A novel method for increasing production of mature proteins in the periplasm of *Escherichia coli*

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Abstract

A novel strategy to obtain high-level production of mature proteins exported to the periplasm of *Escherichia coli* is described. It is based on a modified signal sequence generated by insertion of a coding sequence of the polypeptide precursor of interest at the BamHI site of the commercial vector pQE-30 resulting in an addition of a dodeca-peptide (MRGSH₆GS) at the N-terminus of the precursor. The modification does not affect correct processing of the modified signal nor proper folding of the target protein, resulting in an untagged native product. The method is simple for avoiding onerous optimization of translation initiation and screening of host strains. The usefulness of this method is illustrated by overexpression of DsbC and DsbA. Induced by 0.01 mM IPTG at 37 °C, proteins were overproduced to comprise 20–30% of the total cellular proteins, and more than 95% of the expressed proteins were correctly processed and exported into the periplasm with yields of more than 100 mg per liter culture.

Keywords: DsbA; DsbC; export/secretion; processing; protein overexpression

The development of recombinant DNA technology has made feasible the overexpression of proteins in *Escherichia coli* (Goeddel, 1990). However, each gene presents unique challenges for its overproduction and it is often necessary to take great efforts to optimize the regulatory elements for high-level expression, although different commercial vectors are available with a variety of features.

pQE, a series of commercial vectors, have been widely used for overexpression of proteins in the cytoplasm of *E. coli* for their prominent advantages. They contain a powerful expression cassette composed of a phage T5 promoter, two lac operator sequences, a synthetic ribosome binding site, and an optimized codon sequence of MRGSH₆GS at the N-terminus of the target protein to improve expression up to 50% of total cellular proteins as the manufacturer claimed. The His6 affinity tag is recommended by the manufacturer to be placed at the C-terminus of the recombinant sequence, if the target protein is desired to be exported/secreted out of the cytoplasm. This allows the expressed protein to start with its natural ATG codon and maintains its authentic leader sequence so that to prevent possible interference with correct processing and folding of the target protein. However, the yields of secreted proteins are generally lower than that for proteins targeted

in the cytoplasm (Qiagen protocol, 1992), and the His6-tag is not always as “neutral” as generally assumed (Ledent et al., 1997).

In this communication we have presented an alternative strategy, simple and very efficient, based on an MRGSH₆GS-modified signal by using pQE-30 vector to overproduce target proteins with their native sequences in the periplasm of *E. coli*. To our knowledge, this modified strategy has not been reported before. The method is illustrated by examples of two *E. coli* thiol oxidoreductases, DsbC (Missiakas et al., 1994; Shevehik et al., 1994) and DsbA (Bardwell et al., 1991) for achieving high-level expression and correct processing in the periplasm of *E. coli*.

Results

Effects of temperature and IPTG concentration on expression of mature DsbC and DsbA in the periplasm of E. coli

Two plasmids of pQE-DsbC and pQE-DsbA were constructed to overproduce DsbC and DsbA, respectively, as described in Materials and methods.

As shown in Figure 1, mature DsbC and its precursor move with significantly different mobility in SDS-PAGE. In M15[REP4] cells, the expression of DsbC increases with increasing IPTG concentration from 0.01 to 0.05 mM or higher with about 50% of expressed DsbC precursors processed to mature molecules at 25 °C (Fig. 1, lanes 4–8). More than 95% of the expressed DsbC precursors are correctly processed at 37 °C when induced with the optimal IPTG concentration of 0.01 mM, and the mature protein consists 20–30% of the total cellular proteins (Fig. 1, lane 9).

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Abbreviations: ANS, 8-anilino-1-naphthalenesulfonic acid; CD, circular dichroism; GAPDH, D-glyceraldehyde-3-phosphate dehydrogenase; GdnHCl, guanidine hydrochloride; HPLC, high-performance liquid chromatography; IPTG, isopropyl thio- β -D-galactoside; PCR, polymer chain reaction; PDI, protein disulfide isomerase; TPOR, thiol-protein oxidoreductase.

Fig. 1. Expression and processing of DsbC precursor in M15[REP4] and JM83[REP4]. Cells bearing pQE-DsbC were induced with different concentrations of IPTG at 37 or 25°C. The total cellular proteins were examined on a 12% SDS-PAGE. Lane 1, low-range molecular weight marker; lanes 2 and 14, mature DsbC purified from the cells harboring pDsbC and pQE-DsbC, respectively; lanes 3 and 15, before IPTG induction; lanes 4–8, 9–13, and 22–26, induced with IPTG of 0.01, 0.05, 0.10, 0.5, and 1.0 mM, respectively; lanes 16–21, induced with IPTG of 0.01, 0.015, 0.05, 0.10, 0.50, and 1.0 mM, respectively.

