Replacement of Tyrosine-197 and the Corresponding Tyrosine-195 to Isoleucine in *Cephalosporium acremonium* and *Streptomyces clavuligerus* Isopenicillin N Synthase

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Isopenicillin N Synthase, Site-Directed Mutagenesis, Tyrosine

Isopenicillin N synthase (IPNS) is one of the key enzymes in the penicillin and cephalosporin biosynthetic pathway which catalyses the conversion of δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine to isopenicillin N. The IPNS from *Penicillium chrysogenum* 23X-80–269–37–2, a high penicillin V-producer, was found to possess an isoleucine residue instead of tyrosine at position 195. An attempt to increase the specific activity of IPNS from *Cephalosporium acremonium* and *Streptomyces clavuligerus* was undertaken by altering the corresponding tyrosine residue to an isoleucine at the corresponding location. Unfortunately, no apparent increase in specific activity was encountered when the purified mutant enzymes were analysed and thus, this amino acid difference is likely not responsible for high specific activity in IPNS.

Introduction

Traditional efforts to obtain increased penicillin titers rely heavily on strain improvement programmes, which utilise "brute force" genetic manipulation such as ultra-violet mutagenesis and X-ray treatment (Demain and Elander, 1999). These genetic manipulations could have conferred mutations in either the structural genes encoding the biosynthetic pathway enzymes or other regulatory components (Barredo *et al.*, 1989). One of the crucial enzymes in the penicillin and cephalosporin antibiotic pathway is isopenicillin N synthase (IPNS), which is responsible for the conversion of a linear tripeptide δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV) to isopenicillin N (IPN), a bioactive intermediate.

The IPNS genes from two different *Penicillium chrysogenum* strains, one of which is a high penicillin V-producing strain, have been cloned (Carr *et al.*, 1986; Barredo *et al.*, 1989). When the amino acid sequences of these two IPNS genes were compared, there was one amino acid disparity between them at position 195. The IPNS from *P. chrysogenum* 23X-80-269-37-2, a leucine auxotroph high penicillin V-producer, possesses an isoleucine residue at that particular position as opposed to a tyrosine residue for the IPNS from *P. chrysogenum* AS-P-78. Further comparisons with other IPNS isozymes from *Cephalosporium acremonium* (cIPNS), *Aspergillus nidulans* (aIPNS) and *Streptomyces clavuligerus* (scIPNS) revealed that a tyrosine residue is found at that same corresponding position (Barredo *et al.*, 1989).

As penicillin-overproducing strains of *P. chrysogenum* have been demonstrated to exhibit high IPNS activity (Ramos *et al.*, 1985; Martin, 1987), it would be interesting to investigate the role of this amino acid difference at the molecular level and observe whether or not any increase in IPNS activity could be achieved. Hence, site-directed mutagenesis of the corresponding tyrosine-197 in cIPNS (Y197) and tyrosine-195 (Y195) in scIPNS to isoleucine, respectively, was performed and the purified mutant enzymes analysed. Multiple sequence alignment of the IPNS isozymes was also carried out to indicate that isoleucine-195 is unique to the IPNS from *P. chrysogenum* 23X-80-269-37-2.

Materials and Methods

Origin of strains and multiple sequence alignment of IPNS isozymes

The culture collection reference numbers for *C. acremonium* is American Type Culture Collection

0939–5075/2001/0900–0806 $ 06.00

(ATCC) 11550 and for *S. clavuligerus* is Northern Regional Research Laboratory (NRRL) 3585. All available IPNS amino acid sequences were obtained from GenBank and aligned using the CLUSTAL W Multiple Sequence Alignment Program (version 1.7) (Thompson *et al.*, 1994).

Site-directed mutagenesis and DNA sequencing

The pGKC and pGKSC vectors containing the wildtype cIPNS gene and wildtype scIPNS gene, respectively, were used as the template for site-directed mutagenesis. These vectors are derivatives of pGK, a modified kanamycin resistance glutathione S-transferase (GST) fusion vector (Loke and Sim, 2000) and hence, the IPNS genes have GST as their fusion partner. Mutagenic primers used for the tyrosine-197 to isoleucine (Y197I) replacement in cIPNS were 5′ TACCTCGACCCG**AT**CCCGGAGCCGGCC 3′ and 5′ GGCCGGCTCCGGG**AT**CGGGTCGAGGTA 3′ (base changes in bold and underlined). For the substitution of the corresponding tyrosine-195 to isoleucine (Y195I) in scIPNS, the mutagenic primers were 5′ GTATCTGGAGGAG **ATCC**CGCCGGTGAAG 3′ and 5′ CTTCACCGGCGGG**AT**CTCCTCCAGATAC 3′ (base changes in bold and underlined). The Quikchange™ site-directed mutagenesis strategy (Stratagene) was employed and the number of polymerisation cycles utilised was twenty-five. In order to confirm the presence of the desired mutation, the putative mutant genes were sequenced in its entirety using the ABI PRISM™ BigDye™ terminator cycle sequencing kit (PE Applied Biosystems, USA) and analysed by an ABI PRISM 377 DNA sequencer.

