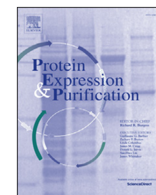
Protein Expression and Purification 133 (2017) 35–40

Contents lists available at ScienceDirect

Protein Expression and Purification

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



Ion exchange resins as additives for efficient protein refolding by dialysis

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A R T I C L E I N F O

Article history:
Received 25 January 2017
Received in revised form
24 February 2017
Accepted 25 February 2017
Available online 1 March 2017

Keywords:
Dialysis
Fructosyltransferase
Ion exchange resin
Inclusion body
Protein refolding
Refolding additives

A B S T R A C T

The most significant drawback of bacterial protein production involving inclusion bodies is the subsequent refolding into bioactive form. Implementation of refolding operations in large-scale applications often fails due to low yields and/or low product concentrations. This paper presents a simple method of integrated refolding by dialysis and matrix assisted refolding that combines advantages of both methods, high product concentrations and high refolding yields. Ion exchange resins (IER) and size exclusion media served as refolding additives and were added to solubilized protein prior to refolding by continuous exchange of dialysis buffer. Refolding experiments were performed with fructosyltransferase (FTF, EC 2.4.1.162) from *Bacillus subtilis* NCIMB 11871 produced as inclusion bodies. Conventional anion exchangers with gel matrix structure enhanced refolding performance by about 43% with final protein concentration of 9 mg/mL and yield improvement is strictly linear dependent on the mass ratio of resins to protein. With the applied setup refolded protein was self-eluted from resin due to pH and salt concentration shift during dialysis. Macroporous resins and gel filtration media showed a negative effect on refolding yields.

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1. Introduction

E. coli system is predestinated for the biotechnological production of recombinant proteins, in particular when the biological activity of the protein product does not require post-translational modifications, such as glycosylation. *E. coli* genetics are well characterized, easy to manipulate and the fermentation process is cost-effective and simple to scale up [1]. However overexpression leads often to formation of protein aggregates without bioactivity, called inclusion bodies (IB). On the one hand they are easy to purify and the protein product accounts for up to 100% of the mass of IBs [2,3]. On the other hand further downstream processing is necessary to refold the protein into bioactive form. During refolding process, the first-order folding reaction competes with second order protein aggregation reaction and the rate of aggregation depends on the protein concentration [4,5]. Consequently, protein aggregation is the key reason for low yields of correctly refolded protein [6].

The literature shows a variety of procedures for efficient recovery of active proteins from inclusion bodies, which generally remove the denaturants and aim to keep the protein aggregation at low rate. The simplest refolding procedure is to dilute the solubilized protein directly or in pulses with refolding buffer. In the dialysis method the solubilized protein is restrained in a membrane and the exchange of denaturing and native buffer occurs gradually [7,8]. The gradual removal of denaturant is also the basic principle of the on-column refolding method, but additionally the protein is bound or trapped in a chromatographic bed to prevent interactions between protein molecules. On-column refolding is achieved by ion exchange, size exclusion, hydrophobic interaction or affinity chromatography [9,10]. More recently it was shown that solubilization and efficient refolding of IBs can be achieved with high hydrostatic pressure of approx. 2 kbar [11,12].

Refolding performance of all techniques can be improved by several chemical additives that either stabilize the protein monomers or inhibit protein aggregation. However, the benefit of these refolding aids for a particular protein has to be determined empirically and they have to be removed in an additional purification step [13,14].

Downstream processing can easily reach 80% of total production costs for therapeutic proteins and therefore offers the largest optimization potential from economic point of view [15]. In spite of this great opportunity for refolding techniques to gain access to

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http://dx.doi.org/10.1016/j.pep.2017.02.011
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large-scale applications, low refolding performance and product concentration abolish the economic feasibility of protein production from IBs, as demonstrated in a comparative study for tissue plasminogen activator [16].

In our previous work [17] we presented an optimized renaturation of fructosyltransferase by dialysis with continuous buffer exchange. Purified IBs contained at least 95% and the renatured product minimum 98% of target protein, respectively. Furthermore, we have shown the presence of soluble, inactive multimers [8] in refolded product by dynamic light scattering method, which reduced the enzyme activity by 17% compared to standard FTF. In this paper, while we refer to our earlier work, the focus is on a novel approach of matrix assisted refolding that improves refolding yields with high product concentration. We used low cost ion exchange resins as solid additives in combination with refolding by dialysis. The method is verified by several resin to protein mass ratios and compared with the use of gel filtration media as refolding additives. All experiments were carried out with the enzyme fructosyltransferase (EC 2.4.1.162).

2. Material and methods

2.1. Recombinant protein expression

The fructosyltransferase (FTF) from *Bacillus subtilis* NCIMB 11871 was expressed as inclusion bodies in *E. coli* BL21 (DE3) Rosetta (Merck Millipore), carrying the plasmid pASK-IBA63b plus (IBA) with the FTF gene. *E. coli* was cultivated in fed-batch process in 5 L fermenter (Minifors, Infors HT) as previously described [17]. Fructosyltransferase is a monomeric enzyme with a size of 51.62 kDa and contains no cysteine residues. The theoretical isoelectric point (pI) of FTF is 5.75, computed by ProtParam tool.

