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α-Enolase binds to human plasminogen on the surface of *Bacillus anthracis*

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ABSTRACT

α-enolase of *Bacillus anthracis* has recently been classified as an immunodominant antigen and a potent virulence factor determinant. α-enolase (2-phospho-D-glycerate hydrolase (EC 4.2.1.11), a key glycolytic metalloenzyme catalyzes the dehydration of D-(+)-2-phosphoglyceric acid to phosphoenolpyruvate. Interaction of surface bound α-enolase with plasminogen has been incriminated in tissue invasion for pathogenesis. *B. anthracis* α-enolase was expressed in *Escherichia coli* and the recombinant enzyme was purified to homogeneity that exhibited a K_m of 3.3 mM for phosphoenolpyruvate and a V_{max} of 0.506 μMmin^{-1} mg^{-1}. *B. anthracis* whole cells and membrane vesicles probed with anti-enolase antibodies confirmed the surface localization of α-enolase. The specific interaction of α-enolase with human plasminogen (but not plasmin) evident from ELISA and the retardation in the native gel reinforced its role in plasminogen binding. Putative plasminogen receptors in *B. anthracis* other than enolase were also observed. This binding was found to be carboxypeptidase sensitive implicating the role of C-terminal lysine residues. The recombinant enolase displayed *in vitro* laminin binding, an important mammalian extracellular matrix protein. Plasminogen interaction conferred *B. anthracis* with a potential to *in vitro* degrade fibronectin and exhibit fibrinolytic phenotype. Therefore, by virtue of its interaction to host plasminogen and extracellular matrix proteins, α-enolase may contribute in augmenting the invasive potential of *B. anthracis*.

1. Introduction

Cell-surface protein-mediated interactions have been known to play a major role in disease-progression. Numerous pathogenic bacterial species intervene with the plasminogen system and a hypothesis has emerged that bacteria use this system for migration across the tissue barriers or for nutritional demands during infection [1]. Plasminogen represents a monomeric proenzyme of the serine protease plasmin that plays a crucial role in fibrinolysis [2]. The ability of gram-positive bacteria to subvert the fibrinolytic activity of human plasminogen for tissue invasion and dissemination in the host makes it a lucrative system for pathogenesis [3–4]. Plasminogen contains five kringle domains, which mediate binding of plasminogen to extracellular matrix, fibrin and lysine-rich receptors that are distributed on mammalian cells. In various pathogenic systems, including bacteria, fungi and protozoa, the invasive phenotype has been correlated with the ability of the organism to bind to laminin, an abundant extracellular matrix glycoprotein [5–7]. Presence of α-enolase on the cell surface was initially studied only on neuronal [8], cancerous and hematopoietic cells, but several reports identified α-enolase to be present on the surface of a wide spectrum of gram-positive and gram-negative bacteria including *Streptococcus pyogenes* [9] and *Streptococcus pneumoniae* [10]. However, the question of how α-enolase is transported through the cell membrane and sorted on the cell surface without the presence of a signal sequence remains intriguing [11]. The presence of α-enolase at the cell surface, where it acts as a receptor instead of an enzyme is not surprising because there is an ever growing list of proteins which are targeted to more than one location in both prokaryotes and eukaryotes with variable biological functions. In the serological proteome analysis of *Bacillus anthracis* secretome, enolase has also been implicated as an immunodominant antigen with perspective protective efficacy [12]. The presence of α-enolase on the surface of bacteria adds a new insight in the generation of antibodies against enolase, post infection. Fungal specific anti-enolase antibodies are found in increased levels in patients with invasive candidiasis.

The potential of *B. anthracis* in recruitment of human plasminogen for invasion and virulence has not been studied till date. Although the surface localization of α-enolase had already been speculated by Chitlaru et al no absolute experimental evidence was provided with confidence. Therefore, the present study was undertaken to ascertain the membrane localization and establish the role of surface α-enolase in *B. anthracis*. We hereby report the first experimental evidence for

Abbreviations: IPTG, isopropyl β-D- thiogalactopyranoside; rBaEn, 6×-histidine tagged recombinant Enolase; hPlg, Human Plasminogen; PMSF, phenyl methylsulfonyl fluoride; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel/electrophoresis; TMB, 3,3′,5,5′-Tetramethylbenzidine; NBT/BCIP, nitroblue tetrazolium dye/ 5-bromo-4-chloro-3-indolyl phosphate; ECM, extracellular matrix

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the acquisition of surface plasmin-like activity by *B. anthracis* and demonstrated the role of surface bound plasminogen, *in vitro*.

2. Materials and methods

2.1. Materials

Plasmid pET-28a (+) from Novagen (Madison, Wisconsin, USA) was used for heterogeneous gene expression. Restriction enzymes were from Fermentas GmbH (Germany). Ni^{2+}-NTA agarose resin was from Qiagen (Hilden, Germany), Nitrocellulose membrane, IPTG, antibiotics and bovine serum albumin were from Amersham Biosciences (Uppsala, Sweden). Anti Histidine murine monoclonal (HIS-probe), alkaline phosphatase and horseradish peroxidase conjugated antibodies, substrate (NBT/BCIP/TMB), human Plasminogen (hPlg), human plasmin, bovine carboxypeptidase B, human fibrinogen, laminin (Engelbreth-Holm-Swarm murine sarcoma basement membrane), human fibronectin and adjuvants (complete and incomplete) were from Sigma (St. Louis, MO, USA). Bradford reagent used for protein estimation was from Bio-Rad (CA, USA). Oligonucleotides were custom synthesized by Microsynth, Switzerland.

Escherichia coli strains DH5α and BL21 (λDE3) (Novagen) were used as hosts for cloning and expression, respectively. A virulent strain of *B. anthracis*, Sterne 34F2 ($pXO1^+$, $pXO2^-$) was used for all the studies.

2.2. Expression and purification of Enolase of B. anthracis

The open reading frame corresponding to enolase of *B. anthracis* was PCR amplified employing forward primer (5′-GACCGGATCCATGTCAACAATTA TTGATGTTTATGCTCGC-3′) and reverse primer (5′-GACCAAGCTTTCGTTT GATGTTATAAAAAGATTTGATACC-3′), containing a BamH I and Hind III restriction site flanking its 5′ end, respectively (underlined) and genomic DNA of *B. anthracis* was used as a template. The amplified DNA fragment was ligated to BamH I/Hind III sites of pET-28a expression vector to obtain pBaEnol plasmid which was subsequently transformed into *E. coli* DH5α cells. The integrity of the cloned gene was verified by automated dideoxy DNA sequencing.

The recombinant protein was expressed as a fusion protein in *E. coli* BL21 (λDE3) cells, with a 6×-His tag (rBaEn). Localization of expressed rBaEn was determined by analyzing various cellular fractions prepared from the induced cells [13]. Optimization of the expression level was carried out by inducing the cultures with different concentrations of IPTG (0.2–1.0 mM) for 4h. Time kinetics of expression was studied by harvesting an aliquot of the culture at different time points post induction with 0.5 mM IPTG.

All purification procedures were performed at 4 °C unless otherwise stated. The expression of recombinant protein from the construct was induced at an optical density of 0.6 at 600 nm by the addition of IPTG (0.5 mM) for 4h at 30 °C. Enolase with His_6-tag at N-terminus was purified from the soluble fraction using Ni^{2+}-NTA affinity chromatography as described by Qiagen. Briefly, the frozen cells were thawed in approximately one-tenth of the total culture volume in buffer A (20 mM potassium phosphate buffer (pH 8.0), 1 mM PMSF, 150 mM NaCl, and lysozyme 20 mg/ml). The cells were lysed by pulse sonication for 30 min (Misonix Inc., USA) and centrifuged at 20,000×*g* for 40 min. The supernatant was loaded onto Ni^{2+}-NTA column pre-equilibrated with buffer B (buffer A + 20 mM imidazole). After loading, the column was washed exhaustively with buffer B. The protein was eluted in buffer C (buffer A + 300 mM imidazole). The purified protein was dialyzed against 10 mM Tris–HCl, pH 8.0. The protein concentration was determined by the Bradford reagent [14] using bovine serum albumin as the standard.

