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Comparison of the Sensitivity of Type I Signal Peptidase Assays

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Abstract Type I signal peptidase cleaves the signal sequence from the amino terminus of membrane and secreted proteins after these protein insert across the membrane. This enzyme serves as a potential target for the development of novel antibacterial agents due to its unique physiological and biochemical properties. Despite considerable research, the signal peptidase assay still remains improvement to provide further understanding of the mechanism and high-throughput inhibitor screening of this enzyme. In this paper, three known signal peptidase assays are tested with an *E. coli* D276A mutant signal peptidase to distinguish the sensitivity of each assays. *In vitro* assay using the procoat synthesized by *in vitro* transcription translation shows that the D276A signal peptidase I was inactive while *in vivo* processing of pro-OmpA expressed in the temperature-sensitive *E. coli* strain IT41 as well as *in vitro* assay using pro-OmpA nuclease A substrate show that D276A signal peptidase I has activity like wild-type signal peptidase. These results suggest that *in vitro* assay using the pro-OmpA nuclease A and *in vivo* pro-OmpA processing assay are more sensitive monitors than *in vitro* assay using the procoat. In conculsion, caution should be used when interpreting the *in vitro* results using the procoat.

Key words: signal peptidase, signal peptidase assays

Introduction

Proteins destined for secretion in both prokaryotic and eukaryotic organisms are initially synthesized as precursor molecules with an extra 15-30 amino acid residues at the amino terminus. This extension sequence, termed signal peptide, is removed by type I signal peptidase, that is localized in the cytoplasmic membrane in bacteria. Two major signal peptidase have been identified with different cleavage specificities in bacteria. Signal peptidase I is responsible for the processing of the majority of the secreted proteins, and signal peptidase II exclusively processes glyceride-modified lipoproteins [1-3].

Signal peptidase I is an attractive target for the development of antibacterial agent because of its unique biochemical and physiological properties. It is essential for the viability of *E. coli* and its active site is relatively accessible on the outer leaflet of the cytoplasmic membrane [4,5]. It is widely distributed in the clinically relevant gram-positive bacteria as well as in *Chlamydia*. One important difference between bacterial and eukaryotic signal peptidase I is their locations within the cells. Unlike bacterial signal peptidase I, the active domain and most parts of the eukaryotic enzyme complex are located in the lumenal side of the microsomes. Thus, any potential inhibitor must cross cytoplasmic membrane and microsomal membrane to access to the active site. Signal peptidase I is a unique protease insensitive to the classic protease inhibitors. Together, these features support the choice of bacterial signal peptidase I as an ideal target for the development of novel antibiotic therapies.

Signal peptidase assays are instrumental in the purification and characterization of signal peptidase activity. Most assay methods for *E. coli* signal peptidase I are noncontinuous and laborous, requiring the separation of the products either by HPLC or SDS-PAGE. Recently, the assays have focused on multiple sample analysis for the development high-throughput inhibitor screening. Some of these assays are listed in Table 1. A fluorogenic peptide has been used for continuous measurement of signal peptidase activity [6]. Although this is a continuous assay, signal peptidases I show nonspecific in the substrate specificity, thus complicating kinetic measurements and suffering from low sensitivity. Thus, the continuous assays are still investigating to provide a useful tool for detailed mechanistic studies of this enzyme and the identification of potential antibacterial agents.

In this study, we have shown the sensitivity of the signal peptidase assays using D276A mutant *E. coli* signal peptidase. This mutant enzyme is inactive in the *in vitro* assay using the procoat as a substrate. In constrast, the enzyme is active using *in vivo* processing of pro-OmpA assay in IT41 temperature-sensitive cells. We have also tested the

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Table 1. Type I signal peptidase assays

Assay	Analysis method	Reference
In vivo		
pro-OmpA expressed in the temperature-sensitive *E. coli* strain IT41	SDS-PAGE/ immunoprecipitation/ fluorography	[10,11]
Complementation of temperature-sensitive *E. coli* strain IT41	Cell growth	[14]
Pre (A13i)-β-lactamase plate assay	β-Lactamase activity	[15,16]
In vitro		
Radiolabeled preproteins synthesized by *in vitro* transcription translation	SDS-PAGE/ immunoprecipitation/ fluorography	[8,9]
Pro-OmpA nuclease A fusion protein	SDS-PAGE/ densitometry	[7,12]
Synthetic peptide	HPLC-UV detector	[17]
	continuous-spectrofluorometric	[18]

activity of D276A signal peptidase I with another *in vitro* assay using the pro-OmpA nuclease A, indicating that the D276A enzyme is active. The study here has directly shown the different results of D276A signal peptidase activity based on the sensitivity of signal peptidase assays, suggesting that caution should be used when interpreting the data.

