Molecular Analysis of Signal Peptidase I Gene in *Streptococcus pneumoniae*

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Abstract: A central event in protein secretion is the type I signal peptidase-mediated cleavage of the N-terminal signal peptide that targets a protein for its destination. In this study, the gene of the signal peptidase I of gram-positive *Streptococcus pneumoniae* (*Spi*) was cloned, expressed and its protein was purified. Switching trial for the proteolytic processing of the outer membrane (OmpA) protein by *Spi* peptidase has drawn attention for the existence of a substrate specificity difference between gram-negative and gram-positive signal peptidases. Analysis of a constructed hybrid protein (pre*Omp*A-*Skc*-His6) along with site-directed mutagenesis in the same construct confirmed the critical role of the -1 and -3 amino acid residues for cleavage by signal peptidase I in *streptococcus pneumoniae*, consistent with the idea that this peptidase contains a serine-lysine catalytic dyad.
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1. Introduction

Most proteins that are completely translocated across the bacterial cytoplasmic membrane, or across the eukaryotic mitochondrial and endoplasmic reticulum, are synthesized as precursors (preproteins) with signal sequence at their N-terminal. This signal sequence is involved in guiding the protein into the targeting and translocating pathway by interacting with the membrane and other components of the cellular secretory machinery (Böhni et al. 1988; Greenburg et al. 1989; Behrens et al. 1991; Wickner et al. 1991; Sung and Dalbey, 1992). Release of the mature part of the protein from the membrane requires proteolytic removal of the signal peptide. This proteolytic processing occurs during or shortly after the translocation event and is catalyzed in both prokaryotes and eukaryotes by enzymes known as signal peptidases. Two major bacterial signal peptidases (signal peptidase I and signal peptidase II) have been identified. Signal peptidase II is specific for precursors of glyceride-modified lipoproteins (Hussain *et al.* 1982; Innis et al. 1984), whereas signal peptidase I is required for processing of nearly all secreted proteins (Wolfe et al. 1983; Tschantz and Dalbey, 1994; Dalbey et al. 1997).

A number of bacterial genes encoding signal peptidase I have been cloned and sequenced from both gram-negative and gram-positive bacteria, including *Escherichia coli* (Wolfe et al. 11982), *Salmonella enterica* serovar Typhimurium (van Dijl et al. 1990), *Haemophilus influenzae* (Fleischmann et al. 1995), *Staphylococcus aureus* (Cregg et al. 1996), *Bacillus subtilis* (van Dijl et al. 1992; Meijer et al. 1995; Tjalsma et al. 1997 and *Streptococcus pneumoniae* (Zhang et al. 1997). Genes from Gram-negative bacteria generally encode larger proteins, approximately 300 amino acids in size, as typified by leader peptidase (*lep*B) of *Escherichia coli* (Wolfe et al. 1983). Genes from Gram-positive bacteria as typified by *sip*S of *Bacillus subtilis* (van Dijl et al. 1992) and *spi* of *Streptococcus pneumoniae* generally encode smaller proteins that are about 200 amino acids in size. Secreted proteins play a central role in the interaction of bacteria with their environment (Wooldridge, 2009).

Despite extensive efforts to predict or experimentally detect proteins that are secreted, the characterization of the bacterial secretome has remained challenging. Until recently, the biochemical characterization of signal peptidase I have concentrated on the enzyme from *E. coli* while biochemical characterization of gram positive signal peptidase I was very limited. In this work, we focused on the expression and purification of signal peptidase I of *Streptococcus pneumoniae*. The substrate specificity difference between gram negative and gram positive signal peptidases was investigated.

2, Materials and Methods

Cloning and expression of signal peptidase I (*Spi*) gene

Two oligonucleotide primers designed to contain *Pst*I (5'-AAATACTGCAGAAGCGAAAAACGCTT-3') and *Bam*HI (5'-GGATCCTTAAAATGTTCCGATACGGGTGAT-3') restriction sites were used to specifically amplify the *Spi* gene using genomic DNA of *S. pneumoniae* (strain NZ303). The expression vector YEp352-*Spi*-His6 was constructed by introducing the PCR-amplified fragment into the YEp352 vector. For expression of *Spi*, *E. coli* strain (XL1-Blue) was transformed by YEp352-*Spi*-His6 and induced with IPTG (1 mM) and

then tested for expression by Western analysis. For expression of *Skc,* recombinant clone pQE-30-Az10 (Muharram et al. 2010) was used for the transformation of *E. coli* cells.

