Journal of Immunological Methods 442 (2017) 1–11

Contents lists available at ScienceDirect

Journal of Immunological Methods

journal homepage: www.elsevier.com/locate/jim

Research paper

Accurate quantitation for *in vitro* refolding of single domain antibody fragments expressed as inclusion bodies by referring the concomitant expression of a soluble form in the periplasms of *Escherichia coli*

Tomoaki Noguchi, Yuichi Nishida, Keiji Takizawa, Yue Cui, Koki Tsutsumi, Takashi Hamada, Yoshisuke Nishi *

Graduate School of Bioscience, Nagahama Institute of Bio-Science and Technology, 1266 Tamura-cho, Nagahama, Shiga 526-0829, Japan

ARTICLE INFO

Article history:
Received 13 October 2016
Received in revised form 21 November 2016
Accepted 30 November 2016
Available online 8 December 2016

Keywords:
Refolding of single chain antibody fragment
CD spectroscopy
Periplasmic fraction
Cytoplasmic insoluble fraction
Dialysis refolding

ABSTRACT

Single domain antibody fragments from two species, a camel V_HH (PM1) and a shark V_{NAR} (A6), were derived from inclusion bodies of *E. coli* and refolded *in vitro* following three refolding recipes for comparing refolding efficiencies: three-step cold dialysis refolding (TCDR), one-step hot dialysis refolding (OHDR), and one-step cold dialysis refolding (OCDR), as these fragments were expressed as 'a soluble form' either in cytoplasm or periplasm, but the amount were much less than those expressed as 'an insoluble form (inclusion body)' in cytoplasm and periplasm. In order to verify the refolding efficiencies from inclusion bodies correctly, proteins purified from periplasmic soluble fractions were used as reference samples. These samples showed far-UV spectra of a typical β-sheet-dominant structure in circular dichroism (CD) spectroscopy and so did the refolded samples as well. As the maximal magnitude of ellipticity in millidegrees (θ_{max}) observed at a given wave length was proportional to the concentrations of the respective reference samples, we could draw linear regression lines for the magnitudes *vs.* sample concentrations. By using these lines, we measured the concentrations for the refolded PM1 and A6 samples purified from solubilized cytoplasmic insoluble fractions. The refolding efficiency of PM1 was almost 50% following TCDR and 40% and 30% following OHDR and OCDR, respectively, whereas the value of A6 was around 30% following TCDR, and out of bound for quantitation following the other two recipes. The ELISA curves, which were derived from the refolded samples, coincided better with those obtained from the reference samples after converting the values from the protein-concentrations at recovery to the ones of refolded proteins using recovery ratios, indicating that such a correction gives better results for the accurate measure of the ELISA curves than those without correction. Our method require constructing a dual expression system, expressed both in periplasm as a soluble form and cytoplasm as an insoluble form; application of the different refolding recipes due to sequence-by-sequence-difference could be precisely monitored using CD spectra with the concomitant soluble samples as a reference.

1. Introduction

Escherichia coli (*E. coli*) is one of the most widely used prokaryotic organisms for the industrial production of proteins for therapeutics, diagnostics, and other biologics. Compared with other established and engineered expression systems, *E. coli* has several advantages: it is inexpensive and can grow rapidly, so it is readily applicable to high cell-density fermentations and scale-up production with the lowest cost. Such advantages are further facilitated with the fact that genetic manipulations of *E. coli* are the easiest among various organisms because its genetics are the best characterized (Baneyx, 1999).

In *E. coli*, there are three ways for production of recombinant proteins: cytoplasmic production, periplasmic production and production directly into the culture medium. In terms of scale and quantity, cytoplasmic and periplasmic production is better choice on the bench top level-experiment than secreted production in the medium.

Abbreviations: CD, Circular Dichroism; ELISA, enzyme-linked immunosorbent assay; TCDR, three-step cold dialysis refolding; OHDR, one-step hot dialysis refolding; OCDR, one-step cold dialysis refolding; CDR, complementarity determining region; HV, hypervariable region; CBB, Coomassie Brilliant Blue; IPTG, isopropyl β-D-1-thiogalactopyranoside; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Gdn-HCl, guanidinium chloride; DTT, dithiothreitol; 2-ME, 2-mercaptoethanol; EDTA, ethylenediaminetetraacetic acid; GSSG, glutathione disulfide; GSH, glutathione; PVDF, polyvinylidene difluoride; PBS-T, phosphate buffered saline with Tween-20 (0.05%); HRP, horse radish peroxidase; CCD, charge coupled device; TOF-MS, time of flight mass spectrometry; BSA, bovine serum albumin; ABTS, 2,2′-azino-di(3-ethylbenzthiazoline-6-sulfonate); Tris, tris (hydroxymethyl) aminomethane; LYZ, lysozyme; HEL, hen egg lysozyme; DSSP, Define Secondary Structure of Proteins; Peri-vector, a vector expressing proteins in the periplasm; Cyto-vector, a vector expressing proteins in the cytoplasm; RMC, refolded molar concentration..

