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Unfolded Histidine-Tagged Protein is Immobilized to Nitrilotriacetic Acid-Nickel Beads, But Not the Nickel-Coated Glass Slide

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Abstract

The adsorption of proteins on the surface of glass slides is essential for construction of protein chips. Previously, we prepared a nickel-coated plate by the spin-coating method for immobilization of His-tagged proteins. In order to know whether the structural factor is responsible for the immobilization of His-tagged proteins to the nickel-coated glass slide, we executed a series of experiments. First we purified a His-tagged protein after expressing the vector in *E. coli* BL21 (DE3). Then we obtained the unfolding curve for the His-tagged protein by using guanidine hydrochloride. Fractions unfolded were monitored by internal fluorescence spectroscopy. The ΔG_{H2O} for unfolding was 2.27 kcal/mol ± 0.52. Then we tested if unfolded His-tagged proteins can be adsorbed to the nickel-coated plate, comparing with Ni^{2+}-NTA (nitrilotriacetic acid) beads. Whereas unfolded His-tagged proteins were adsorbed to Ni^{2+}-NTA beads, they did not bind to the nickel-coated plate. In conclusion, a structural factor is likely to be an important factor for constructing the protein chips, when His-tagged proteins will immobilize to the nickel-coated slides.

Keywords: his-tagged protein, nickel-coated glass slides, protein chip, unfolding

Introduction

For the proteomic analysis, a variety of techniques have recently been developed. A high-throughput analysis with protein chips is a potent technique to screen target molecules in cells or tissues under a given set of physiological conditions. Protein chips are microarrays of proteins adsorbed on the surface of the glass slides in order to facilitate the high-throughput analysis (Fung *et al.*, 2001). In addition, we can take advantage of uniquely biological aspects by analyzing with protein chips. Predominantly, it is possible to differentially display a comprehensive set of proteins in between the control and the experimental samples, so that we can compare protein profiles of the control with the target populations. Therefore, their applications expand potentially to the area of screening for new functional proteins, diagnosis or therapy for diseases. Because of these possible applications, the protein chips have been highly focused and considered as valuable tools.

For those reasons, the well-developed protein chip has apparently been needed. However, the protein chips were little established, unlike DNA chips (Debouck and Goodfellow, 1999; Ramaswamy and Golub, 2002). When compared with the DNA chip fabrication, the protein chip construction is more difficult not only because of the instability of the protein structure retaining its functionality but also no amplification tools for protein like the polymerase chain reaction (PCR) for DNA (Fung *et al.*, 2001).

To date, a diversity of protein chips were experimentally constructed (Arenkov *et al.*, 2000; MacBeath and Schreiber, 2000; Zhu *et al.*, 2001). Among those chips, it is still challengeable to construct the protein chips for histidine (His)-tagged proteins, because His-tagged proteins can bind to bivalent metal ions. We previously constructed the nickel-coated glass slide to immobilize the His-tagged proteins by using a spin coater. In this study, we further determined that a structural factor of His-tagged proteins influences on the immobilization of His-tagged proteins to the nickel-coated glass slides.

Materials and Methods

Expression of His-Tagged WAX 9D Protein: A plant cDNA clone encoding the full-length WAX9D lipid transfer protein (LTP) was expressed as a His-tag fusion protein in *Escherichia coli* BL21 (DE3) and purified by nickel affinity chromatography as described previously (Shin *et al.*, 2006). Briefly, *E. coli* cells were harvested by centrifugation at 7,000 g for 20 min, suspended in a lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM Imidazole, pH 8.0), and lysed by sonication. Then cell lysate was separated from

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cell debris by centrifugation at 15,000 g for 10 min. Finally, the His-tagged fusion protein was separated by nickel affinity column as described in supplier's manual (Invitrogen). For purification of the His-tag protein, the purified His-tagged WAX9D was thrombin-cut and re-purified by nickel affinity column chromatography.

