

# Inhibition by penem of processing peptidases from cyanobacteria and chloroplast thylakoids

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**Abstract** Proteins targeted to the thylakoid lumen of plants and cyanobacteria and the periplasmic space of cyanobacteria are synthesised with N-terminal presequences which are removed following translocation across the membrane. These presequences are thought to direct translocation of the preprotein by a sec-type pathway. Detergent extracts of cyanobacterial and chloroplast membranes contain enzymes which are capable of processing precursors to the mature size. We show that the processing of a range of precursors by both cyanobacterial and chloroplast enzymes is inhibited by the penem SB216357. This is the first report of an inhibitor of these enzymes and indicates that they are type 1 signal peptidases.

**Key words:** Thylakoid processing peptidase; Signal peptidase; Penem; *Phormidium laminosum*; *Pisum sativum*

## 1. Introduction

In cyanobacteria, proteins destined for both the periplasmic space and the thylakoid lumen are synthesised as precursors with N-terminal signal sequences which are removed following translocation [1]. The signal sequences are similar to those which direct proteins to the periplasm of *Escherichia coli* [1,2]. Several components of a preprotein translocation system similar to that found in *E. coli* are known to occur in cyanobacteria, including SecA, D, E, F and Y [3,4], the cytosolic chaperone Ffh [5] and a signal peptidase I [6]. SecA and SecY have been detected immunologically in both thylakoid and cytoplasmic membranes [3,7], suggesting that protein translocation takes place across both types of membrane. None of the genes has been detected in more than a single copy, suggesting that the protein translocation apparatus is identical in the two membranes. Detergent extracts of both thylakoid and cytoplasmic membranes have been shown to process the precursor of the 9 kDa extrinsic (PsbU) protein of photosystem II to the mature size [8,9], providing evidence for the presence of a signal peptidase in both membranes.

A processing peptidase with a specificity similar to the cyanobacterial and *E. coli* enzymes has been identified in thylakoid membranes from pea chloroplasts [10–12]. This enzyme has resisted purification and has not been assigned to any particular class of proteases. Because of the similarity in substrate specificity to the bacterial enzymes, it is likely that this enzyme is also a signal peptidase 1.

The *E. coli* leader peptidase is not inhibited by standard

7protease inhibitors [13,14]. Leader peptidases are mechanistically novel serine proteases that utilize a serine/lysine catalytic dyad similar to that found in  $\beta$ -lactamase [15]. It has been shown recently that *E. coli* leader peptidase is irreversibly inhibited by penem inhibitors containing a  $\beta$ -lactam ring [16].

In this study we have investigated the processing of three precursor proteins by detergent extracts of cyanobacterial thylakoid and cytoplasmic membranes and determined the effect of a penem inhibitor on processing. We have also examined the effect of the penem on the processing of precursors by the pea thylakoid processing peptidase.

## 2. Materials and methods

The moderately thermophilic filamentous cyanobacterium *Phormidium laminosum* strain OH-I-p.Cl 1 was grown to late exponential phase in BG11 medium at 45°C. Membrane fractions were prepared by French pressure cell treatment followed by centrifugation on sucrose step gradients [17]. Cytoplasmic membranes, which are devoid of chlorophyll, were collected from the pooled upper fractions of the gradient. Thylakoid membranes equilibrated at the 42%/60% sucrose interface. Washed, lysed chloroplasts were prepared from 2-week-old pea leaves as in [18]. Processing peptidases were extracted from the membranes by treatment with Triton X-100 as in [10], except that the detergent concentration was 0.15%.

Cloned genes encoding *P. laminosum* plastocyanin [19] and PsbU [2] and the cDNA for the wheat 23 kDa extrinsic protein of photosystem II [20] were transcribed in vitro with SP6 polymerase. mRNA was translated in a wheat germ system (Promega) with [<sup>35</sup>S]methionine (Amersham).

A 20 mM stock solution of the penem SB214357 [21] was prepared in DMSO and diluted immediately prior to use to 20× the final concentration in extraction buffer [10]. For processing experiments 1  $\mu$ l of penem was added to 18  $\mu$ l of detergent extract and preincubated for 15 min at the assay temperature. 1  $\mu$ l of translation product was then added and the incubation continued for 1–4 h. The temperature was 30°C for the *P. laminosum* extracts and 27°C for the pea thylakoid extract. The reaction was terminated by addition of sample buffer and boiling for 5 min.

SDS-PAGE was carried out on 10–20% acrylamide gradient gels containing 4 M urea. Proteins were electroblotted onto nitrocellulose and radioactivity localised by direct exposure to film. Chlorophyll was determined according to the method of [22].

