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Review

Production of high levels of soluble recombinant *Streptomyces clavuligerus* isopenicillin N synthase in *Escherichia coli*

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Abstract

Streptomyces clavuligerus isopenicillin N synthase (scIPNS) gene expression under the control of T7- and *trc*-promoters in pET24d and pTrc99A vectors respectively in *Escherichia coli* was found to be affected by temperature. Although the scIPNS protein is mostly aggregated and inactive in the inclusion bodies when made at 37°C, soluble enzyme is synthesized at 25–28°C. Studies conducted demonstrated that the promoter, as well as the *E. coli* strains used play critical roles in determining the level of soluble scIPNS made. It is also apparent from computational analysis that the protein structure (perhaps influenced by hydrophobic residues at strategic positions) may also affect the solubility of the expressed scIPNS. However, after genetic manipulation (or under appropriate conditions), overproduction of the scIPNS protein by the T7-promoter to a level of ≈ 29% of total soluble protein in *E. coli* BL21(DE3) grown at 25°C was achieved and the recombinant enzyme was found to retain activity. It was also observed that soluble scIPNS expressed at 25°C was converted to the insoluble form after incubation in vitro at 37°C, whereas insoluble scIPNS expressed at 37°C remained aggregated regardless of the incubation temperature in vitro. This suggested that the host's milieu affects the solubility (or folding) of the scIPNS expressed.

Keywords: Isopenicillin N synthase; *Streptomyces clavuligerus*; Expression; Cultivation temperature; Promoter; Host

1. Introduction

Isopenicillin N synthase (IPNS) is involved in a key enzymatic step in the biosynthesis of the β-lactam antibiotics: penicillin and cephalosporin. This non-heme Fe^{2+}-dependent enzyme catalyses the formation of β-lactam isopenicillin N, via the oxidative cyclization of a tripeptide substrate, δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV).

To bring about the creation of novel β-lactam antibiotics, information on the active site of the biosynthetic enzymes is required to facilitate studies targeted at increasing the specific activity and broadening the substrate specificity of the biocatalysts. Biophysical techniques, such as X-ray crystallography, can provide information on the active site, but such analyses need tremendous amount of purified proteins. Thus far, only the crystal structure of *Aspergillus nidulans* IPNS has been elucidated [1]. It would be useful to also obtain sufficient proteins to study the crystal structure of the same enzyme from bacterial *Streptomyces clavuligerus,* i.e. scIPNS, since the gene encoding IPNS was suggested to arose first in a prokaryote and was then horizontally transferred to eukaryotes [2]. Expression of the scIPNS gene in a heterolo-

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Table 1
Expression systems for IPNS isozymes in *E. coli* (modified from Sim and Tan [13])

Source	Promoter	*E. coli* host strain	Induction conditions	Induction temperature	Level of expression	Form	Activity	Reference
C. acremonium	*trc*	NM554, JM109	15 h growth	37°C	25% of total soluble protein	soluble	active	[14]
	lac	TG1	2 h IPTG induction	37°C	5–15% total soluble protein	soluble	active	[13]
	T7	BL21(DE3)	2 h IPTG induction	37°C	40–50% total soluble protein	soluble	active	[13]
P. chrysogenum	*trc*	NM554	15 h growth	37°C	2–3% total soluble protein	soluble	(?)	[14]
A. nidulans	*trc*	NM554	15 h growth	37°C	10–15%	soluble	active	[14]
	lac	NM554	15 h growth	37°C	.40% total soluble protein	soluble	active	[14]
	lac	JM109	15 h growth	37°C	10–20% total soluble protein	soluble	active	[14]
S. clavuligerus	*lac*	MC1022	1–5 h IPTG induction	28°C	undetectable on SDS–PAGE	soluble	active	[4]
(wildtype IPNS)	T7	K38	2.5 h temp induction	37°C	predominantly found in insoluble form	insoluble	inactive	[3]
	lac	BL21(DE3)	15 h IPTG induction	25°C	5% of total soluble protein	soluble	undetectable	This study [a]
S. clavuligerus	T7	BL21(DE3)	15 h IPTG induction	25°C	29% of total soluble protein	soluble	active	This study [a]
(Pro2Ala IPNS)	T7	BL21(DE3)	15 h IPTG induction	37°C	10% of total soluble protein	soluble	active	This study [a]
	trc	BL21(DE3)	15 h IPTG induction	25°C	10% of total soluble protein	soluble	active	This study [a]
	trc	TG1	15 h IPTG induction	25°C	8% of total soluble protein	soluble	active	This study [a]

[a] The recombinant constructs containing the wildtype scIPNS gene cloned under the control of *lac*-promoter (pIPS1) and Pro2Ala scIPNS gene placed behind the *trc*-promoter (pTRC922) and T7-promoter (pHSJ98) were transformed into *E. coli* host strains BL21(DE3) and TG1 simultaneously. Subsequently, the recombinants were cultivated at various temperatures to examine the effects of different promoters, host strains as well as cultivation temperature on the expression of soluble scIPNS.

gous host may present a more feasible means of acquiring the necessary amounts of scIPNS than from its natural producer, *S. clavuligerus*.

Up to the present, there are only two reported cases of cloned scIPNS expression in heterologous host, *E. coli* (Table 1). Durairaj and colleagues (1992) [3] placed the scIPNS gene under the T7-promoter in *E. coli* K38 and reported a high level expression at 37°C but the protein produced was insoluble and inactive. When expressed by the *lac*-promoter based vector in *E. coli* MC1022 [4], soluble scIPNS was only produced at a low level at 28°C. However the possibility of overexpressing the scIPNS enzyme as soluble proteins in *E. coli* has not been extensively investigated.

