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Characterization of Surface Properties of Microbial Transglutaminase Using Aqueous Two-Phase Partitioning Method

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The surface properties of recombinant microbial transglutaminase (MTG) were characterized by varying the pH through the aqueous two-phase system (ATPS) method in order to select the effective refolding pathways. It was found that the MTG activity was lost and intrinsic tryptophan fluorescence was varied at pH values less than 3.8. In this pH region, the surface net charge and surface net hydrophobicity of the MTG, determined by the ATPS method, gradually increased. At around pH 3.5, the local hydrophobicity of the MTG was found to be maximal.

1. Introduction

Transglutaminase (TG protein-glutamine-glutamyltransferase, EC 2.3.2.13) is a family of enzymes that catalyze the acyl transfer reaction between the γ-carboxyamide group of glutamine residues within peptides and the ε-amino group of lysine residues [1]. The functionality of the transglutaminase has long attracted much attention [2-4]. Recently, the gene encoding the mature sequence of microbial transglutaminase (MTG) has been chemically synthesized. MTG was highly expressed as inclusion bodies in *Escherichia coli* and the preliminary refolding of MTG showed activity equivalent to that of native MTG, but with low protein recovery [5,6]. The design and development of the enhanced refolding process is important and necessary.

In biological systems, denatured proteins interact with lipid bilayers and with molecular chaperones during folding. Partially denatured proteins in a molten-globule state have been reported to be a key conformation in interacting with membranes [7]. It has previously been reported that refolding of denatured bovine carbonic anhydrase was assisted by liposome, probably by weak interactions between the proteins and the liposome, similar to protein refolding mediated by molecular chaperones [8]. The effectiveness of the use of aqueous two-phase systems (ATPS) in the design of the liposome-assisted refolding process [8-11] has already been reported.

The purpose of this study is to characterize the surface properties of stressed proteins (acidic pH: from 3 to 6) by selecting the refolding of MTG [11] as a case study. The surface properties of the MTG, such as surface net charge and hydrophobicity and local hydrophobicity, have been quantitatively characterized at various pH values by using an ATPS.

2 Experimental

Transglutaminase minus aspartic acid of the amino terminal group was kindly gifted from Ajinomoto Corp. All other chemicals of analytical grade were purchased from Wako Pure Chemicals (Osaka, Japan). Poly (ethylene glycol) (PEG 1540, 4000, 6000; Mw 1.5 kD, 3kD and 7 kD, respectively) and dextran (Dex) (100~200k; Mw 100-200 kD) were purchased from Wako Pure Chemicals Ltd. (Osaka, Japan).

The basic composition 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 100-200k. The ATPSs were prepared by mixing the stock solutions of 30 wt% PEG and 30 wt% Dex with the transglutaminase solution described above. The pH values of these systems were adjusted by the addition of a high concentration HCl solution of high concentration.

The surface charge （Z）of MTG was analyzed from its partitioning in PEG4000 (9wt%) / Dex 100~200k(9%) ATPS with 100mM Na_2SO_4 or 200mM NaCl, giving positive and negative electrostatic potentials between the two phases [12,13]. The electric potentials of the ATPSs were determined from the partitioning behavior of hydrophilic amino acids such as glutamine and asparagine. The surface properties such as surface net hydrophobicity (*HFS*) and local hydrophobicity (*LH*) of the proteins were analyzed by using the aqueous two-phase partitioning method as was applied for the characterization of the protein surface [12,13]. When the pH is then selected for the pI value at lower ionic strength, the partitioning coefficient of biomolecules was mainly dependent on the hydrophobic effect and the following relationship can be obtained.

$$\ln K = HFS \times HF \quad (1)$$

where the hydrophobic factor (*HF*), which has been defined from the partition coefficient of amino acids [12], is an indicator of the hydrophobicity difference between two-phase systems and the *HFS* value is defined as the surface net hydrophobicity of proteins. Further, the local hydrophobicity of proteins under heat stress was also determined as $LH_{Pr} = \Delta\ln K_{Pr} = \ln K_{Pr,Triton} - \ln K_{Pr.0}$, where $K_{Pr,Triton}$, and $K_{Pr,0}$are the partitioning coefficients of protein in the aqueous two-phase systems with and without 1m M Triton X-405 [12]. The local hydrophobicity of MTG was also characterized by using hydrophobic fluorescence probes, 1-anilino-8-naphthalene sulfonate (ANS) （λex = 400 nm, λem = 470 nm). The surface charge densities of various liposomes are calculated assuming certain structural features of liposomes [14] and ionization of the phosphate group of POPG [15].

