Journal of Biotechnology 192 (2014) 197–203

Contents lists available at ScienceDirect

Journal of Biotechnology

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

Co-expression of disulfide oxidoreductases DsbA/DsbC markedly enhanced soluble and functional expression of reteplase in *Escherichia coli*

Xiao-Fa Zhuo [a], Yi-Ying Zhang [b], Yi-Xin Guan [a,*], Shan-Jing Yao [a]

[a] Department of Chemical and Biological Engineering, Zhejiang University, Hangzhou 310027, China
[b] Department of Structural and Molecular Biology, University College London, London, WC1E 6BT, United Kingdom

ARTICLE INFO

Article history:
Received 19 July 2014
Received in revised form 18 October 2014
Accepted 21 October 2014
Available online 30 October 2014

Keywords:
Reteplase
DsbA/DsbC co-expression system
Soluble expression
Protein folding

ABSTRACT

Reteplase is the third generation of thrombolytic medicine and has many advantages over commercial t-PA. However, over-expressing recombinant reteplase in *E. coli* always accumulates as inclusion bodies due to nine pairs of disulfide bonds formation that is the main obstacle for correct folding. In this paper, in order to enhance soluble expression of recombinant reteplase in *E. coli*, DsbA/DsbC foldases were used to introduce disulfide bonds into the reduced polypeptide chain and catalyze their isomerization to the native disulfide linkage during the folding process. Firstly multiple *E. coli* protein expression systems, i.e. DsbA, DsbC and DsbA/DsbC co-expression were constructed. Subsequently, IPTG and L-arabinose were added to induce expression of foldases and reteplase accordingly, and experimental parameters such as culture temperature and inducer concentration were optimized. As a result, the co-expression system markedly enhanced soluble expression of recombinant reteplase, and up to 60% of reteplase achieved soluble expression especially for the DsbC co-expression system. The fibrin plate method for active reteplase quantification showed that ~70 mg soluble reteplase per liter fermentation broth was obtained with 2.35×10^5 IU/mg thrombolytic activity. Finally, fluorescence spectra indicated that the structural conformation of soluble reteplase was identical to its native state. The soluble expression of recombinant reteplase in *E. coli* was accomplished by co-expression with DsbA/DsbC, which contributes to further research in clinical application and folding mechanism, and provides guidance for production of other proteins with disulfide bonds.

1. Introduction

Reteplase (rt-PA), the third generation of thrombolytics for the treatment of ischemic stroke, is a deletion mutant of wild tissue-type plasminogen activator (t-PA) through genetic engineering (Kohnert et al., 1992). As a key enzyme in the thrombolytic, t-PA converts the zymogen plasminogen into plasmin, which in turn degrades the fibrin network (Collen, 1980; Hoylaerts et al., 1982; Lijnen and Collen, 1987). Human t-PA is a single-chain glycoprotein with 527 amino acid residues (MW of 62–65 kDa), which consists of five separate folding domains: the finger domain, the epidermal growth factor domain, two kringle domains and the C-terminal protease domain (Pennica et al., 1983; Qiu et al., 1998; Teesalu et al., 2002; van Zonneveld et al., 1986). By contrast, rt-PA consists of only the kringle 2 and the protease domains of human t-PA, which is a non-glycosylated deletion variant of t-PA (Martin et al., 1993). The protein sequence of rt-PA is composed of 355 amino acid residues and 9 pairs of disulfide bonds with a calculated MW of 39 kDa. Compared with wild strain, certain structural changes bring about rt-PA some advantages over t-PA such as a prolonged half-life, an increased fibrinolytic potential, more rapid and complete coronary patency, and little adverse effects (Noble and McTavish, 1996; Qureshi et al., 2002; Simpson et al., 2006; Wooster and Luzier, 1999), all of which endow reteplase with a good application prospect.

Expressing reteplase in *E. coli* was achieved about three decades ago, when Kohnert et al. (1992) firstly constructed reteplase plasmids successfully and transformed into *E. coli* host. However, reteplase has 18 free thiols in the amino acid sequence with theoretically 34,459,425 possibilities to form the nine pairs of disulfide bridges, which is the main obstacle to correct folding. Therefore in *de novo* protein synthesis in cells, partially folding reteplase intermediates readily self-associated into disordered aggregates driven by the incorrect disulfide bonds. Previous studies (Kohnert

* Corresponding author. Tel./fax: +86 571 87951982; fax: +86 571 87951982.
E-mail address: guanyx@zju.edu.cn (Y.-X. Guan).

