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Characterization of *Streptococcus agalactiae* α-enolase from *Nile tilapia* (*Oreochromis niloticus*), A Plasminogen Binding Protein on the Surface

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α-Enolase is an important glycolytic enzyme located on the cell surface of various pathogenic *streptococcus* which is capable of plasminogen binding and is contribute to tissue invasion. In the present study, we cloned and purified the α-enolase of *S. agalactiae* from tilapia. The plasminogen-binding ability of α-enolase was confirmed by western-blot. The location of α-enolase was demonstrated by using the methods of cell wall associated protein extraction and flow cytometry. Moreover, the role that α-enolase played in the GBS adherence to EPC cell line was analyzed. These results suggested that α-enolase of *S. agalactiae* was capable for the *S. agalactiae* to invasion by plasminogen activation and the adherence ability of α-enolase highlight its crucial role it played in the pathogenesis of *S. agalactiae*.

Key words: *Streptococcus agalactiae;* α-Enolase; Surface localized; EPC adherence.

Streptococcus agalactiae, also known as group B *streptococci*(GBS), is a widespread pathogen causing invasive disease in newborns, pregnant women, and immunocompromised adults[1,2]. Till now, ten serotypes of *S. agalactiae* has been reported[3] and serotype Ia, Ib and III were considered to be the major cause of fish diseases[4, 5]. In aquaculture, GBS has been reported as an increasingly pathogenic factor infecting many cultured fish such as salmons, channel catfish, tilapia, ya-fish, *etc*.[6-9]. In July 2009, an outbreak emerged in Hainan Province, China. It caused high mortality of the cultured tilapia, leading to a great financial loss to this aquatic farm[10]. In this case, a further understanding of the mechanic of pathogenesis is of great importance.

Once in contact with a new host, a critical step in pathogenesis is for the entering streptococcus to come into contact with an epithelial cell. Gram-positive pathogens express adhesions to mediate the adherence to host tissues[11]. Surface proteins play significant roles in the bacteria colonization and invasion progress by acting as adhesions[12]. As for *S. agalactiae*, surface proteins such as surface immunogenic protein (Sip)[13], C5a peptidase[14] and Alp family proteins[15] have already been characterized. α-Enolase was reported as a surface located protein that is lack of wall-anchoring region in multiple pathogenic *streptococci*. However, the characterization of *S. agalactiae* α-enolase was unreported. α-Enolase is a key glycolytic enzyme in the cytoplasm of prokaryotic and eukaryotic

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cells[17] which had the ability to bind human plasmin(ogen) in aquacultural pathogenic *streptococci* such as *Streptococcus iniae*, *Streptococcus pneumonia*, *Streptococcus pyogenes etc.*[19-21]. Plasminogen is an inactive proenzyme that can be converted to the active enzyme plasmin, which in turn degrade fibrin and activates matrix metalloproteinases in the extracellular matrix (ECM)[16]. In previous study of *Streptococcus pyogenes*, α-enolase could cause tissue invasion by binding plasmin (ogen), thus contributed to the bacterial infection process by disturbing the hosts' homeostasis and vascular potency[22].

To investigate whether *S. agalactiae* á-enolase has the ability of adherence and plasminogen binding which is important to the tissue invasion, we cloned and purified the enzymatically active recombinant á-enolase from tilapia *S. agalactiae* and confirmed the plasminogen binding activity. We furthermore described the *S. agalactiae* α-enolase as a surface-localized protein which could perform as an adhesion factor in *S. agalactiae*. The results highlight the crucial role α-enolase played in the pathogenesis of *S. agalactiae.*

MATERIAL AND METHODS

Bacterial strains and plasmids

S. agalactiae strain HN0303 (GenBank: JF423947.1) was isolated and stored by the authors' laboratory. Cloning vector pMD19-T and expressing vector pET-32a(+) were bought from TaKaRa company, Dalian, China. *Escherichia coli* DH5á and BL21 (DE3) were bought from TianGen Biotech company, China.