Materials and Methods

Bacterial strains and plasmids

E. coli strains MC1061, IT41, SB221, and BL21 (DE3) strains were from the Ohio State University. The plasmid pRD8 carries the *lep* gene while the expression vector pET23d engineers 6 consecutive histidine residues into the cytoplasmic domain of signal peptidase that can purify the mutant from the chromosome-expressed wild-type signal peptidase I [7]. Oligonucleotide mutagenesis was performed using the Quick-change mutagenesis kit (Stragene). The mutant D276A *E. coli* signal peptidase I (GAC changed to GCC at 276 position) was verified by plasmid isolation and sequencing (Sequenase version 2.0).

In vitro activity assay using the procoat substrate

Type I signal peptidase activity was measured by the posttranslational conversion of procoat to coat protein and signal peptide. In this assay, procoat was synthesized in vitro according to the procedure in Zwinziski, *et al.* [8,9]. Cultures (2 ml) of MC1061 expressing different signal peptides were grown to an optimal density at 600 nm of 0.2, induced with arabinose for 2 h, followed by centrifugation for 1 min to concentrate the cells. After resuspending in 0.3 ml of lysis buffer containing 20% sucrose, 10 mM Tris-HCl (pH8.0), 10 mM EDTA, 1% Triton X-100, lysozome (1 mg/ml), deoxyribonuclease (5 μg/ml), ribonuclease (1 μg/ml), and phenylmethylsulfonyl fluoride (5 mM) and incubating for 30 min at room temperature, cell extracts were used directly or diluted (1:10, $1:10^2$, $1:10^3$) and incubated with [^{35}S]-labeled procoat at 37℃ for 30 min. The processing of procoat was analyzed on a 23% polyacrylamide gel.

In vivo signal peptidase I activity assay

The activity of signal peptidase I *in vivo* was measured by examining the processing of outer membrane protein A precursor (pro-OmpA) in IT41, a temperature-sensitive signal peptidase strain [10,11]. Briefly, IT41 was grown at 32℃ in M9 medium containing 0.5% fructose and 50 μg/ml each amino acid (except methionine). After reaching the early-log phase, cultures were shifted to 42℃ for 1h to inactivate the temperature-sensitive signal peptidase. Arabinose (0.2%) was added to the medium to induce synthesis of signal peptidase. Cells were labeled with [^{35}S]methionine for 15 s, and unlabeled methionine was added to a final concentration of 500 μg/ml. At indicated times, samples were removed and quenched in 20% trichloroacetic acid. Samples were immunoprecipitated with antibody directed against outer membrane protein A and analyzed on a 12% SDS-polyacrylamide gel with a 5% stacking gel using a discontinuous buffer system. The gel were fixed and subjected to fluorography.

In vitro activity assay using the pro-OmpA nuclease A substrate

The *E. coli* strain SB 221 bearing the plasmid pONF1 was used to overexpress the pro-OmpA nuclease A substrate, which is a hybrid protein of staphylococcal nuclease A fused to the signal peptide of the outer membrane protein [7]. The cells grown in M9 casamino acids medium and induced by 2 mM IPTG and purified as described in Chatterjee *et al.* [12]. Reaction mixture containing bacterial signal peptidase (final concentration 0.363 μM) was incubated at 37℃ with 9 μL of pro-OmpA (6 μM) in 50 mM Tris, pH 8.0 in the presence of 1% Triton X-100. The reaction was terminated by adding 5X dye supplemented with 100 mM $MgCl_2$, 5% SDS, 25% glycerol, 200 mM β-mercaptoethanol, 200 mM Tris (pH 6.8) and immediately freezing the mixture at -70℃. The samples were analyzed on 17% SDS-polyacrylamide and the gel was stained by Coomassie Brilliant Blue. The concentration of Pro-OmpA nuclease was determined at 280 nm (E at 280 nm = 8.31).

Results

Since signal peptidase is an attractive target for the devel-

opment of antibacterial target, more detailed information on the structure and function of this enzyme needs to be obtained. In order to design more potent inhibitors for bacterial signal peptidase, signal peptidase assays has emphasized to improve sensitivity for the high-throughput analysis. Type I signal peptidase assays are listed in Table 1. In this paper, we have shown different sensitivity of *in vitro* and *in vivo* signal peptidase assays using an D276A mutant *E. coli* signal peptidase.

In vitro activity assay using the procoat substrate

In this assay, procoat was used for a substrate, synthesized by *in vitro* transcription translation. Signal peptidase cleaves procoat to coat protein in a detergent extract of MC1061 cells transformed D276A plasmid. As can be seen in Fig. 1, a 50-fold diluted extract of MC1061 bearing wild-type plasmid (pRD8) catalyzes mostly cleavage of procoat while a 50-fold diluted of MC1061 without plasmid is mostly shown nonprocessed procoat. The background activity is due to the signal peptidase coded by the chromosomal *lepB* gene. The pattern of cell extract containing D276A signal peptidase is comparable as does cell extract of MC1061 without plasmid. With this assay system, we can say that aspartic acid 276 is essential for cleaving the M13 procoat protein to coat protein.

