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Two-step purification of the outer membrane transporter and activator protein ShlB from *Escherichia coli* using internally His_6-tagged constructs

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Abstract

ShlB from *Serratia marcescens* was isolated and purified from a porin-deficient *Escherichia coli* BL21 strain using a combination of detergent extraction, affinity and ion-exchange chromatography. An internal histidine affinity tag was introduced that did not interfere with activity. At each stage of the purification scheme biological activity of the ShlB protein was assessed. Using this scheme, several His_6-tagged mutants of ShlB were purified to electrophoretic homogeneity.

Keywords: Purification; *Escherichia coli*; Outer membrane protein

1. Introduction

The ShlA/B secretion system from *Serratia marcescens* represents the paradigm of a novel type of outer membrane (OM) transport pathway in Gram-negative bacteria. Unlike type I, II, III and IV secretion systems [1], ShlA/B belongs to the two-partner secretion (TPS) family, which differs from the other protein secretion types in terms of the number of protein components involved, the signal sequence-dependent passage across the cytoplasmic membrane (CM) by means of the Sec machinery, energy requirement as well as the periplasmatic translocation mechanism [2]. In vivo, ShlA cytolysin synthesis is regulated by intracellular iron [3]. In vitro, ShlB and a phospholipid component are necessary and sufficient for the activation of ShlA [4]. This holds true for the 1578 amino acid (aa) full-length protein as well as the N-terminal 248 aa fragment of ShlA. Based on CD spectroscopy and tryptic digestion experiments, ShlA was found to undergo secondary and tertiary structural rearrangements, whereas the molecular mass of ShlA-255 (truncated ShlA with 255 N-terminal residues) as determined by electrospray mass spectrometry remains unaltered upon activation [4].

Up to now, TolC is the only outer membrane export protein, for which a crystal structure has been determined. This revealed a novel type of protein structure and secretion mechanism [5]. Due to the results of previous work on the ShlA/B secretion system we are expecting a novel structure for ShlB with a distinct mechanism for ShlB-dependent secretion of ShlA which is different from TolC. Therefore, it is important to determine the crystal structure of ShlB, for which pure ShlB has to be isolated in

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sufficient quantities. Because of the low protein yield with the original purification protocol [4], an internal His_6-tag for affinity chromatography was introduced genetically that did not interfere with hemolytic activity. The His_6-tag ensured correctly targeted OM protein without loss of activity. A comparable tagging strategy was successfully employed for the determination of the FhuA crystal structure [6].

2. Experimental

2.1. Instruments

A Mastercycler Gradient from Eppendorf (Hamburg, Germany) was used for DNA hybridisation. Fermentation of *E. coli* was carried out using a 300-l bioreactor from B. Braun Biotech (Melsungen, Germany). The dot-blot microfiltration apparatus was from Bio-Rad (Munich, Germany). All chromatographic steps were carried out on an Äkta Explorer 100 with Unicorn 4.0 data system from Amersham Biosciences (Freiburg, Germany). The electrophoresis device was a Miniprotean II system from Bio-Rad. A Spectronic Unicam Heλios α from Thermo Spectronic (Cambridge, UK) was used for spectrometric measurements.

2.2. Chemicals

HPSF purified oligonucleotides were from MWG Biotech (Ebersberg, Germany). Restriction enzymes, 1 kB DNA ladder and the ligation kit were from New England Biolabs (Frankfurt, Germany). High-copy pBCKS plasmids were purchased from Stratagene (La Jolla, CA, USA). DNA purification kits were from Qiagen (Hilden, Germany). Anti-His_6 antibodies and the Complete EDTA-free protease inhibitor mix were from Roche Molecular Biochemicals (Mannheim, Germany). GAM-AP conjugate secondary antibody was from Jackson Immuno-Research Laboratories (West Grove, PA, USA). HiTrap Chelating, HiTrap Desalting, MonoQ and MonoS columns were from Amersham Biosciences. Centricon YM-30 centrifugal filters were from Millipore (Schwalbach, Germany). All other fine chemicals and media were purchased from VWR (Dresden, Germany).

3. Insertion of internal affinity tags

Based on the current topology model of ShlB [7], internal His_6-tags were designed as complementary oligonucleotides and introduced into TAB-linker insertion mutants [8] within the proposed external L1/L2 loops. Singular tag insertions at residues 58, 86, 153 and 186 were externally accessible to anti-His_6 antibodies in immobilized whole cells as shown by dot-blot experiments (Fig. 1). Multiple histidine tag insertions at residue position 58 resulted in a targeting defect, where no functional protein could be detected by anti-His_6 antibodies.

