



## Purification of a functional mature region from a SecA-dependent preprotein

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### Abstract

Most of the bacterial proteins that are active in extracytoplasmic locations are translocated through the inner membrane by the Sec translocase. Translocase comprises a membrane “pore” and the peripheral ATPase SecA. Where preproteins bind to SecA and how they activate translocation ATPase remains elusive. To address this central question we have purified to homogeneity the mature and preprotein parts of an exported protein (pCH5EE). pCH5EE satisfies a minimal size required for protein translocation and its membrane insertion is SecA-dependent. Purified pCH5EE and CH5EE can form physical complexes with SecA and can functionally suppress the elevated ATPase of a constitutively activated mutant. These properties render pCH5EE and CH5EE unique tools for the biochemical mapping of the preprotein binding site on SecA.

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Most exported proteins are transported from the cytosol across the inner membrane in bacteria by the Sec translocase [1]. Translocase core comprises a membrane-embedded channel-like structure formed by the SecY/SecE/SecG trimer [2] and the peripheral ATPase SecA [1]. Secretory preproteins are tagged by N-terminal signal peptides, targeted to the membrane by chaperone/pilot-like factors such as the signal recognition particle [3] and SecB [4], bind to membrane-bound SecA [5], trigger multiple rounds of ATP hydrolysis [6,7], and lead to processive preprotein translocation [1].

Mapping of preprotein binding surfaces on SecA has not been possible because traditional model preproteins like proOmpA display low affinity for soluble SecA

[6,8,9]. Synthetic signal peptides were shown to bind tightly to SecA [10] and mutations that prevent this binding have been characterized [11]. In contrast, binding of mature domains has remained elusive. To address this, we resorted to using the model preprotein pCH5EE, a mutant derivative of the major M13 coat protein (hereafter proM13coat), a model substrate with a minimal size required for secretion. Wild type M13 procoat inserts directly into the lipid bilayer by the YidC pathway [12]. Strikingly, introduction of two glutamyl residues after position +2 in the mature region (Fig. 1) renders pCH5EE SecA-dependent for membrane insertion [13].

We now report stable expression of the mature region peptide CH5EE and its purification to homogeneity (>99%). Like pCH5EE, CH5EE interacts physically and functionally with SecA. This peptide can now be used for mapping the mature domain binding site on SecA.

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Fig. 1. pCH5EE and derivative constructs.

## Materials and methods

### Cloning and expression of CH5EE

CH5EE was constructed in two steps. PCR was performed using plasmid pJQ8 (pCH5EE) [13] as a template using the oligo 5'CATATGCAAGCCGGC GAGGAAGAAGGTGACGATCCCGC and reverse primer 5'CGCGAATTCGAGCTCGGTACCCCC3'. The *NdeI/BamHI* restriction fragment was cloned into the expression vector pT7-7. A 0.2 kb *NdeI/BamHI* restriction fragment was subcloned into the pET22b (Invitrogen) giving rise to pIMBB285 that expresses CH5EE-His<sub>6</sub> (pIMBB278). The same product was also subcloned to pET16b where 10 additional histidines were added to the N-terminus giving rise to His<sub>10</sub>-CH5EE-His<sub>6</sub> (pIMBB285). The resulting constructs were transformed in three different *Escherichia coli* Table 1 strains for expression: BL21.19(DE3) [14], BL21/pLysS (Invitrogen), and JM109 (Promega).

### Purification of CH5EE using Ni<sup>2+</sup>-NTA

*Escherichia coli* strain JM109/pIMBB278 (His-CH5EE) was grown in LB medium up to an optical density of  $A_{550}=0.6$ . HisCH5EE expression was induced with IPTG (0.5 mM) for 3 h. Cells were harvested and dissolved in resuspension buffer (RB; 50 mM Tris-Cl, pH 7.6, 10% sucrose, 1 mM PMSF, and 3 ml buffer/g cell pellet) and were lysed by lysozyme (0.4 mg/ml; 1 h; 4 °C) and then treated with DNase (10 µg/ml; 10 min; 20 °C) after addition of MgCl<sub>2</sub> (5 mM). One volume of extraction buffer (ExB; 50 mM Tris-Cl, pH 7.9, 0.5 M NaCl, and 8 M Urea) was added to the resuspended material (final concentration: 25 mM Tris-Cl; 0.25 M NaCl; and 4 M Urea), followed by 15 min incubation (4 °C). Suspensions were treated with a Dounce homogenizer. After centrifugation (4 °C/13,500 rpm, Heraeus rotor 3335), pellets were resuspended in 100 ml of 20 mM Tris-Cl, pH 7.9, 10% sucrose, and 1 mM