## 3. Results and discussion

The presequence of *P. laminosum* plastocyanin has the typical structure of a prokaryotic leader peptide and is known to direct the precursor to the periplasm in *E. coli*, where it is processed, presumably by leader peptidase [19]. *P. laminosum* pre-plastocyanin was processed to the mature size by incubation with a Triton X-100 extract of *P. laminosum* thylakoid membranes (Fig. 1). The band of intermediate size between the precursor and mature proteins is a translation artefact which was consistently present when plastocyanin mRNA

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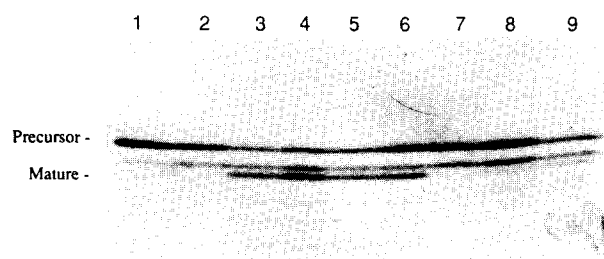


Fig. 1. Processing of *P. lamosum* pre-plastocyanin by a Triton X-100 extract of *P. lamosum* thylakoid membranes (2 h incubation). Lanes: 1, translation product; 2, incubated with extraction buffer; 3, incubated with Triton X-100 extract; 4, as lane 3+5% DMSO; 5, +0.5% DMSO; 6, +0.05% DMSO; 7, as lane 3+1 mM penem; 8, as lane 3+100  $\mu$ M penem; 9, as lane 3+10  $\mu$ M penem.

was translated in vitro. Pre-plastocyanin was also processed by a similar extract of *P. lamosum* cytoplasmic membranes (Fig. 2). Processing by both extracts was partially inhibited by 10  $\mu$ M penem. Substantial inhibition was observed at 100  $\mu$ M. The processing of pre-PsbU was also inhibited by 100  $\mu$ M penem (not shown). These data are consistent with processing catalysed by a signal peptidase 1 and confirm that such an enzyme is present in both cytoplasmic and thylakoid membranes of *P. lamosum*, which is likely to be the product of the cloned *lep* gene [6]. The data also establish the efficacy of the penem as an inhibitor of a type 1 signal peptidase other than *E. coli* leader peptidase.

Like *E. coli* leader peptidase, the thylakoid processing peptidase of pea thylakoids is resistant to a range of protease inhibitors [10]. Furthermore, the specificities of signal peptidases are reportedly very similar [11,12]. We therefore tested the processing of precursors by Triton X-100 extracts of pea thylakoids. Processing of the precursor of the 23 kDa extrinsic protein of photosystem II from wheat was inhibited by 100  $\mu$ M penem (Fig. 3), demonstrating that the chloroplast thylakoid processing peptidase is a type 1 signal peptidase. It has been reported previously that cyanobacterial thylakoidal processing peptidase processes the 23 kDa protein precursor to the mature size [9]. The processing of this precursor by a Triton X-100 extract of cyanobacterial thylakoids was completely

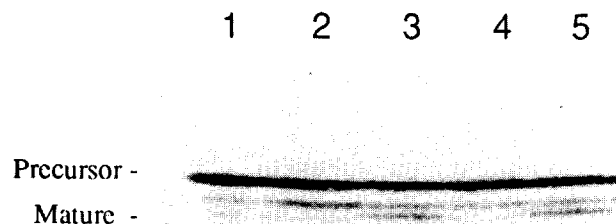


Fig. 2. Processing of *P. lamosum* pre-plastocyanin by a Triton X-100 extract of cytoplasmic membranes. Lanes: 1, translation product; 2, incubated with extraction buffer; 3, incubated with Triton X-100 extract; 4, as lane 3+100  $\mu$ M penem; 5, as lane 3+0.5% DMSO.

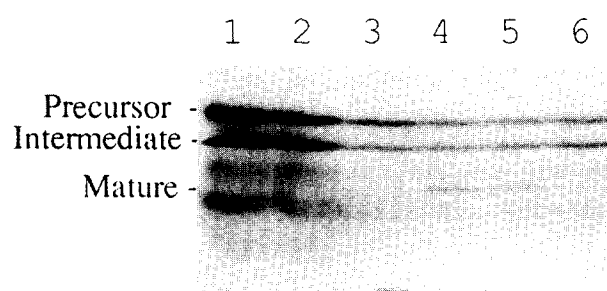


Fig. 3. Processing of wheat pre-23 kDa extrinsic protein by an extract of pea thylakoid membranes. Lanes: 1, translation product; 2, 1 h incubation with extraction buffer; 3, thylakoid extract added immediately prior to solubilisation for electrophoresis; 4, 1 h incubation with extract; 5, as lane 4 plus 0.5% v/v DMSO; 6, as lane 5 plus 100  $\mu$ M penem.

inhibited by 100  $\mu$ M penem (not shown), as observed with pre-plastocyanin.

In this study we have shown that preprotein processing peptidases from cyanobacterial membranes and higher plant thylakoids are inhibited by penem. It confirms the similarity of the enzymes from all these locations. It is the first report of inhibition of these enzymes by any compound, and the data indicate that the activities result from a signal peptidase 1.

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