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Purification and characterization of recombinant *Streptomyces clavuligerus* isopenicillin N synthase produced in *Escherichia coli*

M Durairaj and SE Jensen

Department of Biological Sciences, University of Alberta, Edmonton, AB, Canada

Recombinant isopenicillin N synthase from *Streptomyces clavuligerus* was produced in the form of inactive inclusion bodies in *Escherichia coli*. These inclusion bodies were solubilized by treatment with 5 M urea under reducing conditions. Optimization of refolding conditions to recover active isopenicillin N synthase indicated that a dialysis procedure carried out at a protein concentration of about 1.0 mg ml^{-1} gave maximal recovery of active isopenicillin N synthase. Solubilized isopenicillin N synthase of more than 95% purity was obtained by passing this material through a DEAE-Trisacryl ion exchange column. Expression studies conducted at different temperatures indicated that isopenicillin N synthase was produced predominantly in a soluble, active form when expression was conducted at 20°C, and accounted for about 20% of the total soluble protein. This high-level production facilitated the purification of soluble isopenicillin N synthase to near homogeneity in four steps. Characterization of the purified soluble and solubilized isopenicillin N synthase revealed that they are very similar.

Keywords: recombinant isopenicillin N synthase; expression; penicillin; *Streptomyces clavuligerus*

Introduction

β-lactam antibiotics are widely used for the treatment of bacterial infections because of their clinical effectiveness and low toxicity. Penicillin and cephalosporin-type antibiotics are produced by a number of fungal, as well as Gram-positive and Gram-negative bacterial species. Although the fungal and bacterial producer species come from different taxonomic kingdoms, there is considerable similarity in the biochemical pathways used for penicillin and cephalosporin synthesis. In all species studied to date, isopenicillin N synthase (IPNS) catalyses the oxidative cyclization of an acyclic tripeptide δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV) into isopenicillin N [8] and the penicillin nucleus is then modified in different ways, depending on the producer organism, to yield a variety of β-lactam antibiotics [11]. In view of the industrial importance of this enzyme, significant efforts have been directed towards the characterization of IPNS, and the gene encoding IPNS (*pcbC*) was the first β-lactam synthetase-encoding gene to be cloned [19]. However, the location of the active site, the residues involved in the catalysis and the details of the reaction mechanism of IPNS remain unclear, even after years of intensive biochemical and biophysical studies [13,17,20]. Complete characterization of any enzyme requires large amounts of protein, but obtaining these large amounts from *S. clavuligerus* seemed impractical because of the low specific activity of IPNS in cell extracts [10]. Therefore we expressed *pcbC* at a high level in *Escherichia coli* [6]. However, the IPNS protein was produced in the form of insoluble, inactive inclusion bodies (IB), a phenomenon commonly observed for genes expressed at high level in *E. coli* [12]. While solubilization of IPNS could be achieved under reducing conditions in the presence of high concentrations of urea, the recovery of active IPNS was quite low. Therefore, it was necessary to optimize the conditions used for solubilization and renaturation of IB containing IPNS.

Reducing culture temperature can suppress IB formation and favor production of heterologous proteins in a soluble form [5,18,21]. In this study, we manipulated the culture temperature to enhance production of IPNS in a soluble form. Comparison of the purified soluble and solubilized IPNS indicated that both forms of IPNS are similar, suggesting that either soluble IPNS or solubilized IPNS can be used for characterization studies.

Correspondence: Dr SE Jensen, Department of Biological Sciences, University of Alberta, Edmonton, Alberta, Canada T6G 2E9
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Materials and methods

Materials

Purified bis-ACV was generously provided by Saul Wolfe, Simon Fraser University, Burnaby, BC, Canada. N-terminal amino acid sequence analyses were performed by the Alberta Peptide Institute, University of Alberta. DEAE-Trisacryl chromatography resin was from Reactifs IBF, Villeneuve-la-Garenne, France. Sephadex G-25 resin and Superose 12 HR 10/30 and Mono Q HR 5/5 prepacked high-performance liquid chromatography (FPLC) columns were purchased from Pharmacia, Uppsala, Sweden. Vent DNA polymerase was obtained from New England Biolabs, Mississauga, Canada. *Taq* DNA polymerase was obtained from either Boehringer Mannheim, Laval, Canada or Promega Corporation, Wisconsin, USA. All other chemicals were of reagent grade.

