

Abrridged Region from *Escherichia coli* Periplasmic Stress Sensor DegS Acts as Plasminogen Activator *In Vitro*

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Abstract It is well known that the *Escherichia coli* inner membrane-bound protease DegS is a periplasmic stress sensor for unfolded outer membrane proteins (OMPs). Previous studies have also shown that the outer membrane protease OmpT activates plasminogen *in vitro* and this may be exploited by bacteria in the course of pathogenesis. However, there has been no research on the plasminogen activation ability of the important periplasmic protein DegS. Accordingly, in this study, the whole-length and truncated *degS* genes were separately overexpressed in *Escherichia coli*, the recombinant proteins purified by affinity chromatography, and their plasminogen activator role tested *in vitro*. The results suggested that the whole-length DegS was able to activate plasminogen on a plasma plate. The truncated form of DegS (residues 80-345), designated Δ DegS, also acted as a plasminogen activator, as confirmed by different assays. The serine protease property of Δ DegS was verified based on the complete inhibition of its enzyme activity by PMSF (phenylmethanesulfonyl fluoride). Therefore, the present results indicate that DegS is a plasminogen activator *in vitro*.

Keywords: Periplasmic serine protease, plasminogen activator, *degS* gene

The *degS* gene, characterized as a homolog of *degP* from *Escherichia coli* in 1996, encodes a protein of 355 residues, and deletion of the *degS* gene results in a small-colony phenotype [13]. DegS has been found to be an essential protein that functions to provide σ^E activity for *Escherichia coli* by cleaving the σ^E -specific anti-sigma factor RseA [3].

Recent research has revealed that peptides ending with Omp-like C-terminal sequences bind to the PDZ domain of DegS to activate it, and then the activated DegS cleaves RseA and induces σ^E -dependent transcription. Therefore, these results suggest that DegS acts as a sensor for envelope stress by binding unassembled Omps; DegS activation then involves the relief of inhibitory interactions between its PDZ and protease domain [12]. Reports on the crystal structure of DegS suggest it plays a novel regulatory role for the PDZ domain, including unique reversible protease activation that involves conformational changes [16, 17].

Escherichia coli outer membrane preparations have been found to induce plasminogen activation associated with the expression of OmpT [8], yet only very weak plasmin activity has been detected in *Escherichia coli* cells expressing cloned OmpT [7]. In contrast, OmpT has been shown to be active under extreme denaturing conditions and to have an affinity for denatured substrates, plus it may have a role in the turnover or degradation of membrane proteins in *Escherichia coli* [15]. OmpT has also been proposed to play a role in complicated urinary tract infections [14], and degrade antimicrobial peptides in urine [11].

Furthermore, it has been reported that DegS is necessary for the virulence of extraintestinal *Escherichia coli* that causes meningitis, sepsis, urinary tract infection, and other infections outside the bowel [10].

Plasminogen activation is involved in systemic infection, as inferred from the observations that meningococcal meningitis is associated with enhanced fibrinolytic activity [2]. Thus, the finding of a plasminogen receptor or plasminogen activation function in several invasive bacterial pathogens has led to suggestions that plasmin formation has a role in the tissue damage associated with or needed for invasive infections, including meningitis [1, 9].

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Accordingly, since DegS is necessary for the virulence of extraintestinal *Escherichia coli* causing various infections, and includes a serine protease domain, just like DegP and OmpT, it is important to clarify whether DegS also has the function of plasminogen activation. Therefore, this report explores the plasminogen activation function of recombinant Δ DegS and discusses the probable relation between its necessity as a virulence factor and plasminogen activation function.

MATERIALS AND METHODS

Strains

The bacterial strains used in this study are as follows:

<i>Escherichia coli</i> TG1	[<i>supE hsd Δ5 thi Δ (lac-proAB) F' (traD36proAB⁺ lac^flacZ_M15)]</i>
<i>Escherichia coli</i> BL21(DE3)	[<i>hsdS gal (lcIts857 ind1 Sam7nin5lacUV5-T7 gene 1)</i>]
<i>Escherichia coli</i> DH5 α	[<i>supE44 ΔlacU169 (ϕ80 lacZΔM15) hsdR17 recA1 endA1gyrA96 thi-1relA1]</i>

Protein Expression and Purification

The whole length of *degS* was expressed and purified according to a previously described method [4], except the pET28a vector was substituted for pET22b(+).