Cloning, expression and purification of á-enolase of *S. agalactiae*

S. agalactiae was incubated in the BHI broth culture for 24h and total genomic DNA of *S. agalactiae* was extracted using Tiangen genomic DNA extraction kit according to the manufacturer's instructions (TIANGEN BIOTECH CO., LTD.). á-Enolase sequence was amplified with the former primer 5'- CGCGGATCCATGTCAATTATT ACTGATG -3' and reverse primer 5'-CGGAAGCTTCTATTTTTTTAGGTTGTAG-3', the restriction enzyme site *BamH*I and *Hind*III was underlined. Purified gene was cloned into plasmid pMD19-T and transformed into *Escherichia coli* (*E. coli*) DH5α. Sequencially, the positive plasmid was cloned into pET-32a(+) and transformed into and BL21(DE3). Isopropyl-α-D-thiogalactopyranoside (IPTG) of a final concentration of 1.0mM was added to induce the recombinant α-enolase. Affinity chromatography (Profinity™ IMAC Ni-Changed Resin, BIO-RAD) was used to purify the recombinant protein.

Recombinant α-enolase activity

The reaction was performed as described earlier[22]. Briefly, the reaction was at 37 °C in 100 mM HEPES buffer(pH 7.0), containing 3.3 mM $MgSO_4$, 0.2 mM NADH, 0.3 mM 2-phosphoglycerate (2-PGE), 1.2 mM ADP, 10.3 IU of lactate dehydrogenase, and 2.7 IU of pyruvate kinase in a final volume of 1.0 ml. The reaction started by adding 100 ìl of the sample containing α-enolase. α-Enolase activity was determined by measuring the monitoring the amont of $NADH•H^+$ to NAD^+ in the absorbance at 340 nm, thus estimating the rate of reduction.

Plasminogen binding of recombinant α-enolase

Binding assay was performed as previous study[23]. Purified protein was resolved in a 12% SDS-PAGE gels and electrophoretically blotted onto a PVDF membrane. Blots were incubated at room temperature (RT) for 1h in a blocking TBST((150 mM NaCl, 10 mM Tris–HCl, pH 7.5, 0.05%Tween-20, 3%BSA) and then 10ug/ml human plasminogen (Sigma) was added into the buffer, and incubated overnight at 4!. Anti-plasminogen rabbit antibody (WuXi AppTec Company) was then added for incubation for another 12h at 4! after TBST washed for 3 times. The membrane was incubated with goat-anti rabbit IgG horseradish peroxidase conjugate (WuXi AppTec Company) in blocking solution for 1h at 37°C, afterwards. Detection of binding was achieved by DAB method according to the manufacturer's construction (TIANGEN BIOTECH CO., LTD.).

Preparation of α-enolase antiserum

Two healthy New Zealand male rabbits were chosen to prepare the antiserum of á-enolase. 2ml of equal volume mixture of purified proteins and Freund complete adjuvant containing 1mg of purified protein at the first time. Rabbits were boost with 2ml of equal volume mixture of purified proteins and Freund incomplete adjuvant. Rabbits were sacrificed 3 days after the antibody titer reached

1:16 under anesthesia of ether. The antiserum was collected by centrifugation and then purified by gradient precipitation of ammonia sulfate according to previous study[24].

Extraction of cell wall-associated proteins

S. agalactiae cultivated in 50ml BHI broth for 48 h were harvested (centrifugation at 8000 ×g for 5 min) and the extracellular products were collected for further use. The bacteria were washed twice in TBS (pH 7.5) and then suspended in 1ml TBS. The cells were lysed by a sonicator. The pellets and supernatant were separated by centrifugation at 8000 ×g for 5 min.

Western blot assay

Purified protein was performed on 12.5% SDS-PAGE and transferred on to PVDF membrane. Then the membrane was blocked with TBST containing 3% bovine serum albumin for 1h at RT. After washed three times with TBST for 5 minutes, the membrane was incubated with 1:100 diluted precipitated sera overnight at 4°C. The visualization of the band was as described above. In addtion, the cell wall-associated proteins and extracellular products prepared above were used to confirm the location of nature α-enolase using the same method above.