It is generally believed that the formation of soluble recombinant proteins in *E. coli* is favoured by a lower cultivation temperature than 37°C [5]. Human interferon-α2 (IFN-α2) and interferon-γ (IFN-γ) when expressed in *E. coli* cells grown at 37°C, were both found to be associated with the particulate fraction. However, at growth temperatures of 23–30°C, 30–90% of the recombinant proteins were found to be soluble. In addition to these two proteins, P22 tailspike protein [6], diphtheria toxin [7], ricin A chain [8], basic fibroblast growth factor [9], pro-subtilisin [10] and kanamycin nucleotidyltransferase [11] were all reported to show a similar temperature dependence.

If a high level of expression of a cloned gene is desired, then transcription should be maximized by use of a strong promoter [12]. From the analysis of the expression systems used for producing IPNS isozymes in *E. coli* (Table 1), it was inferred that different promoters made differing levels of scIPNS under the same induction conditions. For example, the levels of *Cephalosporium acremonium* IPNS expressed by T7- and *lac*-promoter based vectors were found to be 40–50% and 5–15% of total soluble protein respectively [13].

E. coli cells contain inherent proteases that are liable to degrade foreign proteins, cause the proteins to unfold and result in aggregate formation. Thus, different strains of *E. coli* harbouring unique sets of proteases can influence the proteins expressed to different extent. This could perhaps explain why *A. nidulans* IPNS when cloned under the same *lac*-promoter in two different *E. coli* strains, NM554 and JM109, was expressed in 40% and 10–20% of total soluble protein respectively [14] (Table 1). It would thus appear that expression of IPNS proteins in *E. coli* cells can be affected by the engineered strains.

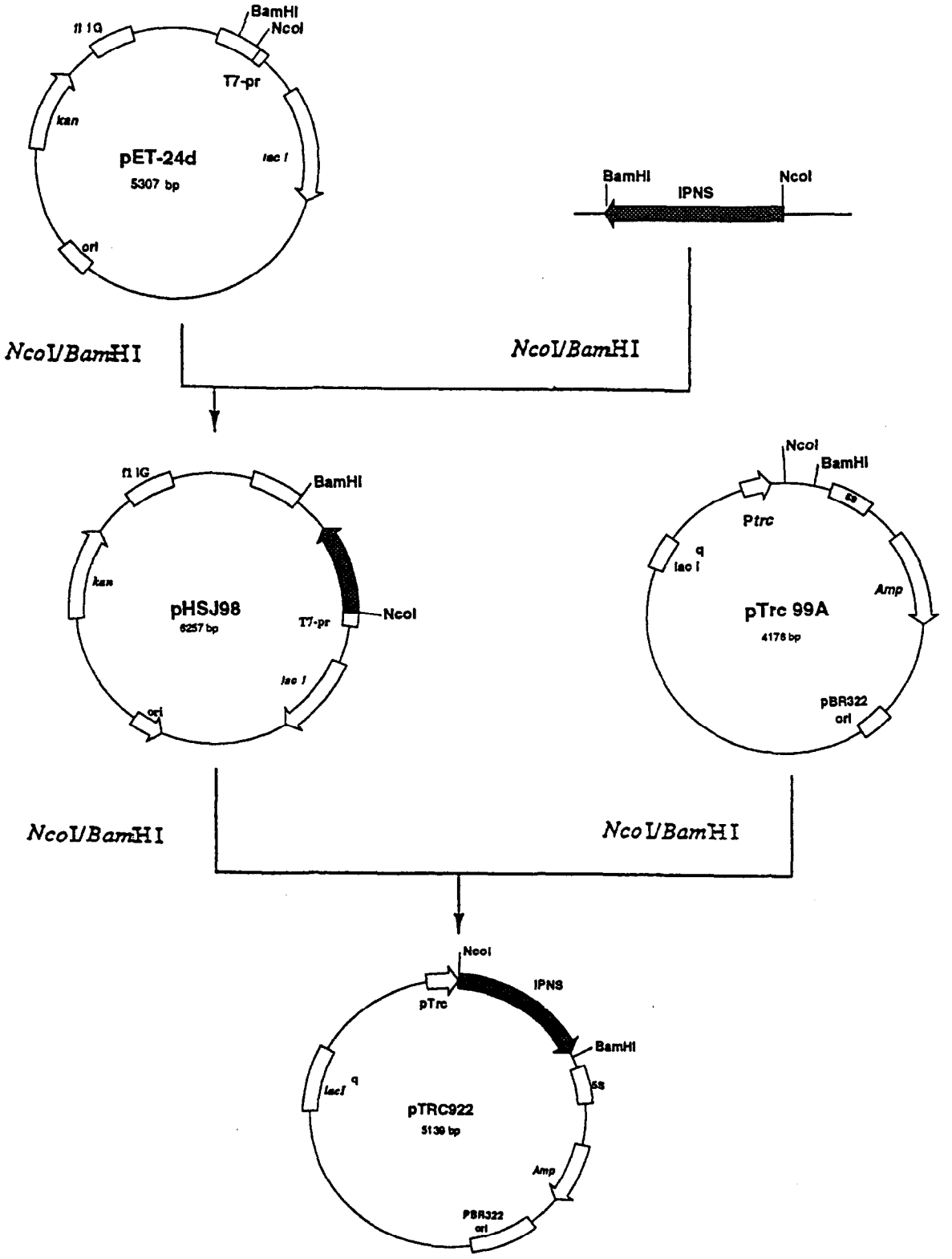
In instances where knowledge of the structure of a protein was not available from X-ray crystallography and NMR spectroscopy, scientists have resorted to the use of protein structure prediction programmes to facilitate the study of the protein functions as well as to carry out site-directed mutagenesis to obtain novel proteins. In this study, two of the frequently used programmes, Chou–Fasman (CF) [15] and Garnier–Osguthrope–Robson (GOR) [16] prediction methods were exploited as a preliminary step to analyse the secondary structure conformation of scIPNS gene after intended biotransformation (i.e. the purported change from proline to alanine at the second amino acid residue for cloning purposes), as well as in comparison with its related fungal IPNS.

The possibility of expressing scIPNS as a soluble protein in *E. coli* by lowering the cultivation temperature was the main objective of this study. Attempts were also made to study the influence of specific promoters and transformed hosts on the level of soluble scIPNS protein obtained.