The cloning vector pBlueScript M13- Δ degS, carrying *AdegS*, was constructed based on two steps. First, *AdegS* was amplified with primer PK1 (5'-CGA ATT CTG GGT GTA ATC ATG GAT CAA CGC GGT-3') and PD2 (common: 5'-CGC CTG CAG TTA ATT GGT TGC CGG ATA TTC-3'). After purification using an UltraPureTM Kit (SBS Co.) and double digestion with BamHI/PstI, *AdegS* was ligated into the multicloning site BamHI/PstI of vector pBlueScript M13 (KS-). The resultant plasmid pBlueScript M13- Δ degS was then sequenced using its T3 and T7 promoters at BOYA Biotech. Co. The DNA sequence and deduced amino acids sequence were compared in NCBI BLAST to verify if the desired fragment had been obtained.

The overexpression vector pRSET A- Δ degS was constructed from pRSET A and pBlueScript M13- Δ degS. First, the pRSET A and pBlueScript M13- Δ degS were both double digested with BamHI/PstI. Second, the linear vector pRSET A and *AdegS* fragment were purified using an UltraPureTM Kit (SBS Co.) and ligated together by a ligation enzyme (Promega). Third, the ligation product was transformed into *Escherichia coli* BL21 (DE3), and the clone with the correct recombinant pRSET A- Δ degS identified by sequencing and selected for Δ DegS expression.

The selected clone was cultured in an LB medium at 37°C until the OD₆₀₀ reached 0.8, and then 1 mM isopropyl-

β -D-thiogalactopyranoside (IPTG) was added to induce the recombinant Δ DegS expression. After 4 h of induction at 20°C, the bacteria cells were collected and destroyed by ultrasonication before centrifugation, and then the supernatant was analyzed by SDS-PAGE. The recombinant Δ DegS was purified under native conditions using a His-bond Ni-affinity resin (Novagen) according to the protocols described in the user's manual.

Enzyme Assay

Fibrin and plasma plates were both used to determine the enzyme activity. The fibrin plates were prepared as follows: 2 ml of a fibrinogen solution [50 mg/ml fibrinogen (human, Calbiochem) in 20 mM Tris-HCl, pH 8.0] was mixed with 40 ml of an agar solution [0.75% (wt/vol) agar in 20 mM Tris-HCl (including 0.15 M NaCl), pH 8.0] at 50°C, and then 8 ml of the above mixture was poured into a petri dish with 40 μ l of a thrombin solution [100NIH thrombin (human, Calbiochem) in 20 mM Tris-HCl, pH 8.0]. After leaving the dish to stand for 30 min at room temperature to allow fibrin clots to form, holes were made on the plate using a hollow plastic tube. The plasma plates were prepared by adding healthy human plasma (Wuhan Blood Center) to 0.75% agar in 20 mM Tris-HCl (including 0.15 M NaCl, 4NIH thrombin, pH 8.0). If required, both plates were heated at 85°C for 30 min to inactivate the plasminogen. A 10 μ l sample solution was dropped into each hole and the plates incubated at 37°C for 15 h. After measuring the diameter of the transparent zone around each hole, the units of enzyme activity were determined according to the standard curve of urokinase (Sigma). The plasminogen (10 μ M) and plasminogen activators (1 μ g) were co-incubated on two heated plates to determine the relation between them.

The fibrinolytic and amidolytic activities were measured separately using chromogenic substrates: suc-Ala-Ala-Pro-Phe-pNA (S-7388, Sigma, Subtilisin and chymotrypsin substrate) and H-D-Val-Leu-Lys-pNA (V-0882, Sigma, plasmin substrate). The reaction mixture (1 ml) contained 20 μ l of the enzyme solution, the 1 mM substrates, and a 100 mM sodium phosphate buffer (pH 7.4). After continuous measurement for 5 min at 37°C using a spectrophotometer equipped with a cuvette temperature controller, the amount of *p*-nitroaniline liberated was determined at 405 nm.

To determine the plasminogen activation ability of Δ DegS directly *in vitro*, the plasminogen (10 μ M) was incubated with and without the purified Δ DegS (1 μ g) in 50 mM Tris-HCl (pH 7.8) at 37°C for different times. Aliquots (10 μ l) were taken from the reaction mixture for SDS-PAGE.

Statistical Analysis

All data are shown as mean \pm SEM, the results were analyzed using a one-way ANOVA, and *P* values <0.05 were considered as statistically significant.

