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Periplasmic production of native human proinsulin as a fusion to *E. coli* ecotin

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Abstract

Native proinsulin belongs to the class of the difficult-to-express proteins in *Escherichia coli*. Problems mainly arise due to its small size, a high proteolytic decay, and the necessity to form a native disulfide pattern. In the present study, human proinsulin was produced in the periplasm of *E. coli* as a fusion to ecotin, which is a small periplasmic protein of 16 kDa encoded by the host, containing one disulfide bond. The fusion protein was secreted to the periplasm and native proinsulin was determined by ELISA. Cultivation parameters were studied in parallel batch mode fermentations using *E. coli* BL21(DE3)Gold as a host. After improvement of fed-batch high density fermentation conditions, 153 mg fusion protein corresponding to 51.5 mg native proinsulin was obtained per L. Proteins were extracted from the periplasm by osmotic shock treatment. The fusion protein was purified in one step by ecotin affinity chromatography on immobilized trypsinogen. After thrombin cleavage of the fusion protein, the products were separated by Ni–NTA chromatography. Proinsulin was quantified by ELISA and characterized by mass spectrometry. To evaluate the influence of periplasmic proteases, the amount of ecotin–proinsulin was determined in *E. coli* BL21(DE3)Gold and in a periplasmic protease deficient strain, *E. coli* SF120.
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Escherichia coli is the best characterized and most widely used bacterial host for the production of recombinant proteins [1,2]. *E. coli* expression systems imply a rapid generation of biomass, low-cost culture conditions, and are very versatile, due the availability of an increasingly large number of cloning vectors and host strains. However, most recombinant proteins applied in therapy and diagnosis are secreted proteins with essential disulfide bonds, and will form inclusion bodies when expressed in the cytoplasm of *E. coli*. This can be a benefit for ease of purification, if *in vitro* refolding conditions lead to the formation of the correct disulfide bonds. Alternatively, the protein can be directed to the periplasm and will be natively folded due to the oxidizing conditions in this extracellular compartment. A major drawback is the limited secretion capacity of the host, and the limited space of this compartment.

Naturally *E. coli* does not secrete high amounts of proteins [3] and the transport to the periplasm or to the culture medium is a particularly complex process [4,5]. Translocation of a protein across the cytoplasmic membrane requires a signal peptide, but the presence of signal sequence alone does not always guarantee efficient protein translocation [6,7]. An alternative is the use of larger fusion partner which may lead to efficient translocation and increase the chances of correct folding.

We recently discovered a novel fusion protein system for the production of disulfide-containing proteins in the periplasm of *E. coli* [8]. The N-terminal domain consists of *E. coli* ecotin including its signal sequence. Ecotin (*E. coli* trypsin inhibitor) is a dimeric, relatively small (16 kDa),

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broad range serine protease inhibitor. It contains one disulfide bond and is exceedingly stable [9]. In a previous study, human pepsinogen was fused to different signal peptides (*pelB*, *dsbA*, or *ompT*), but no recombinant product could be obtained in the periplasm. However, after fusion of pepsinogen to *E. coli* ecotin, native protein was found in the periplasm, and the fusion protein was purified to homogeneity with a yield of 20% [8].

In the current study, we used this new protein fusion tool for the production of native proinsulin in the periplasm. Proinsulin is the precursor of insulin, that consists of two separate peptide chains which are the A-chain (21 amino acids) and the B-chain (30 amino acids) joined by 2 interchain disulfide bonds. A third intrachain disulfide bond is present in the A-chain. In the β-cells of the human pancreas, the precursor of insulin is synthesized as a single peptide chain, preproinsulin. After cleavage of the N-terminal signal sequence, proinsulin is stored in secretary granules. Conversion of proinsulin to insulin proceeds before secretion into blood, and is catalyzed by thiol proteases which remove the connecting peptide (C-peptide) from B- and A-chains [10]. *In vitro*, the connecting peptide of proinsulin is cleaved with trypsin and carboxypeptidase B to obtain native insulin [11].

