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Alternative preparation of inclusion bodies excludes interfering non-protein contaminants and improves the yield of recombinant proinsulin

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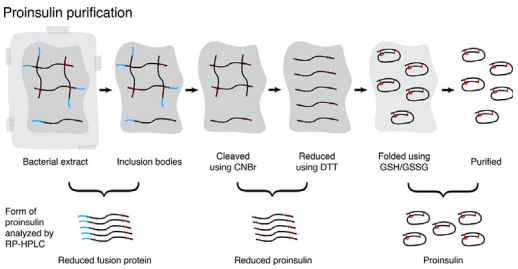
GRAPHICAL ABSTRACT



ABSTRACT

The goal of simple, high-yield expression and purification of recombinant human proinsulin has proven to be a considerable challenge. First, proinsulin forms inclusion bodies during bacterial expression. While this phenomenon can be exploited as a capture step, conventionally prepared inclusion bodies contain significant amounts of non-protein contaminants that interfere with subsequent chromatographic purification. Second, the proinsulin molecules within the inclusion bodies are incorrectly folded, and likely cross-linked to one another, making it difficult to quantify the amount of expressed proinsulin. Third, proinsulin is an intermediate between the initial product of ribosomal translation (preproinsulin) and the final product secreted by pancreatic beta cells

Abbreviations: CNBr, cyanogen bromide; DTT, dithiothreitol; GSH/GSSG, reduced glutathione/oxidized glutathione; PS/DVB, polystyrene/divinylbenzene; RPC, reversed-phase chromatography; RP-HPLC, reversed-phase high-performance liquid chromatography; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TCEP, tris(2-carboxyethyl)phosphine; TFA, trifluoroacetic acid.

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(insulin). Therefore, to be efficiently produced in bacteria, it must be produced as an N-terminally extended fusion protein, which has to be converted to authentic proinsulin during the purification scheme. To address all three of these problems, while simultaneously streamlining the procedure and increasing the yield of recombinant proinsulin, we have made three substantive modifications to our previous method for producing proinsulin:

- Conditions for the preparation of inclusion bodies have been altered so contaminants that interfere with semi-preparative reversed-phase chromatography are excluded while the proinsulin fusion protein is retained at high yield.
- Aliquots are taken following important steps in the procedure and the quantity of proinsulin-related polypeptide in the sample is compared to the amount present prior to that step.
- Final purification is performed using a silica-based reversed-phase matrix in place of a polystyrene-divinylbenzene-based matrix.

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Method details

Step 1: proinsulin expression

The choice of using a prokaryotic versus a eukaryotic expression system for the production of a recombinant protein is frequently dependent on the target protein's folding requirements, the necessity of post-translational peptide bond cleavage and the presence or absence of other post-translational modifications (e.g. glycosylation, phosphorylation, sulfation, amidation, etc.). Production of proinsulin requires both protein folding with simultaneous formation of disulfide bonds (cysteine side chains and disulfide bonds are shown in red in the graphical abstract), and cleavage of a single peptide bond to remove an N-terminal extension peptide (the extension peptide is shown in cyan in the graphical abstract). However, proinsulin does not require any further post-translational processing and previous studies have demonstrated that proper folding, disulfide bond formation and cleavage of the N-terminal extension can be performed in vitro as part of the purification scheme [1]. Therefore, because of the time and cost savings provided by a prokaryotic expression system, *Escherichia coli* remains the organism of choice for heterologous expression of recombinant proinsulin.

Procedure

BL21(DE3) bacteria transformed with the plasmid hPI/pET-9b (both the insert cDNA and corresponding expressed protein sequences are shown in Fig. 1) were grown in LB-Miller medium and protein expression was induced by the addition of 0.4 mM isopropyl-β-D-1-thiogalactopyranoside once the bacteria reached an A_{600} of ~0.8. The bacteria were cultured for an additional 2 h, collected by centrifugation (10,000 × *g* for 10 min at 4 °C), frozen on dry ice and stored at −80 °C. Volumes for all subsequent steps are given for the purification of proinsulin from 1 L of bacterial culture, which routinely generated an initial cell pellet of approximately 3.5 g (wet weight).

Step 2: analytical RP-HPLC

During experiments in which purification of proinsulin was initiated from 5 or 10 L of bacterial culture (using direct scaling from our previous 1 L procedure [1]), it was observed that the final yield of proinsulin did not increase in proportion to the starting culture volume. While investigating potential causes for this problem, we observed that at early stages of the purification procedure there was a significant difference in the integrated peak area for proinsulin-related peptides depending on whether or not the aliquots were chemically reduced prior to analysis by reversed-phase high-performance liquid chromatography (RP-HPLC). An example of these results is shown in Fig. 2 for aliquots from the initial bacterial extract.