of time (1–60 min). The activity of β-gal, α-glu and CAB in the mixed solution was measured after the heat treatment to analyze the remaining fractions of β-gal, α-glu and CAB [19]. The 1 m*M* Triton X-100 solution was then added to the mixed solutions in order to solubilize the liposome membrane and to release the protein entrapped in the inner aqueous phase, and the activity of each enzyme was measured. The translocated amounts of proteins were determined from the mass balance from those values.

2.4. Analytical methods for local hydrophobicity of proteins under heat stress

The local hydrophobicity (LH) of proteins was analyzed by using the aqueous two-phase partitioning method [15]. At the pH corresponding to the p*I* in the low ionic strength buffer, the partition coefficients of proteins and liposomes mainly depend on the hydrophobic effect. When Triton X-405 is added to the PEG/Dex system, Triton X-405 preferably partitions to the top (PEG) phase. The protein which has hydrophobic binding sites with Triton X-405 is likely to partition to the top phase [16]. The difference between partition coefficients of proteins in two-phase systems with and without 1 m*M* Triton X-405 gives a local hydrophobicity of the protein (LH). The LH value of the protein was defined as

$$\mathrm{LH} = \ln K_{\text{with ligand}} - \ln K_{\text{without ligand}} \quad (1)$$

where $K_{\text{with ligand}}$ and $K_{\text{without ligand}}$ are the partition coefficients of the protein in the aqueous two-phase systems with and without Triton X-405, respectively. The local hydrophobicity of enzymes was determined at the various temperatures.

3. Results and discussion

3.1. Translocation of enzymes across phospholipid membrane from outer to inner aqueous phase under heating conditions

The effect of heating conditions on the translocated amounts of β-gal from outer to inner aqueous phase was firstly investigated. Fig. 1 shows the time-course of the translocated amounts of β-gal. Within the specific temperature range of 40–45°C, the translocated amounts of β-gal are increased with increasing temperature and heating time. The values are maximal at around 30 min and then decreased with further increase of heating time. Especially when the solution was heated to 45°C, the amount of translocated β-gal is the largest. Conversely, the translocated β-gal is not detected at temperatures higher than 50 and lower than 40°C. It has been reported that the cytoplasmic β-gal was translocated across the phospholipid membrane [13] and across the inner membrane of *E. coli* cells [14] under suitable heating conditions. The effective heating conditions for increasing the β-gal translocation from inner to outer aqueous phase could be selected as the specific temperature range (42–47°C). The present results on the heat-induced translocation of β-gal from outer to inner aqueous phase are in agreement with previous findings. The change of surface properties of enzymes, accompanying their conformational change, is reported to be an important factor in the model for the heat-induced translocation of β-gal across the phospholipid membrane [13]. In the following section, the change of surface properties, especially local hydrophobicity, was investigated in order to clarify the role of surface properties of proteins.

3.2. Change of surface property of proteins under heat stress conditions

The surface properties of proteins and enzymes under various heat conditions were determined by the aqueous two-phase partitioning method [15]. As shown in Fig. 2, the values of local hydrophobicity of β-gal (LH_G) and their maximum translocated amounts (TR_{max}) are plotted against the heating temperature (T). Bell-shaped curves of both LH_G and TR_{max} are obtained. Two curves indicate the maximum value at the same temperature (45°C), suggesting that the translocation behaviors of β-gal correspond to the change of the local hydrophobicity of protein surface.

The $TR_{G,max}$ values of β-gal (from outer to inner phase) are plotted against the corresponding LH_G values in Fig. 3. The straight lines are obtained in the case of β-gal translocation from outer to inner aqueous phase (O→I, open circles). The data of heat-induced translocation of β-gal from inner to outer aqueous phase across the phospholipid membrane (I→O, closed circles) [13] and from cytoplasm to periplasm across the inner membrane of *E. coli* cells (closed squares) [14] are also shown in Fig. 4. Straight lines with similar slopes are obtained in both

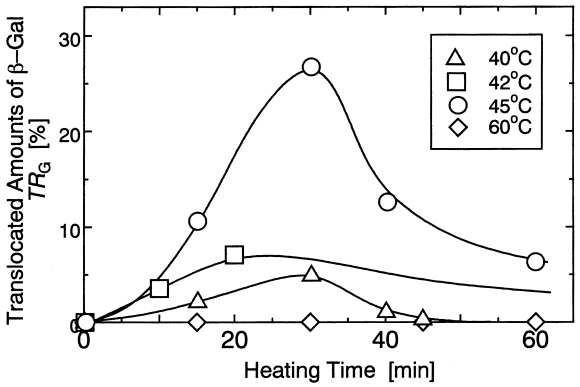

Fig. 1. Time course of translocation amounts of β-gal across phospholipid membrane from outer to inner aqueous phase under heating conditions (40–60°C, 5–60 min).