Equilibrium denaturation: Equilibrium denaturation as a function of guanidium hydrochloride (GdnHCl) concentration was monitored by internal protein fluorescence spectroscopy using a spectrofluorometer (Shimazu). Protein samples at indicated concentrations were equilibrated at various concentration of GdnHCl at 20 °C. Then eguilibrium unfolding was detected by fluorescence spectroscopy with excitation at 280 nm and emission at 310 nm. Nonlinear least-square fit to the equilibrium data was executed by using Origin software (provided by Microcal) and the following Santoro-Bolen equation (Santoro and Bolen, 1988):

$$X_D = \{(X_N + a_1[GdnHCl])+(X_U + a_2[GdnHCl]) \exp[-(\Delta GH_2O/RT + m[GdnHCl]/RT)]\}/ \{1 + \exp[-(\Delta GH_2O/RT + m[GdnHCl]/RT)]\}$$

where X_N and X_U are the intercepts and a_1 and a_2 are the slopes of the baselines at low and high urea concentration, respectively. ΔGH_{2O} is the apparent free energy difference between the folded and unfolded forms of the protein linearly extrapolated to [GdnHCl] = 0, m is the slope describing the dependence of ΔGH_2O on [GdnHCl].

Preparation of Nickel Coated Slides: The glass slides were cleaned up by sonication in ethanol and water alternatively and then completely dried before coating with nickel ion. The cleaned slides were then put on the rotor of the spin coater, spread with 0.8 ml of various concentration of $NiCl_2$ and then spun at various speeds (1000 to 4000 rpm) for different time points. Then coated slides were dried on the hot plates at various temperatures.

Protein Adsorption: The FITC-conjugated His-tagged protein (Ko *et al.*, 2001) was applied to the slides, incubated for 0.5h at room temperature, washed with PBS or methanol and dried for fluorescence detection.

Fluorescence Detection: FITC-conjugated proteins immobilized on the glass slide were detected by a Bio-Imaging Analyzer System (BAS; Fuji Photo Film Co.) at excitation and emission of 490 and 520 nm, respectively.

Western Blots: Protein solution (10 μg each) was resolved by 15% SDS-PAGE, transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore), and probed with His-tag antibodies (Ab) (Cell Signaling). Goat anti-rabbit IgGs (Santa Cruz) conjugated to horseradish peroxidase were used as secondary Ab's and membranes were developed by a chemiluminescent detection method (Park *et al.*, 1998).

Results and Discussion

For assessing the immobilization of His-tagged proteins to the nickel coated glass slides, the vector pET 32a encoding a His-tagged protein gene was transformed into *E. coli* BL21 (DE3). Then the His-tagged protein was expressed, thrombin-cut and purified by the nickel-column chro- matography. The sequence of the His-tagged protein appears in Fig. 1.

We were curious whether the unfolding state of His-tagged proteins affects the immobilization of His-tagged proteins to the nickel ion-coated glass slides. In order to know the query, we examined the GdnHCl(guanidium hydrochloride)-dependent unfolding profile of the His-tagged protein. First, we measured the fluorescence spectra between 0 M and 8 M GdnHCl. As shown in Fig. 2, the fluorescence intensity of the His-tagged protein at 8 M guanidium was higher than at 0 M, indicating that the His-tagged protein is likely to be unfolded at 8 M GdnHCl. Then, the unfolding kinetics was determined by monitoring

M S D K I I H L T D D S F D T D V L K A D G A I L
V D F W A E W C G P C K M I A P I L D E I A D E Y
Q G K L T V A K L N I D Q N P G T A P K Y G I R G
I P T L L L F K N G E V A A T K V G A L S K G Q L
K E F L D A N L A G S G S G H M H H H H H H S S
G L V P R

Fig. 1. The sequence of the thrombin-cut His-tagged protein, the gene of which is encoded in the expression vector pET 32a. The underlined sequences are Histidine-tag.

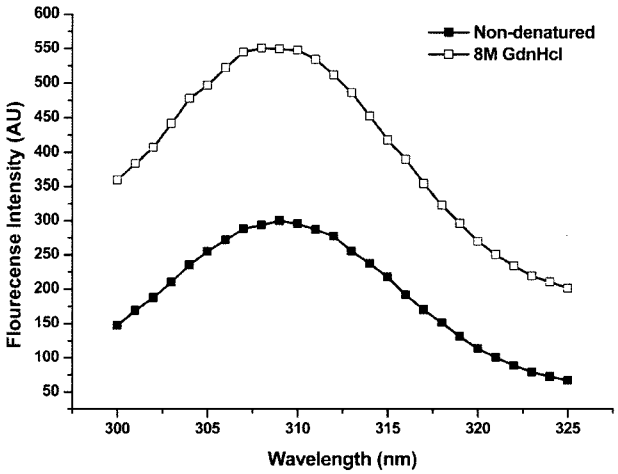

Fig. 2. Fluorescence spectra of the His-tagged protein at 0 M and 8 M guanidium hydrochloride. The emission spectra for the His-tagged protein (excitation at 280 nm) were measured at300 to 325 nm at different concentrations of guanidium hydrochloride.