Location of nature α-enolase analysis

Flow cytometry was applied to detect the location of α-enolase in this study. Briefly[25], *S. agalactiae* was incubated and diluted to a concentration of 10^8cfu/ml. Then α-enolase antiserum was added to the cell culture and co-incubated for 1h. Cells were washed by PBS and incubated with goat anti–rabbit IgG–fluorescein isothiocyanate (FITC) (Sigma) for 1h, followed by paraformaldehyde fixed for 30min and analyzed by flow cytometer.

Adherence assays of α-enolase to EPC cells

Immunofluorescence assay was used to visualize whether α-enolase could specifically adhere to the surface of *Epithelioma papulosum cyprini* (EPC) cells. Briefly, EPC cells was cultured in 24-well cell plates[26], PBS (1mL/well) washed for 3 times then fixated for 20 min with paraformaldehyde (1mL/well), air-dried. α-Enolase (10 mg) was added and incubated at 37°C for 1 h, 1% bovine serum albumin as a negative control. The wells were washed 3 times with PBS and anti-enolase serum was then added, and the cells were incubated at 37°C for 30 min. Then the washed cells were incubated with goat anti–rabbit IgG–FITC at 37°C for 30 min. The samples were washed 3 times and examined using fluorescence microscope.

The adherence rate was determined by plate count method. Briefly, EPC cells were grown on 24 well plates and *S. agalactiae* HN0303 was grown on BHI to a concentration of 10^7cfu/ml. Curtured bacteria were preincubated with anti-enolase serum (negative serum as control) 37°C for 1h. The cultured bacteria were then added to the wells and after incubation for 2 h at 37°C in a CO_2 incubator the adherence was determined by plate count.

RESULTS

Expression and ensymatic activity of *S. agalactiae* α-enolase

The sequence of *S. agalactiae* á-enolase gene was 1308bp in length (Fig.1) and encoded a protein of 435AA residues. After inducing of expression, the recombinant protein we have overexpressed was about 67.2kDa according to the protein analysis. In this study we have successfully built the clone and expression plasmid as Fig.1 showed. Besides, online BLAST showed *S. agalactiae* á-enolase belonged to enolase-like superfamily which represented a similarity of over 90% to enolase of *Streptococcus iniae*, *Streptococcus pyogenes*, and *Streptococcus pneumonia* (Fig.2). Subsequently, the plasminogen binding motif was compared among these strains as shown in Fig.2 in the red frame. We found that the crucial motif FYDKERKVY contribute to plasminogen binding also appeared in the sequence of *S. agalactiae* á-enolase. This result provided us information to investigate whether *S. agalactiae* α-enolase has the ability of plasminogen binding.

Enzymatic activity of recombinant α-enolase

We have identified the enzymatic activity of á-enolase by the coupled-enzyme assay. The absorbance at 340nm decreased with the increasing of the concentration of recombinant α-enolase (Fig. 3). It indicated the recombinant α-enolase could convert phosphoglycerate to phophoenolpyruvate, resulting in the conversion of NADH to NAD in the next step in a dose-dependent manner. The result confirmed the

conversion that α-enolase induces in the glycolysis pathway and proves us evidence to analysis the bioactivity of recombinant α-enolase we have overexpressed.

Plasminogen binding of recombinant α-enolase

According to the sequence analysis results above, western-blot was applied to test the human plasminogen binding activity of recombinant *S. agalactiae* α-enolase. The blot was incubated with human plasminogen and then probed with antibodies against human plasminogen. The result suggested that α-enolase bound to human plasminogen with a least concentration of 10ug/ml (Fig.4). It provided some evidence of the C-teminal motif played in the plasminogen binding process.