2. Materials and methods

2.1. PCR amplification of scIPNS

The polymerase chain reaction (PCR) was used to amplify the *S. clavuligerus* IPNS gene located on the plasmid, pIPSI (a generous gift from Professor S.E. Jensen, University of Alberta, Canada). Using a set of oligonucleotide


BamHI
NcoI
f1 IG
T7-pr
kan
pET-24d
5307 bp
lac I
ori
BamHI
IPNS
NcoI
NcoI/BamHI
NcoI/BamHI
f1 IG
BamHI
kan
pHSJ98
6257 bp
T7-pr
NcoI
lac I
ori
NcoI
BamHI
Ptrc
lac I q
Amp
pTrc 99A
4176 bp
pBR322 ori
NcoI/BamHI
NcoI/BamHI
NcoI
IPNS
pTrc
BamHI
lacI q
pTRC922
5139 bp
Amp
PBR322 ori

primers, a *Nco*I and *Bam*HI site was simultaneously introduced at the 5′ and 3′ ends of the gene respectively. The primers used were 5′-GGTTCCATGGCAGTTCTGATGCCG-3′and 5′-GAAGGATCCTCAGGTCTGGCCGTTCT-3′. Polymerase chain reaction mixtures contained 20 mM Tris–HCl (pH 8.8), 50 mM KCl, 2.5 mM $MgCl_2$, 10 μg gelatin, deoxynucleoside triphosphates (each at a concentration of 100 μm), 5 units of *Taq* DNA polymerase, 50 pmol of each primer, and 0.1 pmol of pIPSI in a final reaction volume of 100 μl. The plasmid was denatured for 3 min at 95°C, and 30 cycles of the PCR were conducted (each cycle consisted of incubation at 95°C for 1 min, 65°C for 1 min, and 72°C for 2 min); this was followed by 10 min incubation at 72°C. The amplified DNA fragment obtained was purified using the QI-Aquick DNA purification kit (QIAGEN).

2.2. Construction of expression vector

The amplified scIPNS gene (≈ 1 kb) was isolated as a *Nco*I/*Bam*HI fragment and cloned downstream of the T7- and *trc*-promoters in pET24d (Novagen) and pTrc99A (Pharmacia) vectors respectively (Fig. 1) and transformed into *E. coli* BL21(DE3) (F^- *ompT* [*lon*] *hsdS*$_B$ ($r_B^- m_B^-$; an *E. coli* B strain) (Novagen). This host strain possesses a DE3 prophage bearing the T7 RNA polymerase gene that operates under the *lac*UV5 promoter and is inducible by isopropyl-thiogalactoside (IPTG). However, only reconstructed clone designated pTRC922 was also transformed into *E. coli* TG1(F′ *traD36* *lacI*^{q}Δ(*lacZ*)*M15* *proA*$^+$*B*$^+$/*supE* Δ(*hsdM*-mcrB)5 ($r_k^- m_k^+$McrB) *thi* Δ(*lac-proAB*) to examine the expression of scIPNS in a different host strain since expression of scIPNS gene in pET24d by T7-promoter would be extremely low due to the absence of the T7 RNA polymerase gene in *E. coli* TG1.

2.3. Expression of scIPNS gene in E. coli

Recombinant cells were inoculated into 50 ml LB medium containing kanamycin (50 μg/ml) and inoculated at the appropriate temperature with agitation at 200 rpm until the optical density (OD_{600}) reached a value of 0.6–1.0, at which time IPTG was added to a final concentration of 1 mM. After further incubation of 15 h, the cultures were harvested by centrifugation, and washed in TDE buffer (50 mM Tris HCl pH 7.2, 100 mM dithiothreitol, 10 mM EDTA). The cell-free extracts were prepared by sonication as described in Sim and Tan [13].

2.4. Purification of scIPNS protein

The scIPNS protein was purified from the cell-free extracts obtained from 200 ml *E. coli* cultures through ammonium sulphate precipitation and DEAE–Sephadex-A50 ion-exchange chromatography. The procedures were carried out as described by Tan and Sim [27] except that the columns were equilibrated using TDE buffer.

2.5. Determination of scIPNS activity by bioassay method

The activity of scIPNS in cell-free extracts was determined by bioassay method using *Micrococcus luteus* ATCC 318 as the test organism and ACV (a generous gift from Professor S. Wolfe, Simon Fraser University, Canada) as the substrate [17]. One unit of activity is the amount of enzyme required to form the equivalent of 1 μmol of isopenicillin N in 1 min.

2.6. Protein determination

Total protein concentration were analysed by the Bradford Assay [18], using bovine serum

Fig. 1. Construction of expression vectors containing the *S. clavuligerus* IPNS gene under the control of the *trc*- and T7-promoters. *Kan*, kanamycin resistance gene; *Amp*, ampicillin resistance gene and *lac* I, *lac* repressor gene.

albumin as the standard. The relative amount of the IPNS protein in the sample was obtained by using a computer program named Bio-Profil Version 5.08 (Vilber Lourmat) which can measure the relative optical densities of the protein bands in lanes of polyacrylamide gels.

2.7. *In vitro analysis of the solubility of scIPNS*

60 μl aliquots of soluble fraction of the cell-free extracts from recombinant *E. coli* cells grown at 25°C were incubated at 25°C in vitro for times varying from 30 min to 2.5 h. A similar set was incubated in vitro at 37°C. Subsequently, all the tubes were centrifuged at 12000 *g* for 20 min at 4°C to separate the cell-free extracts into soluble and insoluble fractions. The protein profiles in all the fractions were thereafter analysed by SDS–PAGE. The insoluble scIPNS acquired by growing the *E. coli* cells at 37°C were also examined in the same manner under in vitro conditions at 25°C and 37°C.

2.8. *Computer analysis of protein sequence*

The Genetics Computer Group (GCG) sequence analysis software package version 7.31 (University of Wisconsin) [19] was used for the analysis of protein sequences. The secondary structure prediction programs based on the algorithms developed by Chou and Fasman (1974) [15] and by Garnier, Osguthrope and Robson (1978) [16] were exploited to analyse the conformation of scIPNS protein.