http://dx.doi.org/10.1016/j.jbiotec.2014.10.028
0168-1656/

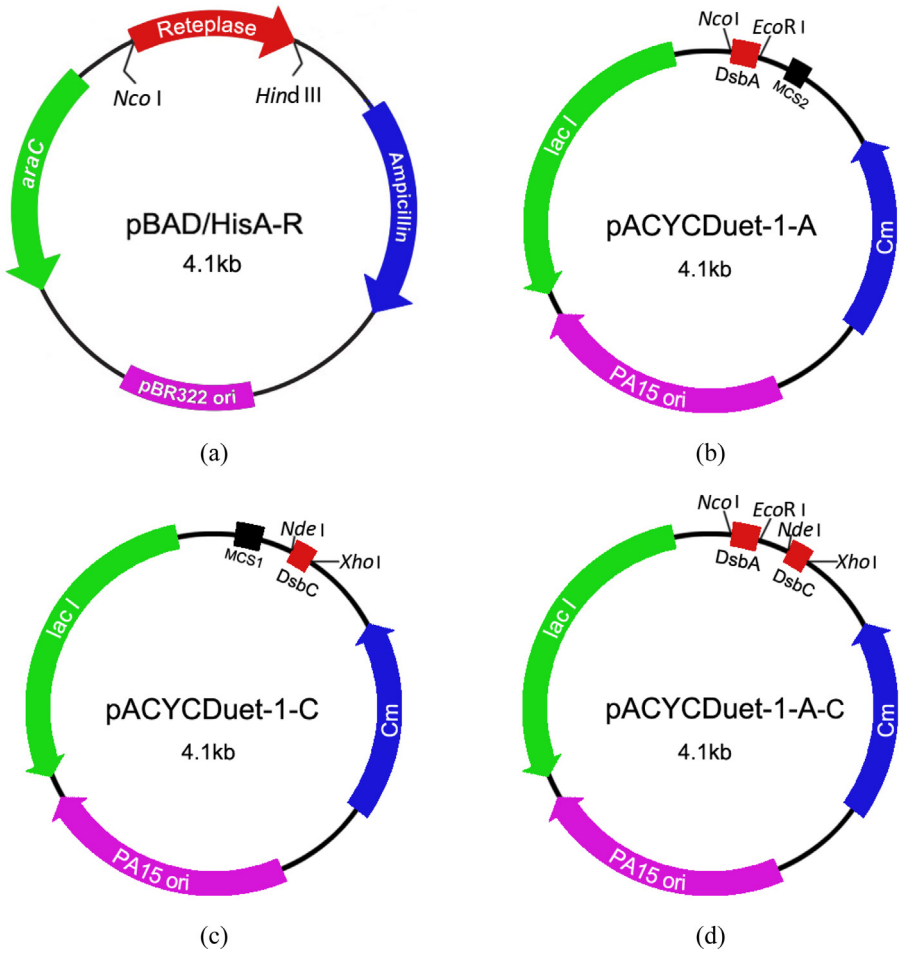

Fig. 1. Structure of recombinant reteplase and DsbA/DsbC plasmids. (a) reteplase. (b) DsbA (C) DsbC. (d) DsbA/DsbC.

et al., 1992; Martin et al., 1993; Zhao et al., 2003) showed that the reteplase protein was mainly expressed as inclusion bodies in *E. coli* and additional steps were needed to refold them *in vitro* to recover the correct conformation, obviously increasing the cost of production. Many others have been attempting to achieve soluble expression of reteplase in a series of alternative hosts, e.g. tobacco plants (Cheung et al., 2009; Goojani et al., 2013), *Leishmania tarentolae* (Hemayatkar et al., 2010), *Laminaria japonica* (Zhang et al., 2008). Although soluble expression of reteplase could be achieved in those hosts, the low expression level and the long production cycle in these processes were still puzzling problems and therefore forced people to resort to *E. coli* system again.

For *de novo* protein synthesis, disulfide bond formation is vital for the correct folding and tertiary structure stability of secreted proteins, because failure to form proper disulfide bonds is likely to cause protein misfolding (Messens and Collet, 2006; Nakamoto and Bardwell, 2004). In *E. coli* host system, the formation of disulfide bonds is usually catalyzed by a range of proteins belonging to the Dsb family. Totally there are six members in Dsb family: i.e. DsbA, DsbB, DsbC, DsbD, DsbE and DsbG. Of all these Dsb proteins, DsbA and DsbC are most extensively studied and widely used. DsbA, a 21 kDa soluble protein, possesses a thioredoxin domain, and an α-helical domain is inserted within the thioredoxin-like fold (Guddat et al., 1998; Schirra et al., 1998). The standard redox potential of DsbA is −119 mV, which makes DsbA a strong thiol oxidant and the immediate donor of disulfide bonds to secreted proteins (Zapun et al., 1993). DsbC has two 23.3 kDa subunits, and each subunit consists of two domains: C-terminal domain with a thioredoxin fold and an N-terminal dimerization domain (Guddat et al., 1998). Importantly, the C-terminal domain contains the Cys98-XX-Cys101 catalytic motif and the two active-site cysteine residues of DsbC are kept reduced in the periplasm (Bessette et al., 1999), which enables DsbC to shuffle misfolded disulfide bonds (Guddat et al., 1998; Messens and Collet, 2006). DsbA and DsbC could facilitate protein folding by catalyzing the formation and isomerization of disulfide bonds. In addition, DsbA has intrinsic activity as a molecular chaperone.

Interactions among several proteins and DsbA/DsbC have been previously reported, and most of them achieved soluble expression by the co-expression of DsbA and DsbC (Maskos et al., 2003). However, almost all of target proteins had less than five disulfide bonds, because the difficulty for protein to obtain soluble expression in *E. coli* augmented significantly with the increase of disulfide bonds in protein. Until now, there has been no report of DsbA and DsbC on reteplase folding both *in vivo* and *in vitro* yet. For reteplase with nine disulfide bonds, DsbC is considered to play an important role for soluble expression in *E. coli*. In this paper, a co-expressing system of DsbA/DsbC in *E. coli* is suggested for the first time to enhance the soluble expression of reteplase due to its specific structure. The fermentation of recombinant *E. coli* is optimized and the structure of reteplase is detected to evaluate the effect of DsbA and DsbC on soluble expression. And last, the mechanism of reteplase disulfide bond formation and isomerization in *E. coli* will be preliminarily explored.