Insulin was the first biotechnology drug, isolated initially from cows and pigs, and introduced in 1922 for the treatment of diabetes. Production of recombinant human insulin in *E. coli* started in 1981 [12]. To date, insulin for therapeutic applications is manufactured in large-scale in *E. coli* and yeast [13]. The formation of the correct disulfide bonds in insulin only occurs at the level of proinsulin. Therefore, production of insulin in *E. coli* is mediated by formation of a single polypeptide chain comprising of proinsulin or a miniaturized derivative thereof [14]. Several strategies for production of proinsulin in the cytoplasm as insoluble inclusion bodies or soluble in the periplasm of *E. coli* have been described [15–19]. The production level of proinsulin as inclusion bodies is very high but refolding with correct disulfide bonds and purification is costly and complex.

Production of native proinsulin in *E. coli* is still a challenging task. Native proinsulin is a quite difficult to express protein, mainly due to severe proteolysis after production in the cytoplasm [20]. Upon secretion into the periplasm, the half-life of proinsulin increased from 2 to 20 min [20]. The oxidizing environment of the periplasm also leads to the formation of proper disulfide bonds in proinsulin. However, the yield is very low as compared to inclusion body production in the cytoplasm [21,22].

An efficient secretory expression of proinsulin was reported by Kang and Yoon [14]. In this study, a modified proinsulin, shortened in the C-peptide, was fused to two Z-domains derived from the staphylococcal protein A, with significantly increased expression yields. A similar approach was recently published using unmodified proinsulin fused to none, a single Z-domain and a double Z domain, to evaluate potential bottlenecks in proinsulin secretion [18]. In another fusion approach, proinsulin was fused to full-length DsbA, including its signal sequence [19].

In the present study, proinsulin was secreted into the periplasm as a fusion to *E. coli* ecotin. As determined by ELISA, and proved by mass spectrometry, the fusion protein was native. The production of ecotin–proinsulin was studied in batch fermentations. After high-density fed-batch fermentation, the fusion protein was extracted from the periplasm by osmotic shock and was purified in one step by affinity chromatography on immobilized trypsinogen.

Materials and methods

Restriction enzymes were obtained from NEB and MBI Fermentas. PCR purification and gel extraction kits were obtained from Qiagen. pCR-Blunt II-TOPO kit and Top10 competent cells were from Invitrogen. *E. coli* BL21(DE3)Gold was obtained from Stratagene and *E. coli* GM2163 (dam^- and dcm^-) was from NEB. *E. coli* SF120 was kindly provided by Prof. George Georgiou's laboratory, Institute of Cellular and Molecular Biology, University of Texas, Austin, USA. Bovine trypsinogen and thrombin were purchased from Sigma. HisTrap FF crude and HiTrap NHS-activated HP columns were from Amersham Biosciences. Antibodies used for ELISA were a gift from Roche Molecular Biochemicals, Penzberg, Germany. Human proinsulin used as a standard was obtained from the NIBSC, National Institute for Biological Standards and Control, United Kingdom. Maxisorp® ELISA plates were purchased from Nunc, Copenhagen. Protein mass standard PeqGold used for SDS–PAGE was from Peqlab, the Mark12™ marker was from Invitrogen. NuPAGE (4–12%) Bis–Tris gels were purchased from Invitrogen. $ZipTip_{C18}$ was purchased from Millipore. Micro BCA™ protein assay kit was purchased from Pierce. RP-HPLC Nucleosil C18 column was from Macherey-Nagel, Germany. All other chemicals were reagent grade.

Construction of the ecotin–proinsulin expression plasmid

pEGP1 containing the ecotin-pepsinogen fusion protein [8] was transformed into *E. coli* GM2163 (dam^- and dcm^-). The plasmid was prepared and digested by BspE1 and Sal1, respectively, to remove the pepsinogen fragment. The digested plasmid encoded *E. coli* ecotin followed by three Gly-Ser repeats. It was dephosphorylated and purified. The proinsulin gene was amplified from pET20b-proinsulin [23]. The forward primer was 5′-GGT TCC GGA TCT GGT TCT GGT TCT CTG GTC CCC CGC GGT AGT CAC CAC CAC CAC CAC CAC CGT TTT GTG AAC CAA CAC CTG TGC GGC-3′ to introduce three Gly-Ser repeats, a thrombin cleavage site, a hexa-histidine tag and an arginine residue just before the start codon of proinsulin. The reverse primer was 5′-AGT GTC GAC TTA GTT GCA GTA GTT CTC CAG CTG GTA-3′. The

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