Polyclonal antiserum against rBaEn was raised in 4–6 weeks old male Swiss Albino mice; immunized intraperitoneally with 25 μg of purified protein with complete Freund's adjuvant (FCA) in 1:1 ratio. Same amount of protein was injected in the mice after 21 days in Freund's incomplete adjuvant (IFA). The serum was collected from the mice after 14 days of the booster immunization.

2.3. Determination of enzyme activity, temperature optima and pH optima

Kinetic properties of the purified rBaEn were determined at room temperature in a 50 mM imidazole buffer supplemented with 8 mM $MgSO_4$ and 30 mM KCl in a total reaction volume of 1 ml. The activity was measured in the reverse (formation of 2-PGA from PEP) direction by monitoring the decrease in PEP absorbance at 240 nm in a continuous spectrophotometric assay on Cecil 800. The change in PEP concentration was determined using an absorption coefficient ($\Sigma_{240\ nm}$) of $1400M^{-1}\ cm^{-1}$. The Michaelis–Menten constant was determined using 0.05 mM-2.5 mM of the substrate concentration, i.e. phosphoenolpyruvate. The kinetic coefficients, K_m and V_{max} of the enzyme were determined using double reciprocal plots of Lineweaver–Burk plot. Optimum temperature for the rBaEn activity was determined by assaying the enzyme activity at different temperatures (4–65 °C) in 50 mM imidazole buffer of pH 6.8. To determine the optimum pH for the rBaEn, the enzyme activity was measured in 50 mM buffer of varying pH range from 4 to 10 (Sodium acetate buffer pH 4.0, 5.0, imidazole buffer pH 6.0, Tris–HCl buffer, pH 7.0, 8.0 and 9.0 and Sodium carbonate buffer, pH 10).

2.4. ELISA for enolase and plasminogen interaction

Wells were coated with either *B. anthracis* cells prepared from the exponentially growing liquid phase culture (0.1–1.0 × 10^8cfu/well) or 500 ng of human plasminogen/human plasmin, in 50 mM carbonate coating buffer, pH 9.6 and incubated for 16 h at 4 °C in triplicates. The wells were washed with 0.05% PBS/Tween 20 and blocked with 100 μl of 2% BSA–PBS for 1 h at 37 °C. 500 ng of hPlg was added in the wells containing immobilized bacilli so as to assess the interaction of *B. anthracis* cells with hPlg (probed with 1:5000 dilution of anti-plasminogen). 500 ng of rBaEn was added to the wells containing hPlg (probed with 1:5000 dilution of anti-His_6) to assess the direct interaction between hPlg and rBaEn. Subsequent incubation was followed with 1:10,000 dilution of horseradish peroxidase conjugated sheep anti-mouse IgG for 1 h at 37 °C. The color was developed by adding TMB and absorbance was measured at 630 nm in a microplate ELISA reader (Bio-Rad).

2.5. Preparation of right-side out membrane vesicles of B. anthracis

A single colony of *B. anthracis* was grown to stationary phase at 37 °C in Brain heart infusion medium. The cells were harvested by centrifugation and washed thrice with 100 mM potassium phosphate, pH 7.0 as described previously [15]. The cells were then incubated (0.6g wet weight/ml) at 37 °C for 60min with 100 mM potassium phosphate, 10 mM $MgSO_4$, 5 mg/ml lysozyme. K_2SO_4 was then added to a final concentration of 150 mM. It was further treated with DNase and RNase (5 μg/ml each) for 20min at 37 °C followed by 10 mM EDTA and readdition of $MgSO_4$ to 20 mM. Three sequential low speed centrifugations (12,000×*g* for 10min, 4 °C) were carried out so as to reduce contamination by intact cells which was further ascertained by carrying out cfu analysis after each centrifugation. Vesicles were isolated by centrifugation (48,000×*g* for 45 min, 4 °C) from the last supernatant and the pellet was resuspended in PBS. The integrity of the membrane vesicles was ascertained by performing lactate dehydrogenase assay. The membrane preparation was stored in small aliquots at −80 °C and was thawed only once before use.

2.6. ELISA with whole cells and membrane vesicles

Wells were coated with approximately 1 × 10^8 (1×) and 2 × 10^8 (2×) whole bacilli/1μg (1×) and 2μg (2×) membrane vesicles in 50 mM sodium carbonate coating buffer, pH 9.6 and incubated for 16h at 4 °C. As positive control, wells were also coated with rBaEn (500 ng). The wells were washed with 0.05% PBS/Tween 20 and blocked with 100 μl of 2% BSA–PBS for 1h at 37 °C.

For competitive ELISA, 500 ng of hPlg was added to the whole bacterial cells and vesicles prior to the addition of anti-enolase antibody and was incubated for 1h at 37 °C. As positive control, 500 ng of hPlg was incubated with rBaEn at 37 °C for 1 h.

Both these sets were probed with 1: 10,000 dilution of anti-enolase polyclonal serum. The bound primary antibody was captured using 1:10,000 dilution of horseradish peroxidase conjugated sheep anti-mouse IgG for 1h at 37 °C. The color was developed by adding TMB and absorbance was measured at 630 nm in a microplate ELISA reader (Bio-Rad).

2.7. Native polyacrylamide gel electrophoresis

rBaEn (1 μg) and hPlg (1 μg) were incubated for 1h at 4 °C to facilitate the complex formation and subjected to a 5% native PAGE in running buffer (83 mM Tris–HCl, pH 9.4 and 33 mM glycine). The protein bands were visualized by staining with Coomassie Brilliant Blue. For reference, only hPlg and rBaEn, 1 μg each, were loaded in separate wells.

2.8. Indirect immunofluorescence assay

An immunofluorescence assay was performed on the whole bacterial cells. Briefly, *B. anthracis* was grown till OD_{600} of 0.8. One ml of the cell suspension was washed thrice with PBS and fixed in carnoyes reagent (acetic acid and methanol in 1:3 ratio). Samples were stored at 4 °C for 1h and were dropped on pre-cleaned chilled coverslips, which were then air-dried and washed with PBS. Cells coated on the coverslips were incubated for 45 min with 1:500 dilution of anti-enolase antibody or anti-His_6 antibody (control) followed by three washes with PBS. Fluorescein isothiocyanate (FITC) conjugated goat anti-mouse IgG (Sigma, Poole, UK; 1:100) was incubated for 45 min. It was then mounted in a solution containing glycerol and PBS in 1:1 ratio. The slides were examined using a Carl Zeiss Confocal Laser Scanning microscope.

2.9. Flow cytometry

For flow cytometric analysis, *B. anthracis* cells were grown till late exponential phase and suspended in phosphate buffered saline (PBS). The cells were treated with mouse polyclonal anti-enolase antisera (1:10,000) and the untreated cells were taken as control. Cells were washed twice with PBS and incubated again with FITC-conjugated goat anti-mouse IgG (1:100) for another 30 min at 4 °C. Cells were washed again and analyzed for fluorescence using Beckman Coulter Cell Lab Quanta™ SC flow cytometry. The cells were detected using forward and log-side scatter dot plots, and a gating region was set to exclude cell debris and larger aggregates of bacteria. 10,000 bacteria (events) were analyzed for fluorescence in a flow cytometer.