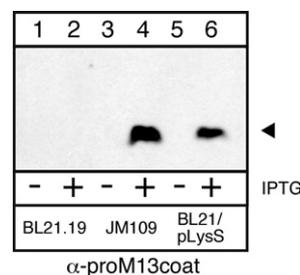


Fig. 2. CH5EE expression. CH5EE protein expression in three different *E. coli* strains (BL21.19, JM109, and BL21/pLysS). Protein expression was induced by addition of 0.5 mM IPTG at 37 °C. Samples were collected before and after 3 h of induction. Protein samples have been resolved by SDS-PAGE on 10% High-Tris gel [11] and immunostained with  $\alpha$ -proM13coat antibody [13]. Lane 1, BL21.19 uninduced whole cell sample; lane 2, BL21.19 after 3 h induction; lane 3, JM109 uninduced whole cell sample; lane 4, JM109 after 3 h induction; lane 5, BL21/pLysS uninduced whole cell sample; and lane 6: BL21/pLysS after 3 h induction.

PMSF and sonicated while the supernatant (Fig 2, lane 3; S1) was kept. After centrifugation (4 °C/13,500 rpm Heraeus rotor 3335), pellets were solubilized in 50 ml of 10 mM triethanolamine, 10% glycerol and the supernatant (lane 4; S2) was kept. After centrifugation (4 °C/13,500 rpm Heraeus rotor 3335), pellets were solubilized in 50 ml 20 mM Tris-Cl, pH 7.9, 10% glycerol, 0.5 M NaCl, 6 M urea, and 1% w/v sodium *N*-lauroyl sarcosine and incubated (15 min; 4 °C), and the supernatant (lane 5; S3) was kept. A final centrifugation (4 °C/13,500 rpm Heraeus rotor 3335) was carried out and the supernatant (lane 6; S4) was incubated (2 h; 4 °C) with nickel-nitrilotriacetic acid-agarose (Ni<sup>2+</sup>-NTA; ratio: 0.25 ml bed volume/liter of culture) treated with equilibration buffer (EB; 20 mM Tris-Cl, pH 7.9, 10% glycerol, 0.5 M NaCl, 6 M urea, 1% w/v sodium *N*-lauroyl sarcosine, and 5 mM imidazol). The resin was separated from unbound flow through material (lane 7; FT) and washed consecutively with WB1 (20 mM Tris-Cl, pH 7.9, 6 M urea, 0.5 M NaCl, 10% glycerol, 0.5% w/v sodium *N*-lauroyl sarcosine, 5 mM imidazol; 5 volumes), WB2 (20 mM Tris-Cl, pH 7.9, 0.5 M NaCl, 10% glycerol, 0.5% w/v sodium *N*-lauroyl sarcosine, and 5 mM imidazol; 5 volumes), and WB3 (20 mM Tris-Cl, pH 7.9, 10% glycerol, 0.5% sodium *N*-lauroyl sarcosine, and 15 mM imidazol; 10 volumes) (lanes 8–10; W1–W3). Proteins were eluted with two bed volumes of elution buffer (EluB; 20 mM Tris-Cl, pH 7.9, 10% glycerol, 0.5% w/v sodium *N*-lauroyl sarcosine, and 300 mM imidazol) and were collected in half column volume fractions (peak fraction shown in lane 11; CH5EE indicated with a filled arrow). Proteins were dialysed against 20 mM Tris-Cl pH 7.9, 6 M urea (12 h; 20 °C), concentrated by ultrafiltration (Ultrafree-15; Millipore) and stored at –80 °C. Protein concentration was estimated by the DC Protein Assay (Bio-Rad), using bovine serum albumin as a standard. Samples were analysed by

Table 1  
Purification of CH5EE from *E. coli* strain JM109 cells

<i>E. coli</i> strain	Culture volume (L)	Wet weight (g)	Total CH5EE in starting material (mg)	Total purified CH5EE (mg)	Purity (%)
JM109	8	8	10.4	1	>99

sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). Protein bands were visualized by silver staining. A similar protocol can be used for the purification of His pCH5EE using strain JM109/pIMBB346 (data not shown). N-terminal sequencing was carried out by Alta Bioscience (UK).

