Protein Expression and Purification 130 (2017) 100–106

Contents lists available at ScienceDirect

Protein Expression and Purification

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

Effects of ionic strength on inclusion body refolding at high concentration

Jakub Gabrielczyk [a, *], Jonas Kluitmann [a], Thorben Dammeyer [b], Hans-Joachim Jördening [a]

[a] Institute of Technical Chemistry, Section of Carbohydrate Technology, Technical University Braunschweig, Hans-Sommer-Straße 10, D-38106 Braunschweig, Germany

[b] Institute of Physical and Theoretical Chemistry, Section of NanoBioSciences, Technical University Braunschweig, Hans-Sommer-Straße 10, D-38106 Braunschweig, Germany

ARTICLE INFO

Article history:
Received 24 August 2016
Received in revised form
11 October 2016
Accepted 13 October 2016
Available online 14 October 2016

Keywords:
Dialysis
Inclusion body
E. coli
Protein refolding
Ionic strength

ABSTRACT

For commercial applications refolding process must be fast, inexpensive and highly efficient. In the past many strategies for protein refolding were introduced. Still, simple refolding methods with high product concentrations are still rare. Refolding experiments were performed with fructosyltransferase (FTF, EC 2.4.1.162) from *Bacillus subtilis* NCIMB 11871 produced as inclusion bodies. Solubilizates were refolded with batch dialysis or by continuous exchange of dialysis buffers with variable ionic strength. By employing dialysis with gentle removal of denaturant the dependence of protein concentration and decreasing refolding yields could be overcome compared to batch dialysis and yields were enhanced by 52% at protein concentrations of approx. 10 mg/mL. The average specific activity of refolded FTF was 123 U/mg, 83% relative to standard FTF. Rising ionic strength of refolding buffers to 600 mM leads to complete renaturation of solubilized protein at equal protein concentration. Buffer composition plays a less significant role on renaturation output. The effect might be correlated with ion charge density of co-solvents.

1. Introduction

Accumulation of protein leads to formation of inclusion bodies because of high protein concentration in cells caused by over-expression. It is accepted that kinetic mechanism for protein folding vs. aggregation *in vivo* is similar to *in vitro* folding process [1]. Other key factors that can facilitate protein aggregation in addition to environmental stress, are charge average and turn forming residue fraction of proteins [2,3]. Inclusion bodies (IBs) consist of insoluble protein aggregates with high density [4] and target protein accounts up to 100% of total mass of IBs [5]. However, they are biologically inactive and that's why they need further treatment.

The general strategy used to recover active protein from inclusion bodies involves 3 steps, purification, solubilization and refolding. The simple and rapid purification of inclusion bodies is the major advantage of selective protein production form inclusion bodies. In the second step aggregated protein has to be solubilized with strong chaotropic agents like guanidine or urea at high concentrations of 6–8 M. And at last solubilized protein has to be refolded which is challenging especially at high protein concentration since refolding is a first-order reaction and protein aggregation a reaction of second or higher order [6,7]. For that reason a large number of refolding additives have been introduced, such as protein stabilizers or aggregation inhibitors [8].

Three different methods can be used for refolding, which basically is the removal of denaturant. The simplest and widely used method is rapid dilution. However yields and/or product concentration are very low. Common yields don't exceed 28% at a maximum initial protein concentration of 0.05 mg/mL [9,10]. Fed-batch dilution and extensive mixing lead to further increase in efficiency of refolding [11,12]. Second method is dialysis, where solubilized protein is captured within a semipermeable membrane. Dialysis can be done batchwise with yields of 95% at protein concentration of 0.135 mg/mL [13] or continuous. With continuous feed of refolding buffer, denaturant is removed gently, which has a beneficial effect on refolding and allows operating at higher concentrations between 2.5 and 5 mg/mL with moderate yields of 60

* Corresponding author.
E-mail addresses: j.gabrielczyk@tu-braunschweig.de (J. Gabrielczyk), jonas.kluitmann@tu-braunschweig.de (J. Kluitmann), t.dammeyer@tu-braunschweig.de (T. Dammeyer), a.joerdening@tu-braunschweig.de (H.-J. Jördening).

http://dx.doi.org/10.1016/j.pep.2016.10.004
1046-5928/

and 55%, respectively [14,15]. Compared with other methods, dialysis is time-consuming and needs bigger amounts of refolding solution. Solid phase methods use matrices, e.g., size exclusion chromatography or ion exchange chromatography, to assist refolding. Matrices either withhold denaturant or protein and at the same time they prevent interactions between protein molecules that lead to aggregation. Therefore very high initial protein concentrations can be loaded on columns, e.g., 42–63 mg/mL with overall yields of 46% and 42% respectively [16,17]. The major drawback of this approach is the low product concentration resulting from dilution of renatured protein by eluent. In both studies overall protein concentration was between 0.38 and 1.12 mg/mL. Additionally solid phase refolding demands high equipment costs, but only simple and cost-efficient refolding processes have the chance to be implemented in large-scale applications [18].

In this study we focused on refolding of inclusion bodies by employing dialysis with the objective of maximizing renaturation yields and product concentration. We investigated the effect of refolding buffers and ionic strength. Furthermore we used in a new approach ion exchange resins as refolding additives. All experiments were carried out with fructosyltransferase (EC 2.4.1.162).