Purification of *S. pneumoniae* signal peptidase I

200 mg of the extracted proteins were mixed with ten volumes of chloroform/methanol (1:1). The mixture was stirred at RT for 18 h. and centrifuged at 3000 g for 5 min. The organic extract was washed by addition of Chloroform, methanol and water to a final ratio of chloroform/methanol/water (8/4/3). The mixture was gently stirred and centrifuged at 14000 rpm for 10 minutes. For the immunoprecepitation, one liter of IPTG-induced *E. coli* cells harboring YEp352-S*pi*-*His*6 were harvested and resuspended in 20 ml of lysis buffer containing 50 mM Na_2HPO_4 and 300 mM NaCl (pH 8.0) and sonicated for 5 min on ice. The lysate was then centrifuged at 50,000 ×*g* for 1 h at 4°C. The resulting supernatant was discarded, and the pellet was resuspended and sonicated for 5 min in 20 ml of lysis buffer with 1% Triton X-100. After centrifugation at 50,000 × *g* for 1 h at 4°C, the supernatant was diluted with 80 ml of lysis buffer and loaded onto a protein A sepharose (PAS) column by the procedure detailed in (Niogret et al. 1996).

Hybrid protein construct and site directed mutagenesis

PCR-amplified fragment of the presequence of *Omp*A gene was fused to the amplified fragment corresponding to the mature part of *Skc* gene. The product was inserted between the *Bam*HI and *Eco*RI sites of YEp352 immediately downstream of the *ADH1* promoter. Site-directed mutagenesis reactions were performed as described in Van Valkenburgh *et al* (1999).

P**urification of leader peptidase from *E. coli***

Cells of E. coli (SZ130) were grown to mid-log phase in LB broth. Cells were harvested by centrifugation, washed once with 5 volumes of ice cold cell buffer containing (10 mM Tris.CI (pH7.5), 22 mM NH_4CH_3COO, 10 mM Mg $(CH_3COO)_2$, 1 mM dithiothreitol), and suspended in an equal weight of cell buffer in liquid nitrogen. Protein of the leader peptidase was then purified by the procedure detailed in ZwizinskiS and Wicknerfj (1980).

Assay for processing of prestreptokinase and the hybrid protein

Reaction mixture (20µl) containing 0.1 µg of signal peptidase I (*Spi*) was incubated at 37°C for 1 h with 2µl of purified prestreptokinase (or the hybrid protein) in 20 mM Tris-HCl (pH 8.0), 0.02% Triton X-100, 5% glycerol and 100 µg of phospholipid. Typically, the reactions were terminated by the addition of SDS sample buffer. Products of the reaction were separated on 12% SDS-PAGE followed by Western analysis.

Miscellaneous

Protein and DNA manipulation were carried out by standard methods Sambrook *et al*. (1989). PCR amplification was carried out using the protocol of Innis *et al*. (1990). Western Blot analysis was conducted according to the procedure of Towbin *et al*. (1979). Cells of *E. coli* were transformed as described in Inoue *et al*. (1990).

3. Results

Expression of *Spi* and *Skc* Genes and Purification of their proteins

Chromosomal DNA of *S. pneumoniae* was used to amplify the ORF of the signal peptidase I gene by PCR. *E. coli* cells harboring the vector YEp352-*Spi*-*His*6 were grown to an A_{600} of 0.7 at 30°C. After induction with IPTG, a protein band at the expected molecular mass of about 20 kDa was visualized by and western analysis using anti-His6 antibody (Fig.1. lane 1). IPTG-induced *E. coli* cells that harboring the recombinant clone pQE-30-Az10 were screened for their expression of streptokinase gene by immunoblotting using anti-His6 antibody where a protein band of 47 kDa was developed (Fig.1, lane 2). Three different procedures were adopted to purify recombinant proteins of *Spi* and *Skc* genes. These protocols were chloroform and methanol extraction, ammonium salt precipitation, and immunoprecipitation. Expressed proteins of *Spi* and *Skc* could not be purified either by chloroform and methanol extraction (Fig. 2. lanes 2, 3) or ammonium salt precipitation (Fig.2. lanes 4, 5). However, immunoprecipitation technique using the column of protein A sepharose (PAS) succeeded in the purification of the expressed S*pi* and *Skc* genes as shown in (Fig.2. lanes 6, 7). Pure single protein bands were visualized by SDS-PAGE and western analysis at the expected molecular masses of 20 kDa and 47 kDa for S*pi* and *Skc,* respectively.

