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Analytical Biochemistry 348 (2006) 307–314
ANALYTICAL BIOCHEMISTRY
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A novel protein refolding method using a zeolite

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Received 20 July 2005
Available online 8 November 2005

Abstract

We have succeeded in developing a simple and effective protein refolding method using the inorganic catalyst, β-zeolite. The method involves the adsorption of proteins solubilized with 6 M guanidine hydrochroride from inclusion body (IB) preparations onto the zeolite. The denaturant is then removed, and the proteins in the IBs are released from the zeolite with polyoxyethylene detergent and salt. All of the IBs tested (11 different species) were successfully refolded under these conditions. The refolded proteins are biochemically active, and NMR analysis of one of the proteins (replication protein A 8) supports the conclusion that correct refolding does occur. Based on these results, we discuss the refolding mechanism.
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Keywords: Zeolite; Refolding; Inclusion body; Denaturant; Detergent; Polyethylene glycol

Many genes of interest are expressed recombinantly in foreign cells. One of the most widely used recombinant systems is *Escherichia coli*, which offers ease of use and is a relatively inexpensive means of obtaining large amounts of target protein [1]. However, the rate of synthesis of the nascent polypeptide in this system is often faster than the rate of folding, resulting in the formation of misfolded protein aggregates that are deposited in the cytoplasm of the bacterial cell as inclusion bodies (IBs)[1] [2]. Many methods for IB solubilization and protein refolding have been reported, for example, methods based on dilution [3], dialysis [4], size exclusion chromatography [5,6], artificial chaperones [7–9], refolding chromatography [10–12], and arginine [13–15]. In the postgenomics era, there is an urgent need to develop a novel protein refolding method that can be applied to any denatured protein.

In general, refolding involves a two-step protocol, namely solubilization of IB by denaturant and then refolding by removal of the denaturant. One of the disadvantages of dialysis and dilution protocols is the large amount of time needed to remove the denaturants. To solve this problem, we investigated new refolding methods that can be performed on the surface of a protein adsorbent and that make the removal of denaturants a much more rapid process.

Zeolites are crystalline porous solids with pores and channel systems in the molecular size range of 0.3–3 nm. They are tectosilicates consisting of corner-sharing AlO_4 and SiO_4 tetrahedra. These physicochemical characteristics are thought to be the basis for their immense importance in catalysis reactions, separation processes, and ion exchange [16–18]. The SiO_2/Al_2O_3 ratio of zeolites can be varied either during synthesis or postsynthetically. The SiO_2/Al_2O_3 ratio is also used to denote the hydrophobicity of zeolites, with higher ratios indicating higher degrees of hydrophobicity and lower ion exchange capacity. In our previous

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[1] *Abbreviations used:* IB, inclusion body; PBS, phosphate-buffered saline; HSQC, heteronuclear single quantum coherence; RPA, replication protein A; DTT, dithiothreitol; PEG, polyethylene glycol; EO, ethylene oxide.

0003-2697/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.ab.2005.10.032

report, we found that zeolites adsorbed proteins in the presence of denaturants [19].

In the current work, we have investigated a simple and effective refolding method using a zeolite. All of the IBs we tested were solubilized, and enzyme activities were recovered.

Materials and methods

Materials

All detergents used were obtained from Calbiochem and Dojindo. $^{15}NH_4Cl$ and D_2O were purchased from Cambridge Isotope Laboratories. Zeolite Y (H-Y, SiO_2/Al_2O_3 ratio 5.7), zeolite L (K-LTL, SiO_2/Al_2O_3 ratio 6.0), zeolite USY (H-USY330, 360, and 390, SiO_2/Al_2O_3 ratios 6.3, 13.7, and 360, respectively), K-ferrierite (K-FER, SiO_2/Al_2O_3 ratio 17.7), β-zeolite (Na-BEA, SiO_2/Al_2O_3 ratio 27), and ZSM-5 (SiO_2/Al_2O_3 ratio 28) were obtained from Tosoh.

Preparations and activity measurements of proteins

All of the coding sequences of proteins used in this study were cloned into pET vectors (Novagen) for expression in *E. coli*.