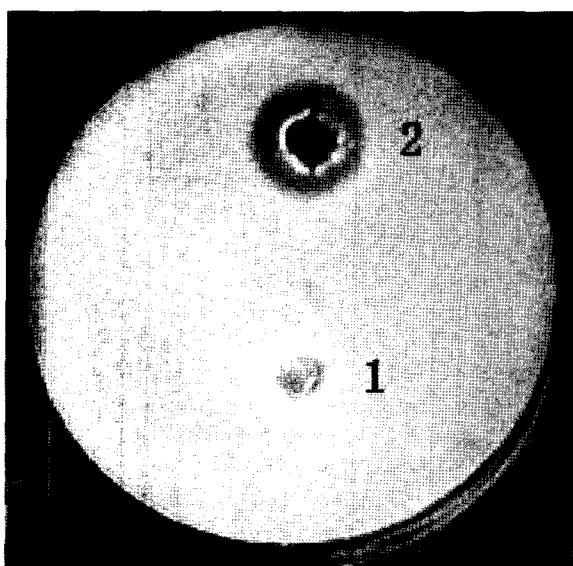


Fig. 1. Enzyme activity of whole-length DegS on plasma plate. 1: Blank; 2: DegS.

RESULTS

Whole-Length DegS Acts as Plasminogen Activator

As shown in Fig. 1, the purified whole-length DegS displayed enzyme activity on the human plasma plate. After the plate was incubated for 16 h at 37°C, a transparent circle formed around the hole in which the purified DegS had been added.

Amplification and Sequencing of $\Delta degS$ Fragments

The open reading frames of the *degQ* and *degS* genes were oriented in the same direction and separated by 92 bases, and appeared to encode proteins with 455 and 355 residues respectively, and the *degS* gene had BamHI and PstI sites in its open reading frame. The *degQ* and *degS* genes were located approximately 72 to 73 min in the *Escherichia coli* chromosome [13]. The results of the colony PCR with primers PK1 and PD2 revealed that all three *Escherichia coli* strains had a $\Delta degS$ fragment. The $\Delta degS$ fragment was purified and digested with BamHI and PstI for insertion into pRSET A. The resultant plasmid pRSET A- $\Delta degS$ was identified by the endorestriction enzymes and sequencing. The $\Delta degS$ fragment was composed of 798 nucleotides and encoded $\Delta DegS$ with 266 residues, which reserved the protease domain and PDZ-like domain of DegS, especially the putative catalytic sites: His⁹⁶, Asp¹²⁶, and Ser²⁰¹ [13].

$\Delta DegS$ Acts as Plasminogen Activator

The recombinant protein $\Delta DegS$ was expressed, purified, and its characteristics investigated. As the $\Delta degS$ fragment

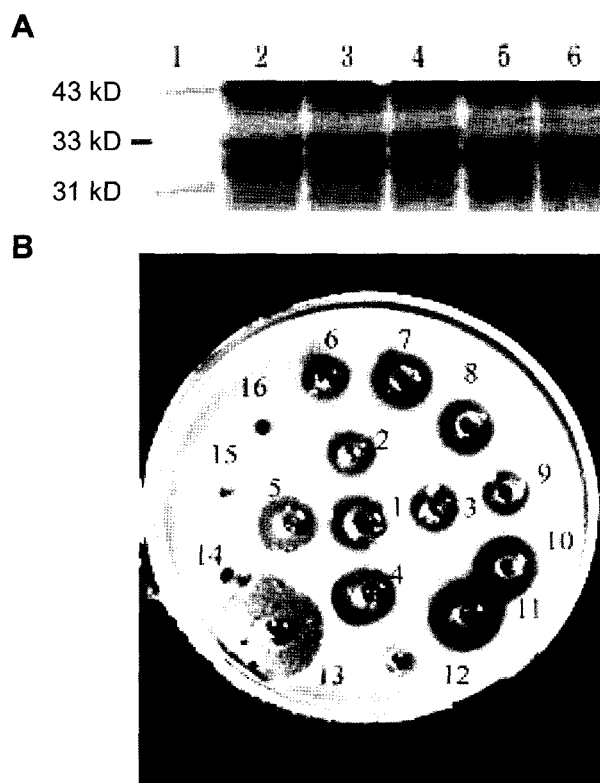


Fig. 2. SDS-PAGE (A) and enzyme activity on fibrin plate (B) of recombinant protein $\Delta DegS$.

