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Protein refolding in an oscillatory flow reactor

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Abstract

We demonstrate that an oscillatory flow reactor is a viable reactor for protein refolding via direct dilution. The mixing characteristics of the oscillatory flow reactor are well described and controllable and, importantly, can be scaled-up to process scale without a loss of mixing efficiency. This makes the oscillatory flow reactor an attractive alternative to conventional stirred-tank reactors for process-scale renaturation.

Introduction

A key challenge in producing active recombinant protein via the inclusion body process route is to refold solubilised, inclusion body protein to give a high yield of native protein with minimal loss to aggregation. The main goal of process scale protein refolding is to develop a cost effective process that is simple, robust and easily scalable. Due to its simplicity and widespread use, protein refolding via direct dilution in a stirred-tank reactor remains the preferred renaturation method at industrial scale. However, large stirred-tank reactors often exhibit low mixing efficiency. The effect of mixing on refolding yield has not been fully elucidated, although there is increasing evidence that yield can be optimised by manipulation of the mixing environment (Goldberg *et al.* 1991). It is likely that mixing controls the rate of change of chemical environment and rapid and efficient mixing minimises protein concentration gradients. Oscillatory flow reactors (OFR) offer efficient and *scalable* mixing environments where efficient eddy mixing within a baffled tube is generated using an appropriate level of fluid oscillation (Mackley 1991). This work reports the first investigation of protein refolding using an OFR and aims to demonstrate that renaturation yield in a laboratory OFR is comparable to that in a well-mixed stirred-tank reactor.

Materials and methods

Materials

Lyophilised, dialysed hen egg-white lysozyme was purchased from Fluka, Poole, UK. Guanidine hydrochloride (GuHCl), oxidised glutathione (GSSG), Tris/HCl buffer and EDTA were from Sigma and were reagent grade quality. Dithiothreitol (DTT) was obtained from Melford Laboratories, Chelsworth, UK. HPLC-grade acetonitrile and trifluoroacetic acid (TFA) were also from Sigma.

Denaturation and reduction of lysozyme

Lysozyme (15 mg ml^{-1}) was denatured in 8 M GuHCl buffer (50 mM Tris/HCl, 1 mM EDTA, 32 mM DTT, pH 8) and incubated at 37 °C for 1 h. The concentration of denatured and reduced lysozyme was determined at 280 nm (Clark *et al.* 1998).

RP-HPLC analysis of lysozyme

Native lysozyme concentration was quantified using a C_5 reversed-phase column (5 μm, 300 Å, 150 mm $\times$ 4.6 mm, Jupiter, Phenomenex, Macclesfield, UK) using HPLC with a linear acetonitrile/water gradient with 0.1% (v/v) TFA [starting at 34% (v/v) acetonitrile, increasing at 1.28% min^{-1} to a final acetonitrile

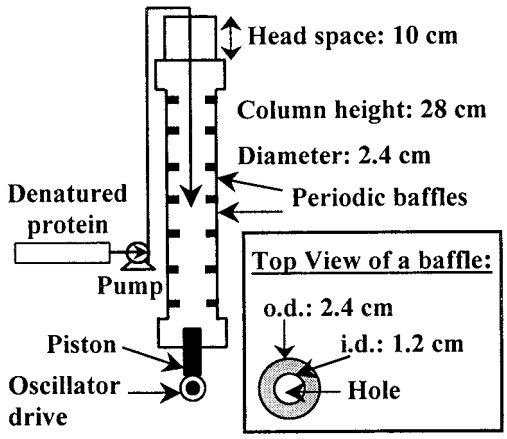

Fig. 1. Diagram of the oscillatory flow reactor (OFR). Fluid is contained within a baffled cylinder and is agitated with a piston to create a uniformly mixed environment.