MTG activity was measured by the calorimetric hydroxamate procedure using N-carbobenzoxy-γ-glutaminylglycine (CBZ-Gln-Gly) [1]. The absorbance at 525 nm was monitored as a measure of the hydroxymation reaction.

3. Results and Discussion

The conformational change of MTG was characterized under the possible stress conditions along the refolding pathway by measuring the intrinsic fluorescence and by using the aqueous two-phase partitioning method. **Figure 1** shows the pH dependence of (a) the activity and intrinsic fluorescence of MTG and (b)

the surface properties of MTG. The pH dependence of the peak wavelength for the intrinsic fluorescence spectrum of MTG was measured after the MTG was incubated in the buffers at various pH values at 4°C for twelve hours. As shown in **Fig.1(a)**, the MTG activity was not changed at pH values between 6.0 and 4.0 during the acidification process and decreased at pH values between 4.0 and 3.5. Although the peak wavelength for the native state of MTG was 357nm, the value was shifted to 341nm at pH values between 3.0 to 4.0 during the acid denaturation (closed circle in **Fig.1(a)**). The peak wavelength of MTG was also measured by denaturing it in an 8M urea solution and was found to be 341nm, indicating that the conformation of the MTG was changed to a structure similar to the urea-denaturing one by reduction of the pH to below 3.0. The blue shift of the fluorescence can herewith be interpreted as the exposure of intrinsic aromatic groups of MTG to its surface thus inducing the conformational change. The MTG activity also decreased with the decrease in the pH value in correspondence with the conformational change. The conformational change was also characterized by using the hydrophobic fluorescence method. The pH dependence of the fluorescence intensity of the hydrophobic probe, ANS, for the MTG solution is also shown in **Fig.1(a)**. At neutral pH from 6.0 to 4.0, the ANS intensity was not changed. However, the values increased and maximized at pH 3.5 and decreased again at pH 3.2. ANS has previously been utilized as a probe to detect partially unfolded protein such as the molten-globule (MG)-like state. The above results shows that the MG-like state of MTG was induced at pH values around 3.5.

As shown in **Fig.l(b)**, the surface properties of MTG (surface net hydrophobicity (*HFS*), local hydrophobicity (*LH*), and surface net charge (*Z*)) were also determined by using the aqueous two-phase partitioning method [12,13]. The *HFS* values increased inversely in correspondence with the decrease of MTG activity at pH values from 4.0 to 3.5. The *LH* value showed a maximum value at pH 3.5 and decreased at more acidic pH values. In our previous report, the two types of hydrophobicities, *HFS* and *LH*, of proteins were shown to be correlated with the accessible surface area (ASA) and with the fluorescence intensity of hydrophobic probe ANS, respectively [16]. In addition, the surface hydrophobicities of the proteins were systematically characterized under various stress conditions (pH, denaturant, temperature and so on), resulting in an

