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Enzyme release from heat-stressed cell membranes as a function of hydrophobicity evaluated by using aqueous two-phase systems

Hiroshi Umakoshi, Ryoichi Kuboi*

Department of Chemical Science and Engineering, Graduate School of Engineering Science, Osaka University, 1–3 Machikaneyama-cho, Toyonaka, Osaka 560, Japan

Abstract

The release behavior of a periplasmic enzyme, acid phosphatase, from heat-stressed *Escherichia coli* cells was characterized by using kinetic analyses when the cells were treated by Triton X-100–EDTA. The hydrophobicity of the cell surface and the release-rate of the enzyme were not influenced by heat treatment at temperatures between 30 and 50°C. However, these values varied above 55°C. The release-rate constants were found to correspond to the net and local hydrophobicity of the outer membrane surface, evaluated by aqueous two-phase partitioning.

Keywords: Aqueous two-phase systems; Hydrophobicity; Enzymes; Acid phosphatase

1. Introduction

Conventional bioprocesses using microbial cells to produce target biomaterials, such as proteins and polypeptides, and to separate them from the cells, usually consist of a series of unit operations [1,2]. The selectivity of target recovery at the primary step of conventional bioprocesses [3] can be an important factor, which determines the efficiency and complexity of the process. Methods for cell disruption using chemical, enzymatic, and/or osmotic stress [4–10] have some advantages as compared to mechanical methods, and are favorably used as a primary step in practical bioprocesses [11–13]. This is because the stress of such methods against the bacterial cells is milder than that of mechanical methods, so that the specific release of products from the cells can be easily achieved with minimal contamination from undesirable products.

Such methods also have some disadvantages: (i) the optimal condition for selective recovery of target product is selected by trial and error; (ii) the yield of target product may be relatively low. These problems may be caused by the lack of quantitative understanding of the cell properties and their potential functions induced by the environmental stress. The possibility to design more selective bioprocesses based on quantified cell properties has been reported [14,15]. The effective design of bioprocesses for the selective recovery of the enzymes can be achieved by evaluating the cell properties and, furthermore, by utilizing their stress–response functions.

In the previous study, a method to optimize the enzyme release process across the outer membrane of *E. coli* cells under mechanical (ultrasonic method) [16] and chemical stress (Triton X-100–EDTA treatment) [17] was reported, using kinetic analyses

*Corresponding author

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of enzyme release. The possibility to recover cytoplasmic enzymes selectively has recently been demonstrated by using translocation phenomena across the inner membrane of *E. coli* cells induced under suitable heat stress [18]. It has also been shown that the hydrophobicity of the target and the phospholipid membrane is a key factor for control of the translocation (release) process of some enzymes [19]. The hydrophobic interaction between the target enzyme and the cell membrane is thought to play an important role in the release processes of the target enzyme across the inner [18,19] and outer membrane of cells [17].

In the present study, the release process of a periplasmic enzyme, acid phosphatase (AcP), across the outer membrane of heat-stressed *E. coli* cells was investigated in the presence of Triton X-100–EDTA [17]. The dependence of the enzyme release process on the evaluated surface hydrophobicity of heat-stressed cells was investigated and discussed.

2. Experimental

2.1. Materials

The *p*-nitrophenylphosphate (*p*-NPP), used for substrate of acid phosphatase (AcP), was purchased from Wako Pure Chemicals (Osaka, Japan). Casamino acid was from Difco Laboratory (Detroit, MI, USA). Nonionic detergent, Triton X-100, and poly(ethylene glycols) (PEG1540, 4000, and 6000) were from Wako. The salts and other chemicals used were all of analytical grade.

2.2. Cultivation and heat treatment of bacterial cells

The strain used in this study is *E. coli* W3110. The basic cultivation media of *E. coli* cells were modified M9 media (pH 7.4, in g/l): Na_2HPO_4, 7.0; KH_2PO_4, 3.0; NaCl, 5.0; NH_4Cl, 1.0; casamino acid, 1.0; $MgSO_4$, 0.75; glycerol, 50. Following overnight growth at 30°C in a 100-ml Erlenmeyer flask (130 rpm) with a working volume of 20 ml, the culture was used as an inoculum for each 100 ml medium in a 300-ml shaking flask. The cells were harvested in the stationary growth phase by centrifugation (11 000 *g*, 10 min), washed once with 50 m*M* Tris–HCl buffer, and resuspended in the buffer at the cell concentrations, C_0, of 25 mg/ml. The suspensions of heated cells, which were exposed to heat stress (10 min; 40, 42, 47, 50 and 55°C), were also prepared.

