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Refolding single-chain antibody (scFv) using lauroyl-L-glutamate as a solubilization detergent and arginine as a refolding additive

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ABSTRACT

Therapeutic potential of immunoconjugates has opened a new window for antibody-based biopharmaceuticals. Greater tissue penetration and hence enhanced cell toxicity are obtained with a smaller version of antibodies. While the whole antibody can be readily produced via mammalian expression system, antibody fragments often require refolding of insoluble proteins. Here we report a new refolding method for antibody fragments using a novel amino acid–based detergent as a solubilizing agent and arginine-assisted refolding. Inclusion bodies of antibody fragments were solubilized by 2.5% lauroyl-L-Glu (C12-L-Glu) and successfully refolded by multi-step dilution into a buffer solution containing arginine hydrochloride and thiol/disulfide-exchange reagents. Adjustment of temperature was found to be critical for increase in the refolding yield. Although each protein requires appropriate optimization, solubilization by C12-L-Glu and dilution refolding assisted by arginine can generate the native, functional antibody fragments. The procedure should enable us to utilize bacterial expression systems for the large-scale manufacturing.

Introduction

Monoclonal antibodies hold great promise as effective human therapeutics. One of the major disadvantages of applying the whole antibodies for immunoconjugates is their large size, which reduces accessibility to the target sites [1–3]. Smaller version of antibodies can overcome this problem. One of them is a construct linking two variable domains (light and heavy chain) of antibody with flexible linkers, called single-chain antibodies (scFvs) [4,5]. A number of functional molecules have been fused to scFv, creating novel multi-functional proteins, including biosensors for detection of target molecules [6], cytokines for immunotherapy [7,8], and radioisotopes for imaging and cytotoxicity [9,10]. The last application is particularly attractive against solid tumors, such as breast cancers, due to smaller sizes allowing efficient tissue penetration [11–14]. A small size also opens a window for intracellular application of antagonistic antibodies [15,16]. It becomes increasingly apparent, however, that these engineered antibody fragments, unlike the intact antibodies, cannot be readily produced using a mammalian secretion system, due to both folding and aggregation problems [12,17,18]. Thus, most antibody fragments, including scFv, are produced in bacteria, often as inclusion bodies [17,18]. Refolding of proteins in general, and of these fragments in particular, is still done on a trial-and-error basis. Therefore, an effective and robust in vitro refolding method would be a valuable asset for not only scFvs but also other proteins [19].

We have recently developed a new refolding method using a novel amino acid–based detergent, lauroyl-L-glutamate (C12-L-Glu) [20]. This detergent is highly effective for protein solubilization, but yet readily dissociates from the detergent-protein complex upon dilution [20]. Solubilization of insoluble proteins from IBs by the detergent and dilution of the solubilized protein resulted in effective refolding of two model proteins, which was further improved when arginine was included in the refolding solvent. Here we applied a new refolding method for antibody fragments, i.e., two model scFv

Abbreviations used: C12-L-Glu, lauroyl-L-glutamate; scFv, single-chain antibody; SDS–PAGE, sodium dodecylsulfate–polyacrylamide gel electrophoresis; DTT, dithiothreitol; CMC, critical micelle concentration; SEC, size exclusion chromatography; GSH, reduced glutathione; GSSG, oxidized glutathione; IB, inclusion body; AISD, additive-introduced step-wise dialysis.

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molecules, based on solubilization by this detergent and dilution refolding assisted by arginine. The procedure should enable us to utilize bacterial expression systems for the discovery of novel engineered antibodies and the large-scale manufacturing.

Materials and methods

Materials

Lauroyl-L-Glu (C12-L-Glu), and arginine hydrochloride were obtained from Ajinomoto Co. Inc. (Kawasaki, Japan). All other chemicals were of biochemical research grade.

Production and expression of inclusion bodies

Escherichia coli stain BL21(DE3) was used as the expression host: expression plasmids contained T7 promoter followed by HyHEL-10 single chain scFv [21,22] or anti-fluorescein scFv [23]. The host cell was grown overnight in a 250 ml conical flask. The whole cell culture obtained, including both media and cells, was directly introduced into sonicater for cell disruption. The cell homogenate was centrifuged (Tomy-Seiko, Tokyo, Japan) at 7600*g* on 5 °C to collect inclusion bodies. The collected inclusion bodies (IBs) were suspended in 100 ml of 1% Triton X100-containing washing buffer and then centrifuged at 8000*g* for 15 min on 5 °C. The pellet of inclusion bodies was suspended with acetone and then centrifuged at 8000*g* for 15 min on 5 °C. The resultant pellet was washed twice with 100% acetone. The IBs thus obtained were used as a starting material for refolding experiments as described in detail below.

Analytical method

Analytical size exclusion chromatography was performed using Superdex 75 10/300 GL column (10 × 300 mm, 23.6 ml, GE Healthcare, Tokyo, Japan). The column was equilibrated with 100 mM sodium phosphate buffer containing 200 mM arginine-hydrochloride (pH 6.8) [24] and sample (25–50 μl) was applied at a flow rate of 0.8 ml/min at room temperature. The eluted materials were detected by UV absorbance at 280 nm. The concentrations of anti-lysozyme HyHEL-10 scFv [22] and anti-fluorescein scFv [24] were determined by absorbance at 280 nm using an absorption coefficient of 2.02 and 1.5 mg/ml/cm, respectively.