#### Binding assay of the purified CH5EE protein to SecA

Optical biosensor measurements were carried out on an IBIS II Surface Plasmon Resonance instrument (Echocemie). Cross-linking of CH5EE (3  $\mu$ g in 50  $\mu$ l from a 30–60  $\mu$ g/ml stock in 10 mM Hepes, pH 8.5) was added onto carboxymethylated dextran-coated gold sensor discs (CMD6 or CMD20; Xantec) and was cross-linked via NH<sub>2</sub>-specific *N*-ethyl-*N'*-(dimethylaminopropyl)carbodiimide/*N*-hydroxysuccinimide. The surface was equilibrated with buffer H and regenerated with 100 mM HCl. Data were collected for 400 s and were analysed using IBIS Kinetic Analysis software [11].

#### Suppression of the SecA<sub>W775A</sub> ATPase by CH5EE

SecA<sub>W775A</sub> (0.2  $\mu$ M; buffer B, 1 mM ATP) was supplemented with increasing amounts of CH5EE, or signal peptide (3K7L) as indicated. Basal ATPase was determined (30 min; 37 °C) [6]. ATPase activities are expressed as percentage of the activity of SecA<sub>W775A</sub> in the absence of CH5EE.

## Results and discussion

#### Cloning and expression of CH5EE

Cloning CH5EE in certain expression vectors (Materials and methods) resulted in two constructs that express CH5EEHis<sub>6</sub> (pIMBB278) and His<sub>10</sub>CH5EEHis<sub>6</sub> (pIMBB285). These constructs were transformed in several different strains to identify optimal strain/plasmid combinations. Levels of expression from three different *E. coli* strains (BL21.19, BL21/plysS, and JM109) are shown (Fig. 2). A polypeptide corresponding to CH5EE was barely visible when gels were stained with Coomassie blue (data not shown), suggesting that expression is low. To visualize CH5EE we resorted to using immunostaining with an  $\alpha$ -proM13coat antibody [13]. The levels of CH5EE-His<sub>6</sub> expression obtained with strain JM109 (Fig. 2, lane 4) were significantly (~3-fold) better than those with BL21/pLysS (Fig. 2, lane 6) and no expression was observed with BL21.19 (Fig. 2, lane 2). Therefore, we used JM109/pIMBB278 for subsequent large-scale purification of CH5EE. Introduction of a second histidiny tag (His<sub>10</sub>-CH5EE-His<sub>6</sub>) gave similar results (data not shown) and failed to improve on the low expression yield.

We concluded that different genetic backgrounds affect the levels of expression of CH5EE even if the growth conditions and the amount of IPTG used for the induction of cells are identical. These levels are not altered in a double His-tagged CH5EE construct (data not shown).

#### Purification of CH5EE

From 8 L of JM109/pIMBB278 *E. coli* cells, grown and induced as indicated under Materials and methods, 8 g of cells was harvested. CH5EE was purified as described under Materials and methods. The soluble material after the last centrifugation step (lane 6; S<sub>4</sub>) was applied to Ni<sup>2+</sup>-NTA preequilibrated resin. CH5EE eluted from this resin was highly pure (>99%). As we can observe from the silver stained gel of the purification (Fig. 3), during extensive washes of inclusion bodies with urea and detergent solutions to remove contaminants a significant amount of CH5EE is lost (lanes 3–5, 7; S<sub>1</sub>–S<sub>3</sub>, FT). Additional loss of material occurs during washes on the Ni<sup>2+</sup>-NTA resin and the final dialysis step needed to remove sarcosyl and to exchange the protein into urea buffer. This reduction in yield is necessary to obtain a highly pure product. We measured total purified protein after the concentration step (Materials and methods) by the DC Bio-Rad method and around 1 mg of highly pure CH5EE were obtained per 8 L of JM109/pIMBB278 *E. coli* cells. The purified CH5EE material can be immunostained with an  $\alpha$ -His tag antibody (data not shown) and has the correct aminoterminal (Met-Gly-Ala-Gly-Glu-Glu-Glu) as determined by N-terminal sequencing. We therefore conclude that the produced polypeptide is of the correct size and does not undergo proteolytic modification.