Binding assays to detect the antiserum and natural α-enolase

Western-blot was employed to investigate the immunogenicity of *S. agalactiae* α-enolase. Purified protein was gained through affinity chromatography, eluting under a concentration of 150mM imidazole. α-Enolase antiserum was collected after sacrificing the

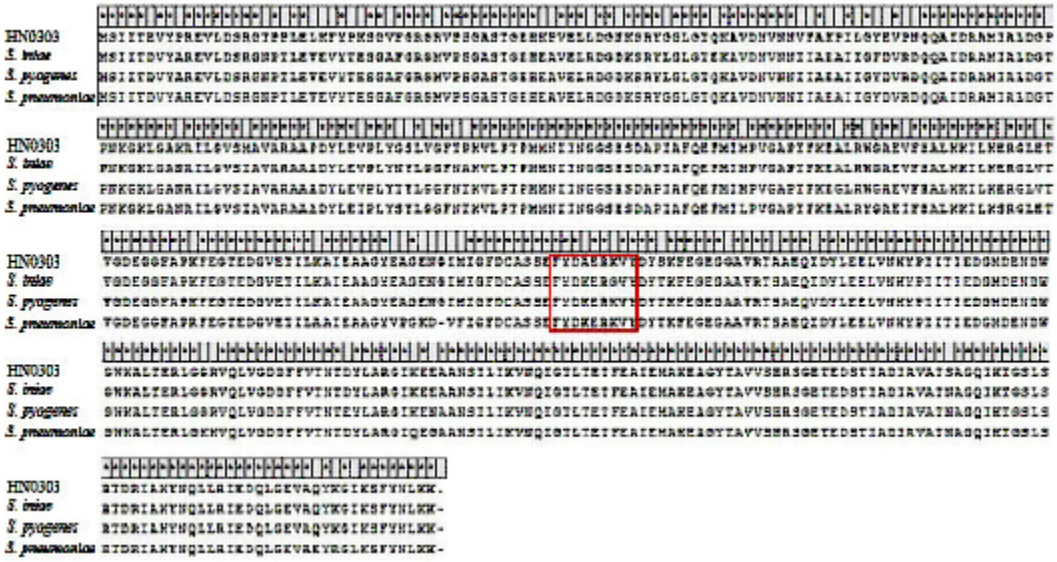


Fig. 1. Clustal W alignment of the amino acids sequences of *S. agalactiae* α-enolase and enolase of *Streptococcus iniae*, *Streptococcus pyogenes*, and *Streptococcus pneumonia*. The red box shows the internal plasminogen-binding motif

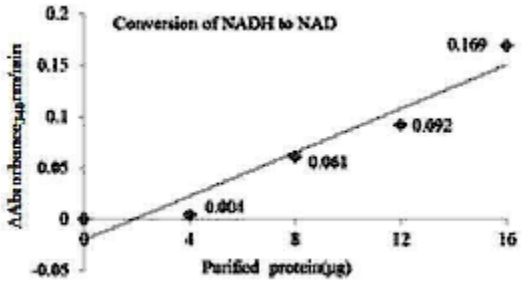


Fig. 2. Enzymatic activity of purified α-enolase. The decrease of absorbance at 340nm depicting the catalytic activity of α-enolase by the conversion of NADH to NAD

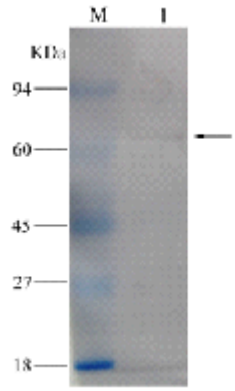


Fig. 3. Binding of recombinant α-enolase to human plasminogen. M, protein marker; 1, recombinant protein binding to human plasminogen.

rabbits. The result showed the antiserum could respond efficiently to the recombinant α-enolase (Fig.5). Sequentially, the antiserum was used to identify the location of natural α-enolase using immunoblot method. The result suggested that α-enolase was presented in the *S. agalactiae* which could be detected both on the cell wall and the cytoplasm with a molecular weight of 45kDa. However, in the extracellular products α-enolase cannot be detected (Fig.6).