Expression plasmids were transformed into *E. coli* strain Rosetta2 (DE3) (Novagen) or BL21 Star (DE3) (Invitrogen). The bacterial cells were grown from a single colony overnight in 100 ml SuperBroth (Qbiogene). After transfer to a 900-ml culture and growth for 1.5 h at 37 °C, the cells were induced with isopropyl β-D-thiogalactoside to a final concentration of 1 mM and incubated for 4–6 h at 37 °C. The cells were harvested by centrifugation at 6000*g* for 15 min, and the pellets were resuspended in 100 ml lysis buffer [1× phosphate-buffered saline (PBS) containing lysozyme] and sonicated. The lysates were centrifuged at 8000*g* for 20 min. The pellets were washed three times with 1× PBS and resuspended in 100 ml of 6 M guanidine hydrochloride, 20 mM β-mercaptoethanol, and 1× PBS, and the mixture was incubated for 2–3 days at room temperature.

Protein concentration was determined by the Bradford method [20] with bovine serum albumin as a standard, and protein activities were measured as described previously [21–26].

NMR spectroscopy

^1H–^{15}N heteronuclear single quantum coherence (HSQC) spectra for replication protein A 8 (RPA 8) were acquired on a Bruker DRX-600 spectrometer equipped with a 5-mm inverse detection triple resonance probe with *x*, *y*, and *z* gradient coils. NMR experiments were performed using 100 μM ^{15}N-labeled RPA 8 complexed with RPA 30 (27–142) in 25 mM sodium phosphate (pH 7.5), 150 mM KCl, 5 mM dithiothreitol (DTT), and 5% D_2O. Spectra of ^{15}N-labeled RPA 8 derived from IBs were acquired in 0.5 M guanidine hydrochloride, 25 mM sodium phosphate (pH 7.5), 20 mM β-mercaptoethanol, and 5% D_2O. All NMR investigations on RPA 8 were carried out at 283 K. Suppression of the residual water signal was achieved using Watergate [27].

Refolding of IBs

IBs were denatured in 6 M guanidine hydrochloride, 1× PBS (pH 7.4), and 20 mM β-mercaptoethanol for several days at room temperature. Then 100 mg of a given protein adsorbent was suspended in 1 ml of the denatured protein solution. The solutions were then vortexed before being incubated for 30 min on a Rotary Culture RCC-100 mixer (Iwaki Glass). After the incubation, the samples were centrifuged at 10,000 rpm for 5 s. Subsequently, all of the supernatants were discarded. The protein adsorbents were washed four times, each time with 1 ml of 1× PBS and 20 mM β-mercaptoethanol, before being resuspended in 1 ml of 50 mM Hepes (pH 7.5), 0.5 M NaCl, 0.5% (w/v) polyethylene glycol (PEG) 20,000, 20 mM β-mercaptoethanol, and 1% (v/v) Tween 20. The mixtures were vortexed and then incubated for 10 h on a Rotary Culture RCC-100 mixer at 4 °C. After the incubation, the samples were centrifuged at 10,000 rpm for 1 min. The supernatants were stored and treated as refolded protein solutions.

Refolding of IBs by continuous elution

Method 1

The first elution of this method was as described above for the refolding of IBs. Protein adsorbents were then resuspended in 1 ml of 50 mM Hepes (pH 7.5), 0.5 M NaCl, 0.5% (w/v) PEG 20,000, 20 mM β-mercaptoethanol, and 1% (v/v) Tween 20. The mixtures were incubated for 10 h on a Rotary Culture RCC-100 mixer at 4 °C. After the incubation, the samples were centrifuged at 10,000 rpm for 1 min. The supernatants were stored and treated as refolded protein solutions. This procedure was performed two times.

Method 2

The first elution of this method was as described above for the refolding of IBs. Protein adsorbents were then washed four times, each time with 1 ml of 1× PBS and 20 mM β-mercaptoethanol, and then resuspended in 1 ml of 6 M guanidine hydrochloride, 1× PBS (pH 7.4), and 20 mM β-mercaptoethanol. The solutions were vortexed before incubation for 30 min on a Rotary Culture RCC-100 mixer. After the incubation, the protein adsorbents were washed four times, each time with 1 ml of 1× PBS and 20 mM β-mercaptoethanol, and then resuspended in 1 ml of 50 mM Hepes (pH 7.5), 0.5 M NaCl, 0.5% (w/v) PEG 20,000, 20 mM β-mercaptoethanol, and 1% (v/v) Tween 20. The mixtures were vortexed and incubated for 10 h on a Rotary Culture RCC-100 mixer at 4 °C. After the incubation, the samples were centrifuged at 10,000 rpm for 1 min. The supernatants were stored and treated as refolded protein solutions. This procedure was performed two times.

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