2.3. Treatment of E. coli cells with Triton X series and EDTA

After the cell suspension (final concentration, 10 mg/ml) was diluted with Tris–HCl buffer (50 m*M*, pH 8.0) containing EDTA (final concentration 5 m*M*), an adequate amount of Triton X-100 solution was added to obtain a final concentration of 20 m*M*, as previously described [17]. The solution was stirred at 25°C for 0.1–5 h. A 1-ml portion of the solution was withdrawn and centrifuged (4300 *g*, 5 min). The activity of various enzymes in each supernatant was analysed ($R_{i,Triton}$ [U enzyme/mg cell]; i represents enzyme types). The precipitates containing the Triton-treated cells were resuspended in the same volume of Tris buffer and were disrupted using the ultrasonic method (input power, 40 W; solution volume, 1 ml; disruption time, 3 min) in order to determine the total activity of AcP per unit weight of packed cells ($R_{i,max}$ [U enzyme/mg cell]). The released fraction of AcP ($RF_{i,Triton}=R_{i,Triton}/R_{i,max}$) was then determined as the ratio of released amounts of enzymes ($R_{i,Triton}$ and $R_{i,max}$) and was used as a parameter for the kinetic analysis. The release-rate constants of periplasmic AcP, k_{AcP} (i= AcP), of the stressed *E. coli* cells were determined from the following equation as previously reported [18].

$$\ln D_{i,Triton}^{-1} = \ln(1 - RF_{i,Triton})^{-1} = k_i t \quad (1)$$

where t is the time of Triton X-100–EDTA treatment.

2.4. Surface properties of bacterial cell membrane (the aqueous two-phase partitioning methods)

The surface net hydrophobicity (HFS_{Cell}) and local hydrophobicity (LH_{Cell}) of cells were determined by the following method using aqueous two-phase systems (ATPS) [20–22]. The partitioning of bacterial cells is mainly dependent on the hydro-

phobic effect at the isoelectric point (pI) and at low ionic strength. The HFS_{Cell} values can then be determined from the slope of the equation:

$$\ln K_{Cell} = HFS_{Cell} \times HF,$$

where HF is the hydrophobic difference between the two phases in ATPS [23] and K_{Cell} is the partition coefficient of cells. The local hydrophobicity of cells, LH_{Cell}, was determined as the increment change of partitioning coefficient of cells in the presence of 1 mM Triton X-100, $\Delta \ln K_{Cell,Triton}$ ($=\ln(K_{Cell,Triton}/K_{Cell,0})$; $K_{Cell,Triton}$ and $K_{Cell,0}$ are the partition coefficient of cells in ATPS with and without Triton) at pH = pI.

2.5. Measurements

The enzyme activity of AcP (EC 3.1.3.2) as a marker enzyme for periplasmic one [24,25] was measured in a sodium acetate–acetic acid buffer (pH 4.5) at a wavelength of 340 nm using *p*-NPP as the substrate [26]. The cell concentration was determined by measuring absorbance at 660 nm. All measurements were carried out using a Shimadzu UV160-A spectrophotometer (Kyoto, Japan).

3. Results and discussion

3.1. Release of periplasmic enzyme across the outer membrane of heat-stressed E. coli cells

The effect of heating of *E. coli* cells on the release of periplasmic AcP during Triton X-100–EDTA treatment was investigated on the basis of the first-order kinetics [17] of the time course of $\ln D_{1,Triton}^{-1}$ of the AcP (Fig. 1). The slope of the line can be defined as the release-rate constant, k_{AcP}. The release-rate constant was not changed although the cells were exposed to heat stress at 30–50°C. Conversely, the k_{AcP} value was sharply reduced at 55°C. Such phenomena may be caused by a cell-response against the change of the heat conditions.