* Corresponding author at: Faculty of Bioscience, Nagahama Institute of Bio-Science and Technology, 1266 Tamura-cho, Nagahama, Shiga 526-0829, Japan.
E-mail address: y_nishi@nagahama-i-bio.ac.jp (Y. Nishi).

http://dx.doi.org/10.1016/j.jim.2016.11.014
0022-1759/

Antibody fragments and their derivatives for production in *E. coli* have been expressed and produced in *E. coli* following the same ways as described above (Boss et al., 1984; Cabilly et al., 1984; Bird et al., 1988; Huston et al., 1988; Condra et al., 1990; Buchner and Rudolph, 1991; Arbabi-Ghahroudi et al., 1995; Maeda et al., 1996; Tsumoto et al., 1998; Umetsu et al., 2003). Production of antibodies and their derivatives is often given in the form as 'cytoplasmic aggregates (inclusion bodies)' in large quantity, rather than as native forms (soluble forms) in the cytoplasm or periplasm, and the production is divergent depending on the degree of sequence heterogeneity (Maeda et al., 1996; Guo et al., 2003; Arakawa and Ejima, 2014). Once cytoplasmic aggregates were obtained, several different techniques are employed for refolding, including dilution-based refolding (Buchner and Rudolph, 1991), stepwise dialysis refolding (Condra et al., 1990; Maeda et al., 1996; Tsumoto et al., 1998; Umetsu et al., 2003), ion-exchange chromatography-based refolding (Guo et al., 2003), and size-exclusion chromatography-based refolding (Yang et al., 2005; Fursova et al., 2009; Kudou et al., 2011).

Finding the most optimal refolding processes is necessarily required, as there is no general rule to show an optimal protocol for *in vitro* refolding of antibody and its fragments. Trial and error type of experiments is required for finding the most optimized protocol and quantitative evaluation must be accompanied with such an experiment; correcting measurements of the concentrations of the refolded proteins are particularly important.

In reviewing the literature describing the production of antibody's fragments following *in vitro* refolding from aggregates, there are the ways for quantitation of refolding yields by directly calculating A_{280R}/A_{280D}, where A_{280R} represents the protein concentrations in the refolded solution, and A_{280D} represents those in the denatured solution before refolding (Tsumoto et al., 1998; Guo et al., 2003), or measuring the protein concentrations, not by UV but by Bradford's assay (Chen et al., 2006). The peak area of ion-exchange HPLC was compared with the concentration of the authentic proteins, which had previously been prepared (Fujii et al., 2007). In other protocols, the refolding yield is monitored with binding to antigens (Maeda et al., 1996; Yang et al., 2005; Kudou et al., 2011). In papers dealing with refolding proteins other than antibodies or antibody fragments, there are several methods, *i.e.*, 'functional assays' for quantitation, in addition to the common simple absorbance (A_{280}) assay (Yang et al., 2004). They included the assays, such as quantitation based on the enzymatic activity of an authentic sample (Hevehan and De Bernardez Clark, 1997), the amount of the cell lysate (Chang et al., 2001), or the receptor-ligand binding activity against a ligand (Xie et al., 2003).

In the present work, we tried to set up to establish a more standardized quantitation protocol for *in vitro* refolding than ever used before. The target antibody fragments, a camel V_HH (PM1) and shark V_{NAR} (A6), both being single domain antibody fragments, were used for standardization; two expression vector cassettes were constructed: one is for cytoplasmic production (Cyto-vector) and another is for periplasmic production (Peri-vector). This dual expression system is prerequisite for our quantitating assay. With the 'Cyto-vector', the proteins without a signal sequence directing to the periplasm were exclusively produced as 'aggregates' in the form of inclusion bodies in the cytoplasm, *i.e.*, 'cytoplasmic inclusion bodies', which were to be refolded. With the 'Peri-vector', the proteins with a signal sequence, which were recovered in a soluble form in the periplasm, *i.e.*, 'periplasmic soluble form', were used as reference samples. In the latter case, two types of 'an insoluble form' are possibly produced; one is in the cytoplasm ('cytoplasmic insoluble form') and another is in the periplasm ('periplasmic insoluble form') or both. For testing the refolding recipes quantitatively by our dual system, we chose three different dialysis refolding recipes; three-step cold dialysis refolding (TCDR), one-step hot dialysis refolding (OHDR), and one-step cold dialysis refolding (OCDR). For determining refolding efficiencies, Circular Dichroism (CD) spectrometric analyses were employed for quantitation.