2.10. Blot overlay assays

Whole cell lysates of *B. anthracis* were prepared by sonicating the culture obtained at an absorbance of 0.8 (at 600 nm) and were subjected to electrophoresis. The gel was then trans-blotted (Bio-Rad Transblot Apparatus Model) onto a nitrocellulose (NC) membrane in blotting buffer (25 mM Tris–HCl, 192 mM glycine and 10% ethanol, pH 8.0)

[16]. The NC was overlaid with hPlg (1 μg) for 1 h with constant shaking in the presence or absence of 0.1M lysine. The blot was probed with anti-plasminogen antibody (1:5000) followed by incubation with secondary antibody conjugated with alkaline phosphatase. The immunoreactive bands were visualized by NBT/BCIP substrate solution in alkaline phosphatase buffer.

2.11. Immunoblotting of various cellular fractions of B. anthracis

Immunoblot analysis was performed with right-side out membrane vesicles along with other cellular fractions of *B. anthracis*. For fractionation, 1 ml of *B. anthracis* was lysed by pulse sonication for 15 min (Misonix Inc., USA) and centrifuged at 20,000×*g* for 45 min. The supernatant and pellet were subjected to SDS-PAGE (12%) and transferred to NC membrane. It was then analyzed for the presence of α-enolase. The blot was incubated with anti-enolase antiserum (1:10,000) for 1h at 37 °C followed by a further incubation with sheep anti-mouse conjugated with IgG alkaline phosphatase (1:10,000). The immunoreactive bands were visualized by NBT/BCIP as substrate in alkaline phosphate buffer.

2.12. Interaction with extracellular matrix (ECM) proteins

2.12.1. Interaction of α-enolase and laminin

Wells were coated with different concentrations of laminin in 50 mM sodium carbonate coating buffer, pH 9.6 and incubated for 16h at 4 °C. The wells were washed with 0.05% PBS/Tween 20 and blocked with 100 μl of 2% BSA–PBS for 1h at 37 °C. The wells were further incubated with 500 ng rBaEn. Anti-enolase polyclonal antiserum (1:10,000) was then added and incubated for 1h at 37 °C. The bound antibody was probed with 1:10,000 dilution of horseradish peroxidase conjugated sheep anti-mouse IgG for 1h at 37 °C. The color was developed by adding TMB and absorbance was measured at 630 nm in a microplate ELISA reader (Bio-Rad).

2.12.2. Degradation of human fibronectin by B. anthracis cells

Approximately 1 × 10^8 *B. anthracis* cells were washed, mixed with 100 μl of PBS, PBS-containing hPlg (1 μM) plus streptokinase (50 U), or PBS-containing hPlg (1 μM), incubated 1h at 37 °C with gentle shaking. After incubation, the cells were washed with PBS and resuspended in 100 μl buffer [Tris–HCl, 50 mM; NaCl, 100 mM, and $CaCl_2$, 5 mM; pH 7.0] in the presence or absence of human fibronectin (Fn: 30μg/ml) and further incubated for 40h at 37 °C with gentle shaking. All samples were centrifuged and 20 μl of supernatant were electrophoresed on a 10% polyacrylamide SDS-PAGE. After electrophoresis, proteins were transferred to a nitrocellulose membrane and probed with polyclonal rabbit anti-human fibronectin antibody, 1:2500 (Sigma).

2.13. Assay of plasminogen bound to B. anthracis by fibrin lysis

A microtitre plate fibrin lysis assay was performed as described previously [17]. Briefly, *B. anthracis* cells (10^6/5 × 10^6/10^7) were centrifuged at 12,000×*g* for 5min and washed with phosphate buffered saline. Cells were incubated with 50 μg/ml of plasminogen for 3 h at 37 °C, washed and resuspended in PBS. Fibrin gel (1.25%) was prepared using low melting agarose and once it is cooled to room temperature, 2 mg/ml of human fibrinogen, 50 μg/ml of human plasminogen and 0.05U/ml of thrombin were added. The gel was poured in a 96 well microtitre plate and allowed to set for 4h at 37 °C in a humidified chamber. Increasing number of *B. anthracis* cells incubated with plasminogen were added to the precasted fibrin gel for 12 h at 37 °C and untreated cells (without plasminogen incubation) were taken as control. For reference, 50 ng/ml of plasminogen was also incubated with increasing concentrations of human streptokinase (5 U to 100 U), a known plasminogen activator. Fibrin lysis was visualized and quantitated spectrophotometerically at 405 nm.

To rule out the possibility of fibrinolysis mediated by secreted metalloproteases (MMPs) from *B. anthracis* [18], inhibitors of MMPs; 1,10 phenanthroline, 2, 2 Biprydyl and EDTA (1 mM each) were incubated with bacterial cells prior to the addition of hPlg. Fibrin lysis was quantitated by taking absorbance at 405 nm.

2.14. Plasminogen activation by B. anthracis cells

The ability of *B. anthracis* to activate human plasminogen into plasmin was monitored in a quantitative assay using the chromogenic substrate D-Val-Leu-Lys-*p*-nitroanilide (Sigma). Approximately 2 × 10^8 *B. anthracis* cells were washed with PBS, mixed with 50 μg/ml hPlg in the presence or absence of streptokinase (50U). After incubation, the cells were centrifuged, washed, and resuspended in 200 μl of chromogenic substrate D-Val-Leu-Lys-*p*-nitroanilide (2 mg/ml). After 12 h incubation at 37 °C, the cells were pelleted and the absorbance of the supernatant was measured at 405 nm in a microplate reader. To assess the effect of metalloprotease inhibitors on plasminogen activation, cells were incubated with MMP inhibitors (MMPI); 1,10 phenanthroline, 2,2 Bipyridyl and EDTA.

3. Results and discussion

3.1. Primary sequence analysis

The *B. anthracis* α-enolase exhibits high homology with *S. pneumoniae* and *S. pyogenes* with 83% and 84% similarity. The Clustal W analysis was employed to align the bacterial enolases and Boxshade server 2.0 was used to identify the conserved domains responsible for catalytic activity and metal ion binding. The analysis revealed the presence of **SHRSGETED** as the catalytic motif. Analysis of deduced amino acid sequence of *B. anthracis* enolase showed the presence of neither a signal sequence nor a hexameric **LPXTGX** motif, the domains considered to be crucial for translocation through the cell wall and for anchoring surface proteins to the gram-positive bacterial cell wall.

3.2. Purification of B. anthracis α-enolase and enzyme kinetics

The PCR amplification of α-enolase from *B. anthracis* resulted in the amplification of the expected 1.4kb fragment, which was cloned into the expression vector. SDS-PAGE profile showed a 47 kDa band corresponding to rBaEn with N-terminus Hexa-histidine tag upon