A. 1: protein molecular marker; 2–4: pRA- $\Delta degS$ 4 h after addition of isopropyl- β -D-thiogalactopyranoside (IPTG); 5–6: pRSET-A 4 h after addition of IPTG. B. 1–3: pRA- $\Delta degS$ 1 h after addition of IPTG; 4–6: pRA- $\Delta degS$ 2 h after addition of IPTG; 7–9: pRA- $\Delta degS$ 4 h after addition of IPTG; 10–11: pRA- $\Delta degS$ 6 h after addition of IPTG; 12: Blank; 13: Urokinase (50IU); 14–16: pRSET-A 4 h after addition of IPTG.

did not include any promoter, start codon, SD sequence, or stop codon, it was expressed using the vector pRSET A, which includes an SD sequence, TATA box, start codon ATG of lacZ, and His tag, plus its T7 promoter facilitates the overexpression of foreign genes with the addition of isopropyl- β -D-thiogalactopyranoside (IPTG). The resultant vector pRSET A- $\Delta degS$ was transformed into *Escherichia coli* BL21(DE3) and the expression products analyzed by SDS-PAGE (Fig. 2A) and the plasma plate method (Fig. 2B). There were 96 nucleotides from the lacZ ATG to the BamHI site and 21 nucleotides from the PstI site to the stop codon TGA in pRSET A. The expression product, encoded by the total 915 nucleotides, showed a band with the molecular mass of about 33 kDa in SDS-PAGE. This protein was expressed in the cytoplasm of the host and its enzyme activity detected on the plasma plate, as shown in Fig. 2B.

$\Delta DegS$ was overexpressed after being induced by isopropyl- β -D-thiogalactopyranoside (IPTG) for 4 h, and the recombinant $\Delta DegS$ purified by His-Bond Ni-

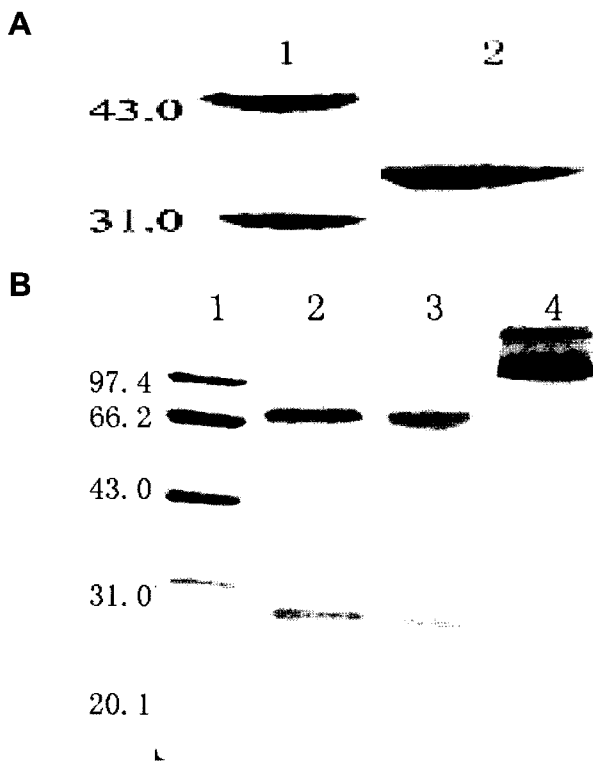


Fig. 3. SDS-PAGE of purified Δ DegS (A) and products degraded from plasminogen by Δ DegS (B).

A. 1: Protein molecular marker; 2: purified Δ DegS (5 μ g); B. 1: Protein molecular marker; 2, 3: Plasminogen incubated with Δ DegS for 1 & 3 hours; 4: Plasminogen (control).

affinity chromatography. The purified product also exhibited a molecular mass of about 33 kDa in SDS-PAGE (Fig. 3A).

The enzymatic characteristics of the purified Δ DegS were investigated by the fibrin plate method using two chromogenic substrates. The fibrinolytic and amidolytic activities were both completely inhibited by PMSF (phenylmethanesulfonyl fluoride), yet unaffected by the others (Table 1), indicating that Δ DegS possessed serine protease activity.

The recombinant Δ DegS functioned on the unheated fibrin and plasma plates, yet not on the heated fibrin and plasma plates in which the plasminogen was inactivated by heating in advance, which is similar to urokinase and tissue plasminogen activators. Therefore, the interaction

between Δ DegS and plasminogen was investigated using a heated plasma plate (Table 2). On the heated plasma plate, neither Δ DegS nor urokinase exhibited any enzyme activity, yet when mixed with plasminogen they both exhibited a higher fibrinolytic activity than the plasminogen itself, proving that Δ DegS acts as a plasminogen activator, like urokinase, although its enzyme activity was higher than that of urokinase.

To further evaluate the plasminogen activation activity of Δ DegS, plasminogen and Δ DegS were incubated together and electrophoresed. A zymogram analysis of the samples showed a clear modification of the molecular mass of plasminogen. As shown in Fig. 3B, the band of the apparent molecular mass of 94 kDa corresponding to plasminogen progressively disappeared, accompanied by the appearance of two bands with apparent molecular masses of 68 kDa and 27 kDa, corresponding to the conversion of plasminogen into plasmin.