3. Results

3.1. *PCR amplification of scIPNS gene using designed oligomers*

Previous expression of *C. acremonium* IPNS driven by the T7-promoter in the vector pET24d showed that the protein was expressed in the soluble form at high levels (48% of total soluble proteins) [13]. This vector contains a unique *Nco*I site at the translation initiation site. Therefore the creation of a *Nco*I site at the translation initiation codon of the *S. clavuligerus* IPNS gene should allow efficient expression of the scIPNS gene. Introduction of a *Nco*I site required that the second codon CCA be altered to GCA, thus converting the amino acid proline to alanine. This alteration was achieved by the polymerase chain reaction. Amplification of scIPNS gene located on pIPS1 plasmid was carried out using a set of designed oligonucleotide primers such that the exponentially synthesized product would be an intact Pro2Ala scIPNS gene with the preservation of the ATG translation start and TGA stop codon (Fig. 1).

3.2. *Construction of overexpression plasmids*

To investigate the influence of the T7- and *trc*-promoters on the expression of scIPNS gene in *E. coli*, two expression vectors were simultaneously constructed, viz pHSJ98 and pTRC922 (Fig. 1), incorporating the T7- and *trc*-promoters respectively. The ≈ 1 kb *Nco*I/*Bam*HI gene fragment was then cloned into pET24d to form the pHSJ98 construct and the scIPNS gene from recombinant pHSJ98 was subsequently subcloned into the pTrc99A vector. This was to ensure that the identical scIPNS gene of the same sequence was examined when comparing the strength of the 2 promoters to drive scIPNS synthesis. A diagram showing the relative distances between the promoter, ribosome binding site (RBS) and the scIPNS gene in pHSJ98 and pTRC922 is shown in Fig. 2. The scIPNS gene was positioned 69 bp and 43 bp downstream of the T7- and *trc*-promoters respectively. However, the cloned gene in both constructs were equally separated from the RBS by 8 bp. The dual recombinant plasmids, pHSJ98 and pTRC922, were subsequently transformed into *E. coli* host cells and the synthesis of the protein was analysed by SDS–PAGE (Fig. 3) and densitometric scanning (Fig. 4).

3.3. Effect of cultivation temperature on scIPNS expression in pHSJ98

The recombinant clone pHSJ98 was induced for 15 h with IPTG at various temperatures and high level expression of scIPNS protein was achieved at incubation temperatures of 25°C and 28°C. As illustrated in Fig. 3, the cell-free extracts obtained from the cultures incubated at different temperatures contained an overexpressed protein band of ≈ 38 kDa which is absent in the non-recombinant *E. coli* BL21(DE3) culture.

Based on the analysis of the SDS–PAGE in Fig. 3, it was observed that at 25°C, a higher level of scIPNS protein was made by the T7-promoter in the soluble form as indicated by the prominent band in the third lane. As the incubation temperature was increased gradually, the protein seemed to become increasingly insoluble. At higher temperatures of 30°C, 34°C, 37°C and 42°C, the protein was predominantly associated with the particulate fraction with little or no scIPNS present in the soluble form. Densitometric scanning results of the various soluble fractions also showed a decrease in solubility when the culturing temperature was increased (Fig. 4(i)).

The authenticity of the scIPNS protein expressed was verified by immunoblotting using immunized serum (data not shown) and the specific activity of the recombinant scIPNS (unpurified) was found to be of a higher value than the enzyme extracted from *S. clavuligerus* NRRL 8535 (0.0015 units per mg soluble cell protein)[4]. The activity of the insoluble scIPNS was not measured and assumed to be negligible as all the insoluble IPNS expressed were reported to be inactive before solubilization [3,28].

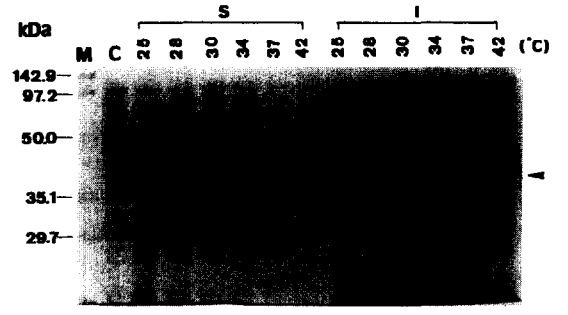


Fig. 3. SDS–PAGE analysis of the cell-free extracts obtained from pHSJ98 incubated at various temperatures. The cell-free extract (soluble fraction) of *E. coli* BL21(DE3)/pET24d was used as a control (C). The soluble (S) and insoluble (I) fractions of the cell-free extracts acquired at 25°C, 28°C, 30°C, 34°C, 37°C and 42°C are labeled accordingly. *M*, protein standard of various molecular sizes in kDa. The arrow indicates the position of the scIPNS protein band.

3.4. Effect of cultivation temperature on scIPNS expression in pTRC922

The finding that T7-promoter driven expression of scIPNS gene was dependent on the induction temperature raises the possibility that

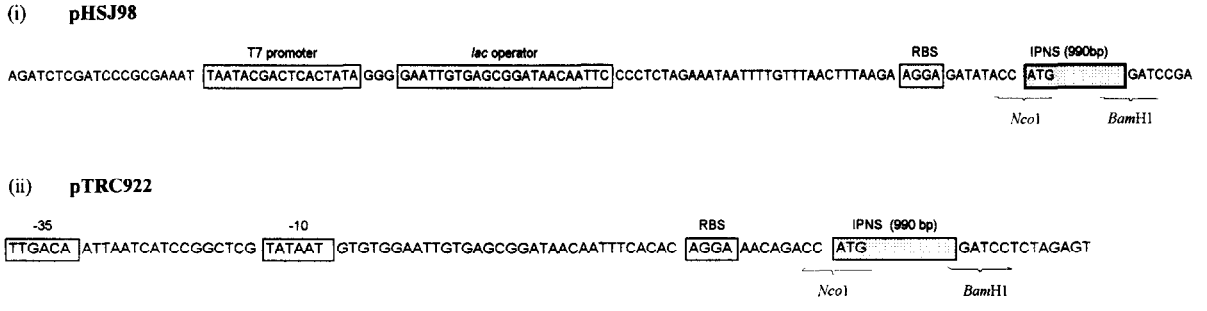


Fig. 2. A detailed diagram featuring the relative positions of the cloned scIPNS gene with respect to the promoters and ribosome binding site (RBS) in the recombinant vectors pHSJ98 and pTRC922 accordingly in (i) and (ii). The relevant sequences of pET24d and pTrc99A around the transcription initiation point of scIPNS gene are also shown.

expression of the enzyme under the *trc*-promoter may be regulated in a similar fashion by the cultivation temperature.