Heat stress of cells has previously been reported to induce a variety of responses, such as the change of the surface properties of the outer and inner membrane [18,21,27] and the induction of translocation of

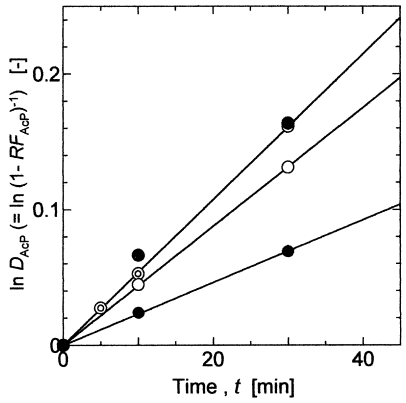

Fig. 1. Time course of the logarithm of the remainder fraction, D_{AcP}^{-1}, of AcP by 20 mM Triton X-100–5 mM EDTA treatment of *E. coli* W3110 cells and heat-stressed cells (30–55°C, 10 min). Symbols: (⊙) control; (○) heated at 42°C; (shaded circles) 47°C; (black circles) 55°C.

specific cytoplasmic enzyme across the inner membrane [18,28,29] (M. Kitagawa, Y. Matsumura, T. Tsuchido, private communication). It has also been reported that cytoplasmic β-gal was released (translocated) to the periplasm across the inner membrane of cells under heat stress [18]. The mechanism of the heat-induced translocation of the β-gal has also been verified by using liposomes as a model system of the inner membrane [19]. The translocated amounts of β-gal under such heating conditions have been shown to depend on the local hydrophobicity of the target enzyme and the inner membrane, which can be evaluated by an aqueous two-phase partitioning method. Similarly, the release-rate of AcP may be affected by the surface properties of the outer membrane of *E. coli* cells because the periplasmic AcP is released to the surrounding media across the outer membrane, which were partly permeabilized by nonionic detergent Triton X-100–EDTA treatment.

In the following, the heat-induced variations of the surface properties of the outer membrane of cells were investigated.

3.2. Variation of surface properties of outer membrane of heated cells on the enzyme release

Among the possible properties of the cell surface, which may be expected to affect enzyme release, the hydrophobicity of cells is thought to be the most

dependent on the release process of the enzyme. This is because the nonionic detergent Triton X-100 was used, and the electrostatic effect can be negligible. Two hydrophobicity parameters, surface net and local hydrophobicity of *E. coli* cells stressed under various conditions, were determined by the aqueous two-phase partitioning method in order to investigate the effect of the surface properties of cells on the release-rate constant. The properties of the stressed cells, which were evaluated at various temperatures, were surface net (HFS_{Cell}) and local hydrophobicity (LH_{Cell}), and release-rate constant, k_{AcP} (Fig. 2). As shown in Fig. 2(a), the values of k_{AcP} were not changed at temperatures of 30–50°C, and decreased at temperatures above 50°C. The local hydrophobicity of cell surface, LH_{Cell}, gradually increased with increasing heating time within the experimental range (Fig. 2(b)). Conversely, the HFS_{Cell} values, indicating the surface net hydrophobicity of cells, were not changed below 50°C, and were sharply increased at higher temperatures. In this way, heating at relatively low temperature (30–50°C) was found to have no effect on either the enzyme release or the surface net hydrophobicity.

In the previous report on the heat-induced translocation (release) of cytoplasmic enzyme across the inner membrane [18], the role of local hydrophobicity of both the cell membrane and the releasable target enzyme was discussed. The release-rate of the enzyme was well correlated with the hydrophobicity values. The same tendency was obtained for the release of periplasmic enzymes across the outer membrane of cells.

3.3. Dependence of the surface properties on the release behavior of periplasmic enzyme

In Fig. 3, the release-rate constants of AcP, k_{AcP}, were compared with the hydrophobicity value of the cell surface. As shown in Fig. 3(a), the surface net hydrophobicity of the cell, HFS_{Cell}, was negatively correlated with the release-rate constant of AcP. In relation to local hydrophobicity, LH_{Cell}, all the data seem to be scattered around a single curve (Fig. 3(b)). A decrease of the k_{AcP} values can then be