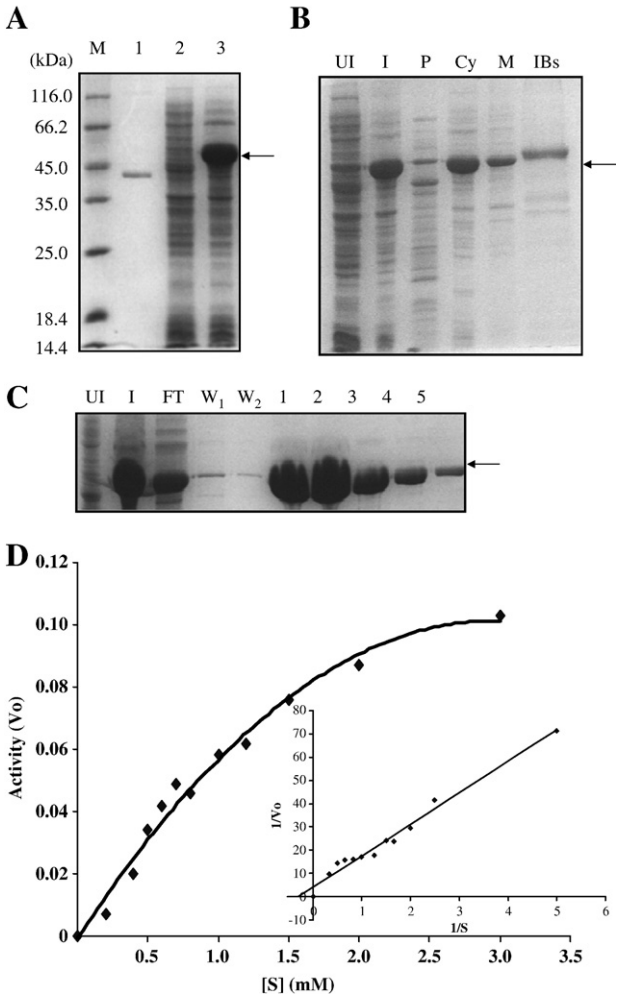


Fig. 1. A: Expression of recombinant enolase from *B. anthracis* in *E. coli*. *E. coli* cells harboring plasmid pBaEnol under the control of T7 promoter were induced with 0.5 mM IPTG. Lane 1, a 40 kDa purified protein of *B. anthracis*. Lane 2, uninduced cell lysate. Lane 3, induction with IPTG. M indicates the protein molecular mass standards. Arrow points to the rBaEn. B: Localization of the rBaEn. Different cellular fractions (50 μg) were prepared and analyzed on 12% SDS-PAGE. Lanes represent 25 μg each of uninduced (UI), induced (I), periplasmic (P), cytoplasmic (Cy), membranous (M) and inclusion bodies (IBs) fractions. Arrow towards right depicts the expressed protein. C: Purification of rBaEn using Ni^{2+}-NTA chromatography. Lane UI, uninduced cell lysate harboring pBaEnol. Lane I, cells induced with 0.5 mM IPTG. Lane FT, flow through. Lanes W_1 and W_2 represent two subsequent 50 mM immidazole washes. Lanes 1–5, protein eluted with 300 mM immidazole. D: Kinetic analysis of the rBaEn. Michaelis–Menten plot. The enzyme activity was measured using different substrate (PEP) concentrations (0.2 mM–3.0 mM) under standard reaction conditions for 20 min. V_0 indicates the initial velocity of the reaction and [S] is the substrate concentration. The inset depicts Lineweaver–Burk plot. Values represent mean of at least 3 independent estimations.

induction with IPTG (Fig. 1A, Lane 3). Analysis of various cellular fractions indicated that the rBaEn was expressed predominantly in the soluble fraction (Fig. 1B). The affinity purified recombinant protein migrated as a single band and was purified to homogeneity (Fig. 1C, Lane 3–5). A single immunoreactive band at 47 kDa, probed with anti-6×-His antibody confirmed the authenticity of the expressed recombinant protein (data not shown).

The purified enzyme remained active within the temperature range of 25 °C to 45 °C with the highest activity at 37 °C and pH 6.8 (data not shown). The recombinant enzyme followed the classical Michaelis–Menten kinetics, K_m and V_{max} was calculated using Lineweaver–Burk plot (Fig. 1D). The K_m of rBaEn was calculated to be 3.3 mM for phosphoenol pyruvate with a V_{max} of 0.506 μMmin^{-1} mg^{-1}. The K_m observed for PEP, 3.3 mM, falls within the range (140 μM for type-III isoenzyme from rat [19] to 7.2 mM for enolase present in plastids of *Ricinus communis* [20]) reported for enolase from different sources. However, as it is known that K_m value is usually higher for the reverse reaction in comparison to the forward reaction, PGA to PEP (30 μM in human enolase [21] to 1.0 mM in *E. coli* [22]); therefore, the observed K_m for PEP is even higher than the highest value reported for that of PGA.

3.3. Interaction of human plasminogen with B. anthracis whole cells, membrane vesicles and rBaEn

ELISA performed with whole cell bacilli coated on the polystyrene plates demonstrated a concentration dependent increase in binding of *B. anthracis* to plasminogen. Saturation observed after 5 × 10^7 cells confirmed the specificity of the interaction (Fig. 2A). Similarly, ELISA established the interaction of human plasminogen with purified α-enolase (Fig. 2B). In none of the cases, binding with human plasmin was detected (data not shown) and the data was in agreement with the previous studies which showed a significantly higher affinity of enolase for plasminogen than plasmin [9].

To establish the direct association of α-enolase with surface of *B. anthracis*, ELISA performed with two different concentrations of whole cell bacilli and membrane vesicles were probed with anti-enolase antibodies (Fig. 3A). The results implicated the surface localization of α-enolase. In a competitive ELISA, the binding of surface enolase to anti-enolase antibody was completely abrogated when the cells were preincubated with human plasminogen. In this case, the surface enolase was not accessible to the antibodies as it interacted with plasminogen, resulting in a significant inhibition. The wells containing hPlg (as positive control) showed high absorbance when allowed to interact with α-enolase and subsequently probed with anti-enolase antibody. Therefore, we concluded that α-enolase is present on the membrane of *B. anthracis* and binds to human plasminogen on the surface.

Native-PAGE also showed a significant shift in the mobility of rBaEn due to the formation of a high molecular weight complex between enolase and plasminogen in a dose dependent manner (Fig. 3B).

3.4. Effect of lysine and carboxypeptidase on binding of B. anthracis enolase to human plasminogen

Blot overlay assay revealed several other putative plasminogen receptors in addition to enolase (Fig. 4A). Binding of plasminogen to eight other bacterial components (not identified as yet) with estimated molecular masses of « 86 kDa, 47 kDa (α-enolase), 39 kDa, 36 kDa, 34 kDa, 31 kDa, 27 kDa and19 kDa (depicted by arrows) coincided with the previous studies with *Candida albicans*. The eight *C. albicans* plasminogen-binding proteins identified are primarily intracellular proteins exported by non-classical routes and bound adventitiously to the cell surface [23]. Thus, the presence of several bands in *B. anthracis* sonicate implicate the existence of other plasminogen-binding proteins in *B. anthracis*.

Plasminogen kringle domains often mediate interactions with lysine residues of cellular receptors. To evaluate their possible involvement in the interaction of plasminogen with α-enolase, binding assay was carried out in the presence of 0.1M L-lysine. The binding of rBaEn and other putative receptors was inhibited completely in the presence of lysine (Inset, Fig. 4B), underlining the pivotal role of exposed lysine residue(s) in rBaEn for binding to the plasminogen. Also, the binding of carboxypeptidase treated purified recombinant enolase and *B. anthracis* cells, to plasminogen was found to be reduced to 20% in the presence of 10 U of carboxypeptidase; indicating that the binding is partially mediated through the C-terminal lysine residues of enolase (Fig. 4B). The inhibition was found to be dose dependent.