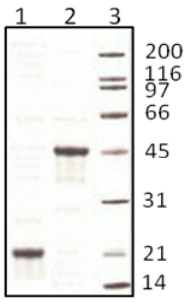

Fig.1: Cloned *S. pneumoniae* signal peptidase I (*Spi*) and streptokinase (*Skc*) genes. Total proteins were extracted from transformed *E. coli* cells (Xl1-Blue), subjected to 12% SDS-PAGE and immunoblotted by Western analysis using anti-His6 antibody. Lane, 1: *expressed Spi*; Lane, 2: *Skc* expressed from clone pQE-30-Az10; Lane, 3: protein marker.

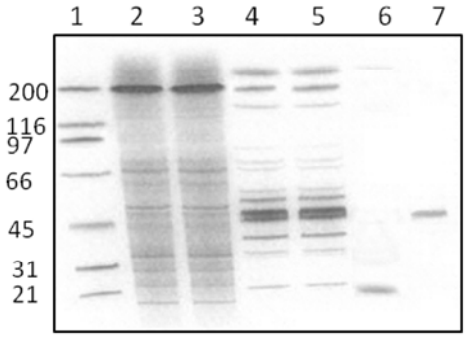

Fig. 2: Purification of recombinant *Spi and Skc* proteins. Expressed proteins of both genes *were extracted* from transformed cells of *E. coli*. Lane, 1: protein marker; Lanes, 2 and 3: chloroform- methanol extracted proteins of recombinant *Spi* and *Skc*, respectively. Lanes, 4 and 5: precipitated proteins of recombinant *Spi* and *Skc* with ammonium sulfate, respectively. Lanes, 6 and 7: precipitated proteins of recombinant *Spi* and *Skc* with protein A sepharose (PAS) and anti-His6, respectively. Proteins were dissolved in SDS loading buffer, loaded onto 12% SDS-PAGE and stained by Coomassie blue, R-250. Molecular masses of the purified proteins from both recombinant clones were compared by protein marker placed in lane 1.

Comparative analysis of signal peptidases in gram-positive and gram-negative bacteria

The proteolytic processing of prestreptokinase protein by purified *Lep* (Fig. 3a. lane, 2) instead of *Spi* and *Omp*A protein by *Spi* instead of *Lep* was conducted. As observed in Fig.3b, *Spi* and *Lep* peptidases were able to cleave the signal peptide from the protein of prestreptokinase yielding the mature form of 41 kDa (lanes, 4, 5). In case of *Omp*A protein, the mature protein form was developed with a molecular mass of about 32 kDa with the *Lep* peptidase only (Lane, 7), whereas the precursor form (35 kDa) was developed with the *Spi* enzyme (lane, 6). This means that the processing of *Omp*A could not be switched from the *Lep* to *Spi* enzyme. This result has shed light on the presence of the substrate specificity difference between signal peptidases from both bacterial groups. To address this point, a hybrid protein was constructed in which the signal peptide of the *Omp*A protein was fused to the mature part of the streptokinase protein (pre*Omp*A-*Skc*-His6) and appended with six histidine residues at its C-terminus. Expressed protein of this hybrid yielded a band of 46 kDa (Fig. 4. Lane, 3). This constructed protein was analyzed as a substrate for *Spi* peptidase. This is to investigate if the amino acid residues in the signal peptide are enough for the protein maturation with *Spi* or if it needs extra molecular information in the mature part of the protein. Processing results of the hybrid protein with *Spi* developed a protein band of the precursor form at 46 kDa (Fig. 4. Lane, 5). On the other hand, a faint protein band developed at 41 kDa which corresponding to that of the mature form of streptokinase indicating a partial processing of this protein by *Spi*. Appearance of extra protein band at 10kDa (Lanes 4, 5) maybe interpreted as a self-cleavge activity of *Spi* peptidase