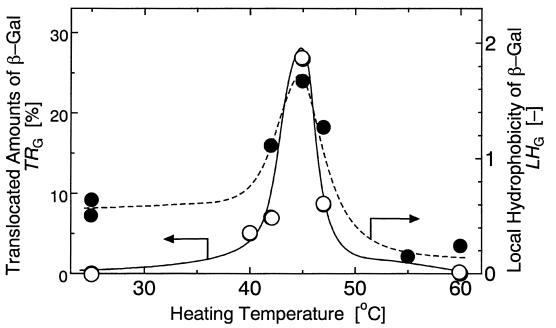

Fig. 2. Effect of heating temperature on the maximum translocation amounts of β-gal and their local hydrophobicity under heat stress.

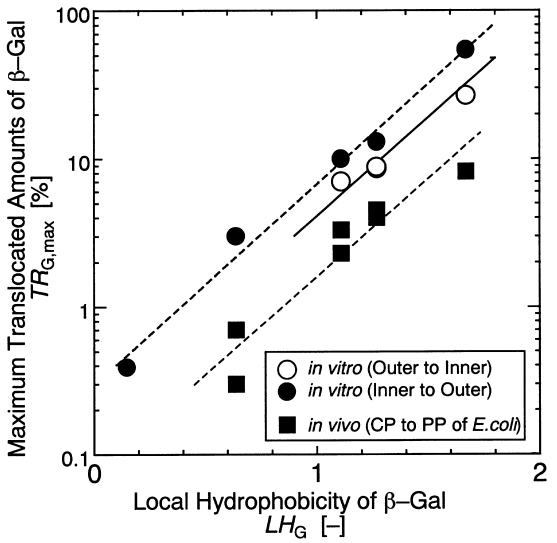

Fig. 3. Relationship between local hydrophobicity of β-gal and the maximum translocation amounts. Symbols: (○) translocation from outer to inner aqueous phase across phospholipid membrane; (●) from inner to outer [13]; (■) from cytoplasm (CP) to periplasm (PP) across the inner membrane of *E. coli* cells [14].

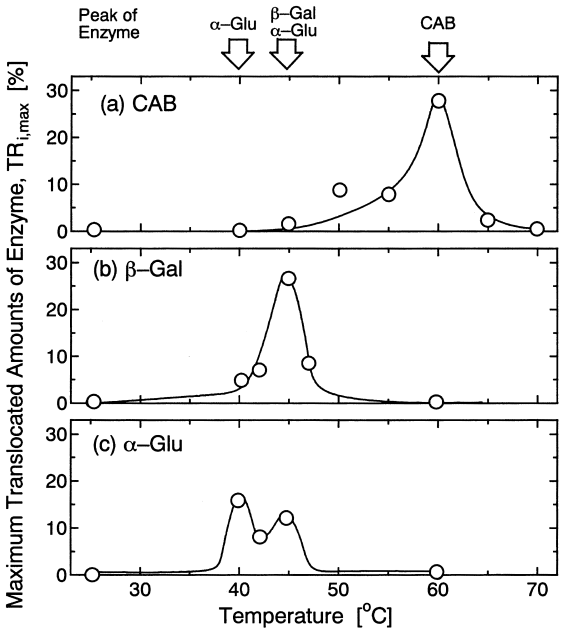

Fig. 4. Dependence of maximum translocation amounts of (a) CAB, (b) β-gal, and (c) α-glu on the heating temperature. Arrows on the top of this figure show the peak of curve of each enzyme.

cases, although the absolute values of $TR_{G,max}$ at constant LH_G are significantly different. The difference between the case of I→O and O→I may be caused by the difference of interacting efficiency of the protein–lipid. The reduction of in vivo $TR_{G,max}$ may be due to the presence of many other intracellular proteins and heat shock proteins [5,14,17,18]. Thus, the local hydrophobicity of the enzymes under heating conditions, which can be determined by the aqueous two-phase systems (ATPS) method [15], is found to be an important factor in heat-induced translocation across phospholipid and/or biological membrane.