Fluorescence quenching assay was performed as described previously [23]. In brief, 1 ml of 0.6 μM fluorescein (Wako-Junyaku, Osaka, Japan) in 100 mM Tris–HCl (pH 8.0) was titrated with 7.9 μM of the refolded scFv in the same buffer, and the solution was incubated for 1 h at 23 °C. Upon excitation at 492 nm, the fluorescence emission intensity was collected at 510 nm; all experiments were conducted in a quartz cell at 23 °C using an Hitachi FL-2500 spectrofluorometer (Hitachi, Japan).

Sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was preformed with Laemmli buffer system under reducing condition with 10–20% gradient separating gels. Protein bands were visualized by CBB.

Results

Anti-lysozyme antibody scFv refolding using C12-L-Glu

First model antibody fragment tested was a scFv (HyHEL-10 scFv) derived from a monoclonal antibody against hen egg white lysozyme. HyHEL-10 scFv was expressed in *E. coli* primarily as IBs: scFv was undetectable in the supernatant of lysed cells. The starting material was prepared according to the previous report [22]. Solubilization of IBs was done in 2.5% C12-L-Glu detergent in the presence of reducing agent as described in the flowchart (Fig. 1, step-1). In practice, however, a 15 mg portion of HyHEL-10 scFv IBs in wet weight was first suspended using vortex in 0.5 ml of 20 mM sodium phosphate (pH 8.5) containing 5% C12-L-Glu to accelerate dispersion of the IBs. This was immediately mixed with 20 mM sodium phosphate for a final volume of 1 ml and hence a final detergent concentration of 2.5%. To this solution a stock 100 mM dithiothreitol (DTT) solution was added to make a final DTT concentration of 1.2 mM. The solution was incubated for 30 min at 37 °C, sufficient for complete solubilization of the proteins and reduction of cysteine groups that may have been spontaneously oxidized after IB extraction. The solution was centrifuged at 10,000*g* for 10 min on 4 °C. The concentration of solubilized scFv was estimated to be 6 mg/ml from the SDS–PAGE band intensity relative to the standard protein of known concentration (IgG was used as a standard): little scFv was observed in the pellet fraction. A 0.2 ml portion of the above scFv sample was mixed with 0.3 ml of 20 mM sodium phosphate (pH 8.0) to make the final C12-L-Glu concentration of 1.0% (Fig. 1, step-2) still above the critical micelle concentration (CMC) of C12-L-Glu: the CMC is about 0.3% [20]. The mixture was incubated for 30 min at 5 °C. Next, 20 μl of the above mixture was diluted 20-fold into 380 μl of 20 mM sodium phosphate (pH 8.0) containing 0.84 M arginine hydrochloride, 1.05 mM oxidized and reduced form of glutathione, and varying concentration of C12-L-Glu (step-3). While maintaining scFv and

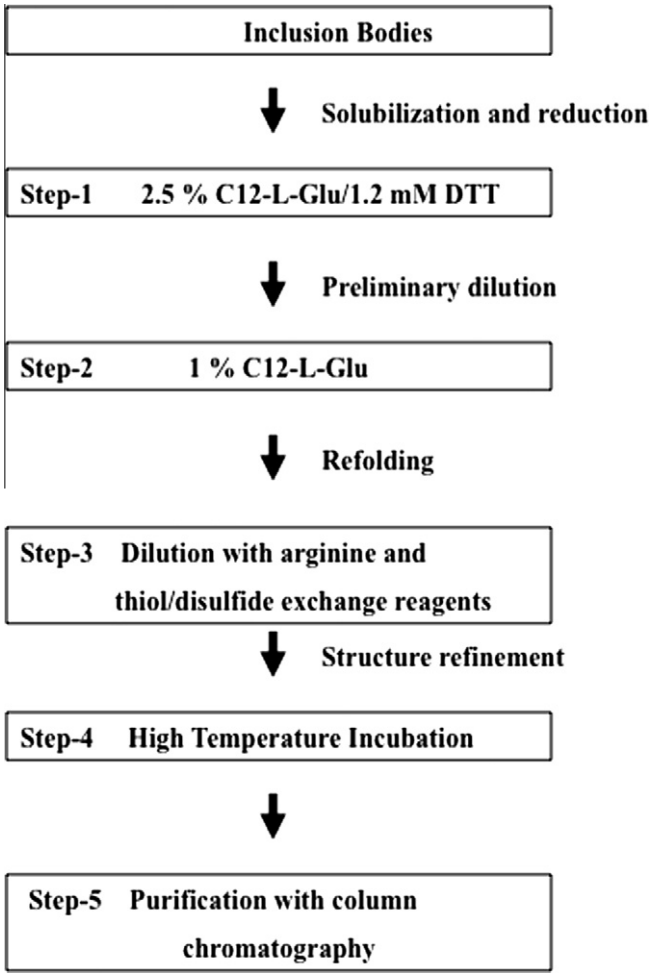

Fig. 1. Scheme of the refolding procedure for scFv using C12-L-Glu and arginine hydrochloride. See text for detail.