It was again obvious from the results (Fig. 4(ii)) that at higher incubation temperatures, the yield of soluble protein was lower as demonstrated by the reduction in the level of soluble scIPNS made from 10% to 7.0% when the temperature was shifted from 25°C to 37°C. Using immunoblotting techniques, specific antibodies generated were used to verify the presence of the scIPNS protein made by the *trc*-promoter (data not shown).

3.5. Comparison of the strength of T7- and trc-promoter to drive the synthesis of the scIPNS protein

The amount of soluble scIPNS expressed as percentage of soluble protein by the T7- and *trc*-promoter at 28, 34 and 37°C are charted in Fig. 4(iii) to compare the production of the soluble enzyme under different transcription signals.

At 28°C, T7-promoter driven scIPNS synthesis reached a level of 12.7% of total protein obtained which is ≈ 2.5 fold higher in magni-

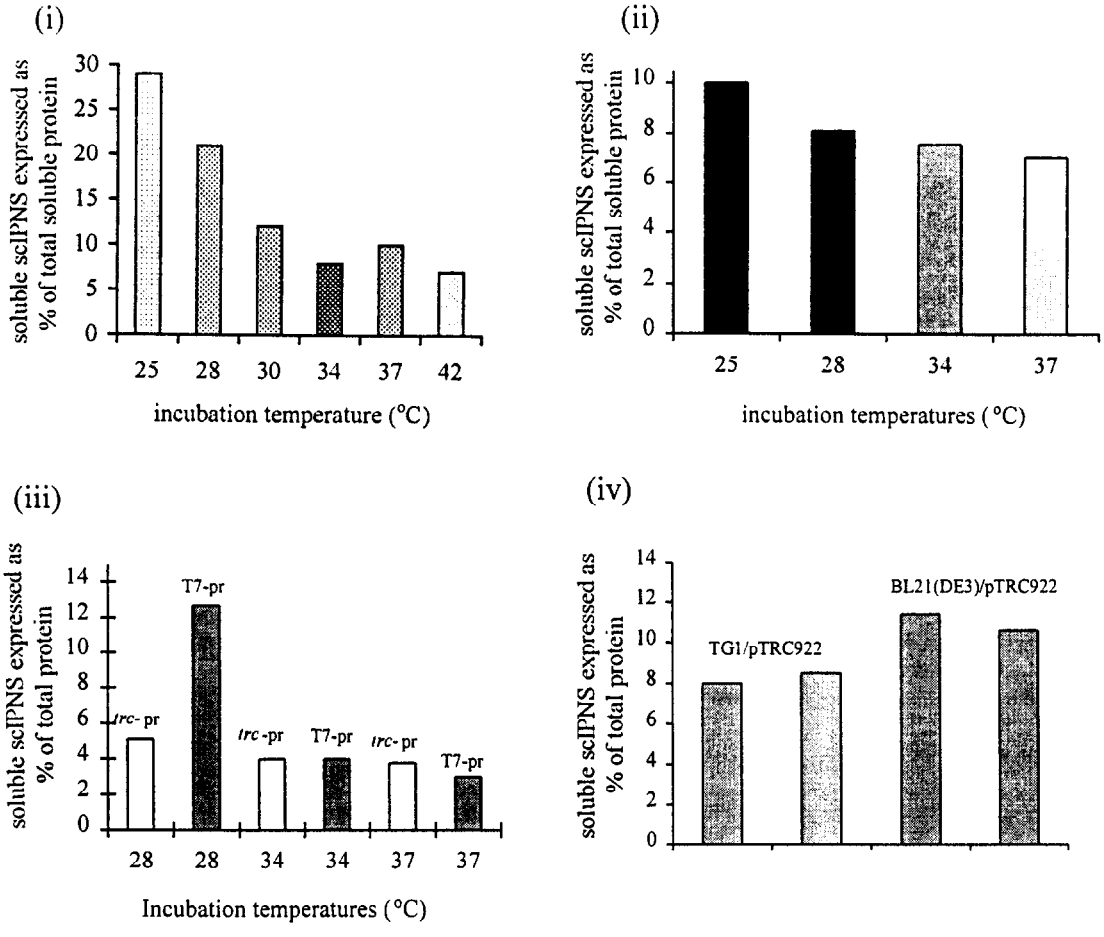


Fig. 4. The following charts show the results of the densitometric scanning of cell-free extracts of the respective recombinants present at the relevant induction temperatures; (i) soluble scIPNS expressed as percentage of the total soluble protein of BL21(DE3)/pHSJ98 obtained at various temperatures; (ii) *trc*-promoter driven expression of soluble scIPNS at various temperatures; (iii) densitometric scanning of the soluble fraction of the cell-free extracts derived from T7- and *trc*-promoters at 28°C, 34°C and 37°C (pr: promoter) and (iv) comparison of the level of soluble scIPNS made by *E. coli* BL21(DE3)/pTRC922 and TG1/pTRC922.