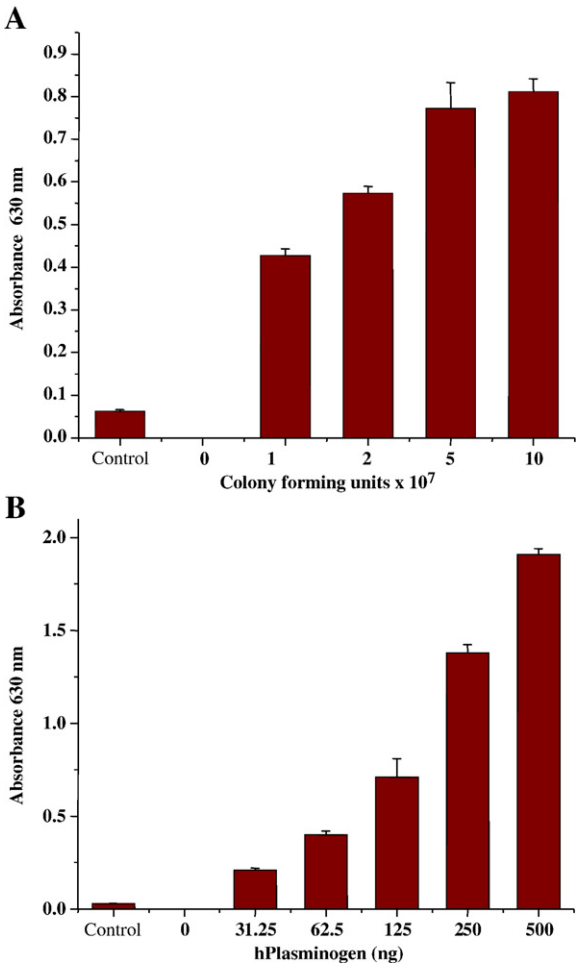


Fig. 2. A and B: Interaction of *B. anthracis* and purified rBaEn with human plasminogen. Increasing concentrations of (A) colony forming units of *B. anthracis*, (B) human plasminogen/human plasmin, were coated in 50 mM carbonate coating buffer, pH 9.6 in triplicates. 500 ng of hPlg was added in the wells containing bacilli and probed with 1:5000 dilution of anti-plasminogen while 500 ng of rBaEn was added to the wells containing hPlg and probed with 1:5000 dilution of anti-His_6 antibody. 1:10,000 dilution of horseradish peroxidase conjugated sheep anti-mouse IgG was used as secondary antibody.

3.5. Interaction of B. anthracis enolase to laminin, an ECM protein

Microbial agents have evolved a variety of mechanisms to adhere to cells and to the extracellular matrix, and these mechanisms play a major role in the pathogenesis as well as in the severity of the cognate diseases. *B. anthracis* can colonize the host and also has a propensity to cross blood brain barrier leading to the most severe neurological complication, meningoencephalitis. The results presented here clearly demonstrate that α-enolase of *B. anthracis* binds to laminin, a major component of the extracellular matrix (Fig. 5A). Thus, it is highly speculative that the ability of *B. anthracis* to bind to laminin might represent an important mechanism by which it can lead to tissue invasion and blood dissemination.

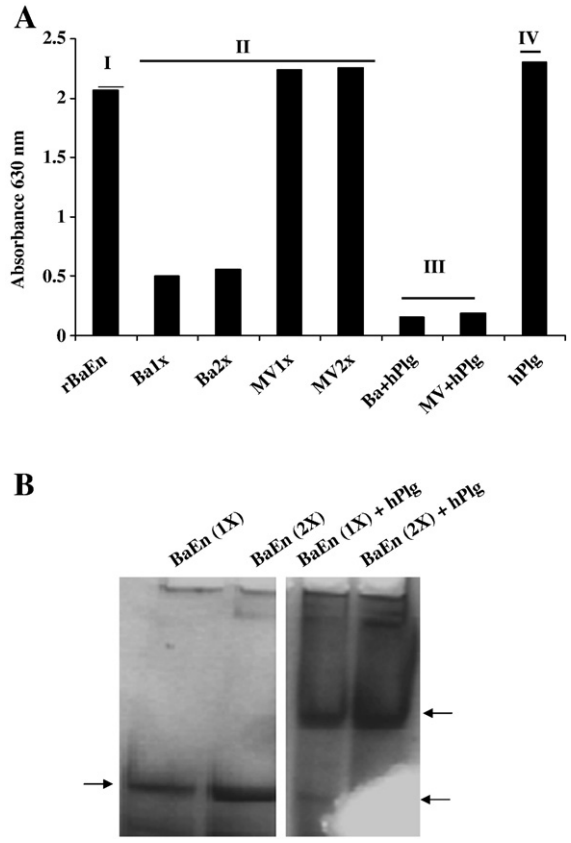


Fig. 3. A: Interaction of *B. anthracis* and human plasminogen is mediated by α-enolase on the surface. (II) 500 ng of rBaEn/1×10^8 (1×) and 2×10^8 (2×) whole bacilli/1 μg (1×) and 2 μg (2×) membrane vesicles were coated in 50 mM carbonate coating buffer, pH 9.6 in triplicates. The samples were probed with anti-enolase antibody (1:10,000). (III) For competitive ELISA, 500 ng of hPlg was added to the whole bacterial cells and membrane vesicles and was incubated for 1 h at 37 °C and were then coated on the wells. The complex was allowed to interact with α-enolase. I depict 500 ng of coated rBaEn and IV represented coated hPlg interaction with α-enolase; as positive controls. All the wells were probed with anti-enolase antibodies (1:10,000) followed by 1:10,000 dilution of horseradish peroxidase conjugated sheep anti-mouse IgG as secondary antibody. B: Native-PAGE to assess the interaction of human plasminogen and *B. anthracis* enolase. rBaEn (1 μg, 1× and 2 μg, 2×) was loaded onto 5% native polyacrylamide gel as shown by arrows on the left side of the gel. High molecular weight complex was evident (arrow towards upper right side) when rBaEn (1× and 2×) was incubated with hPlg (1 μg). The lower arrow towards right side reflects free rBaEn which was unable to bind to hPlg.

3.6. Degradation of human fibronectin by B. anthracis cells with a surface proteolytic plasmin-like activity

B. anthracis cells when incubated with plasminogen were able to degrade human fibronectin even in the absence of plasminogen activators such as streptokinase. Following electrophoresis, fibronectin and fibronectin degradation products were specifically detected with polyclonal anti-fibronectin antibodies (Fig. 5B). Therefore, it is conceived that components on the bacterial cell surface activate plasminogen independent of any exogenous plasminogen activators and thus enhance the proteolytic potential of the bacterial cell surface.

3.7. Functional consequence of plasminogen binding to enolase on B. anthracis cell surface: fibrinolysis by plasminogen activation

Initiation of a number of infections involves adherence of bacteria to the host cells followed by spreading to the local tissue. One of the mechanisms involved in bacterial migration through the human extracellular matrix is tissue degradation and remodeling by the pathogen [3–4,24–25]. In addition to producing their own proteolytic enzymes, some pathogens also take advantage of the mammalian host's fibrinolytic system, which is normally responsible for the breakdown of fibrin and extracellular matrix [26]. The ability of *B. anthracis* cells to acquire surface bound plasmin activity was investigated using a microtitre fibrin plate assay. The cells were able to retain bound plasminogen after washings and as is evident from the fibrin lysis and increase in the absorbance, this plasminogen could be activated to plasmin without the external addition of plasminogen activators (Fig. 5C). A decrease in the absorbance of fibrin gel was observed with a decrease in concentration of human streptokinase incubated with 50 μg/ml human plasminogen (positive control) (Fig. 5D). Fibrinolysis observed in the absence of established plasminogen activators indicated that *B. anthracis* possesses an elaborate plasminogen activator system. The contribution of major exported metalloproteases in plasminogen activation and fibrinolysis was ruled out by including several metalloprotease inhibitors in the experiment. The results concluded that plasminogen could also be activated even in the presence of MMPIs (Fig. 5E, inset).

