Site-Directed Mutagenesis of Proline-285 to Leucine in *Cephalosporium acremonium* Isopenicillin N – Synthase Affects Catalysis and Increases Soluble Expression at Higher Temperatures

Paxton Loke and Tiow-Suan Sim*

Department of Microbiology, Faculty of Medicine, National University of Singapore, MD4/4A, 5 Science Drive 2, Singapore 117597. Fax: 65-7766872. Email: micsimts@nus.edu.sg

* Author for correspondence and reprint requests

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The conversion of δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV) to isopenicillin N is dependant on the catalytic action of isopenicillin N – synthase (IPNS), an important enzyme in the penicillin and cephalosporin biosynthetic pathway. One of the amino acid residues suggested by the *Aspergillus nidulans* IPNS crystal structure for interaction with the valine isopropyl group of ACV is proline-283. Site-directed mutagenesis of the corresponding proline-285 to leucine in *Cephalosporium acremonium* IPNS resulted in non-measurable activity but an increased soluble expression at higher temperatures in a heterologous *E. coli* host.

Introduction

The penicillins and cephalosporins are widely used antibacterial therapeutics, characterised by their high efficacy and low toxicity (Demain and Elander, 1999). One of the key enzymes in the penicillin and cephalosporin biosynthetic pathway is isopenicillin N synthase (IPNS), a member of the extended family of mononuclear iron-dependent enzymes (Schofield *et al.*, 1997). IPNS catalyses the formation of a bicyclic ring structure, isopenicillin N (IPN), from the linear tripeptide δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV). IPN is the first β-lactam intermediate formed in the pathway and it possesses both the β-lactam and thiazolidine rings.

Crystal structure analyses have ascribed the IPNS catalytic centre to a HisXAsp(53–57)XHis motif (Borovok *et al.*, 1996) which encompasses the amino acids histidine-214, aspartate-216 and histidine-270 (*Aspergillus nidulans*, IPNS numbering) (Roach *et al.*, 1995, 1997). Other amino acid residues implicated in ACV substrate-binding include the R-X-S motif (arginine-279 and serine-281), tyrosine-189 and arginine-87 (Roach *et al.*, 1997). Furthermore, the same motifs for catalysis and for binding the valine carboxylate portion of ACV are supported by site-directed mutagenesis investigations of *Cephalosporium acremonium* IPNS (cIPNS) (reviewed in Sim and Loke, 2000). Four other residues, namely leucine-231, valine-272, proline-283 and leucine-223 were also suggested to interact with the valine isopropyl group of ACV via van der Waals forces (Roach *et al.*, 1997).

In this study, the corresponding proline-285, one of the residues postulated for van der Waals interaction, was changed to leucine by site-directed mutagenesis in cIPNS to study its effects on catalysis and solubility. Proline residues are very rigid and its presence results in kinks in polypeptide chains (Branden and Tooze, 1991). Hence, it would be interesting to see if the removal of the kink at P285 would be detrimental to IPNS catalysis. The results obtained revealed that the mutant P285L exhibited an increased soluble expression at higher temperatures in a heterologous *E. coli* host but its activity was nullified.

Materials and Methods

Site-directed mutagenesis and DNA sequencing

Mutagenesis reactions were based on the Quikchange site-directed mutagenesis kit strategy (Stratagene) according to the manufacturer's instructions. The PCR mutagenesis condition employed was 20 cycles of 95 °C (30s), 55 °C (1 min) and 72 °C (14 min). The recombinant expression construct pXW523 (Tan and Sim, 1996) harbour-

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ing the wildtype cIPNS gene was used in this investigation and the primer pair for mutagenesis was 5′ GCCAGTCACTGCTCTTCTTCGTCAAC 3′ and 5′ GTTGAC GAAGAAGAGCAGTGAC-TGGC 3′(base change in bold and underlined). Putative mutant plasmids were extracted from *E. coli* using plasmid mini-prep kits (Promega) and sequenced using the ABI PRISM™ BigDye™ terminator cycle sequencing kit (PE Applied Biosystems) to verify that only the specific P285L mutation has occurred. The precipitated sequencing products were analysed by an ABI PRISM 377 DNA sequencer.

Heterologous expression and IPNS enzymatic assays

Growth and induction of *E. coli* BL21(DE3) cultures for the heterologous expression of wildtype and mutant P285L cIPNS and enzymatic assays were performed as previously described (Tan and Sim, 1996). The cIPNS gene is under the control of a T7 promoter in pXW523. Protein determination was carried out using the Bradford assay (Bio-Rad) and scanning densitometry was employed to determine the relative amounts of cIPNS proteins expressed using the Bio-Rad GS-700 Imaging Densitometer.

Results and Discussion

Expression of the mutant P285L was initially carried out at 37 °C, similar to the expression conditions for wildtype cIPNS. Densitomeric readings revealed that the soluble expression level of the mutant P285L was 26% of total soluble protein, which was comparable to the wildtype cIPNS level of 25.3%. Enzyme bioassays were carried out using the mutant P285L soluble fractions expressed at 37 °C and surprisingly, no enzymatic activity was detected as compared to the wildtype cIPNS (Table I). Thus, this work supports the importance of this residue for cIPNS activity and also verifies an earlier observation that the IPNS (which has a similar substitution at position 285) from a mutant strain N-2 of *Acremonium chrysogenum* (syn *C. acremonium*) is probably an inactive protein (Ramsden *et al.*, 1989).