Under this condition, 150 mg purified DsbC was obtained from 1 L culture in a shaking flask. IPTG at higher than 0.01 mM results in the decrease of mature species (Fig. 1, lanes 10–13).

Similarly, as in M15[REP4], DsbC precursors are almost completely processed to mature molecules and exported into the periplasm of JM83[REP4] induced with 0.01 mM IPTG at 37°C (Fig. 1, lane 16). However, the processing efficiency drops to 50% when IPTG increases to 0.015 mM (Fig. 1, lane 17), indicating that the concentration of IPTG is a critical factor for processing efficiency of expressed proteins.

DsbA is overexpressed and correctly processed in M15[REP4] at 37°C even without induction by IPTG (Fig. 2, lane 1). The high processing efficiency of >95% is approached with 0.01 mM IPTG induction (Fig. 2, lane 2), but sharply decreases to <10% if IPTG concentration increases to 0.05 mM or higher (Fig. 2, lanes 3–6). In JM83[REP4] cells, a processing efficiency of >95% is also approached with 0.01 mM IPTG induction (Fig. 2, lane 20); how-

ever, the total amount of expressed DsbA increases with higher IPTG concentrations (Fig. 2, lanes 16–19, 21–24). Using a compromised IPTG concentration of 0.05 mM, 120 mg DsbA was obtained at 37°C from 1 L culture in a shaking flask.

Mature DsbC and DsbA are also successfully overproduced in the periplasm of DH5 α [REP4], JM101[REP4], and TG1[REP4] induced with 0.01 mM IPTG at 37°C for 8 h as shown in Figure 3.

Characterization of expressed DsbC

The first four amino acid residues at the N-terminus of DsbC prepared from the cells harboring pQE-DsbC were determined to be Asp-Asp-Ala-Ala, which are consistent with the authentic sequence (Missiakos et al., 1994; Shevehik et al., 1994), indicating correct processing of the DsbC precursor.

The intrinsic fluorescence, ANS fluorescence, and CD spectra of DsbC were all identical with that of DsbC prepared from the cells

Fig. 2. Expression and processing of DsbA precursor in M15[REP4] and JM83[REP4]. Cells bearing pQE-DsbA were induced with different concentration of IPTG at 37 or 25°C. The total cellular proteins were examined on a 12% SDS-PAGE. Lanes 1 and 14, before IPTG induction; lanes 2–6, 7–11, 15–19, and 20–24, induced with IPTG of 0.01, 0.05, 0.10, 0.50, and 1.0 mM, respectively; lane 12, low-range molecular weight marker; lane 13, mature DsbA purified from the cells harboring pQE-DsbA.

Fig. 3. Expression and processing of DsbC and DsbA precursors in different cell strains containing REP4 plasmid. Cells were induced with 0.01 mM IPTG for 8 h at 37 °C. The total cellular proteins were examined on a 12% SDS-PAGE. Lane 1, mature DsbA purified from the cells harboring pQE-DsbA; lanes 2 and 7, JM101; lanes 3 and 8, DH5 α ; lanes 4 and 9, TG1; lanes 5 and 10, JM83; lanes 6 and 11, M15; lane 12, mature DsbC from the cells harboring pDsbC; lane 13, low-range molecular weight marker.

harboring pDsbC, respectively (data not shown). The above data show that mature DsbC purified from the cells harboring pQE-DsbC has the same secondary structure, the microenvironment of the aromatic residues, and the surface hydrophobicity as that of DsbC from the cells harboring pDsbC.

Enzymatic activities and chaperone activity of mature DsbC

Mature DsbC purified from the cells harboring pQE-DsbC has the same disulfide isomerase (580 ± 37 U/g) and thiol-protein oxidoreductase (TPOR) (56 ± 2 U/g) activities as that of DsbC from the cells harboring pDsbC. As shown in Figure 4, the two DsbC preparations assist the reactivation of denatured GAPDH with a very similar profile, indicating that they have the same chaperone activity.