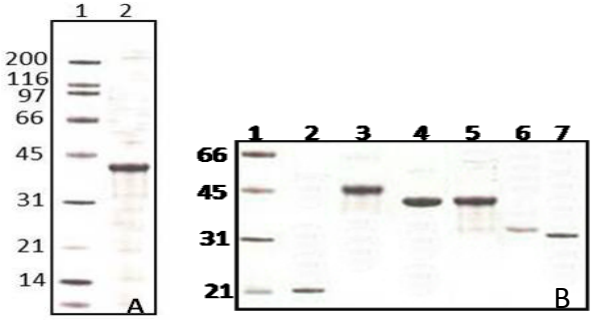

Fig. 3: a. Purification of leader peptidase (*Lep*) of *E. coli*. Lane, 1: protein marker; lane, 2: purified *Lep* peptidase with a molecular mass at 39 kDa. **b**. Proteolytic processing analysis of prestreptokinase and *Omp*A proteins by *Spi* and *Lep* peptidases. Lane, 1: protein marker; Lane, 2: purified *Spi*; Lane, 3: purified prestreptokinase with a molecular mass at 47 kDa; Lane, 4: *Spi* peptidase with prestreptokinase; lane, 5: *Lep* peptidase with prestreptokinase; Lane, 6: *Omp*A with *Spi* peptidase; Lane, 7: OmpA with *Lep* peptidase. In Lanes, 2-7: Total proteins extracted from transformed *E. coli* cells (Xl1-Blue), subjected to SDS-PAGE and immunoblotted by Wesern analysis using anti-His6 antibody.

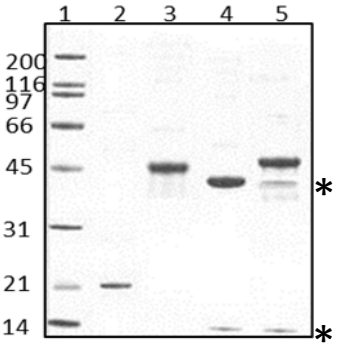

Fig. 4: Proteolytic processing analysis of pre*Omp*A-*Skc*-His6 by *Spi*. Lane, 1: protein marker; Lane, 2: purified *Spi*; Lane, 3: Expressed precursor of pre*Omp*A-*Skc*-His6 with a molecular mass of 46 kDa; Lane, 4: *Spi* peptidase with native prestreptokinase; lane, 5: *Spi* peptidase with pre*Omp*A-*Skc*-His6. Extracted total proteins were analyzed by 12% SDS-PAGE and immunodetected by Western analysis using anti-His6 antibody.

Site directed mutagenesis of the hybrid protein

The alanine residues at -1 and -3 in the signature sequence of the signal peptide of the *Omp*A in the hybrid protein were substituted by serine and lysine (A18S and A20K) using site directed mutagenesis. This was carried out to let this hybrid protein have the same amino acid residues at the -1 and -3 as in the native protein of streptokinase. The new protein construct pre*Omp*A-*Skc* A^{18S} A^{20K}-His6 containing the mutated signature sequence of *Spi* was incubated for testing its proteolytic processing by *Spi* enzyme (Fig. 5). In this figure, a strong mature protein band of 41 kDa (Lane, 2) was appeared compared to the faint one appeared previously. This result indicates the role of the two amino acid residues in the -1 and -3 for processing by *Spi*. The Development of three protein bands with molecular masses ranged between 4 and 10 kDa (Fig. 5. lanes, 2, 3) confirmed the self-cleavage activity of *Spi* that reported previously in (Fig.4).

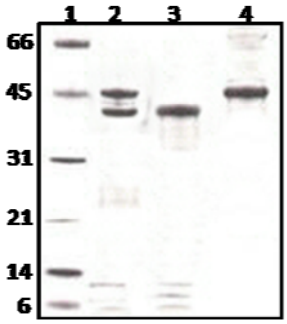

Fig. 5: Proteolytic processing analysis of pre*Omp*A-*Skc* A^{18S} A^{20K}-His6 by *Spi*. Lane, 1: protein marker; Lane, 2: pre*Omp*A-*Skc* A^{18S} A^{20K}-His6 with *Spi*; Lane, 3: native precursor of streptokinase with *Spi*; Lane, 4: native precursor of streptokinase alone. Extracted total proteins were immunodetected by anti-His6 antibody.