The ability of *B. anthracis* cells to bind and activate hPlg was measured using an assay based on the ability of plasmin to cleave the chromogenic substrate in a reaction that can be followed spectrophotometrically at 405 nm. In this experiment, the basal level of proteolytic activity was determined using *B. anthracis* cells treated with PBS only. As shown in Fig. 5E, increased proteolytic activities could be

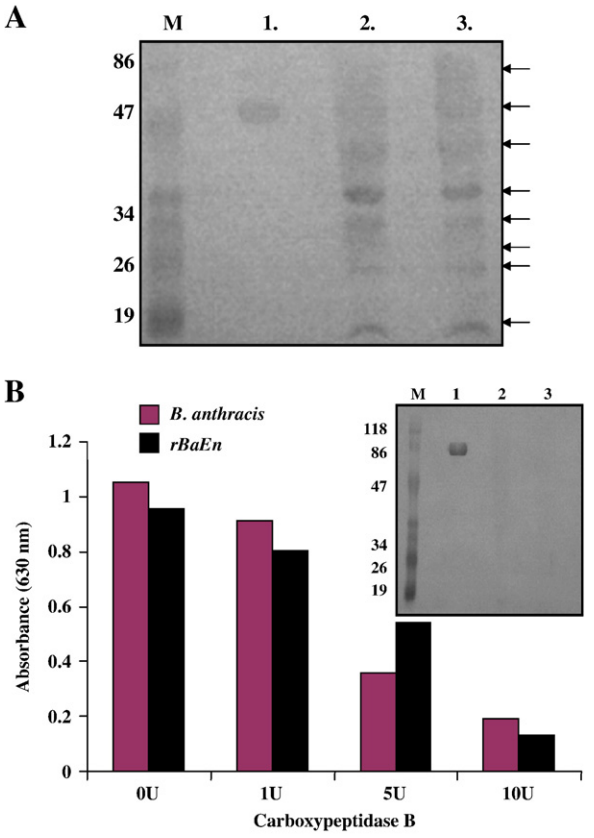
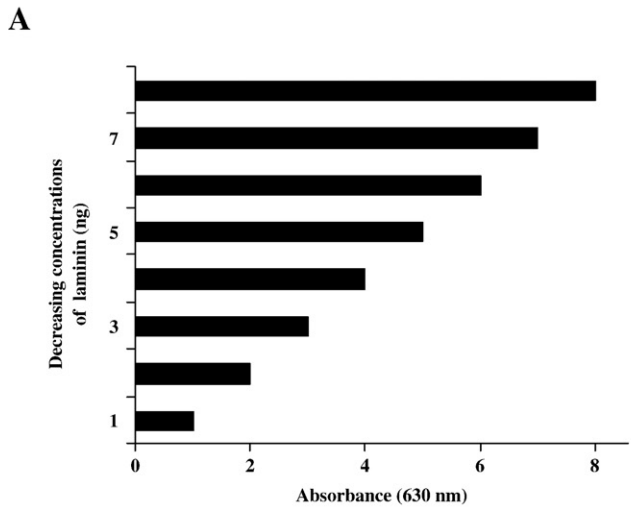
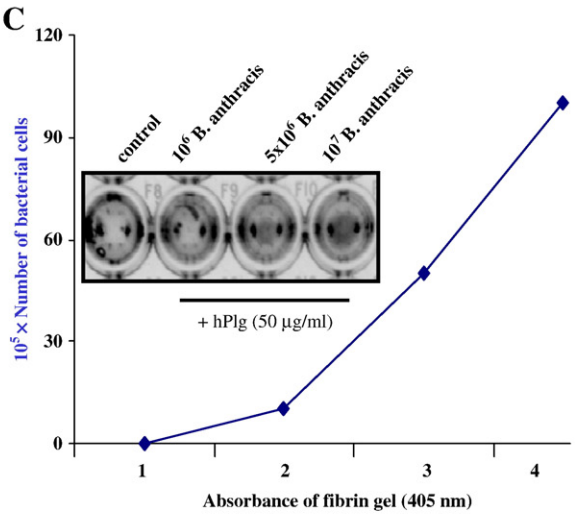
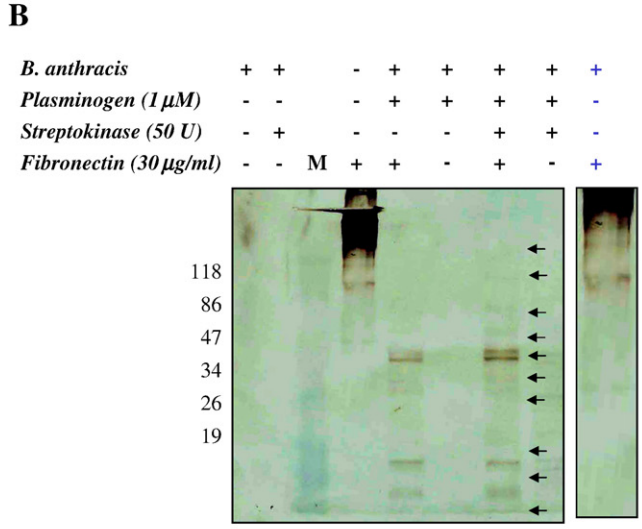
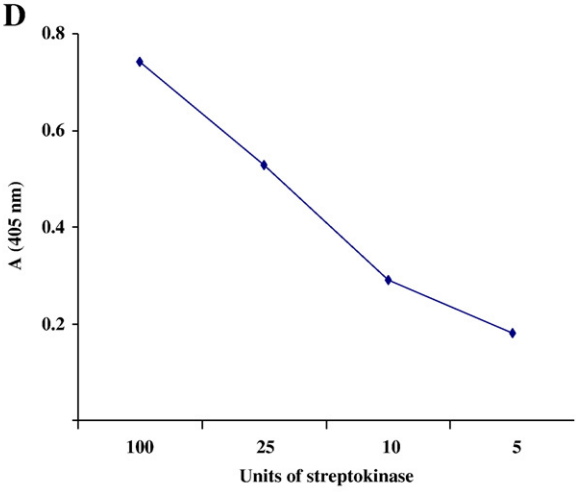


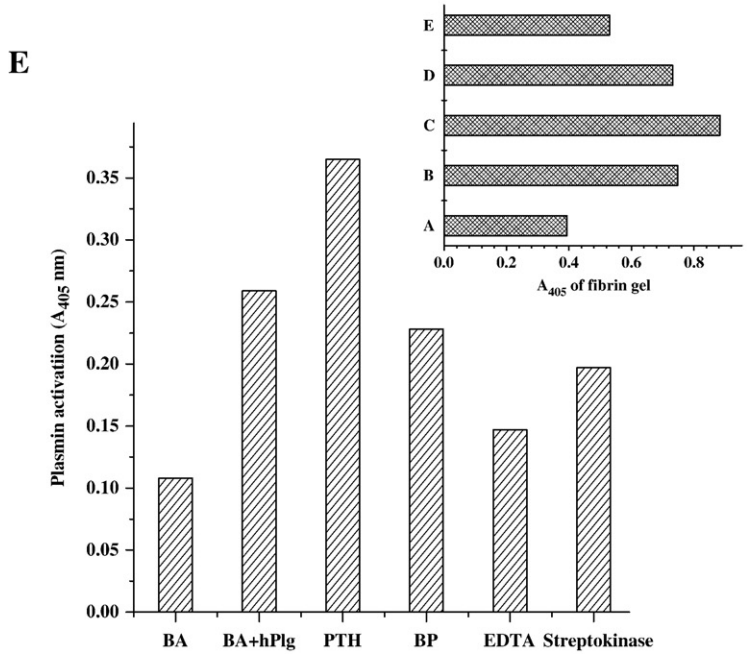
Fig. 4. A: Blot overlay assay to identify plasminogen receptors: *B. anthracis* cell lysate and purified rBaEn was run on a 12% SDS-PAGE and was trans-blotted onto NC membrane followed by incubation with hPlg. Blot was probed with 1:5000 dilution of anti-plasminogen antibody. Lane M, molecular mass standards. Lane 1, rBaEn. Lane 2 and 3, *B. anthracis* cell lysate. Arrows point towards the putative plasminogen receptors. B: Binding of *B. anthracis* α-enolase to human Plasminogen was lysine and basic carboxypeptidase sensitive: Binding of 500 ng rBaEn and *B. anthracis* cells (10^8) to human plasminogen was carried out in the absence and presence of increasing units of carboxypeptidase B. The wells were probed with anti-enolase antiserum (1:10,000) followed by sheep anti-mouse IgG-alkaline phosphatase conjugated (1:10,000). The inset showed an immunoblot. Lane M, molecular mass standards. Lane 1, 1 μg human plasminogen, ~92 kDa. Lane 2 and 3, *B. anthracis* cell lysate incubated with 1 μg hPlg in the presence of lysine. M indicates the protein molecular mass standards.