Characterization of expressed DsbA

DsbA prepared from the cells harboring pQE-DsbA was determined to have a correct N-terminal Ala-Gln-Tyr sequence (Bardwell et al., 1991). Figure 5 shows the ability of DsbA to stimulate oxidative refolding of denatured and reduced RNase A very similar as published (Akiyama et al., 1992).

Secondary structure prediction of modified signal peptides

Structure prediction by GOR method shows that the dodecapeptide, MRGSH₆GS, added at the N-terminus of the signal peptide of DsbC or DsbA precursor, forms a random coil with positive charges and does not affect the typical α -helix conformation of the positive-charged signal peptide (Pugsley, 1993) that follows. To establish further the base of the present strategy for general applications, the same secondary structure predictions have been carried out for another ten signal peptides from SWISS-PROT data bank. They are signal peptides of Bla (AC, P00810), DsbE (P33926), DsbG (P77202), LamB (P02943), MalE (P02928), OmpA (P02934), OmpF (P02931), OmpT (P09169), PhoA (P78051), and PPI

Fig. 4. Effect of DsbC on the reactivation of denatured GAPDH upon dilution. The reactivation of guanidine hydrochloride-denatured GAPDH at 140 μ M was initiated by 50-fold dilution into 0.1 M phosphate buffer (pH 7.5) in the presence of mature DsbC purified from the cells harboring pQE-DsbC (■) or pDsbC (□) at different molar ratios to GAPDH as indicated. After cooling on ice for 30 min followed by incubation at 25 °C for 3 h, the sample was assayed for GAPDH activity relative to an equivalent amount of native GAPDH.

(P20752), respectively, which are often utilized as leader sequences to produce recombinant proteins in an export/secretion pathway. The results show that the addition of the sequence of MRGSH₆GS at the N-terminus of each of the above 10 signal peptides without exception has no effect on the secondary structure of the respective signal peptide.

Discussion

The present strategy shows the following features compared to the conventional export/secretion methods:

Fig. 5. DsbA-stimulated oxidation of reduced and denatured RNase A. Reduced and denatured RNase A was incubated with (■) or without (□) 0.4 mg/mL mature DsbA purified from the cells harboring pQE-DsbA in the presence of 0.9 mM GSSG and 0.6 mM dithiothreitol. Sample was withdrawn at intervals and assayed for RNase activity relative to an equivalent amount of native RNase.

(1) Overexpression of untagged native proteins in *E. coli* periplasm is simply achieved by insertion of a precursor coding sequence of interest into the BamHI site of the commercial pQE-30 vector in the right reading frame. Using the optimal IPTG concentration of 0.01 mM, DsbC, and DsbA, composed of 20–30% of the total cell proteins were expressed and exported into the periplasm with a processing efficiency of higher than 95%. DsbA and DsbC were produced in all the employed cell strains, JM83, M15, DH5 α , JM101, and TG1 containing REP4 plasmid, with a similar high efficiency of expression and processing, indicating a low host specificity of the constructed expression plasmids. This strategy takes all advantages of the regulatory elements of pQE-30 vector and therefore avoids onerous optimization of translation initiation and screening of host strains, which are necessary for design of conventional export/secretion systems. It has been noticed that DsbA was poorly expressed with less than 1% of the total cellular proteins when the coding sequence of its precursor was cloned in pKK223-3 vector (Pharmacia, Uppsala, Sweden) (data not shown).

(2) Although osmotic shock has been commonly used to isolate target proteins located in the periplasm, contamination with proteins from other compartments is inevitable if a target protein is to be prepared on a large-scale (Le & Trotta, 1991). Small amounts of unprocessed DsbC precursor was indeed found in the periplasm fraction after osmotic shock. This “leaked out” contaminant can be removed with metal-chelating or ^{RGS}·His6 antibody affinity chromatography (data not shown).

(3) A large amount of data have shown that mutations in a signal sequence resulting in changes of hydrophobicity and/or charges may block export and/or processing of the target protein (Pugsley, 1993). However, in the present modification of the signal peptides of DsbC and DsbA, the sequence of MRGSH₆GS directly added to the N-terminus of the precursors does not interfere at all with the correct processing of the precursors and the proper folding of the mature molecules in the periplasm of *E. coli*. Secondary structure prediction of the modified signal sequences provided a tentative explanation for the availability of this method in general.

