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Expression and purification of *Pseudomonas aeruginosa* SecA N-terminal domain: Stimulation of ATPase activity of the SecAL43P mutant protein

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Abstract

Pseudomonas aeruginosa is a ubiquitous Gram-negative bacterium which secretes a wide range of hydrolytic enzymes, toxins, and virulence factors into the extracellular medium. Although *P. aeruginosa* possesses numerous specific systems for the export of proteins across its double-membrane envelopes, the Sec system is still the major and essential mechanism. However, very little is known about its molecular basis. We constructed, cloned, and expressed the N-terminal 236 amino acids of PaSecA domain (PaSecAN236), and SecAL43P mutants of *P. aeruginosa* in *Escherichia coli* BL21.19 (*secA*^{ts}). Here, we describe the purification of PaSecAN236 by using osmotic shock as the first step to efficiently release targeted protein from cells, followed by cation-exchange and size exclusion columns to obtain homogeneous PaSecAN236. The purified PaSecA N-terminal domain was functional in stimulating the ATPase activity of mutant SecAL43P protein of *P. aeruginosa*.

Keywords: Osmotic shock; SecA; ATPase

The *secA* gene was first identified and characterized in the Gram-negative bacterium *Escherichia coli* [1]. The *E. coli* SecA, a homodimer of 102-kDa subunits in solution, along with SecYEG and other Sec proteins are intrinsic components of the protein secretion machinery. SecA binds precursor proteins, hydrolyzes ATP, and uses the energy of hydrolysis to translocate proteins across the cytoplasmic membrane. Previous studies in our laboratory have shown that *E. coli* SecA forms ring-like pore structures upon interaction with anionic phospholipids as observed by electron microscopy and by atomic force microscopy (AFM),[1] and may play an important structural role [2]. *Pseudomonas aeruginosa* is noted for its environmental versatility, causing disease in certain immunosuppressed individuals, and its resistance to antibiotics. Although it has evolved numerous specific systems for the export of the proteins across its double-membrane envelope, the Sec system is still the dominant and essential mechanism [3–5]. However, very little is known about the molecular basis of the Sec system in this bacterium. Therefore, we explored the Sec system of *P. aeruginosa* by starting with a truncated SecA N-terminal 236 amino acids fragment, whose counterpart N-terminal 234 amino acids from *Bacillus subtilis* (BsSecAN234) has been shown to be able to restore *E. coli* MM52 (*secA51*^{ts}) growth at a non-permissive temperature and to form a functional higher molecular complex with EcSecAL43P protein [6]. In this study, we cloned, expressed and purified the *P. aeruginosa* SecA N-terminal domain, which was functional in stimulating the ATPase activity of defective PaSecAL43P mutant protein.

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[1] *Abbreviation used:* AFM, atomic force microscopy.

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Materials and methods

Bacterial strains, plasmids, and media

Escherichia coli DH5α was used for plasmid isolation and for subcloning DNA fragments. Chromosomal DNA was isolated from *P. aeruginosa* PAO1. *E. coli* BL21.19 [*secA*(*am*) *supF*^{ts}*trp*(*am*) *zch::*Tn*10 recA::cat clpA::kan*], a SecA truncation mutant [7], harboring pET20b/*PasecAN236* was used for overproduction of the N-terminal 236 amino acid residues of *P. aeruginosa* SecA. Unless indicated otherwise, cells for SecA overproduction were cultured in TAG medium (10 g/L Tryptone, 5 g/L NaCl, A salts [8], and 0.5% glucose) supplemented with 100 μg/ml ampicillin.

Construction of pET20b/PasecAN236 plasmid

The DNA fragment encoding N- terminal 236 amino acid residues of *P. aeruginosa* SecA was amplified with PCR method using the oligo forward primer 5′-AAGGAG ATATACATATGTTTGCGCCTTTG-3′, reverse primer 5′-ATCAGCTTGTTGAAAGCTTAGTACAGCTCG-3′ and plasmid pET20b/*PasecA* as a template. The product digested with *Nde*I and *Hin*dIII was ligated with pET20b vector that was treated with same restriction enzymes to give pET20b/*PasecAN236* plasmid.

SDS–PAGE and Western immunoblotting

Protein samples were subjected to 12% SDS–PAGE, transferred to a PVDF membrane and visualized by rabbit antibodies, prepared by injecting rabbits with purified *P. aeruginosa* SecA protein.