2.2. IB isolation and solubilization

Cells were harvested by centrifugation (20 min, 5,000 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 (3 runs of 5 min, 50% amplitude and 70% output power) with Sonoplus HD 2070 (Bandelin). Cell lysate was centrifuged (10 min, 10,000 g, 4 °C) and washed in detergent buffer. Crude IB pellet was 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. Fig. 1 illustrates qualitatively the purification process and the high recovery and purity of target protein in IBs. Washed IBs were solubilized at ambient temperature in denaturing buffer (8 M urea, 0.1 M Tris, pH 8) and clarified by centrifugation (20 min, 25,000 g, 4 °C). Final concentration of solubilized protein was 10 mg/mL.

2.3. Solid phase refolding additives

Purolite ion exchange resins employed in this study (Table 1) were supplied by Purolite GmbH, Germany and all others by Lenntech BV, Netherlands (Tables 1 and 2). Size exclusion media Sephadex G-75 and Sephacryl S-100 HR were supplied by VWR International GmbH, Germany and Bio-Gel P2 by Bio-Rad Laboratories GmbH, Germany (Table 2).

2.4. Refolding procedure

Resins were washed in 163 mM citrate-phosphate buffer of pH 6 (ionic strength equal to 300 mM) and retained liquid was removed by vacuum filtration. 400 mg of dried resins were dispersed in 3 mL

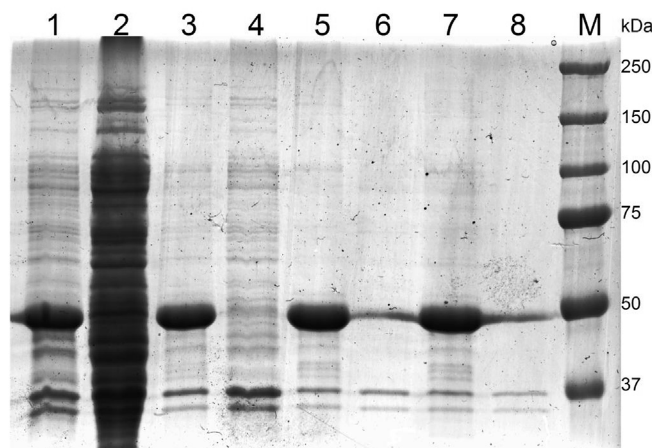

Fig. 1. SDS-PAGE: Exemplary purification of 2 g wet biomass according to protocol. Lane 1, cell lysate in detergent buffer (1:10 dilution); lane 2, supernatant of cell lysate (1:1); lane 3, IB pellet in detergent puffer (1:5); lane 4, supernatant of washed IB in detergent buffer; lane 5, IB resuspended in washing buffer (1:5) 1st time and lane 7, 2nd time; lane 6, supernatant (1:1) of 1st and lane 8, 2nd washing; lane M, molecular weight marker.

of solubilized protein and transferred into cellulose based dialysis tubing (MWCO 14 kDa, Visking). For continuing tests 200, 400, 800 and 1,200 mg of resin per assay was used. Refolding experiments were performed in triplicate by dialysis with continuous buffer exchange against citrate-phosphate buffer at 4 °C, setup was adopted from Maeda et al. [18] and is illustrated in Fig. 2. Dialysis chamber (2.35 L) was filled in advance with 200 mL of the solubilization buffer and the total volume of solubilized protein was approximated to 50 mL. The chamber was considered to be ideally mixed. Duration of the process was calculated by equation for elimination of a reactant with fixed values for residual urea concentration (c_t) of 10 mM and $k = \dot{F}/V = 0.054\ h^{-1}$ that corresponds to a feed rate of 0.127 L/h:

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

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

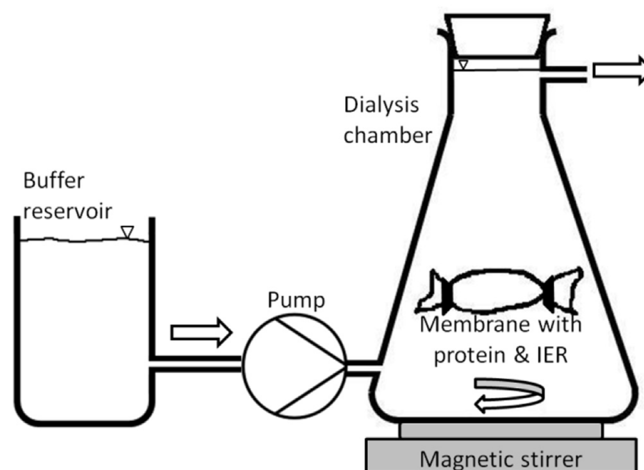

Fig. 2. Schematic diagram of the dialysis setup with continuous buffer exchange. Dialysis chamber (2.35 L) was filled in advance with 200 mL of solubilization buffer and dialyzed against citrate-phosphate buffer (163 mM) at 4 °C until a residual urea concentration of 10 mM was reached.

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