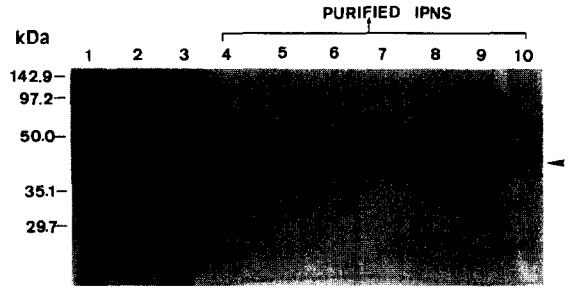


Fig. 5. Purification of scIPNS obtained from *E. coli* BL21(DE3)/pHSJ98 cells induced at 28°C. Lane (1) shows the protein standard of various molecular sizes (kDa). The soluble fraction of the cell-free extracts of *E. coli* BL21(DE3)/pHSJ98 is shown in lane (2). In lane (3), the ammonium sulphate precipitated fraction is shown. The 7 fractions from DEAE Sephadex-A50 purified column are shown in the remaining lanes. The position of the scIPNS band is indicated by an arrow.

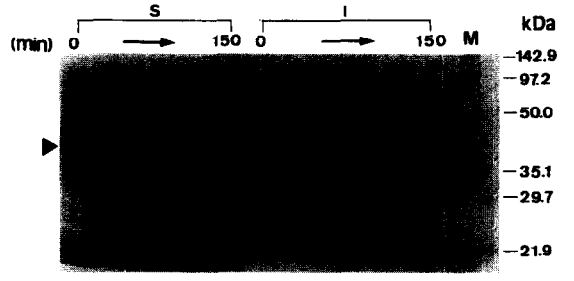


Fig. 6. SDS–PAGE analysis of the soluble scIPNS in vitro at 37°C. The soluble (S) and insoluble (I) fractions obtained after incubation of the cell-free extracts at 37°C in vitro for 0, 30, 60, 90, 120 and 150 min are shown accordingly. *M*, protein standards of various molecular sizes in kDa. The position of the scIPNS protein is indicated by an arrow.

tude than the 5.1% of soluble scIPNS derived from the *trc*-promoter expression. This shows that T7-promoter can signal a higher level of transcription and thus translation of scIPNS protein at 28°C than the *trc*-promoter. However it was interesting to note that at higher incubation temperatures of 34°C and 37°C, the level of soluble scIPNS expressed by the T7-promoter, an assumed stronger promoter, was comparable to that produced by the *trc*-promoter.

3.6. Effect of E. coli host strains used for the expression of scIPNS by pTRC922

E. coli TG1 and BL21(DE3) recombinant cells harbouring the pTRC922 construct carrying the scIPNS gene under the *trc*-promoter was induced at 28°C and cell-free extracts prepared. The results obtained (Fig. 4(iv)) revealed that the production level of scIPNS protein by the *trc*-promoter was higher in *E. coli* BL21(DE3) ($\approx$ 11%) than in TG1 ($\approx$ 8%).

3.7. Purification of scIPNS protein from recombinant pHSJ98

The high level expression of scIPNS under the T7-promoter facilitated the purification of the protein via a 2-step procedure. The scIPNS protein was successfully purified to homogeneity from the cell-free extracts of pHSJ98 by ammonium sulphate precipitation followed by DEAE–Sephadex-A50 ion-exchange chromatography. Analysis of the salt-precipitated proteins by SDS–PAGE analysis (Fig. 5) indicated that the scIPNS was the major component in the 55–85% ammonium sulphate saturated fraction. Enzymatic activity of the various fractions obtained after each purification of the scIPNS protein is shown in Table 2. The relative intensity of the major band increased with the specific activity of the enzyme sample. The major band of the sample with the highest specific activity accounted for about 89% of the purified protein. Thus it is possible to obtain 7.3-fold purification of scIPNS by using the above two step purification scheme.

Table 2
Purification of recombinant *S. clavuligerus* IPNS from *E. coli*

Purification step	Protein concentration (mg/ml)	Isopenicillin N formation (nmol/ml)	Specific activity (μmol/mg · min)	Purification fold
crude cell-free extract	0.305	88.99	0.029	1
ammonium sulphate precipitate	0.054	19.83	0.037	1.3
DEAE–Sephadex A-50	0.016	34.51	0.214	7.3