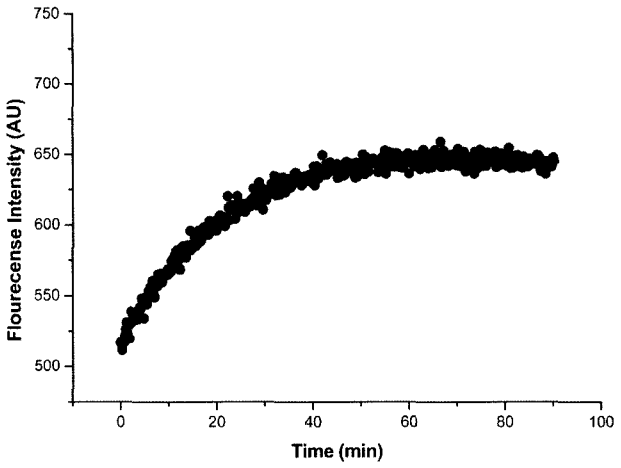

Fig.3. Unfolding rate of the His-tagged protein in the presence of 8 M guanidium hydrochloride. Unfolding was monitored by fluorescence intensity (Ex. 280nm; Em. 310 nm). Protein solutions of 2.4 nM His-tagged protein were mixed to 8 M guanidium hydrochloride in PBS. Temperature was 25°C.

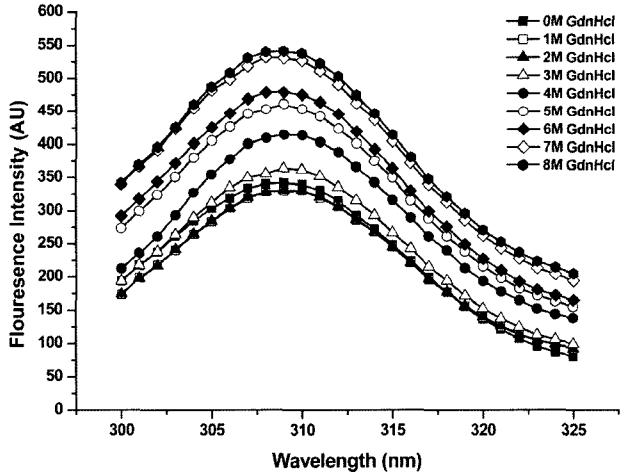

Fig. 4. Fluorescence spectra of the His-tagged protein at 0-8 M guanidium hydrochloride. The emission spectra for the His-tagged protein (excitation at 280 nm) were measured at 300 to 325 nm at various concentrations of guanidium hydrochloride. The concentration of the His-tagged protein was 2.4 nM and temperature was 25°C.

fluorescence intensity (Ex: 280 nm; Em: 310 nm) as a function of time immediately after 2.4 nM His-tagged protein was added to 8 M GdnHCl solution. In Fig.3, the folding/unfolding equilibrium was completely reached 60min after addition of the protein. Therefore we incubated the protein for more than 1 h in the unfolding buffer to reach the complete unfolded state.

Fluorescence spectra of the His-tagged protein were then obtained at various concentration of guanidium hydrochloride (Fig. 4). To know reversibility of unfolding/