A
Decreasing concentrations of laminin (ng)
7
5
3
1
0
2
4
6
8
Absorbance (630 nm)


C
120
90
60
30
0
control
10^6 B. anthracis
5x10^6 B. anthracis
10^7 B. anthracis
F8
F9
F10
+ hPlg (50 µg/ml)
10^5 × Number of bacterial cells
1
2
3
4
Absorbance of fibrin gel (405 nm)


B
B. anthracis + + - + + + + +
Plasminogen (1 µM) - - - + + + + -
Streptokinase (50 U) - + - - - + + -
Fibronectin (30 µg/ml) - - M + + - + - +
118
86
47
34
26
19


D
0.8
0.6
0.4
0.2
A (405 nm)
100
25
10
5
Units of streptokinase


E
Plasmin activation (A405 nm)
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
BA
BA+hPlg
PTH
BP
EDTA
Streptokinase
E
D
C
B
A
0.0
0.2
0.4
0.6
0.8
A405 of fibrin gel

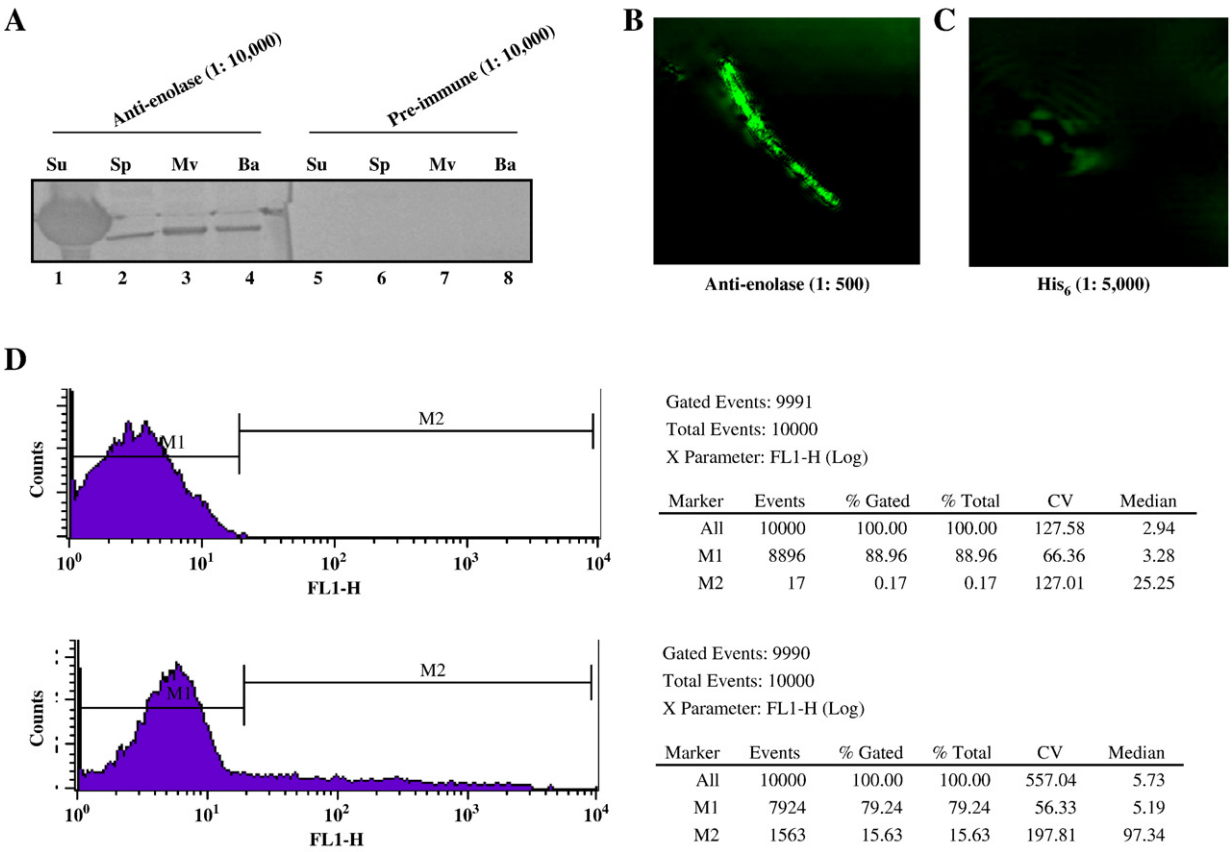


Fig. 6. A Immunoblotting of *B. anthracis* cellular fractions: *B. anthracis* cells were lysed and cellular fractions were analyzed by western blotting using anti-enolase polyclonal antiserum. Lane 1, Su—cytosolic fraction after sonication. Lane 2, Sp—pellet obtained after sonication containing membrane proteins. Lane 3, Mv—right-side out membrane vesicles. Lane 4, Ba—*B. anthracis* total cell lysate. B and C: Fluorescence Microscopy: Panel B: *B. anthracis* cells probed with anti-enolase antiserum (1:500) or Panel C: His_6 monoclonal (1:5000); followed by labeling with FITC-conjugated goat anti-mouse IgG. D: Flow cytometric analysis of *B. anthracis* cells expressing α-enolase on surface: *B. anthracis* cells were stained with anti-enolase antibody (1:10,000) as primary antibody. Untreated cells were taken as control. Fluorescence, after staining with FITC-labeled secondary antibody, was analyzed using FL1 and displayed as histogram. The histogram area in M_1 is from control cells whereas; area in M_2 is from anti-enolase treated *B. anthracis* cells.

detected when bacteria were incubated with hPlg in the absence of plasminogen activator, streptokinase. Also, MMPIs did not abolish plasminogen activation suggesting that the effect is not mediated by metalloproteases secreted by *B. anthracis*. These experiments demonstrated the ability of bacterial cells to bind to plasminogen on the surface which thereby arms the bacilli with a broad spectrum of substrates for proteolysis mediated by plasmin. Thus, the capture of plasminogen by surface α-enolase represents a potent virulence mechanism.

3.8. α-enolase is present on the surface of B. anthracis

Once the role of enolase as a ligand for plasminogen was established, we examined the localization of this molecule in *B. anthracis*. By using specific anti-enolase antibodies, the distribution of α-enolase in various subcellular fractions of *B. anthracis* was examined by western blotting. The results showed the presence of the protein in both the cytosolic and cell membrane fraction (Fig. 6A, Lane 1 and 3); while no corresponding immunoreactive band was seen with pre-immune serum (Fig. 6A, Lane 5 and 7).