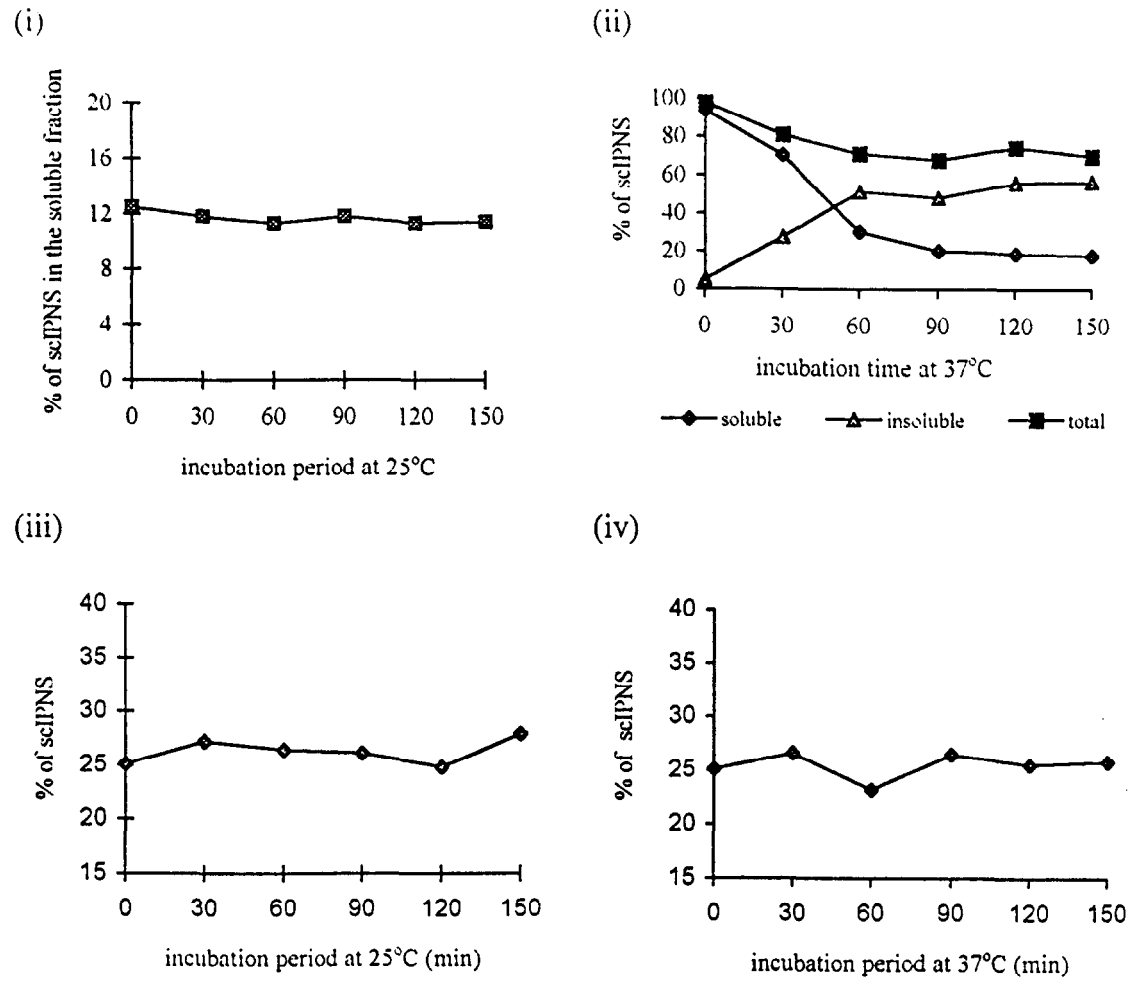


Fig. 7. The following graphs show the densitometric scanning results of the in vitro analysis of solubility of scIPNS; (i) the level of scIPNS that are present in the soluble fraction of the cell-free extracts after incubation in vitro at 25°C; (ii) the % of soluble scIPNS, insoluble and total scIPNS obtained after incubation of soluble protein expressed at 25°C in vitro at 37°C; (iii) the results obtained from the in vitro incubation of insoluble scIPNS at 25°C for 150 min and (iv) the level of aggregated scIPNS present in the cell-free extracts after incubation of the insoluble fraction in vitro at 37°C.

3.8. In vitro analysis of the solubility of scIPNS

The soluble scIPNS derived from cell cultures cultivated at 25°C when incubated in vitro at the same temperature remained soluble throughout the test period of 2.5 h (Fig. 7(i)). However SDS–PAGE and densitometric scanning results (Figs. 6 and 7(ii)) showed that the soluble scIPNS incubated at 37°C in vitro was found to be associated with the aggregated fractions with increasing incubation time. Within 60 min incubation, only about 29.9% of the total soluble scIPNS remained in the soluble fraction whereas 51.3% appeared to be transformed into the aggregated form. Summation of the scIPNS in both fractions after various intervals indicated that after incubation of 150 min (Fig. 7(ii)), only 70.4% of the total scIPNS was detected and 29.6% of the enzyme was unaccounted for.

The results obtained from the in vitro incubation of insoluble scIPNS expressed at 37°C at the two temperatures, 25°C and 37°C (Fig. 7(iii) and 7(iv)) showed that at both temperatures, the amount of scIPNS in the insoluble fractions

remains relatively constant throughout the 2.5 hours incubation. Unlike in the earlier case, there is no loss of protein after incubation in vitro.

3.9. Analysis of predicted structure conformation of scIPNS proteins

Analysis of the schematic plots of structures of wildtype and Pro2Ala scIPNS (diagrams with the secondary structures not shown) using the CF and GOR prediction programmes revealed an increase in hydrophobicity near the N-terminal end in both of the plot structures indicated by the increase in the number of ◇ symbols which denotes hydrophobicity. However, the single amino acid change has no observable effect on the secondary structure from residues 180 to 280 which has been deduced to be the active site of the enzyme from studies of the crystal structure of the IPNS protein from *A. nidulans* [1].

Gross examination and comparison of the degree of hydrophobicity and hydrophilicity from the plot structures of the *S. clavuligerus* (scIPNS) and *C. acremonium* (cIPNS) enzyme indicated that only between the 150th and 200th amino acid residue, is there a pronounced difference in terms of summation of the observed hydrophobicity and hydrophilicity.

4. Discussion

One of the problems encountered when producing foreign proteins in *E. coli* is that the product often accumulates as insoluble aggregates (inclusion bodies) within the producing bacteria. Enzymes that are expressed in the insoluble form are less desirable since solubilization of the enzymes requires the use of strong chaotrophic reagents such as urea and guanidium HCl which can lead to loss in enzyme activities [20]. Thus it is important to develop strategies for efficient production of soluble scIPNS protein in *E. coli*.

As reported here, soluble enzyme is synthesized at 25–28°C, but the protein is mostly aggregated in inclusion bodies when made at 37°C. Since the optimal growth temperature of *S. clavuligerus* is 28°C, it appears that the folding and stability of the protein is affected by temperature, or the protein milieu in the host strain grown at different temperatures. Furthermore, the production of some mammalian proteins, interferon-α2 [5], interferon-γ [5] and fibroblast growth factor [9], have also been reported to be insoluble when made in *E. coli* grown at 37°C. However, the exact mechanism involved whereby the proteins are produced in insoluble form at elevated temperature and as soluble forms at lower temperature is still unclear.

Although the level of protein manufactured by the T7-promoter was far higher than that of the *trc*-promoter, temperature-dependent soluble expression was achieved for both promoters and found not to be directly dependent on the concentration of protein. Thus far, no direct correlation between the insoluble recombinant protein made and protein concentration or production rate has been found [5]. It is also possible that variations of other cultivation conditions will yield even higher levels of soluble protein than what is presently achieved.