A critical step in the protocol presented here was the introduction of urea in the resuspension buffers during the different steps of the purification. Introduction of urea removes higher order contaminants and aggregated forms of CH5EE (lanes 3–5; S<sub>1</sub>–S<sub>3</sub>) and allows the isola-

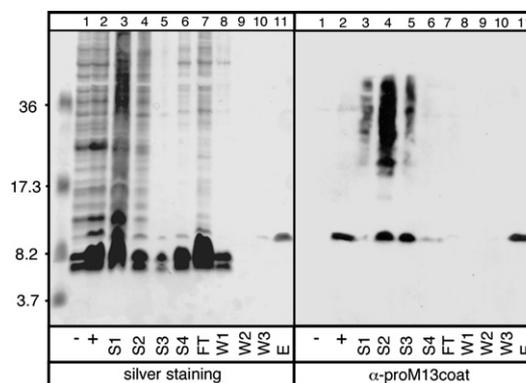


Fig. 3. SDS–PAGE analysis of CH5EE purification. Protein samples were separated by SDS–PAGE on a 10% High-Tris gel [11] and stained as indicated. MW markers (Bio-Rad): carbonic dehydratase (36 kDa), lysozyme (17.3 kDa), aprotinin (8.2 kDa), and insulin (3.7 kDa).

tion of highly pure (>99%) protein. This purification procedure can be used as a general protocol for proteins that remain in inclusion bodies and it is difficult to handle otherwise. The same purification protocol can be used to purify the procoat CH5EE (data not shown) improving significantly on the previous purification of pCH5EE [13].

#### CH5EE binds to SecA

To determine whether purified CH5EE is functional in interacting with SecA, we immobilized CH5EE on an optical biosensor (Fig. 4) using previously established assays [7,11]. SecA binds to CH5EE, while a control protein does not. The change in refractive index was followed as a function of time and until equilibrium was reached at three concentrations of ligate followed by a dissociation phase in the presence of buffer alone.

Interaction of CH5EE with SecA was further corroborated by functional suppression of the ATPase activity of SecAW775A, a mutant derivative that is functional for translocation but has a highly elevated basal ATPase [15]. CH5EE is added in different molar ratios to SecAW775A. Binding of CH5EE suppresses SecAW775A ATPase to comparable levels as with 3K7L (Fig. 4).

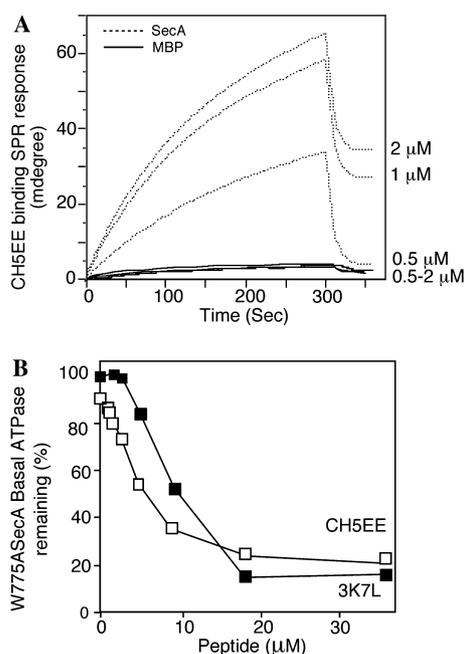


Fig. 4. Binding of CH5EE to SecA. (A) Binding of SecA to a CH5EE biosensor. Polypeptides (0.5–2 μM in 50 mM HEPES, pH 8, 50 mM KCl, and 5 mM MgCl<sub>2</sub>) were added to CH5EE immobilized on a biosensor chip and refractive index change was followed with time as described previously [11]. Sensograms showing the binding response at the indicated protein concentrations are shown. MBP, maltose binding protein used as a non-binding control. (B) ATPase suppression assay. SecAW775A (0.2 μM in 50 mM Tris–Cl pH 8, 50 mM KCl, 5 mM MgCl<sub>2</sub>; and 1 mM ATP) was supplemented with the indicated amounts of CH5EE, or signal peptide. Basal ATPase was determined (30 min; 37 °C). ATPase activities are expressed as percentage of the SecA W775A activity in the absence of peptides.

These experiments indicated that, in contrast to OmpA, CH5EE is capable of forming stable complexes with SecA in solution. The availability of this unique reagent now allows us to study the nature of the interaction of translocating substrates with SecA, to identify possible parts of mature domains that are being recognized as “signals,” and to proceed with the biochemical mapping of the binding site on SecA.

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