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# Improving the refolding efficiency for proinsulin aspart inclusion body with optimized buffer compositions

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ABSTRACT

Successfully recovering proinsulin's native conformation from inclusion body is the crucial step to guarantee high efficiency for insulin's manufacture. Here, two by-products of disulfide-linked oligomers and disulfide-isomerized monomers were clearly identified during proinsulin aspart's refolding through multiple analytic methods. Arginine and urea are both used to assist in proinsulin refolding, however the efficacy and possible mechanism was found to be different. The oligomers formed with urea were of larger size than with arginine. With the urea concentrations increasing from 2 M to 4 M, the content of oligomers decreased greatly, but simultaneously the refolding yield at the protein concentration of 0.5 mg/mL decreased from 40% to 30% due to the increase of disulfide-isomerized monomers. In contrast, with arginine concentrations increasing up to 1 M, the refolding yield gradually increased to 50% although the content for oligomers also decreased. Moreover, it was demonstrated that not redox pairs but only oxidant was necessary to facilitate the native disulfide bonds formation for the reduced denatured proinsulin. An oxidative agent of selenocystamine could increase the yield up to 80% in the presence of 0.5 M arginine. Further study demonstrated that refolding with 2 M urea instead of 0.5 M arginine could achieve similar yield as protein concentration is slightly reduced to 0.3 mg/mL. In this case, refolded proinsulin was directly purified through one-step of anionic exchange chromatography, with a recovery of 32% and purity up to 95%. All the results could be easily adopted in insulin's industrial manufacture for improving the production efficiency.



## 1. Introduction

Since 1920s, diabetic patients were treated with insulin purified from bovine or porcine pancreas. After nearly 100 years, insulin and its analogues are still the first choice for this disease, but are now produced through genetic engineering techniques [1–4]. With the number of diabetic patients continuously increasing at an alarming rate, it is reported that the requirement for insulin will increase to more than 16,000 kg per year [5]. The productivity of current insulin manufacturing systems would not be sufficient to meet the future market demands. So, it is substantially necessary to improve the insulin productive and production efficiency to meet the increasing need from the diabetic patients and other related diseases.

*Escherichia coli* and *yeast* are two main expression systems for recombinant insulin production that express insulin as a single peptide of proinsulin with the C-peptide retained to increase the expression level [6–8] which is subsequently removed by trypsin and carboxypeptidase B to get correct insulin molecule [9]. Compared with *yeast*, the *E. coli* host system allows advantages such as high yield, low cost, simple media and easy to handle, but it expresses proinsulin as inactive inclusion bodies [10]. Hence, the refolding of proinsulin inclusion bodies has been recognized as the key unit for insulin's production.

Proinsulin contains three disulfide bonds of Cys7–Cys72, Cys19–Cys85, and Cys71–Cys76 [11]. The formation pathway of these disulfide-bonds has been mentioned in several articles [12]. Different formation speeds and various optimal redox potentials were found for the three disulfides bonds [13,14]. In the early

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refolding strategies for proinsulin inclusion body, all cysteines were completely reduced and inactivated by Sulfitolysis prior to refolding [15]. This method was tedious and still had a low refolding yield. In fact, proinsulin refolding could directly start from the reduced denatured state, usually with some additives to suppress the precipitate [16]. It was also found that different leader and C-peptides would have great impacts on the expression level *in vivo* and refolding yield *in vitro* [16–18].

Arginine and urea are the two most widely used additives both presenting obvious advantage of inhibiting precipitation during protein refolding [19–21]. However, they may have different effects on the soluble refolded structures. Chen et al. found arginine could inhibit not only the precipitate but also soluble aggregate for G-CSF refolding, while urea could only suppress the precipitation [22]. Although arginine routinely showed better efficacy than urea for most proteins refolding, it must be removed by additional desalting step to decrease the conductivity if ion exchange chromatography was adopted for the following purification step.

For the inclusion body protein containing multiple disulfide bonds, redox system is another important factor on refolding yield. Redox buffers consisting of reduced and oxidized glutathione (GSH and GSSG, respectively) have become standard. However, refolding with cysteine/cystine yielded about two times more native proinsulin compared with refolding in the presence of GSH/GSSG [11]. Recently, a diselenide bond containing variant of glutathione (GSeSeG) combined with GSH was reported to have advantages over the oxidation for disulfide during protein refolding [23], which could provide markedly improved efficiencies in the folding of a number of proteins, including hirudin, lysozyme, human epidermal growth factor and even bovine serum albumin [24]. However, such diselenide oxidant has not been reported to be adopted for insulin's production. With a relatively low pKa, redox containing diselenide could catalyze disulfide formation even at acid pH value and often bring a faster folding speed than the traditional redox of GSH/GSSG.