Materials and methods

Bacterial strains and media

Escherichia coli M15[REP4] (Qiagen, Hilden, Germany), JM83 (ATCC), DH5 α (ATCC), JM101 (ATCC), and TG1 (ATCC) were the host strains for expression studies. JM83 [REP4], DH5 α [REP4], JM101[REP4], and TG1[REP4] were constructed, respectively, by transformation with the regulatory plasmid REP4 extracted from M15[REP4]. REP4 is necessary for maintaining the viability of host cells harboring the expression plasmids derived from pQE-30. In all the experiments, cells harboring recombinant plasmids were grown in 2 $\times$ YT broth containing 25 μ g/mL kanamycin and 100 μ g/mL ampicillin.

Construction of expression plasmids, pQE-DsbC and pQE-DsbA

pDsbC, containing the full length coding sequence of DsbC precursor, is a generous gift from Dr. Rudi Glockshuber, Eidgenössische Technische Hochschule Honggerberg, Switzerland. pUCBM21-DsbA, containing the full length coding sequence of

DsbA precursor, was kindly given by Professor Shengli Yang of the Shanghai Research Center of Biotechnology, Academia Sinica (Zheng et al., 1997).

Primer 1 (5'GGGATCCATGAAGGTTTAT3') and primer 2 (5'TGGATCCTTATTTACCCGCTG3'), with BamHI sites (underlined) at the 5' terminus, were synthesized to amplify the coding sequence of DsbC precursor. The PCR products were first inserted into Bluescript II/SK(+) vector (Stratagene, La Jolla, California) at BamHI site and confirmed by DNA sequencing, and then subcloned into pQE-30 vector (Qiagen) at the same restriction site to create pQE-DsbC, which encodes MRGSH₆GS-DsbC precursor. Similarly, primer 3 (5'CGGATCCATGAAAAAGATTTGGCTGGCG3') and primer 4 (5'CAAGCTTTTCTCGGACAGATA3') with BamHI and HindIII sites (underlined) at their respective 5' terminus, were used to amplify the coding sequence of DsbA precursor. The amplified fragment was cloned into pQE-30 via BamHI and HindIII to construct pQE-DsbA, which encodes MRGSH₆GS-DsbA precursor.

Expression and purification of DsbC and DsbA

The overnight cultured seed cells bearing pQE-DsbC or pQE-DsbA were diluted 100-fold and incubated for another 2 h. Expression was then induced with IPTG of different concentrations at 25 or 37 °C for 6–8 h. The expression and processing of target proteins were examined using 12% SDS-PAGE.

Target proteins released from the periplasm of harvested cells by osmotic shock (Shevehik et al., 1994) were dialyzed against 10 mM Tris buffer (pH8.0) overnight, and then centrifuged for 20 min at 15,000 g at 4 °C. The supernatant was loaded onto a Q-Sepharose Fast Flow column (Pharmacia) and eluted with a gradient of 0–0.4 M NaCl in the same buffer. The main peak was collected, desalted, and lyophilized.

Determinations of enzyme and chaperone activities

GAPDH was prepared from rabbit muscle according to Liang et al. (1990). Activities of PDI and TPOR of expressed proteins were assayed according to Lambert and Freedman (1983). The oxidative activity of DsbA was determined according to Akiyama et al. (1992).

The reactivation of GdnHCl-denatured GAPDH upon dilution in the presence of DsbC at different concentrations was taken as a measurement of chaperone activity of DsbC according to Cai et al. (1994).

Other determinations

Protein concentrations were determined spectrophotometrically at 280 nm with the following absorption coefficients ($A_{1\text{cm}}^{0.1\%}$): 0.7 for DsbC (Missiakas et al., 1994), 1.1 for DsbA (Wunderlich et al., 1993), and 0.98 for GAPDH (Scheek & Slater, 1982).

The N-terminal amino acid sequences of DsbC and DsbA were determined on a MilliGen/Biosearch model 6600 ProSequencer linked to a Waters (Franklin, Massachusetts) HPLC system with simultaneous detection at 269 and 313 nm.

CD spectra from 200 to 250 nm were determined with a Jasco 720 spectropolarimeter at 25 °C. Intrinsic and ANS fluorescence spectra were measured in a Hitachi F-4010 spectrofluorometer at 25 °C with excitation wavelength of 280 and 373 nm, respectively.

Secondary structure prediction

Secondary structures of segments of native signals and the corresponding MRGSH₆GS-modified signals followed by the N-terminal 20 amino acid residues of their mature molecules, respectively, were predicted using the GOR method (Garnier et al., 1996).

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