Heterologous expression, purification of GST-IPNS enzymes and enzymatic assays

Expression of the wildtype and mutant cIPNS and scIPNS were carried out as previously described (Tan and Sim, 1996; Sim *et al.*, 1996). The duration of expression was 15 h post-induction at 37 °C for cIPNS and 25 °C for scIPNS. Expression at 25 °C post-induction for the Y197I cIPNS mutant was carried out when soluble expression was not obtainable at 37 °C. Protein purification was performed using the MicroSpin™ GST purification columns (Amersham Pharmacia), which allow for the rapid purification and analysis of fusion proteins by binding to glutathione sepharose 4B and eluting by reduced glutathione according to the manufacturer's instructions. Protein determination was performed using the Protein Assay Reagent (Bio-Rad) according to the manufacturer's instructions. Enzymatic assays using nutrient agar plates seeded with *Micrococcus luteus* ATCC 381 were performed four times according to Baldwin *et al.* (1987).

Results and Discussion

Multiple sequence alignment

Sequence comparison of thirteen IPNS isozymes revealed that isoleucine at position 195 is exclusive to the IPNS from *P. chrysogenum* 23X-80-269-37-2 (accession no. M15083). All other IPNS possess a

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C. acremonium (X03148)      LSSVV-LIRYPYLDPYPEPAIKTADDGTKLSFEWHEDVSLITVLYQ 227
S. clavuligerus (M19421)    LSSVS-LIRYPYLEEYPP--VKTGPDGQLLSFEDHLDVSMITVLFQ 223
S. lipmanii (M22081)        LSCRSLMIRYPYLDPYPEAAIKTGPDGTRLSFEDHLDVSMITVLFQ 227
S. griseus (X54609)         LSAVT-LIHYPYLEDYPP--VKTGPDGTKLSFEDHLDVSMITVLFQ 223
S. jumonjinensis (M36687)   LSSVS-LIRYPYLEEYPP--VKTGADGTKLSFEDHLDVSMITVLYQ 223
N. lactamdurans (X57310)    LSSVS-LIRYPYLEDYPP--VKTGPDGEKLSFEDHFDVSMITVLYQ 221
Flavobacterium (X17355)     LSAVS-LIRYPFLENYPP--LKLGPDGEKLSFEHHQDVSLITVLYQ 220
L. lactamgenus (X56660)     LSAVS-LIRYPFLENYPP--LKLGPDGEKLSFEHHQDVSLITVLYQ 220
A. nidulans (A10846)        LASVV-LIRYPYLDPYPEAAIKTAADGTKLSFEWHEDVSLITVLYQ 225
A. chrysogenum (M33522)     LSSVV-LIRYPYLDPYPEPAIKTADDGTKLSFEWHEDVSLITVLYQ 227
S. cattleya (D78166)        LSAVS-MIRYPYLDPYPEAAIKTGPDGTRLSFEDHLDVSMITVLSK 224
P. chrysogenum (X17436)     LSSVV-LIRYPYLNPYPPAAIKTAEDGTKLSFEWHEDVSLITVLYQ 225
P. chrysogenum (M15083)     LSSVV-LIRYPYLNPIPPAAIKTAEDGTKLSFEWHEDVSLITVLYQ 225
                                                  195
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Fig. 1. Multiple sequence alignment of IPNS isozymes. The shaded box shows a tyrosine residue in all IPNS except the IPNS from *P. chrysogenum* (M15083), which has an isoleucine residue (bold) at position 195.

Table I. Activity of the purified fusion wildtype *Cephalosporium acremonium* isopenicillin N synthase (wt cIPNS) and *Streptomyces clavuligerus* isopenicillin N synthase (wt scIPNS) and the respective mutant enzymes determined by bioassay[a] using *Micrococcus luteus* ATCC 381 as the test organism.

Enzyme type	Purified fusion protein concentration [mg/ml]	Total activity (Units[b])	Specific activity (Units/mg total purified proteins)	Relative specific activity (%)
wt cIPNS	0.70 ± 0.02	0.284 ± 0.045	0.41 ± 0.059	100% ± 14%
Y197I	0.84 ± 0.01	0.113 ± 0.019	0.135 ± 0.015	33% ± 4%
wt scIPNS	0.64 ± 0.05	0.38 ± 0.035	0.594 ± 0.054	100% ± 9%
Y195I	0.75 ± 0.06	0.13 ± 0.033	0.173 ± 0.044	29% ± 7%

[a] The IPNS reaction was carried out according to Baldwin *et al.* (1987) with the addition of purified enzyme preparations to a cofactor mixture containing 1 mM bis-ACV, 2 mM DTT, 1 mM L-ascorbate and 0.1 mM $FeSO_4.7H_2O$ in 50 mM Tris-HCl (pH 7.7) buffer at 26 °C and terminated by methanol. The reaction mix was dispensed into wells made in bioassay plates, which are nutrient agar plates seeded with overnight cultures of *M. luteus*.
[b] 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.

tyrosine residue at that corresponding position (Fig. 1).