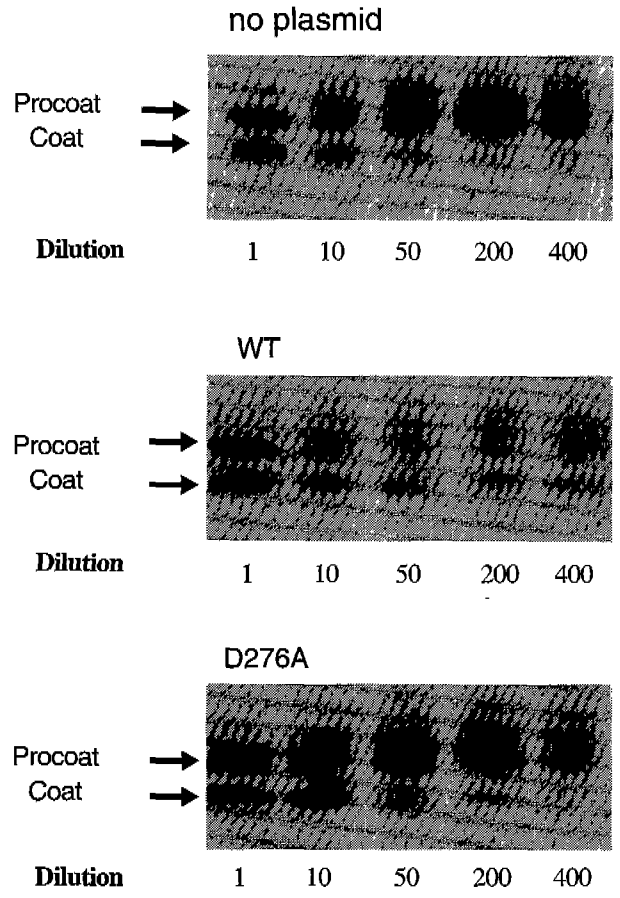

Fig. 1. *In vitro* signal peptidase I processing of the M13 prcoat protein. Cultures expressing the wild-type or D276A mutant signal peptidase proteins were tested for signal peptidase I activity by measuring the posttranslational processing of the M13 procoat at various dilutions of cell extract. Cell extracts were prepared as described in the "Materials and Methods". Undiluted or diluted extracts were incubated for 30 min at 37℃ with *in vitro* synthesized [^{35}S]procoat. The procoat was separated from the processed coat protein by using a 23% SDS-polyacrylamide gel.

In vivo signal peptidase I activity assay

As a second assay, the activity of D276A signal peptidase was tested using *in vivo* assay. Briefly, this assay is based on the fact that overproduction of signal peptidase accelerates the processing of the precursor (pro-OmpA) to outer membrane protein A at the nonpermissive temperature in IT41. The cells containing either wild-type or a plasmid encoding the D276A mutant were pulse-labeled with an [^{35}S] methionine and chased with an excess of unlabeled methionine. At the indicated times, aliquots were removed and analyzed by immunoprecipitation to OmpA, SDS-polyacrylamide gel electrophoresis, and fluorography. IT41 D276A signal peptidase is processing rapid with a $t_{1/2}$<10 S, as seen in the wild-type signal peptidase (Fig. 2). This indicates that this aspartic acid 276 is not an important residue in the signal peptidase activity.

In vitro activity assay using the pro-OmpA nuclease A substrate

Pro-OmpA nuclease A is a hybrid protein of the *Stphy-*

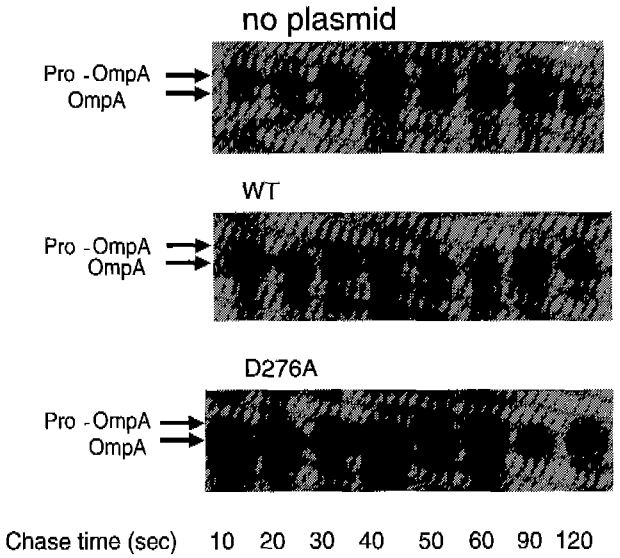

Fig. 2. *In vivo* processing of pro-OmpA at the non-permissive temperature in IT41 bearing no plasmid or encoding wild-type or D276A signal peptidase I molecules. IT41 without plasmid, or with plasmids encoding wild-type or D276A signal peptidase I were grown at 30℃ to the mid-log phase. After inducing signal peptidase I by the addition of arabinose (0.2%), cultures were shifted to 42℃ for 1 h. Pro-OmpA processing was determined as described in the "Materials and Methods".