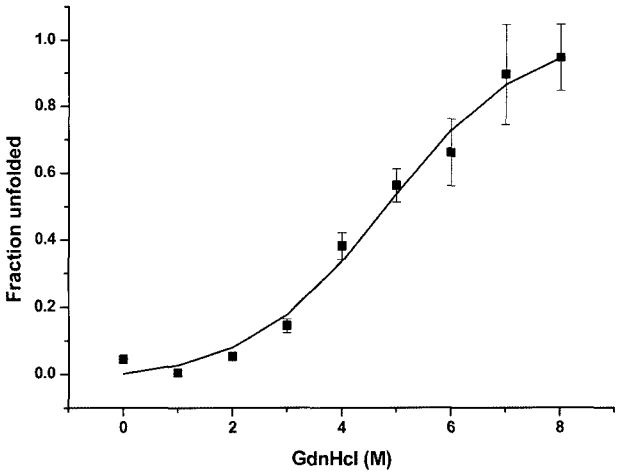

Fig. 5. Folding profile. The unfolded fraction was detected by fluorescence intensity (Ex. 280nm; Em. 310 nm). The data was fitted by the Santoro-Bolen equation as described in Materials and Methods. Three independent experiments were carried out and average values were plotted. The ΔH_2O for unfolding was 2.27 kcal/mol ± 0.52.

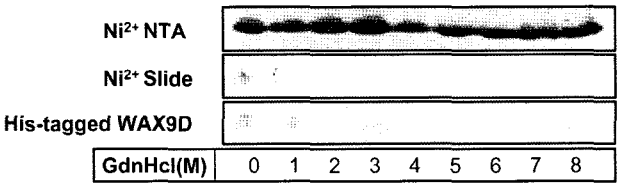

Fig. 6. Immobilization of the His-tagged protein to Ni^{2+}-NTA beads (top panel) and the nickel ion-coated glass slides (middle panel: the His-tagged protein, bottom panel: the His-tagged WAX9D). The His-tagged protein was adsorbed to Ni^{2+}-NTA beads and the nickel ion-coated glass slide and then unbound protein was washed with PBS (phosphate buffered saline). Then the adsorbed proteins in Ni^{2+}-NTA beads and the nickel ion-coated glass slide were detected by Western blots and an image analyzer, respectively.

folding process, the protein was refolded by dialysis against a refolding buffer. Interestingly, the fluorescence spectrum of the refolded protein was similar to that of protein (data not shown), indicating that the unfolding/ folding processes were reversible.

The folding profile for the His-tagged protein was generated by replot of fluorescence intensities at various concentration of GdnHCl (Fig. 5). Fitting was performed at all three different data sets by using the Santoro-Bolen equation (see Materials and Methods). The two-state model (U↔N) is possibly applicable to fit the data. The fitting generated ΔGH_2O as 2.27 kcal/mol±0.52.

Then we tested immobilization of the His-tagged protein to Ni^{2+} ion-coated slide at various guanidium solutions. As

a control, we also immobilized the same protein to the Ni^{2+}-NTA (nitrilotriacetic acid) beads. For immobilization to Ni^{2+}-NTA beads, 2.4 nM His-tagged protein was added to 10 ml NTA beads at 0-8 M GdnHCl, incubated for 1 h and washed thoroughly. Then bead-immobilized protein was detected by Western Blots (Fig. 6, Top panel). Of interest, the His-tagged protein was immobilized in 0-8 M GdnHCl. In comparison, the immobilization of the His-tagged protein to the nickel coated glass slide was detected at 0 and 1 M GdnHCl (Fig. 6, Middle panel). Of interest, the immobilization profile for His-tagged WAX9D was similar to that for the His-tagged protein (Fig. 6, Bottom panel). These data indicate that the folding state is likely to greatly influence on the immobilization of His-tagged proteins to the nickel ion-coated slides, but not on NTA beads.

For His-tagged proteins to immobilize to a solid support, Ni^{2+} ion-chelated imidodiacetic acid (IDA) or nitrilotriacetic acid (NTA) have typically been used (Arnold, 1991; Ko *et al.*, 2001). Previously, we observed that the His-tagged proteins were immobilized to the nickel coated slides prepared with only $NiCl_2$ in the absence of NTA or IDA (Hyun *et al.*, 2002). Although the detailed mechanism remains unknown, we found in this study that the glass slide coated with only $NiCl_2$ can not be utilized in experimental conditions for unfolded proteins.

The nickel-coated slides prepared here by the spin-coating method have many advantages for the protein chip fabrication; 1) low cost, 2) simple constructing process, and 3) more importantly, maintenance of the target domains for test proteins during the protein chip fabrication. However, to extend its application area, the nickel-coated glass slide will be improved to more tightly bind His-tagged proteins in extreme biochemical conditions.

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