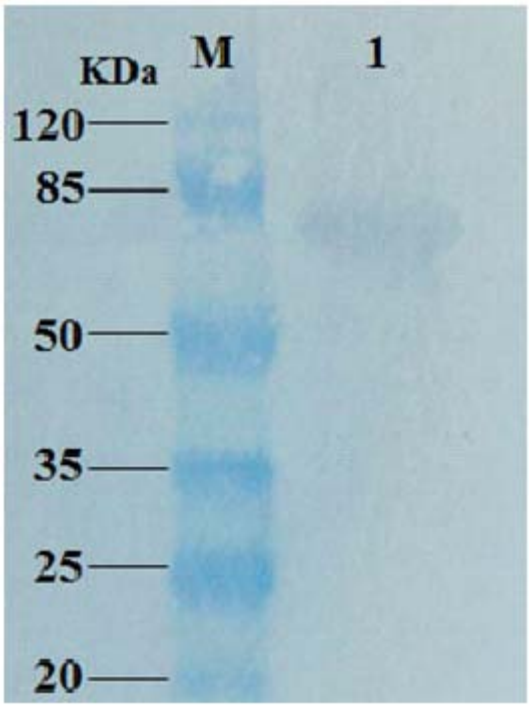


Fig. 4. Western blot of α-enolase. M, protein marker; B, recombinant α-enolase binding to immune antiserum

Location of nature α-enolase analysis

Further identification of natural á-enolase location was analyzed by flow cytometry. The surface located α-enolase could bind to the specific antiserum prepared above and then could be

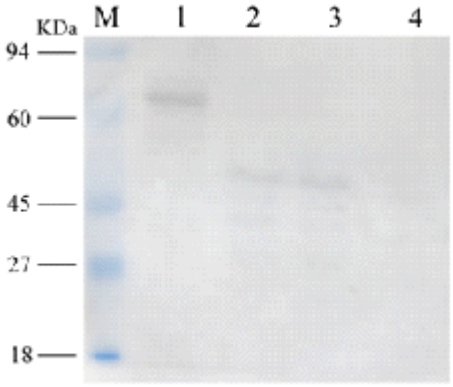
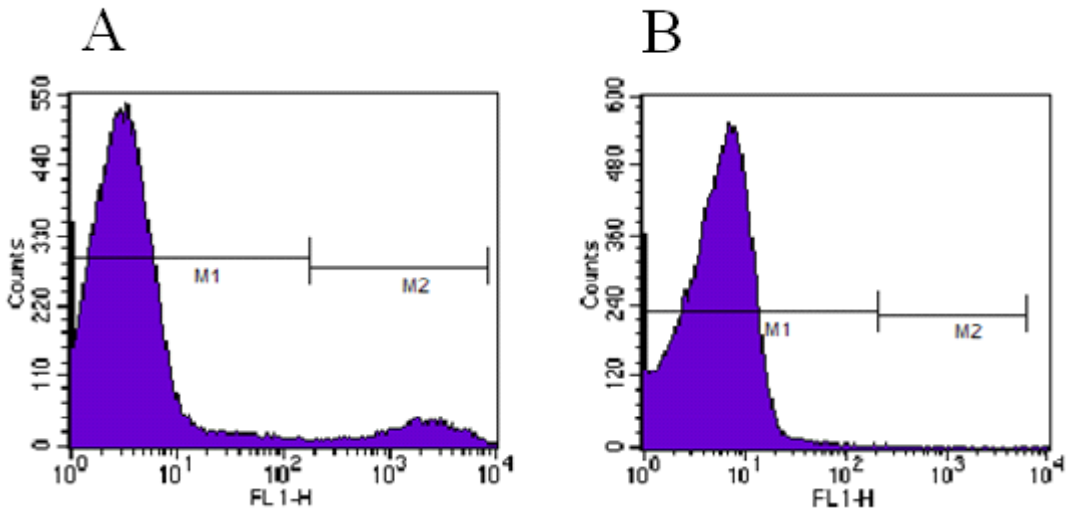


Fig. 5. Exrtacellular detection of α-enolase. M, protein marker; 1, recombinant α-enolase; 2, cytoplasm and soluble proteins; 3,cell fragments and insoluble proteins ; 4, extracellular products. α-enolase could be detected in the cytoplasm and on the surface of S. agalactiae.