2. Material and methods

2.1. Cloning and protein expression

The fructosyltransferase expression plasmid pASK_FTF11871 was constructed by PCR-amplification of the *Bacillus subtilis* NCIMB 11871 *ftf* gene [19] with oligonucleotides fw1NcoI 5′-GGCCCATGGCC AAAGAAACGAACCAAAAGCC-3′ and rv1XhoI 5′-CACCTCGAGTTTGTTAACTGTTAATTGTC-3′ to attach the restriction endonuclease cleavage sites *Nco*I and *Xho*I (underlined). The signal peptide was deleted to enhance the yield of fructosyltransferase (EC 2.4.1.162). The PCR-reaction was carried out using high fidelity Phusion DNA Polymerase (Thermo Scientific) and PCR-product was cloned in plasmid pASK-IBA63*b* plus (IBA) to generate a C-terminal translational fusion with Strep-tag® II. The ligation reaction was transformed into NEB5alpha competent cells (New England Biolabs) and positive clones verified by sequencing with forward and reverse primers (Eurofins MWG Operon). For expression the respective plasmid was transformed to *E. coli* BL21 (DE3) Rosetta (Merck Millipore). Fructosyltransferase is a monomeric enzyme; it has a size of 51.62 kDa and contains no cysteine residues.

E. coli were cultivated in fed-batch process in 5 L fermenter (Minifors, Infors HT). Batch medium consisted of LB medium, ampicillin (200 mg/L) and chloramphenicol (10 mg/L) at 37 °C, pH 7. Feed medium was composed of glucose (300 g/L), tryptone peptone (100 g/L), yeast extract (50 g/L), KH_2PO4 (20 g/L), $MgSO_4 \times 7H_2O$ (10 g/L), $(NH_4)_2HPO_4$ (6 g/L) and ampicillin (372 mg/L). Stirrer speed and oxygen feed were regulated by the system and dissolved oxygen maintained at 40%. Expression was induced with anhydrotetracycline (200 μg/L) at OD_{600} of 0.6.

2.2. IB isolation and solubilization

Cells were harvested by centrifugation (20 min, 5000 g, 4 °C) and resuspended in detergent buffer (1 M urea, 0.1 M Tris, 25 mM deoxycholate and 1% (v/v) IGEPAL CA-630) with final concentration of 100 g wet biomass per liter. Cell suspension was disrupted by sonication in ice bath (Sonoplus HD 2070, Bandelin) and centrifuged (10 min, 10,000 g, 4 °C). Crude IBs were resuspended, 200 g wet mass per liter, for 1 h at ambient temperature in washing buffer (1% (v/v) Triton X-100, 2.5 mM EDTA) and centrifuged. Washing step was repeated three times.

If not stated otherwise, 1–50 mg of pelletized IBs were solubilized at ambient temperature in 1 mL of 8 M urea and 0.1 M Tris at pH 8, and clarified by centrifugation (20 min, 25,000 g, 4 °C).

2.3. Purification of tagged FTF from E.coli extract

Cells were resuspended in 163 mM citrate-phosphate buffer (pH 6) and disrupted by sonication in ice bath. Solution was centrifuged for 10 min at 10,000 g and 4 °C. Supernatent was further clarified for 20 min at 25,000 g and 4 °C. Manufacturer's Protocol (PR03, IBA) was applied for purification via Strep-Tactin affinity column with a volume of 5 mL. Eluate containing target protein was dialyzed against 163 mM citrate-phosphate buffer by batch dialysis.

2.4. Refolding procedure and buffers

Refolding was done by batchwise or continuous exchange of dialysis buffer with cellulose membrane (MWCO 14 kDa, Visking) at 4 °C. For batch dialysis the buffer was replaced three times over a period of 48 h and a buffer to sample volume ratio of 100:1. Dialysis with continuous buffer exchange, setup described by Maeda et al. [20], was employed in 2.35 L or 0.55 L (nominal volume 2 L and 0.5 L, respectively) dialysis chamber initially containing 9% of solubilization buffer. Duration of the process was calculated by equation for elimination of a reactant with fixed values for residual urea concentration (c_t) ≤ 10 mM and k ($\dot{F}/V$) = 0.054 h^{-1}:

$$c_{t,urea} = c_{0,urea} \cdot e^{-kt}$$

$\dot{F}$ - feed rate (L/h) V - volume of dialysis chamber (L) t – dialysis time (h).

Dialysis chamber was considered to be ideally mixed and the residual urea concentration equal or lower than 10 mM. Refolded FTF solutions were clarified by centrifugation (20 min, 25,000 g, 4 °C) and filtration (0.22 μm).

Refolding buffers of varying concentrations, pH 6, were phosphate buffer (Na_2HPO_4/KH_2PO_4 and ratio of 12:88), citrate-phosphate buffer (citric acid/Na_2HPO_4, 37.4:62.6) citrate buffer (citric acid/sodium citrate, 11.5:88.5) and succinate buffer (succinic acid/NaOH, 35.3:64.7). The degree of dissociation in polyprotic acids and resulting ionic strength was calculated using HySS program [21].

2.5. Analysis of refolded protein

Protein concentration was determined by UV spectral analysis using Nanodrop 2000c (Thermo Scientific) with Protein A280 method and E&MW option (E = 70,250, M_w = 51.62 kDa). The yield of correct refolded enzyme was calculated from the protein concentration in supernatant after dialysis relative to concentration of solubilized protein prior to dialysis. The relative standard deviation of the change in volume is 4.7% (n = 40). Qualitative analysis of protein purity was performed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and 10% gel. Proteins were visualized using Coomassie Blue. Image processing was done with ImageJ program.

2.6. Dynamic light scattering (DLS)

DLS measurements were performed with a Malvern Zetasizer Nano ZS and calculated by Zetasizer Software version 7.03. Protein samples [0.2–0.5 mg/mL] were measured at 20 °C in disposable polystyrene cuvettes at a fixed position of 4.65 mm and automatic attenuation. Measurement angle was 173° Backscatter. Size distribution and polydispersity (Pd) were averaged over three