As a slight increase of heterologous soluble expression was observed for the mutant P285L at 37 °C, the study was extended to investigate whether or not soluble expression could still be obtained at higher induction temperatures. Thus, the mutant P285L and wildtype cIPNS (as control) were expressed at higher temperatures of 40 °C, 45 °C, 50 °C and 55 °C. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analyses of the soluble protein fractions of wildtype cIPNS and mutant P285L at the various temperatures are shown in Figures 1 and 2 respectively. At 40 °C, the soluble expression level of wildtype cIPNS dropped to 14.5% of the total soluble proteins while that of the mutant P285L remained high at 25.6% (Table II). Interestingly, soluble expression of up to 9.7% of total soluble protein for mutant P285L could still be obtained at 45 °C whereas that of the wildtype was almost negligible, thus demonstrating a higher soluble ex-

Table I. Activity of the wildtype and mutant P285L cIPNS enzymes determined by the bioassay method using *Micrococcus luteus* ATCC 381 as the test organism.

Enzyme type	Soluble protein concentration [mg/ml]	Total activity (Units)	Specific activity (Units/mg total soluble proteins)
Wildtype cIPNS	10.40	5.80	0.56
Mutant P285L	12.44	n.m[b]	n.m.

[a] One unit of activity is the amount of IPNS required to form the equivalent of 1 μmol of isopenicillin N per ml per minute at 26 °C.
[b] Not measurable (n.m.)

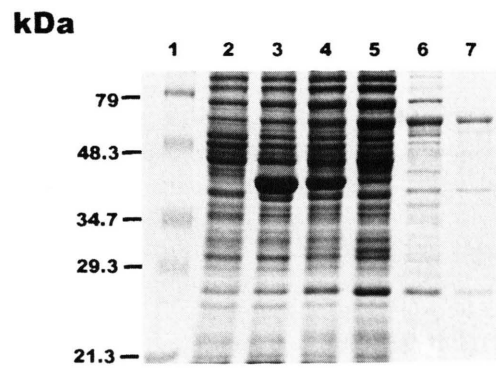

Fig. 1. SDS-PAGE analysis of the soluble protein fractions of wildtype cIPNS expressed at various temperatures obtained from *E. coli* BL21(DE3). Lane 1, molecular weight markers (in kDa); lane 2, *E. coli* BL21(DE3)/pET24d; lanes 3–7, wildtype cIPNS expressed at 37 °C, 40 °C, 45 °C, 50 °C and 55 °C, respectively. The cIPNS protein is 38.4 kDa in size.

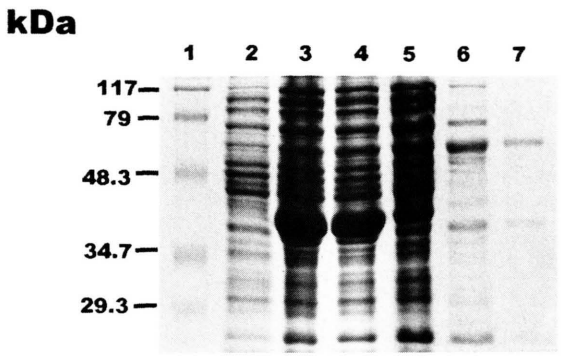

Fig. 2. SDS-PAGE analysis of the soluble protein fractions of mutant P285L expressed at various temperatures obtained from *E. coli* BL21(DE3). Lane 1, molecular weight markers (in kDa); lane 2, *E. coli* BL21(DE3)/pET24d; lanes 3–7, mutant P285L expressed at 37 °C, 40 °C, 45 °C, 50 °C and 55 °C, respectively.

pression for the mutant P285L at higher temperatures in a heterologous *E. coli* host. This suggests that mutant P285L may exhibit a higher thermostability than wildtype cIPNS due to the site-specific alteration of proline-285 to a leucine residue. However, it is unfortunate that the catalytic activity was lost at the expense of increased soluble expression levels at higher temperatures. The notion of sacrificing activity for the sake of stability was previously demonstrated in T4 lysozyme where alterations at the catalytic residues reduced or eliminated enzymatic activity but increased the thermal stability of the protein (Shoichet *et al.*, 1995). Undisputably, other amino acid substitutions that can increase soluble expression or thermal stability but not affect drastically the activity of the protein may be necessary for the rational improvement of cIPNS.

Table II. Soluble heterologous expression of wildtype cIPNS and mutant P285L enzymes.

Expression temperature (15 hours induction)	Average% of total soluble protein	
	Wildtype cIPNS	P285L mutant
37 °C	25.3%	26%
40 °C	14.5%	25.6%
45 °C	2.9%	9.7%
50 °C	< 1%	< 1%
55 °C	< 1%	< 1%

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