A, sample group; B, control group. M1, mean fluorescence intensity (MFI) of unlabeled bacteria, M2,MFI of labeled bacteria that treated with rabbit antienolase serum

Fig. 7. Location of α-enolase on the *S. agalactiae* by flow cytometry analysis

detected by the flow cytometry through the FITC fluorescent brightness (Fig.7). From the result we could see the bacteria pre-incubated with anti-enolase serum showed significant increase from 0.59% to 7.45%, indicating specific adherence of antibody to the surface exposed antigen. Besides, the immunoblot analysis result above supported the surface location of flow cytometry.

Adherence assays of α-enolase to EPC cells

Immunofluorescence was applied to confirm whether α-enolase could adhere to the surface of EPC cell line. The result indicated that most of α-enolase could adhere to the surface of EPC as shown in Fig.8. Moreover, we employed plate count analysis to confirm whether *S. agalactiae* α-enolase contributed to *S. agalactiae* adherence. We found that in the present of anti-enolase serum, the adherence to the EPC cells was 31.98% of the control group which pre-incubated with normal rabbit serum. The result suggested that the α-enolase could act as an adherence factor of *S.agalactiae*. Together with these information we concluded that *S.agalactiae* α-enolase was a cell surface located protein which contributed to the adherent ability of the *S.agalactiae*.

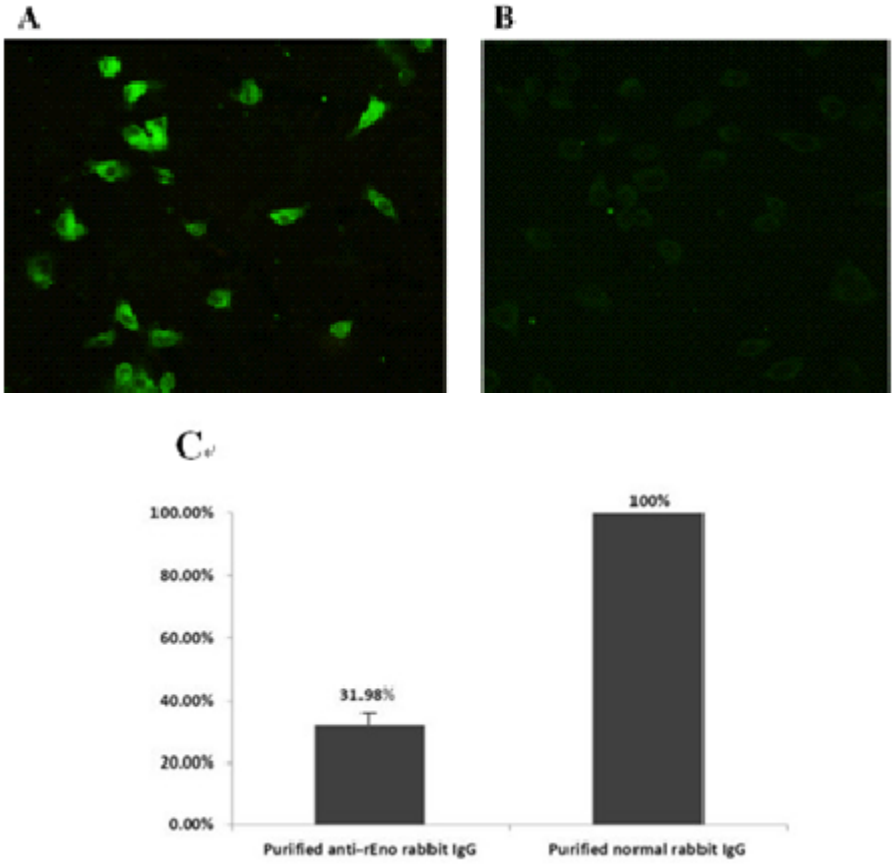


Fig. 8. Adherence of α-enolase to EPC cell. A, Immunofluorescence analyses showing binding of the purified enolase to EPC cells; B, EPC cells treated with antienolase serum which showing a relative low fluorescence; C, adherence rate of α-enolase by plate count method