It was found that although the level of soluble scIPNS produced by the T7- and *trc*-promoters were comparable at 37°C, the amount made by the former was ≈ 2.5-fold higher at 28°C. Based on the results obtained so far, it is not possible to give a definite explanation for this observation.

The *E. coli* host cells used in the present study, BL21(DE3) differs from TG1 in that the former does not produce an outer membrane protease due to a mutation in the *ompT* gene [22] and it lacks the *lon* protease [23]. On the other hand, Swamy and Goldberg (1981) [24] indicated that *E. coli* TG1 contains the full complement of proteases in *E. coli*. This difference in protease level may account for the higher level of expression of scIPNS in *E. coli*

BL21(DE3) than TG1. However, the difference in the amount of scIPNS produced is not marked, implying that other more important factors are involved which limits the high level expression of the cloned scIPNS gene in the two hosts.

Besides scIPNS, β-lactamase, GAG-9 and to a lesser extent polio virus protease were reported to be more soluble in some strains of *E. coli* than others [21]. Thus, the roles that host cells play in determining whether a protein form aggregates is interesting but mostly unknown.

In addition, the experiment conducted to examine the solubility of scIPNS in vitro also suggests the involvement of the host as well as the environmental conditions in affecting the formation of aggregates. The fact that soluble scIPNS expressed at 25°C was transformed into the insoluble form upon incubation in vitro at 37°C and not at 25°C suggests that temperature is the pre-eminent factor involved. Temperature could directly affect the inter- and intra-molecular linkages which prevents the protein from assuming its normal, soluble conformation, thus resulting in insoluble aggregate formation. Or the temperature could indirectly affect the solubility of certain proteins, such as heat shock proteins, proteins involved in binding of prosthetic group or chaperones, which might in turn interact with the native scIPNS protein and render it insoluble. Shoemaker et al. (1985) [25] suggested that some form of inter-protein interaction was involved in aggregate formation. However, it is likely that the in vivo formation of scIPNS protein aggregates in *E. coli* is an irreversible process, as the insoluble scIPNS protein expressed at 37°C remains aggregated after incubation in vitro at 25°C at 37°C. The difference in the solubility of scIPNS at 25°C and 37°C could also be attributed to the dissimilar protein profiles made by the transformed host at the two temperatures, though the difference was not observable from SDS–PAGE. Further investigations are needed to clarify the above notion. The fact that some proteins were lost after incubation in vitro indicated that a proportion of the scIPNS might have undergone degradation by proteases in *E. coli* that were active during incubation at 37°C.

To acquire more information on the degree of involvement of host and environmental factors in the solubilization process, further investigation involving the use of purified scIPNS for incubation in lysates of non-transformed *E. coli* BL21(DE3) cultivated at different temperatures have to be examined.

The increase in hydrophobicity near the N-terminal end of Pro2Ala scIPNS could be responsible for the increased solubility ($\approx$ 10% of total soluble protein) of the Pro2Ala scIPNS protein at 37°C cloned under the T7-promoter as compared to the insoluble protein obtained by Durairaj and colleagues [3] when the wildtype gene was expressed by the same promoter at the same temperature.

According to the 'hydrophobic collapse' model of protein folding, the driving force behind folding is hydrophobic amino acid clustering to avoid water, with the eventual secondary and tertiary structure further stabilized by hydrogen bonding and electrostatic forces [26]. Thus the altered hydrophobicity of Pro2Ala scIPNS might affect the folding process and vary the forces involved, effecting a change in the interaction between residues and thus the resultant mature protein might have a conformation different from the wildtype which probably accounts for the increased solubility.

There are more reports of soluble IPNS cloned from fungal sources than those cloned from bacterial species. However the reason for the greater solubility of the fungal compared to the bacterial IPNS has not been investigated. In 1991, Baldwin et al. [14] obtained different levels of expression from 3 fungal genes, namely the IPNS from *C. acremonium, Penicillium chrysogenum* and *A. nidulans*, when cloned under the same *trc*-promoter (Table 1). The only parameter that was varied in the expression system used was the structure of the IPNS. Thus even within the fungal family, the IPNS gene structures could be very different at the tertiary level which might be critical for solubility, such

that the fungal IPNS are generally more soluble than their bacterial counterparts. If so, the amino acids residing in positions 150 to 200 of scIPNS that have pronounced differences in terms of hydrophobicity and hydrophilicity as compared to the fungal cIPNS, could be targeted for computer modeling and site-directed mutagenesis subsequently carried out to test the effect of biotransformation on protein solubility. Thus, besides the cultivation conditions as well as the expression systems used to manufacture bacterial scIPNS, the gene structure itself seems to play a role in determining whether the protein will form inclusion bodies.

Based on the experimental results (summarized in Table 1) obtained after studying the effects of *cultivation temperature*, *promoter* and *host strains* on the expression of scIPNS proteins in *E. coli*, the following conclusions could be derived: (i) decreasing the cultivation temperature from 37°C to 25°C increases the production of soluble scIPNS from ≈ 10% to ≈ 29% under the control of T7-promoter in *E. coli* BL21(DE3). This shows that temperature does affect the level of expression of soluble protein; (ii) T7-promoter is a powerful promoter that could make significant levels of soluble scIPNS provided optimum working conditions are applied concomitantly; (iii) though *trc*-promoter driven expression of soluble scIPNS is affected by the *E. coli* host strains used, the difference in expression in the two hosts, *E. coli* TG1 and BL21(DE3) is only marginal. Thus it is possible that T7-promoter driven expression of soluble scIPNS could also be affected by the host strain used and the degree to which different strains affect the expression has to be worked out and (iv) Pro2Ala scIPNS protein appears to have an increase in hydrophobicity at the N-terminal end as indicated by both structure analysis programmes used in this study, therefore this change could also contribute to the higher level of soluble protein expressed. Expression of a wildtype gene under the T7-promoter using the same cultivation conditions would indicate whether the amino acid altered has an effect on expression of soluble protein. In conclusion, the improved expression that we are reporting now seems to be due to many factors rather than a single factor.

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