Expression and purification of PaSecAN236

BL21.19 (*secA*^{ts}), harboring pET20b/PasecAN236, was cultured in TAG medium containing 100 μg/ml ampicillin at 30 °C. PaSecAN236 was induced with 0.5 mM IPTG at OD 600 nm of 0.8 and was overexpressed at 20 °C overnight. Osmotic shock [9] was first used to release overexpressed N-terminal peptide, PaSecAN236, as follows. Cell pellet, 3.6 g wet weight, harvested from 1 L culture was washed twice with 50 ml of 20 mM phosphate buffer (pH 6.0) containing 20% sucrose and 0.5 mM EDTA and incubated on ice for 15 min. Cell pellet was collected by centrifugation at 6000*g* for 10 min, resuspended in 50 ml of ice-cold deionized water and incubated for another 15 min on ice. After centrifugation at 6000*g* for 10 min, the recovered supernatant fraction was then diluted to 100 ml in the buffer containing 25 mM phosphate buffer (pH 6.0) and 1 mM DTT, and applied to a SP-Sepharose (Amersham Bioscience Corp.) 26/10 packed cation-exchange column. Protein was eluted with a segmented gradient from 0 to 1 M NaCl in the same buffer at a flow rate of 1.5 ml/min. The protein peak eluting at about 0.17 M NaCl was pooled (fractions 26–32), concentrated by centrifugation in Centricon units (Amicon, with a 10 kDa cut-off membrane) and loaded onto the Sephacryl-200 HR (Amersham Bioscience Corp) 16/90 packed column in 100 mM $(NH_4)_2HCO_3$buffer containing 1 mM DTT at a flow rate of 1 ml/min (Fig. 2B). The purified proteins (fractions 15–20) were pooled, aliquoted and stored at −80 °C. Protein concentration was determined by the Bradford assay (Bio-Rad) using BSA as a standard.

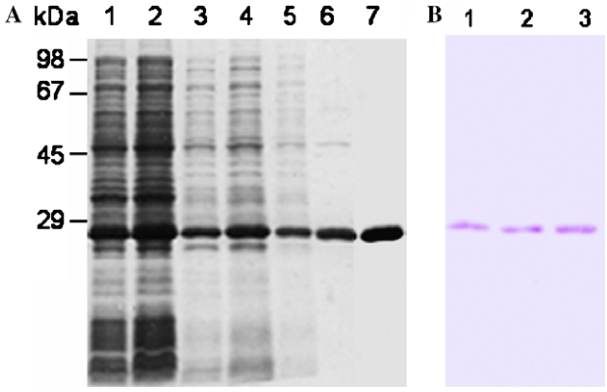

Fig. 1. Purification of N-terminal PaSecAN236 domain. The SecA-containing fractions at each purification step were subjected to the 13% SDS–PAGE. (A) Coomassie brilliant blue staining: lanes 1 and 2, 3 and 6 μl of total cell proteins from a volume of 54 ml; lanes 3 and 4, 6 and 12 μl of the 1st osmotic shock fraction from a volume of 50 ml; lane 5, 12 μl of 2nd osmotic shock fraction from a volume of 50 ml; lane 6, 12 μl combined peak fractions after SP-Sepharose column from a volume of 42 ml; lane 7, 12 μl of peak fraction (#17) after Sephacryl-200 column. (B) Western blot of PaSecAN236 proteins using anti-PaSecA serum: lane 1, total cell protein; lane 2, osmotic shock fraction; lane 3, purified PaSecAN236.

ATPase activity assay

The ATPase activity assays were carried out by the Malachite color assay [10]. Reaction mixtures in 50 μl contained 2 μg of purified proteins were first incubated in reaction buffer containing 50 mM Tris–HCl (pH 7.6), 20 mM KCl, 20 mM NH_4Cl, 0.1 mM DTT, and 2 mM $Mg(OAc)_2$ in the presence or absence of liposomes (*P. aeruginosa* lipid mixtures) at 30 °C for 30 min. Two millimolar ATP (final concentration) was added after the incubation and reactions were continued for another 30 min. Eight hundred microliters of color reagent (0.3375% Malachite green and 1.05% ammonium molybdate in 0.75 N HCl) was added to the reaction mixtures and incubated at room temperature for 1 min. Reactions were stopped by adding 100 μl of 34% trisodium citrate (pH 8.3). ATPase activity was determined by measuring OD at 660 nm after 40 min incubation at room temperature.

Results and discussion

Expression of PaSecAN236 in E. coli BL21.19 (secA^{ts})

To achieve a better expression of PaSecAN236 protein under the control of T7 promoter, we employed TAG