Heterologous expression, fusion purification and enzymatic analysis

Expression of the recombinant GST fusion vector pGKC (cIPNS) and pGKSC (scIPNS) was performed at 37 °C and 25 °C respectively for 15 hours. The soluble expression of the mutant Y197I cIPNS and Y195I scIPNS was achieved at 25 °C. For the comparative determination of enzymatic activity, the wildtype cIPNS and scIPNS and the respective mutants were purified as fusion proteins with GST (SDS-PAGE analyses are shown in Figs 2 and 3, respectively).

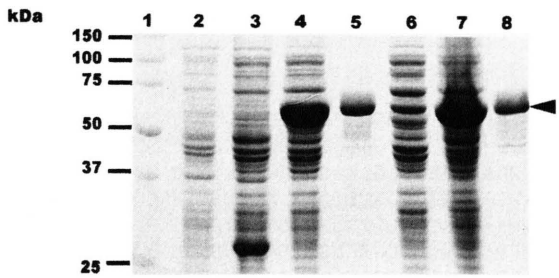

Fig. 2. SDS-PAGE analysis of the soluble protein fractions of wildtype cIPNS and mutant Y197I obtained from *E. coli* BL21(DE3). Lane 1, molecular weight markers; lane 2, *E. coli* BL21(DE3); lane 3, *E. coli* BL21(DE3)/pGK vector, lane 4, wildtype cIPNS (pGKC) after expression at 37 °C; lane 5, purified fusion GST-cIPNS; lanes 6 and 7, mutant Y197I after expression at 37 °C and 25 °C respectively; lane 8, purified fusion GST-Y197I. The arrowhead indicates the position of the fusion protein.

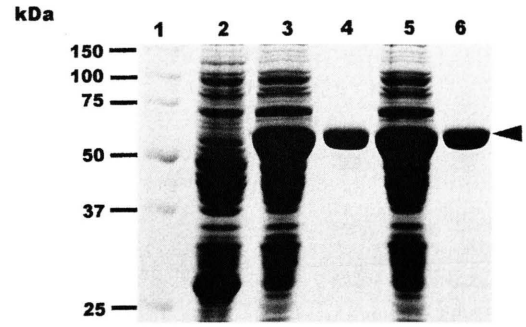

Fig. 3. SDS-PAGE analysis of the soluble protein fractions of wildtype scIPNS and mutant Y195I obtained from *E. coli* BL21(DE3) after expression at 25 °C. Lane 1, molecular weight markers; lane 2, *E. coli* BL21(DE3)/pGK vector, lane 3, wildtype scIPNS (pGKSC); lane 4, purified fusion GST-scIPNS; lane 5, mutant Y195I; lane 6, purified fusion GST-Y195I. The arrowhead indicates the position of the fusion protein.

The mutants Y197I and Y195I exhibited a relative specific activity of 33% and 29% respectively when purified fusion proteins were used (Table I). No increment in specific activity for the mutants was observed, thus indicating that this particular tyrosine residue in IPNS when altered to isoleucine is not responsible for any improvements in catalytic activity. Thus, high penicillin V yields from *P. chrysogenum* 23X-80-269-37-2 might possibly be due to effects on genes of the biosynthetic pathway apart from IPNS or effects on regulation. Furthermore, the introduction of this particular amino acid difference was suggested to occur during the strain improvement programme (Gracia-Dominguez *et al.*, 1991).

An interesting observation arises, in that if isoleucine-195 does diminish IPNS catalysis as shown in the substitutions of the corresponding tyrosine residues to isoleucines in cIPNS and scIPNS, the consequence for the IPNS from *P. chrysogenum* 23X-80-269-37-2 should be unfavourable. Nevertheless, high levels of penicillin V could still be obtained from *P. chrysogenum* 23X-80-269-37-2. Conversely, it may also be possible but more unlikely that this particular amino acid difference is distinctive only for the IPNS from *P. chrysogenum* 23X-80-269-37-2 and does not affect its catalysis in this specific *P. chrysogenum* strain. As such, only a comparison of the specific activity of IPNS from *P. chrysogenum* 23X-80-269-37-2 and *P. chrysogenum* AS-P-78 would confirm this suggestion.

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