lococcus aureus nuclease A attached to the signal peptide of the *E. coli* outer membrane protein A (OmpA). The wild-type and D276A signal peptidase were purified using a nickel affinity column according to the procedure in Klenoic *et al.* [13] (data not shown). An aliquote containing 0.364 μM (undiluted) of each purified signal peptidase proteins, after dilution (1:10, $1:10^2$, $1:10^3$) with a buffer (20 mM Tris-HCl, pH 8.0) was incubated with the pro-OmpA nuclease A substrate at 37℃ for 30 min and analyzed by SDS-PEAG and Coomassie Brilliant Blue staining. The D276A mutant signal peptidase has activity corresponding to wild type signal peptidase activity (Fig. 3).

Discussion

Type I signal peptidase assays has developed to improve the purification of signal peptidase and characterization of signal peptidase activity for the structural and functional information. Nowadays, since signal peptidase I is an ideal antibacterial target, development of signal peptidase assays has focused on increasing sensitivity for the high-throughput analysis.

In this paper, we have directly shown that the result of signal peptidase activity should be cautious when interpreting data. The D276A mutant enzyme was inactive with *in vitro* assay using the procoat synthesized by *in vitro* transcription translation while the enzyme was active with *in vivo* assay based on pro-OmpA expressed in the temperature-sensitive *E. coli* strain IT41 and *in vitro* assay using the pro-OmpA nuclease A substrate. These results clearly indicate that *in vitro* assay using an pro-OmpA nuclease A substrate and *in vivo* processing data provide more reliable and sensitive monitors than *in vitro* assay using the procoat synthesized by *in vitro* transcription translation. Among three signal peptidase assays, we can suggest that the *in vitro* assay using pro-OmpA nuclease A is the best tool in the characterization of signal peptidase activity. It is sensitive and takes advantage of cold method when comparing with the *in vivo* assay used isotope. However, this assay is not useful for high-throughput screening of inhibitor libraries since it is not continuous and time-consuming. Recently, continuous assays are still investigating based on the cleavage of small synthetic peptide substrate by HPLC or fluorometer [17,18]. In conclusion, this paper clearly indicates that the interpretation of *in vitro* data using a procoat should be cautious.

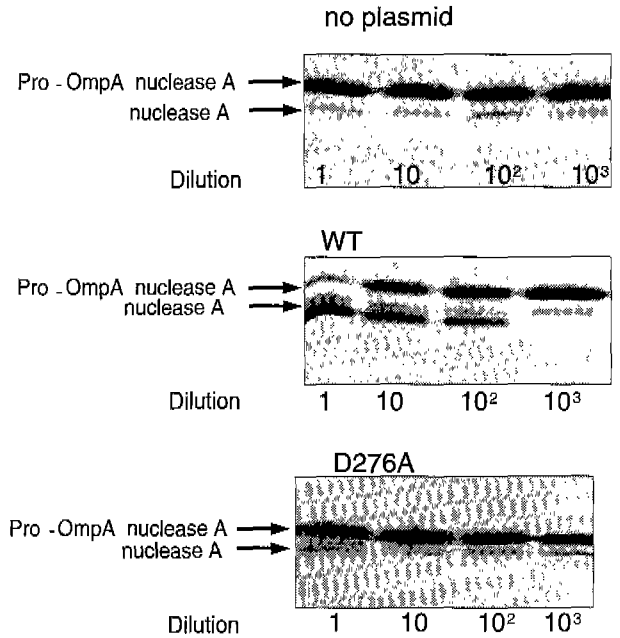

Fig. 3. *In vitro* signal peptidase I processing of the pro-OmpA nuclease A protein. Processing of pro-OmpA nuclease A is initiated by the addition of 1 μl of signal peptidase I enzyme and incubation for 1 h at 37℃. The reaction was described in the "Materials and Methods" and used either directly (without dilution) or after dilution (1:10, $1:10^2$, $1:10^3$) with a buffer (20 mM Tris-HCl, pH 8.0). The processing of pro-OmpA nuclease A to nuclease A was analyzed by SDS-PAGE using a 17% polyacrylamide gel and stained by Coomassie brilliant blue staining.

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