Only His_6-tag mutants within the proposed L1 loop (positions 58 and 86) retained at least 95 wt.% activity in an in vivo hemolysis assay (Table 1).

4. Over-expression and detergent extraction of ShlB from OM preparations

The genes encoding for the His_6-tagged ShlB proteins were subcloned into pBCKS high-copy vectors. ShlB was overproduced in *E. coli* BL21

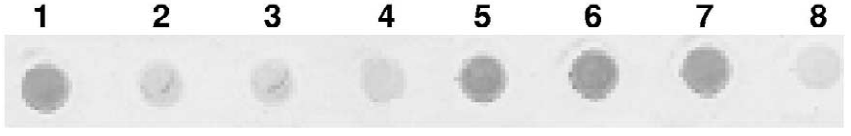

Fig. 1. Dot-blot with anti-His_6 antibodies applied to nitrocellulose-immobilized whole cells and detected with GAM-AP conjugate secondary antibodies resulting in a violet precipitate. Lane 1, BL21/pST58H_6+11; lane 2, BL21/pST58H_6+18; lane 3, BL21/pST58H_6+26; lane 4, BL21/pST58H_6+40; lane 5, BL21/pST86H_6+11; lane 6, BL21/pST153H_6+11; lane 7, BL21/pST186H_6+13; lane 8, BL21/pES14 (*shlB shlA*) control, without His_6-tag. *Suffixes indicate the number of additional residues introduced into the resulting ShlB protein.

Table 1
Functional analysis of His_6-tagged insertion mutants: (A) in vivo hemolysis on blood agar plates, size of lysis halos after 24-h incubation; (B) in vivo hemolysis of 8% (v/v) erythrocyte suspension, A_{405}/A_{405}(total lysis) of 20 μl ShlA* crude extract

E. coli BL21 transformed with	(A) In vivo hemolysis on blood agar plates	(B) In vivo hemolysis (%) of erythrocyte suspension
pES14 (*shlB shlA*)	++	100
pES15 (*shlB*)	–	0
$pST58H_6+11^a$ (*shlB*^{b} *shlA*)	++	95
$pST58H_6+18^a$ (*shlB*^{b} *shlA*)	–	0
$pST58H_6+26^a$ (*shlB*^{b} *shlA*)	–	0
$pST58H_6+40^a$ (*shlB*^{b} *shlA*)	–	0
$pST86H_6+11^a$ (*shlB*^{b} *shlA*)	++	95
$pST153H_6+11^a$ (*shlB*^{b} *shlA*)	+/−	5
$pST186H_6+13^a$ (*shlB*^{b} *shlA*)	+/−	5

++, >5 mm; +, 1–5 mm; +/−, <1 mm; −, 0 mm.
[a] Suffixes indicate the number of additional residues introduced into the resulting ShlB protein.
[b] Symbolizes His_6-tag insertion.

omp8, in which all major porins are deleted [9]. Bacteria were grown in LB medium at 37 °C, supplemented with chloramphenicol. At $A_{600}=0.5$, transcription of ShlB was induced by adding 1 m*M* IPTG. Biomass was collected from 200-l cultures grown for 3.5 h after induction (Fig. 2).

Cell pellet (493 g) was collected by centrifugation for 10 min at 5000 *g*. Cells were suspended in 5×300 ml 50 m*M* Tris–HCl, pH 8.0 and disintegrated with a French pressure cell. The OM fraction (Fig. 3, lane 2) was prepared by selective solubilization of the CM in 0.2% Triton X-100 [10]. During the entire isolation procedure, 50 m*M* protease inhibitor mix (Complete, EDTA-free) was present. The OM pellet was resolubilized in 5×300 ml extraction buffer (50 m*M* Tris–HCl, pH 8.0, 0.5% LDAO) and shaken overnight at 8 °C. Compared to OG used in the original protocol, with LDAO as detergent, solubilization of ShlB from OM preparations could be improved by a factor of 10 and yielded up to 90% of total ShlB in solution. After centrifugation for 90 min at 108 000 *g*, ShlB in the