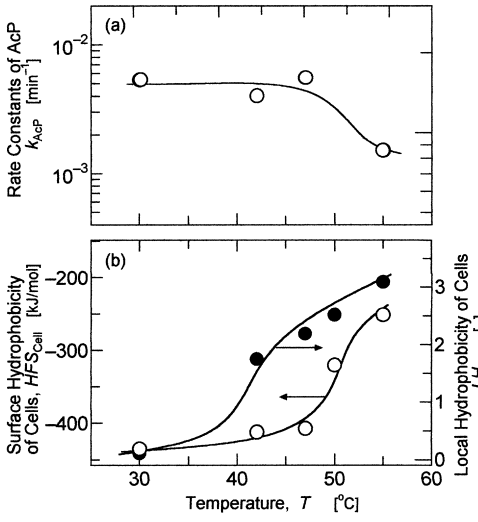

Fig. 2. Dependence of (a) release-rate constants of periplasmic AcP, k_{AcP}, and (b) the surface net and local hydrophobicity of *E. coli* W3110 cells on the heating temperature. The k_{AcP} values were determined after 20 m*M* Triton X-100–5 m*M* EDTA treatment of cells heated at various temperatures for 10 min. Open and filled symbols indicate the HFS_{Cell} and LH_{Cell} values, respectively.

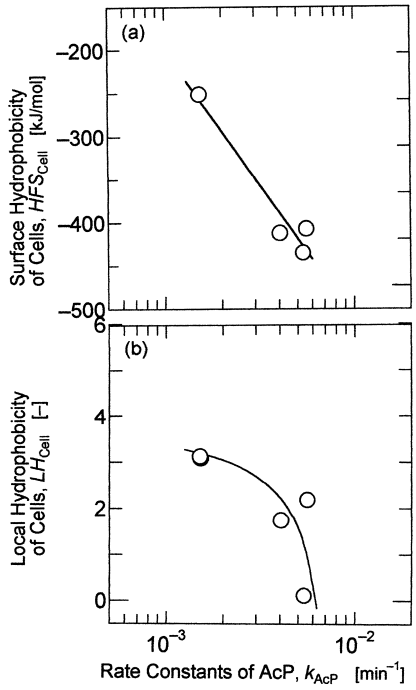

Fig. 3. Relationship between release-rate constant, k_{AcP}, of AcP and (a) surface hydrophobicity of cells, HFS_{Cell}, or (b) local hydrophobicity, LH_{Cell}. The k_{AcP} values were determined after 20 m*M* Triton X-100–5 m*M* EDTA treatment of cells heated at various temperatures for 10 min.

observed with increasing hydrophobicity values. The relationship among the heating conditions, release-rate constants, and the surface hydrophobicities of the heated cells has thus been presented.

It has been reported that the translocation behavior of cytoplasmic β-gal across the inner membrane of *E. coli* cells under heat stress was correlated with the local hydrophobicity values of the inner membrane of the *E. coli* cells and target β-gal [18]. In the case of release of periplasmic AcP to the surrounding media across the outer membrane under milder chemical stress (in the presence of Triton X-100–EDTA), the release-rate constant, k_{AcP}, was also found to depend on the evaluated hydrophobicity of heat-stressed cells. The similar dependence of the release process of the periplasmic AcP across the outer membrane of heat-stressed cells on the surface hydrophobicity under mechanical (disruptive) stress was also observed [18].

It is considered that the efficiency of the target recovery can be improved by using the heat-induced translocation phenomena of the cytoplasmic enzymes, which are dependent on the hydrophobic properties of the inner membrane of the cells [18]. The effective release of periplasmic enzyme with the Triton X-100–EDTA treatment can also be achieved by considering the hydrophobicities of the outer membrane of cells, which were evaluated above.

In conclusion, the evaluation and control of the surface hydrophobicity of the inner and outer membrane surface of *E. coli* cells and the target protein surface, by exposure to suitable heat stress, are effective for the design and development of selective release processes of cytoplasmic and periplasmic enzymes.

4. Notation

$D_{i,Triton}$, remainder fraction of enzyme i after Triton X-100–EDTA treatment ($=1-RF_{i,Triton}$) (—)

HF, hydrophobic factor between two-phases [19] (mol/kJ)

HFS_{Cell}, surface net hydrophobicity of bacterial cells determined by the aqueous two-phase partitioning method [16,17,26] (kJ/mol)

i, kinds of enzyme (i = AcPacid phosphatase)

k_i, release-rate constant of enzyme i when cells were treated by Triton X-100–EDTA (min^{-1})

LH_{Cell}, local hydrophobicity of bacterial cells determined by the aqueous two-phase partitioning method [16,17,26] (—)

$RF_{i,Triton}$: released fraction of enzyme i after Triton X-100–EDTA treatment (—)

t, time for Triton X-100–EDTA treatment (min)

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