3.3. Heat-induced translocation of other enzymes

The possibility of appling the translocation phenomena to the bioseparation process using heat stress was also investigated. The heat-induced translocation of other proteins (α-glu and CAB) was investigated using the same method. In general, heat-induced translocation is also observed in the case of CAB and α-glu. The dependence of TR_{max} values of the enzymes on the heating temperature is shown in Fig. 4. The values of translocated amounts of α-glu and CAB are maximized, respectively, at 40–45°C (Fig. 4a) and 60°C (Fig. 4c). The LH values of the enzymes at various temperatures have also been determined by the ATPS method [20] and found to be well correlated with the corresponding TR_{max} values. The difference of peak of TR_{max}–T curves among the enzymes is expected to be caused by the difference of local hydrophobicity, LH, which is accompanied by their conformational change under heat-stress conditions. LH values of the liposomes and proteins were already found to be the key factors in heat-induced translocation phenomena [14], and the translocation model was successfully described using LH values in our previous work [13]. It is suggested from the results that the difference in heat-induced translocation phenomena of proteins can be exploited for protein separation, as shown in our previous work [21], by selecting suitable heat-stress conditions.

The possibility of the selective separation and recovery of the target enzyme is discussed in the following section on the basis of the heat-induced

translocation of the enzymes across the phospholipid membrane.

3.4. Selective separation and recovery of target from enzyme mixture

The translocation behavior of enzymes from a solution containing three enzymes was investigated. After the enzyme solution was firstly mixed with the liposome solution, the mixture was exposed to suitable heat conditions. Selective translocation of target enzyme from outer to inner aqueous phase across the liposome membrane can be carried out. The separation of liposomes entrapping the target from other proteins can be easily accomplished by using, for example, gel-permeation ATPS method.

The enzyme solution containing CAB, β-gal, and α-glu at the same concentration (6.0 mg/ml) was heated under various heating conditions (40–60°C, 15–60 min). The translocated amounts of three enzymes at various conditions are summarized in Table 1. At 40°C, 2–7% α-glu is translocated to the inner phase of liposomes, although the other enzymes are not. Both 2–3% α-glu and 7–10% β-gal are translocated at 45°C. At 60°C, only CAB is translocated to the inner phase. The suitable temperature for the specific translocation of each enzyme in the mixture containing three enzymes is in agreement with that of each enzyme in single system (Fig. 4). The translocation amounts in the former case (Table 1) are, however, reduced compared to those in the latter case (Fig. 4). This is presumably because not only target but also other proteins coexist in an experimental solution. The heat-induced protein translocation is thought to be induced by attractive interaction between the hydrophobic binding site of liposome and that of the protein surface, as shown previously [13]. The variation of local hydrophobicity, fluidity and particle size of the liposome have been previously studied under heat stress [21]. Because the number of the local hydrophobic sites on the liposome surface is limited, some of these proteins at the molten globule state, with lower local hydrophobicity, cannot translocate simultaneously through the same binding site. The translocation behavior of each protein was therefore suggested to be competitive. The target protein could thus be separated and recovered selectively from the mixture by selecting suitable heat-stress conditions based on the results of Fig. 4.

In conclusion, the possibility of selective separation and recovery of the target from the protein mixture by stress, using the response functions of liposome and various proteins under the thermal conditions, has been presented. It is suggested that the efficiency of such a separation method can be improved by exploiting a continuous-mode method and by optimizing the stress condition on the basis of surface properties of liposomes and proteins, such as membrane fluidity of the liposome, net charge, local and surface net hydrophobicity of various proteins with and without salts.

Table 1
Summary of heat-induced translocation of three enzymes (CAB, β-gal, and α-glu) across phospholipid membrane from outer to inner aqueous phase

Temperature (°C)	Heating time (min)	Translocation amounts, $TR_{i,max}$ (%)		
		CAB	β-Gal	α-Glu
25	0	0	0	0
40	15	0	0	2.0
40	30	0	0	7.1
40	60	0	0	0
45	15	0	6.2	2.3
45	30	1.2	10.0	3.0
45	60	0	0	0
60	15	2.3	0	0
60	30	4.5	0	0
60	60	1.0	0	0

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