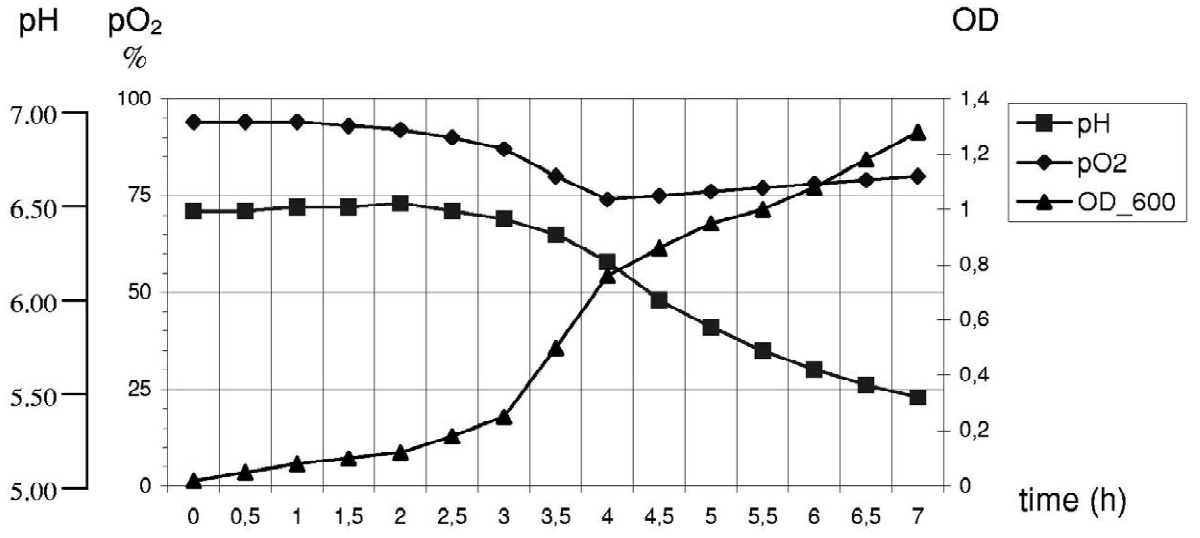

Fig. 2. Fermentation of *E. coli* BL21omp8/pSB86H_6. History plot showing cell density as $O.D._{600}$ (▲), pH (■) and P_{O_2} (♦) conditions in a 200-l fermentation.

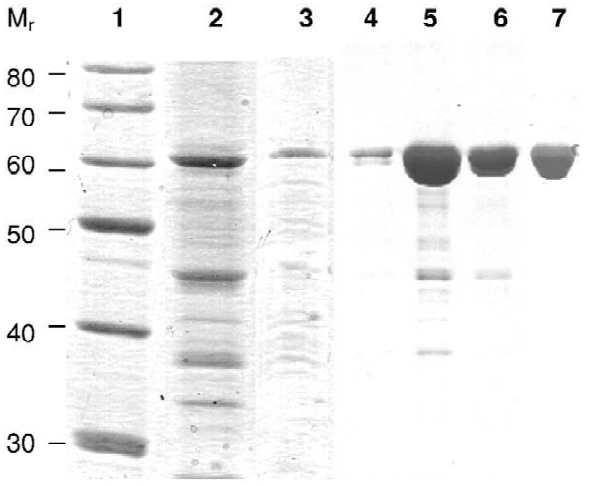

Fig. 3. Chromatographic purification of ShlB86H$_6$. Lane 1, 10 kDa ladder molecular mass marker; lane 2, OM preparation; lane 3, OM after LDAO extraction; lanes 4–6, peak fractions after affinity chromatography; lane 7, after serial ion-exchange chromatography.

supernatant contained in LDAO micelles was only slightly contaminated by other OM proteins (Fig. 3, lane 3).

5. Chromatographic purification of ShlB mutants

In the first step, 50 ml of the ShlB-containing solution was applied to the Ni^{2+}-charged HP5/5 chelating column pre-equilibrated with binding buffer (50 m*M* Tris–HCl, pH 8.0, 0.1% LDAO, 20 m*M* imidazole) using a 50 ml Superloop. To remove protein contamination and LPS, ShlB-loaded columns were extensively washed with 20 bed volumes of washing buffer (50 m*M* Tris–HCl, pH 8.0, 0.1% LDAO, 50 m*M* imidazole). ShlB was eluted with a linear gradient (50 m*M* Tris–HCl, pH 8.0, 0.1% LDAO, 50–400 m*M* imidazole). ShlB protein eluted at 150–200 m*M* imidazole. Peak fractions contained 2–10 mg/ml of the ShlB crude product (Fig. 3, lane 4–6).