DISCUSSION

Adherence of bacteria and host cells is the initial step of the infection of diseases. In this study, we have cloned and expressed the recombinant α-enolase of *S.agalactiae* isolated from Hainan, China. The results in this study provide evidence that *S.agalactiae* α-enolase is a plasminogen binding and cell surface located protein that has the same functions in other

pathogenic *streptococci* reported earlier[19, 23]. The plasminogen binding activity suggested it was one of the factors that contributed to tissue invasion thus causing infection of the hosts.

Online BLAST sequence analysis indicated *S.agalactiae* α-enolase showed high similarities to other strains of pathogic *agalactiaes*, mostly above 99%. Recent reports demonstrated that the two lysine residues on the C-teminal end were essential to the plasminogen binding ability. The sequence of a motif with nine amino acids FYDKERKVY was crucial for the plasminogen binding activity according to Jones' report[18]. Bergmann *et al.*[19] suggested that two lysine redidues(underlined) of this motif played an important role in binding plasminogen in *S. penunoneae* enolase. Besides, the study on the group A streptococci showed the two lysine residues on the C-terminal, K434 and K435 contribute significantly to the plasminogen-binding activity of intact group A streptococci[21]. In our study, however, the *S.agalactiae* α-enolase had a sequence of FYDAERKVY, which had eight amino acids in common with the *S. penunoneae* enolase. In addition, the similar sequence was also found in the *Streptococcus iniae* and *Streptococcus pyogenes*. These results indicated that the plasminogen binding process of *S.agalactiae* might mediate by the C-teminal lysine residue which was surface exposed on the α-enolase protein.

Localisation study was used to determine the cellular location of α-enolase in the *S.agalactiae* using specific antibody against purified *S.agalactiae* α-enolase. Western blot analysis illustrated that α-enolase was located in the cytoplasm, cell wall and extracellular of *S.agalactiae*, which was similar to Jones' experiments[18]. Early reports have suggested α-enolase was located on the surface of pathogenic bacteria[20,23]. The contradiction that the cell surface location of the protein and the lack of typical anchor motif is still unexplainable. Chhatwal *et al.*[27] indicated it might be caused by associating with receptors on the cell thus executing its function related to infections. Further study to clarify the exact mechanism of anchorless protein transfer to the cell membrane are needed be carried out.

In the adherence assay, we found that α-enolase could efficiently adhere to the surface of EPC cells and also in the cytoplasm. In addition, the adherence could be inhibited by about 70% in the presence of anti-enolase serum. Early reports about the binding ability of *Mycoplasma bovis* α-enolase[28] suggested the significant role it played in the colonization of *Mycoplasma bovis*. Besides, other analysis showed α-enolase was not only an adhesion-related factor, but also an autoantigen in connective tissue diseases[29]. From these results we speculated that α-enolase might be an important factor related to the pathogen of *S.agalactiae*.

Pathogenic bacteria could invade the hosts' tissue by adhering to epithelial cells, escaping from host immunity or interacting with extracellular matrix. In conclusion, we have characterized the α-enolase of *S.agalactiae.* The results provided evidence that á-enolase could act as a plasminogen receptor which was located on the surface of *S.agalactiae.* As a result, the surface located α-enolase may be responsible for the ability to cross tissue barrier and to cause infection by plasminogen activation. In addition, it was also an adherence factor of *S.agalactiae*. These findings suggested that α-enolase was one of the virulence factors of *S.agalactiae*. Since it is conservative among all the serotypes, further study would need to investigate protection mechanism.

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