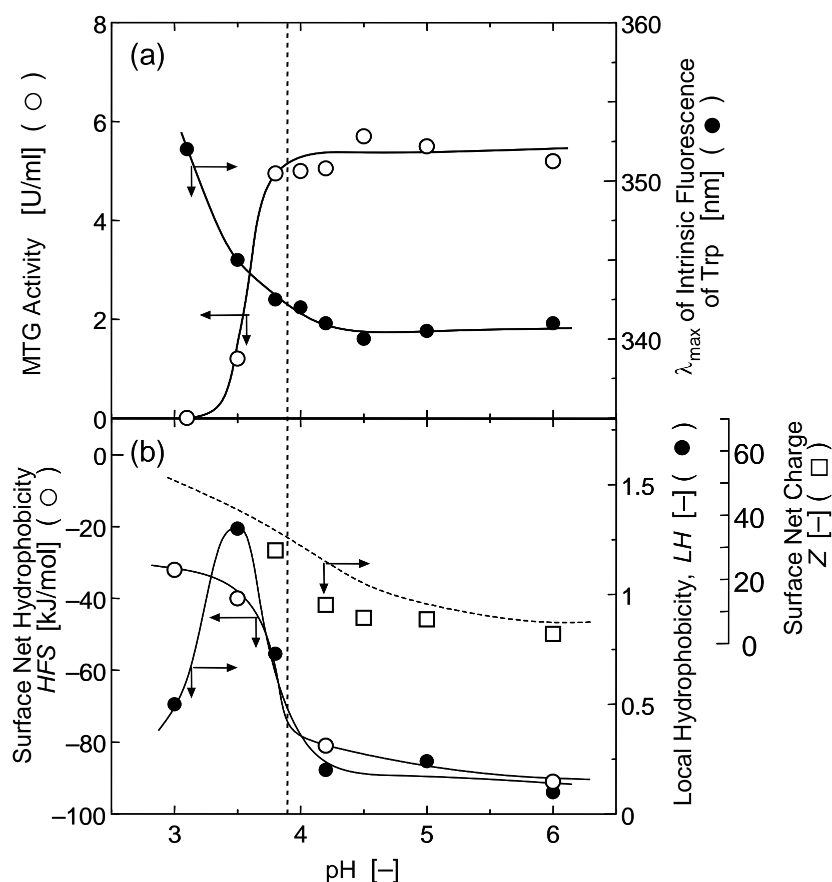

Fig.1 pH Dependence of (a) activity and conformation of MTG and (b) surface properties determined by the aqueous two-phase partitioning method

increase in the *HFS* values for the proteins in the unfolded state and the *LH* values were maximal at the specific stress condition required to induce the conformational change to the MG-like state. It has been reported that the pH shift could induce the conformational change of MTG and acidification of the solution at pH 4.2 changes the MTG conformation to the MG-like state [17]. The above results obtained with MTG against the pH shift corresponded well with the previous findings [6]. The above results may imply that the conformation of MTG was changed to the MG-like state at which the intrinsic clustered hydrophobic residues were exposed to the protein surface at pH 3.5 and changed to the fully unfolded state in which the hydrophobic residues were dispersedly exposed to its surface. The specific pH condition to induce the characteristic conformation of MTG was found to be around 3.5 during the acidification.

The electrostatic interaction can also be an important factor affecting the conformational change of MTG. The values of the surface net charge, *Z*, are shown in **Fig.1(b)**, together with those calculated from the charged amino acids. The *Z* value was not significantly changed at pH values above 4.2 and increased with a decrease in the pH value, especially, at pH 4.0. The measured values were smaller than the calculated ones at pH values from 4.0 to 6.0, implying that the charged residues were buried inside the protein molecule because the conformational change was not fully achieved in the above pH region. These phenomena corresponded with the results obtained from the surface hydrophobicities. It is considered that the drastic change in the interaction between the (charged) amino acids was induced at pH values around 4.0 and may inhibit the conformational change from unfolded to the native state of MTG during its refolding process. It is, however, expected that practical refolding can be suitably achieved at pH 4.0 because the repulsive force between the proteins is easily induced at this pH as compared to that at pH 6.0. The charge density of MTG, defined as the charge per protein surface, is 0.025 C/nm^2 at pH 6.0 and is much smaller than those for lysozyme (0.15 C/nm^2) and ribonuclease (0.065 C/nm^2), implying that the aggregates of MTG may easily be formed during the refolding at pH 6.0.

The control of hydrophobic and electrostatic interaction was thus found to be important during the conformational change of MTG.

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