DISCUSSION

An unheated plasma plate contained all plasma elements, including activated plasminogen; however, plasminogen was not active in a heated plasma plate. Δ DegS showed enzyme activity on an unheated plasma plate, yet no activity on a heated one, indicating that it dissolved fibrin indirectly through plasminogen activation. On the heated plasma plate, a mixture of Δ DegS and plasminogen exhibited a two-fold higher enzyme activity than plasminogen alone (Table 2). Thus, since Δ DegS cleaved plasminogen into plasmin, and when mixed with plasminogen exhibited a higher enzyme activity than plasminogen alone on a heated plasma plate, it is clear that Δ DegS acted as a plasminogen activator.

In addition to the whole-length DegS acting as a plasminogen activator, the truncated fragment (Δ DegS) also showed a plasminogen activation function *in vitro*, although less, suggesting that the enzyme activity of Δ DegS was rooted in the whole-length DegS. Furthermore, it had been reported that the His⁹⁶, Asp¹²⁶, and Ser²⁰¹ of DegS are the putative catalytic sites for the cleavage of RseA [13], and when comparing Δ DegS and DegS, the putative active sites of DegS were also conserved in Δ DegS, signifying that the plasminogen activation activity of DegS was likely

Table 1. Effect of inhibitor on enzyme activity of Δ DegS (%).

Activities (%)	PMSF 1 mM	Pepstatin A 10 μ g/ml	Benzamidine 1 mM	EDTA 5 mM	Aprotinin 1 mM	Leupeptin 1 mM	Control ^a
Fibrinolytic	0	111.0	115.6	110.1	121.1	110.4	100
Amidolytic	0	98.5	124.5	105.0	100.2	114.7	100

Abbreviation: PMSF (phenylmethanesulfonyl fluoride).

^aAbsolute values of fibrinolytic and amidolytic activities of control were 1.15 cm (R) on fibrin plate and 0.017 of AOD₄₀₅, respectively.

Table 2. Activation of plasminogen by Δ DegS.

Plate	Enzyme activity	Δ DegS (10 μ l)	Plg (10 μ l)	UK (10 μ l)	Δ DegS+Plg (10 μ l)	UK+Plg (10 μ l)
Heated plasma plate	Dissolving area (mm ²)	0	38.47	0	64.81	45.15
	Relative activity (%)	0	100	0	168.50	117.40
Unheated fibrin plate	Dissolving area (mm ²)	103.81	94.98	226.87		
	Relative activity (%)	100	91.5	218.50		

Abbreviations: UK (Urokinase); Plg (Plasminogen).

due to its protease domain, especially the putative catalytic sites: His⁹⁶, Asp¹²⁶, and Ser²⁰¹.

DegS is essential for the virulence of extraintestinal *Escherichia coli* that causes certain diseases, especially urinary tract infections [10]. DegP, a periplasmic protease like DegS, is essential for the clearance of denatured or aggregated proteins from the inner-membrane and periplasmic space in *Escherichia coli*. Furthermore, it cleaves the major pilin subunit of Pap pilus, PapA [6], and P pili that are important virulence factors in uropathogenic *Escherichia coli* [5]. All this reveals the complicated relation between DegS, DegP, and the filamentous apparatus, such as P pili, in the virulence of uropathogenic *Escherichia coli*.

The conserved carboxyl-terminal sequence in the pilin subunits, although not a cleavage substrate for DegP, activates the protease, and the activating peptide is recognized by DegP's PDZ domains [5]. DegS also has a PDZ domain, and the peptides ending with Omp-like C-terminal sequences bind the DegS PDZ domain, and then the activated DegS cleaves the RseA [12]. Other studies have indicated that RseA plays an important role in both the degradation of membrane proteins and urinary tract infections when activated by Omp T in *Escherichia coli* [14, 15].

Thus, from previous reports and the current results, it would appear that DegS, a periplasmic protease, can be cleaved or activated by peptides, such as the C-terminal sequence in pilin subunits and Omp T, to activate plasminogen by binding with plasminogen receptors on its filamentous surface appendages, especially in the case of virulent uropathogenic *Escherichia coli*.

In conclusion, the present study found that DegS acted as a plasminogen activator *in vitro*; thus, future studies will focus on the plasminogen activation function of DegS *in vivo* and the relationship between DegS and plasminogen receptors in *Escherichia coli*.

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