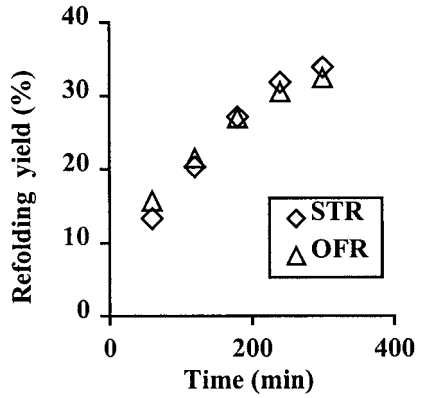

Fig. 2. Refolding yield (native protein by HPLC: initial denatured protein) using the OFR at intense mixing ($Re_o = 1580$) is comparable to that of a standard stirred-tank reactor (STR) under intense mixing condition (Re = 6353).

concentration of 46% (v/v)] was used to elute the samples, at a solvent flow rate of 1 ml min^{-1}. Absorbancy was measured at 280 nm.

Oscillatory flow reactor

Figure 1 shows a schematic diagram of the oscillatory flow reactor (OFR). The column is separated into eight cavities by seven flat ring baffles. Each baffle has an external diameter of 24 mm and an internal diameter of 12 mm. A piston, driven by an oscillator drive (cam rod), is used to oscillate the fluid within the column at a controllable frequency and amplitude. The denatured protein was fed through a needle that was placed within the fourth cavity of the column counting from the top. Mixing intensity is characterised by an oscillatory Reynolds number, Re_o, defined as:

$$Re_o = \frac{D\omega x_o}{\nu},$$

where D (m) is the tube diameter, ω (rad s^{-1}) is the angular frequency of the oscillator drive, x_o (mm) is the oscillatory amplitude (measured from centre-to-peak) and ν (m^2 s^{-1}) is the kinematic viscosity (Mackley 1991). A wide range of mixing intensities can be achieved simply by altering Re_o. At $Re_o \leqslant 400$, the flow pattern resembles axi-symmetric laminar flow. At $Re_o > 400$, the flow pattern is more turbulent-like (Mackley 1991).

Fed-batch mode refolding in an OFR

Denatured lysozyme (15 mg ml^{-1}) was fed into the OFR at a flow rate of 0.09 ml min^{-1} for 120 min using a peristaltic pump (Watson Marlow 101 U/R), to give a final protein concentration of 1 mg ml^{-1}. The initial volume of refolding buffer (4 mM GSSG, 50 mM Tris/HCl, 1 mM EDTA, pH 8, 20 °C) was 140 ml. Refolding was conducted under intense oscillation giving $Re_o = 1580$ (f = 3.5 Hz, $x_o = 3$ mm). Upon completion of feeding, the solution was left for 3 h with oscillation at $Re_o = 1580$. One ml samples were withdrawn hourly and quenched with 100 μl 10% (v/v) TFA for RP-HPLC analysis. The experiment was duplicated.

Fed-batch stirred-tank refolding

Refolding experiments conducted in a baffled stirred-tank with vigorous agitation provided a control for the OFR tests. Denatured lysozyme (15 mg ml^{-1}) was fed into a 200 ml baffled tank containing 140 ml refold buffer (4 mM GSSG, 50 mM Tris/HCl, 1 mM EDTA, pH 8, 20 °C) at a flow rate of 0.09 ml min^{-1} for 120 min using a peristaltic pump (Watson Marlow 101 U/R), to a final protein concentration of 1 mg ml^{-1}. The reaction mixture was stirred vigorously at an impeller Reynolds number (Re) of 6353 using a 4-blade Rushton impeller (higher mixing intensity would not increase the refolding yield, data not shown). Upon completion of feeding, the solution was left for 3 h with stirring. One ml samples were withdrawn hourly and quenched with 100 μl 10% (v/v) TFA for RP-HPLC analysis.

Results and discussion

The refolding yield obtained in the OFR was comparable to that obtained in the stirred-tank, as shown in Figure 2. This result shows that OFR is a viable refolding reactor. The mixing characteristics of a stirred reactor often vary significantly with scale. As such the scale-up of dilution processes may result in sub-optimal recovery of native protein. However, the mixing characteristics of an OFR are well defined and scale predictably (Smith 1999). The correct OFR mixing intensity can be identified at laboratory-scale and applied to a larger scale operation without significant generation of poorly mixed zones. This characteristic makes OFR an attractive potential alternative to conventional stirred-tank reactors for process-scale renaturation.

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