3.9. Fluorescence microscopy

α-enolase present in *S. pyogenes*, *Streptococcus mutans* and *S. pneumococci* are plasminogen-binding protein that, besides being cytosolic, has been shown to be surface-displayed [10,27,28]. Therefore, fluorescence microscopic analysis using anti-enolase antibodies was carried out to assess the possible surface localization of α-enolase on *B. anthracis*. The study revealed that *B. anthracis* enolase is secreted

Fig. 5. A: Laminin binding to *B. anthracis* α-enolase: Serial dilutions of laminin was allowed to interact to 500 ng of α-enolase and the interaction was assessed by probing the complex with anti-enolase antiserum (1:10,000) followed by detection with anti-mouse IgG conjugated with alkaline phosphatase. The absorbance of the substrate was measured at 630 nm in an ELISA reader. B: Degradation of fibronectin: Approximately 1×10^8 *B. anthracis* cells were mixed with PBS-containing hPlg (1 μM) plus streptokinase (50 U), or PBS-containing hPlg (1 μM), incubated for 1 h at 37 °C. The cells were washed and further incubated in the presence or absence of human fibronectin (Fn: 30 μg/ml) for 40 h at 37 °C with gentle shaking. The supernatant obtained after centrifugation were electrophoresed on a 10% SDS-PAG and were transferred to a nitrocellulose membrane and probed with polyclonal rabbit anti-human fibronectin antibody, 1:2500. The color was developed using NBT/BCIP as substrate solution. M represents migration of the molecular mass standards on the gel. C: Microtitre fibrin plate assay: *B. anthracis* cells were incubated with hPlg and were then added onto the precasted fibrin gel. The graph showed an increase in the absorbance of fibrin gel, measured at 405 nm. Arrow in the inset depicts clotting of fibrinogen with increase in the number of bacterial cells. D: Plasminogen activation by streptokinase: As a positive control, plasminogen activator, streptokinase is added in conjunction to hPlg instead of bacterial cells. The absorbance of the fibrin gel was measured in each well at 405 nm and plotted with increasing units of streptokinase. E: Effect of metalloproteases on fibrinolysis: Fibrinolysis assay was done in a similar way as described in Materials and methods. The graph in the inset depicts A, Bacterial cells (2×10^8) incubated with PBS; B, Cells (2×10^8) with hPlg (50 μg/ml); C, Cells (2×10^8) with hPlg (50 μg/ml) and MMPI (1,10 Phenanthroline; 2,2 Bipyridyl (D) and EDTA (E), 1 mM each. The absorbance of fibrin gel in each well was measured at 405 nm in a spectrophotometer and is plotted on *X*-axis in each case. The figure depicts activation of plasminogen to plasmin by *B. anthracis*. *B. anthracis* cells were incubated with PBS (background control)/hPlg alone or hPlg with MMPI (1, 10 Phenanthroline; 2,2 Bipyridyl; abbreviated as PTH and BP in the graph) or hPlg in the presence of streptokinase (50 U) as a positive control. Plasmin activity was measured by incubation of cells with the chromogenic substrate and determination of the absorbance at 405 nm.

and attached to the outer surface of the bacterial cells (Figs. 6B and C), in addition to its presence in the cytosol. Thus, its dual location in cytosol and on cell surface indicated its pivotal role in glycolysis and pathogenesis, respectively. However, the most challenging and important issue that needs to be addressed further is to discern the mechanism of its export to the cell surface.

3.10. Flow cytometric analysis

In order to provide confirmatory evidence for the localization of α-enolase on the bacterial cell surface, the antibodies raised against the purified rBaEn were used to probe *B. anthracis* cells and the staining reaction was visualized by flow cytometry. Control cells that were not treated with anti-enolase polyclonal antiserum but with FITC-conjugated secondary antibody gave no fluorescence as against the cells stained with specific anti-enolase antibody, thus, demonstrated the cell surface localization of the cognate antigen (Fig. 6D). A total of 15% bacterial cells imparted the fluorescent signal; the relatively low signal observed could be attributed to scarce distribution of enolase on the surface as is the case with that of *S. pneumoniae* [29]. However low but the expression was sufficient to endow the bacilli with plasminogen-binding potential. It is also plausible that other surface proteins might affect the availability of exposed enolase epitopes and therefore interfere with the reactivity of antibody. Moreover, the expression levels of enolase could be influenced by environmental conditions as is shown for the surface enolase protein in *S. pneumoniae* [30] where the gene showed decreased transcription in several conditions but it is very difficult to *in vitro* simulate the conditions experienced by bacteria *in vivo*.

4. Concluding remarks

The present study describes characterization of a 47 kDa protein, α-enolase as a plasminogen and laminin binding molecule on the surface of *B. anthracis*. α-enolase is one of the key glycolytic enzymes catalyzing the conversion of PGA to PEP in the catabolic direction in the penultimate step of Embden Meyerhoff Parnas pathway; nevertheless, its presence on the surface of cells is not without precedent. α-enolase has been shown to be expressed on the surface of many gram-positive bacteria and has recently been speculated to be present on the surface of *B. anthracis*. The question that how proteins lacking any signal peptide are exported on the cell surface is unresolved; the factors that affect their export and membrane association remain a mystery and is an important area for research. Studies report SecA2 dependent pathway for enolase secretion in *Listeria monocytogenes* [31]. Another group even speculates that the surface enolase does not come from inside the bacterium but is scavenged from other cells that have undergone programmed cell death [32].

As the present study has been carried out on the non-capsulated strain of *B. anthracis* (Sterne strain, $pXO1^+$, $pXO2^-$), the access of cell surface enolase–plasminogen complex in a capsulated bacterium to the host tissues can be questioned. However, studies in *S. pneumoniae* depicted that enolase is not only present in proximity to the cell wall but is also present at the outer edge of the capsular polysaccharide [10]. Immunoelectron micrographs generated for *S. pneumoniae* also revealed the presence of enolase on the surface irrespective of the status of capsule expression. It was concluded that surface exposed enolase is relatively non abundant but the efficiency of plasminogen binding is high and this amount is sufficient to contribute to pneumonococcal pathogenesis [29,33]. Another study carried out by Andreas Knaust and colleagues on Neisseria meningitides compared binding of plasminogen with wild type strain and isogenic capsule deletion strain where the binding capacity of the unencapsulated strain was enhanced but the expression of the capsule did not prevent plasminogen from interacting with plasminogen receptors [34]. Therefore, it can be deduced that surface enolase will be available for plasminogen binding even in the encapsulated strain of *B. anthracis* ($pXO1^+$, $pXO2^+$).

Here, in addition to the functional characterization and localization of α-enolase, we also describe the fibrinolytic potential of *B. anthracis* mediated by surface enolase, as a plasminogen receptor. The use of plasminogen system is not restricted to bacterial pathogens. The invasive yeast *C. albicans* has α-enolase homologs that function as plasminogen receptor and potentiate the transcytosis of *C. albicans* across a monolayer of human brain microvascular endothelial cells [23,35]. It can therefore be postulated that the plasminogen–enolase association may play a critical role in the virulence of *B. anthracis* by causing a direct damage to the host cell extracellular matrix, possibly by enzymic degradation of extracellular matrix proteins or other protein constituents. In the present study, it has been concluded that α-enolase can mediate the binding of *B. anthracis* to laminin, a major component of the basal membrane of the vasculature. This might function as a guidance mechanism, first allowing *B. anthracis* adherence to the extracellular matrix, initiating tissue colonization, followed by plasminogen activation and laminin degradation in restricted areas. Hence, expression of α-enolase at the cell surface seems to be a common mechanism by which *B. anthracis* can induce destruction of the extracellular matrix, favoring their invasion and dissemination and attempts to block this interaction could be of clinical relevance.

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