In the second step, any remaining contamination and degradation products of ShlB were removed using an anion-exchange MonoQ HR5/5 column connected in tandem to a cation-exchange MonoS HR5/5 column. Peak fractions of the Ni-chelate chromatography were pooled and transferred into storage buffer (50 m*M* Tris–HCl, pH 8.0, 0.1% LDAO) using HiTrap™ Desalting columns. The pooled protein fractions were concentrated to 20 mg/ml protein (5 ml total volume) using Centricon 30000 filters. Approximately 10 mg of ShlB was loaded onto the serially connected ion-exchange columns pre-equilibrated with storage buffer. According to the previously published purification protocol for wt ShlB [4], degradation products of ShlB could be separated with a linear gradient from 0 to 1 *M* NaCl. The rational of the tandem ion-exchange chromatography was to elute ShlB in early fractions at a ion-exchange buffer pH (50 m*M* Tris–HCl, pH 8.0, 0.1% LDAO) near the theoretical p*I* of ShlB (p*I*=8.7), while the main degradation bands (Fig. 3, lane 5), identified by Edman sequencing (data not shown), with a theoretical molecular mass of M_r 21 000 (p*I*=6.0) and M_r 40 000 (p*I*=9.5) were retained on MonoQ and MonoS, respectively. ShlB with a molecular mass of M_r 61 000 eluted between 150 and 250 m*M* NaCl (Fig. 3, lane 7) and amounted to 6 mg.

6. Analytical procedures

Protein concentrations were determined using BCA protein assay reagents [11]. Activity was assessed by incubating the ShlB samples for 1 min with 20 μl of inactive ShlA* hemolysin crude extract. The activity of the resulting ShlA was determined with 1 ml of an 8% (v/v) erythrocyte suspension. The released hemoglobin was measured spectroscopically at 405 nm. Hemolysis time constants were extrapolated to 15-min incubation in order to calculate hemolytic units [12], which were related to 1 mg of total protein (Table 2).

7. Conclusions

Using a combination of an affinity tag insertion, detergent extraction, affinity and ion-exchange chromatography, we obtained active, highly purified His$_6$-tagged ShlB in high yields, which should be suitable for protein crystallization. The high ShlB

Table 2
Hemolytic activity of $ShlB86H_6$ during the purification procedure

	HU/mg protein
OM preparation	60
After detergent extraction	320
After affinity chromatography	750
After serial ion-exchange chromatography	920

$ShlB86H_6$ containing probes were tested in an in vitro activation assay for their ability to activate 20 μl of ShlA* crude extract and related to 1 mg of total protein. One hemolytic unit (HU) is the amount of hemolysin that leads to release of 50% of the total hemoglobin in 1 ml of a 8% (v/v) erythrocyte suspension within 15-min incubation time.

yield was due to the high expression level, low OM protein contamination (Fig. 3, lane 2) and selective solubilization with LDAO detergent (Fig. 3, lane 3). SDS–PAGE showed ShlB at M_r 62 000. Sequencing of lower bands revealed only minor contamination by ShlB degradation products. The main band contained only processed, correctly targeted ShlB, as shown by Edman sequencing (data not shown). Degradation bands were separated from the main band due to differing partial charge profiles using ion-exchange chromatography.

For the first time, the transporter and activator protein ShlB was purified to such an extent and amount that crystallization is possible. The result of this work is a prerequisite to understand the structural requirements for ShlA protein transport across the outer membrane and concomitant conversion of ShlA from an inactive protein to a cytolysin.

8. Nomenclature

aa	amino acid
CD	circular dichroism
CM	cytoplasmic membrane
GAM-AP	goat anti-mouse alkaline phosphatase conjugate antibody
HU	hemolytic unit
IMAC	immobilized metal affinity chromatography
IPTG	isopropyl-β-D-thiogalactopyranoside
LDAO	*N*,*N*-dimethyl-dodecylamine-*N*-oxide
OG	octyl-β-D-glucopyranoside
OM	outer membrane
PCR	polymerase chain reaction
TPS	two-partner secretion

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