Because of the great commercial value, detailed refolding procedure for recombinant proinsulin were rarely reported in publications, in spite that insulin has been industrially produced and clinically administered for decades. In this study, by characterizing the components during the refolding of proinsulin aspart, the precursor of a rapid-acting insulin analogue of insulin aspart, we clearly identified the disulfide-linked aggregates and disulfide-isomerized monomers (monomers with incomplete or incorrect disulfide bonds) as the only two off-path folding components, namely by-product. The impacts of additives including arginine and urea, as well as several redox pairs and oxidants containing sulphur or selenium on the refolding yield were systematically investigated and compared. Different from what literature reported before, only oxidant was found to be necessary for the native disulfide bonds formation for proinsulin and the refolding yield would be increased to 80% with a new oxidant of diselenide. Although arginine exhibited better effects on proinsulin refolding than urea, similar refolding yield could be achieved with urea at a slightly decreased protein concentration but resulting in a simpler downstream procedure. In this case, a recovery of 30% for proinsulin could be achieved after one step purification of anion exchange chromatography, with purity above 95% determined through SDS-PAGE. All the results lay a foundation for improving the insulin production efficiency and could be easily adopted in insulin's industrial production.

## 2. Materials and methods

### 2.1. Preparation and solubilization of proinsulin aspart inclusion body

The *E. coli* BL21 cells carrying proinsulin aspart plasmid was first grown at 37 °C in 500 mL shake-flasks each containing 100 mL LB medium supplemented with 100 μg/mL kanamycin monosulfate. When cell density reached $OD_{600}$ of 0.8–1.0, 750 mL LB inoculant was transferred into a 20 L bioreactor (NBS, US) containing 15 L fermentation medium (*yeast* extract 5 g/L, tryptone 10 g/L, sodium chloride 10 g/L, glycerol 5 g/L) supplemented with 100 μg/mL kanamycin monosulfate. Proinsulin aspart expression was induced at $OD_{600}$ of 4.0–5.0 with 1 mM isopropyl-D-thiogalactopyranoside (IPTG). After 4 h induction, cells were harvested by centrifugation at 8000 *g* for 15 min at 4 °C. The cell pellets were then resuspended in 20 mM Tris–HCl (pH 8.0) containing 1 mM EDTA in the ratio of 1:10 (w/v) and were lysed by high pressure homogenization for 3 runs at pressure of 800 bar. Cell debris and soluble materials were removed by centrifugation at 15,000 *g* for 20 min at 4 °C. The pellets containing inclusion body were washed three times with 20 mM Tris–HCl (pH 8.0), containing 1% Triton X–100, 2 M urea, 1 mM EDTA, and finally 33 g inclusion body was recovered through centrifugation. For the refolding experiment, 1 g inclusion body was solubilized in 6 mL denaturing buffer (20 mM Tris–HCl, pH 8.5, containing 6 M guanidine chloride and 100 mM β-mercaptoethanol) and left for 7 h at room temperature with continuous stirring. Insoluble material was removed by centrifugation at 15,000 *g* for 15 min and the supernatant was buffer-exchanged with 8 M urea 1 mM EDTA, pH 3.0 through desalting column (HiTrap desalting 25 × 16 mm ID, GE Healthcare). The desalted sample adjusted to 10 mg/mL protein concentration with the same buffer and used as the starting material for further study.

### 2.2. Dilution refolding with different additives

Refolding was initiated at the protein concentration of 0.5 mg/ml by 20-fold dilution of the denatured protein into refolding buffers of 20 mM glycine-NaOH, pH 10.0, with various refolding additives at a predetermined concentration, including arginine (0.1, 0.2, 0.5 and 1.0 M), urea (1, 2, 3, and 4 M), and different redox pairs or oxidants, including GSSG/GSH, cystine/cysteine, seleno-L-cystine (CSeSeC) and selenocystamine dihydrochloride (CASeSeCA). The refolded samples were incubated at 4 °C overnight. Refolding yield was the ratio of the correctly refolded proinsulin mass to the denatured proinsulin mass (proinsulin accounted for 70% of total protein mass in inclusion body) and was calculated with the following equation.

$$\text{Refolding yield} = P_1/(P_0 \cdot 70\%\ C)$$

$P_1$, peak area of the correctly refolded proinsulin;
$P_0$, peak area of standard proinsulin at 1 mg/mL;
C, protein concentration of the refolding solution;
Peak area was determined through RP-HPLC with same sample volume.

### 2.3. Purification of refolded proinsulin

The refolded proinsulin in the present of 2 M urea was loaded onto an anion exchange column (XK 200 × 16 mm ID, GE Healthcare) containing 40 mL Q-Sepharose Fast Flow (GE Healthcare) equilibrated with buffer A (20 mM glycine-NaOH, pH 10.0) and connected to AKTA Purifier system. The column was then washed with buffer A until UV baseline was reached. The bound proinsulin was then eluted by 20% buffer B (20 mM glycine-NaOH, 1 M NaCl, pH 10.0). The eluted peak was collected and subject to SDS–PAGE analysis.