4. Discussion

Bacterial protein secretion is a highly orchestrated process that is essential for bacterial survival, infection, and virulence and in the interaction with their environment ((Wooldridge, 2009). Steps of protein insertion into membranes involve topographic changes such as binding to a membrane or transit of polypeptides across the bilayer. Many membrane proteins in both prokaryotic and eukaryotic cells are synthesized in precursor form with an NH2-terminal leader sequence of 15 to 30 amino acid residues. Leader sequences are removed during, or shortly after, the assembly event by enzymes termed leader peptidases. The study of leader peptidases may provide clues to the mechanism of membrane protein assembly (Behrens et al. 1991; Sung and Dalbey, 1992; Zhang et al. 1997; von Heijne, 1983). Generally, the secretion of many of proteins in both Gram-positive and Gram-negative bacteria is mediated by the evolutionarily conserved general secretory (Sec) system (Powers et al. 2011).

A number of genes encoding signal peptidases I have been cloned and sequenced from both gram-positive and gram-negative bacteria (Wolfe et al. 1982; van Dijl et al.1990; Fleischmann et al. 1995; Cregg et al. 1996; van Dijl et al. 1992; Meijer et al. 1995; Tjalsma et al. 1997; Zhang et al. 1997). In fact, some of the conserved regions and critical residues involved in active sites are present in the enzymes of both bacterial groups. However, considerable differences also exist. These differences include the primary sequences, the size, and the topology of the enzymes. Why are signal peptidases from two bacterial groups so different although they catalyze a similar reaction?

Our data addressed that signal peptidases in both bacterial groups of gram-negative and gram-positive show a substrate specificity difference. This is indicated by the protein of the *Omp*A (known substrate of *E. coli* signal peptidase I) was not cleaved *in vitro* by purified *Spi* peptidase, although both enzymes were able to process prestreptokinase *in vitro* (Fig. 3b). This result, along with the presence of some conserved and critical residues in the active sites of the peptidase from both bacterial groups (van Dijl et al. 1988; Wang and Dalbey, 2010) allowed for a comparative analysis between gram-positive and gram-negative enzymes. This analysis implied the construction of a hybrid protein (preOmpA-Skc-His6). Partial proteolytic processing of this hybrid protein appeared with *Spi* enzyme as indicated by the appearance of faint band (Fig. 4) corresponding to the mature form of streptokinase (41 kDa).

A main goal of the comparative analysis was to understand why *Spi* and *Lep* peptidases exhibit nonoverlapping substrate specificities. Studies in eubacterial systems have shown that the type I signature consists of a serine, lysine, arginine, and two aspartic acid residues that are important for the function of leader peptidase from *E. coli* and SipS from *B. subtilis* (Tschantz et al. 1993; van Dijl et al. 1995). Therefore, a new form of the preOmpA-Skc-His6 protein was constructed in which the amino acid residues at -1 and -3 were substituted to be the same as in the native protein of streptokinase. The new construct was then examined with the purified *Spi* peptidase I. Incubation of the purified *Spi* peptidase I with the new construct increased the intensity of the faint protein band that appeared with the first construct. These data revealed that the -1 and -3 amino acids probably help to position the signal peptide relative to the active site through their interactions with distinct binding pockets on the enzyme's surface (Paetzel et al. 1998; Chen et al. 1999).

Another striking common feature of *S. pneumoniae* signal peptidase that was noticed in this study is self-cleavage activity (Fig. 5) that developed

products with molecular masses of 4, 8 and 10 kDa. Self-cleavage has been found to be a unique property among LexA-like proteases. Sequence analysis demonstrated that the regions around self-cleavage sites of signal peptidase I and LexA-like proteases have some common properties (von Heijne,1983; van Dijl et al. 1995; Perlman and Halvorson, 1983; Jain et al. 1994). We have shown that amino acids at the -1 and -3 positions are important for recognition and proper cleavage of the signal peptide by *Spi* peptidase. Whether all bacterial signal peptidases catalyze self-cleavage like LexA-like proteases remains to be addressed. Also, identifying factors that govern the substrate specificities exhibited by *Spi* need to be identified.

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