2. Materials and methods

2.1. Materials

Vector pET26b (+) (Merck, Tokyo, Japan) was used as a template for constructing a 'Peri-vector' and a 'Cyto-vector'. *E. coli* BL21-CodonPlus (DE3)-RIPL (Agilent Technologies, Inc., Hachiouji, Tokyo, Japan) was used as a host cell for expression and production of antibody-fragments. Two antibody-fragments, PM1 and A6 were used in this experiment. PM1 is a camel single-chain antibody fragment V_HH, a completely synthesized fragment based on the sequence previously described as cAbBCII10-L-L-L (PDB entry 1ZMY), which is a grafted version of three CDRHs from cAbLys3, a selected V_HH clone against hen egg white lysozyme, onto a cAbBCII10 framework and thus shows binding against HEL (Saerens et al., 2005). A6 is a synthetic shark single-chain antibody fragment V_{NAR}, which was selected following *in vitro* panning against bovine serum albumin from our V_{NAR} phage-library composed of a scaffold from baby brownbanded bamboo shark with CDR3 randomization (data not shown). Both sequences were first constructed in the phagemid pCANTAB 5E. The amino acid sequences of PM1 and A6 are shown in Supplementary Figs. 1A and B, respectively.

2.2. Vector constructions for periplasmic expression and cytoplasmic expression

For constructing the 'Peri-vector', the sequences cloned into pCANTAB 5E phagemids were digested with *Nco*I and *Not*I, and the *Nco*I-*Not*I fragments were ligated into pET26B (+)(ΔMA) (Supplementary Fig. 2). The 'Peri-vector', a periplasmic version of pET26B (+)(ΔMA), was constructed by deleting the C-terminal MA of the PelB leader sequence; the sequence was changed from MKYLLPTAAAGLLLLAAQPAMA to MKYLLPTAAAGLLLLAAQPA. In the *Nco*I-*Not*I fragments of PM1 and A6 from pCANTAB 5E, the C-terminal MA of the g3 signal sequence was attached to the N-termini of PM1/A6 sequences. This M functions as a first methionine after ligated into pET26B (+)(ΔMA). If we use an intact pET26B (+) for expression in periplasm, MA from the *Nco*I-*Not*I fragments of PM1/A6 from pCANTAB 5E, and MA of the C-terminus of the PelB leader from intact pET26B (+) line tandem, *i.e.*, MAMA. Tandem MAMA may be misread by signal peptidase, as the sequence AXA is a cleavage site for signal peptidases (Chi and Lee, 2004).

For constructing a 'Cyto-vector', the *Nco*I-*Not*I fragments of PM1 and A6 were ligated into pET26B (+)(ΔPelB) without the PelB leader sequence (MKYLLPTAAAGLLLLAAQPAMA), whose sequence was completely deleted from the vector (Supplementary Fig. 3). In the *Nco*I-*Not*I fragments of PM1 and A6 from pCANTAB 5E, the C-terminal MA of the g3 signal sequence was always left at the N-termini of PM1 and A6 sequences so that this M functions as a first methionine after ligated into pET26B (+)(ΔPelB).

2.3. Expression and purification of PM1 and A6 from periplasmic fractions

'Peri-vecors' with PM1/A6 fragments were electroporated into competent BL21-CodonPlus (DE3)-RIPL cells. A culture stock was plated onto agarose LB plates containing 20 μg/mL of kanamycin (Km). A single colony was picked up from a plate and cultured in 10 mL of liquid 2YT medium (20 μg/mL of Km) at 37 °C with shaking at 200 rpm overnight (starter).

One mL of the starter culture was transferred into 1 L of 2YT medium (20 μg/mL of Km) and cultured. When the culture reached OD_{600} at 0.5, it was split into three parts: 920 mL for large-scale culture and two portions of 40 mL each for analysis by SDS-PAGE. IPTG was added to the 920 mL culture and one of the 40 mL cultures (final 0.1 mM) but not to the other 40 mL culture. Cultures were further incubated at 18 °C for 21 h with shaking at 200 rpm. Then, the cells were spun down at 4 °C and the supernatant was decanted and 7.5 mL of ice-cold