Bacterial cells, plasmid constructs and culture conditions

High level expression of *pcbC* was achieved using pMDOP7VW, a plasmid construct identical to pMD0P7 [6] except that the *pcbC* gene was amplified using Vent instead of *Taq* DNA polymerase. The entire *pcbC* gene in pMDOP7VW was sequenced and found to correspond exactly to the *pcbC* gene sequence from *S. clavuligerus*. Expression of *pcbC* in the pMDOP7VW construct is under the control of a T7 promoter which requires T7 RNA polymerase for transcription. The gene encoding T7 RNA polymerase is carried on a second plasmid, pGP1-2, and is under the control of the heat-inducible λP_L promoter. *E. coli* K38 strain and T7 promoter-based expression vectors were kindly provided by S Tabor, Harvard Medical School, Cambridge, MA. All expression studies were carried out in an expression medium consisting of 2% tryptone (Difco, Detroit, MI, USA), 1% yeast extract, 0.5% NaCl, 0.2% glycerol and 50 mM potassium phosphate buffer (pH 7.2), and supplemented with ampicillin at 100 μg ml^{-1} as recommended by S Tabor (personal communication).

Effect of temperature on pcbC gene expression

E. coli K38 cells carrying the pMDOP7VW construct were cultivated in 4 × 100 ml of expression medium in 500-ml flasks at 30°C and 280 rpm until the OD_{600} reached 1.5. The flasks were then transferred to 42°C for the induction of T7 RNA polymerase. After 30 min of induction, each of the four flasks was incubated at a different temperature, 20, 24, 30 or 37°C, for 2 h. Crude cell extracts and IB preparations were prepared from each culture by cell breakage using ultrasonic disruption followed by centrifugation as described in [6].

Solubilization and refolding of IB

One volume of IB suspension was added to four volumes of denaturing solution (6.25 M urea, 50 mM Tris-HCl (pH 7.2), 1.25 mM EDTA and 62.5 mM DTT), incubated for 2 h at 21°C and then desalted by Sephadex G-25 gel filtration chromatography [6]. The desalted extract was assayed to determine protein concentration and IPNS activity. Alternatively, IB suspension was solubilized as described above and then dialyzed against a 100-fold excess of TDEG buffer (50 mM Tris-HCl (pH 7.2), 1 mM DTT, 0.01 mM EDTA and 10% glycerol) for 8 h at 4°C.

Purification of solubilized IPNS

E. coli K38 cells carrying the pMDOP7VW construct were cultivated in 3 L of expression medium at 30°C and 280 rpm until the OD_{600} reached 1.5 in a 5-L (Microferm, New Brunswick Scientific, NJ, USA) fermentor. The aeration rate was maintained at 4 L min^{-1}. The culture was then warmed to 42°C for 30 min followed by further incubation for 2 h at 37°C. The cells were concentrated using a Pellicon Tangential Flow Ultrafiltration System (Millipore, Marlborough, MA, USA), harvested by centrifugation at 10000 × *g* for 10 min and then used to prepare IB suspension.

Ion exchange (DEAE-Trisacryl) chromatography: Crude solubilized IPNS resulting from the refolding of IB material by dialysis was loaded on a DEAE-Trisacryl column (1.6 × 30 cm) equilibrated with starting buffer (200 mM Tris-HCl (pH 8.0), 0.01 mM EDTA, 1 mM DTT and 10% glycerol) and eluted with a linear Tris-HCl gradient consisting of 250 ml each of starting buffer versus limiting buffer (300 mM Tris-HCl (pH 8.0), 0.01 mM EDTA, 1 mM DTT and 10% glycerol). Protein elution from the column was monitored using a LKB 2238 UVICORD SII detector (Pharmacia, Uppsala, Sweden) at 280 nm and a LKB 2210 1-channel recorder. Five-millilitre (100 drop) fractions were collected and assayed for IPNS activity and characterized by SDS-PAGE analysis.

Purification of soluble IPNS

E. coli K38 cells carrying the pMDOP7VW construct were cultivated exactly as described for the production of solubilized IPNS except that after the 42°C heat induction the culture was incubated at 20°C for 2 h. Harvested cells were used to prepare crude cell extract containing soluble IPNS as described above.

Fractionation by ammonium sulfate precipitation: Solid ammonium sulfate was added gradually to 8 ml of crude extract at 4°C with stirring, and material precipitating between 50 and 70% saturation was collected by centrifugation at 17000 × *g* for 30 min. The pellet was suspended in 8 ml of TDEG buffer and dialyzed overnight against a 100-fold excess of TDEG buffer.

Ion-exchange (DEAE-Trisacryl) chromatography: Dialyzed material resulting from ammonium sulfate fractionation was loaded onto a DEAE-Trisacryl column (1.6 × 30 cm) and eluted as described earlier for solubilized IPNS. Fractions displaying IPNS activity were pooled and concentrated by ultrafiltration using an Amicon PM 10 membrane (Danvers, MA, USA) to 1.20 of their original volume.

Gel filtration (Superose 12 HR 10/30) chromatography: Concentrated IPNS containing material from the DEAE-Trisacryl column was applied repeatedly in 0.25-ml portions to a Superose 12 column equilibrated with TDEK buffer (100 mM Tris-HCl (pH 7.5), 1 mM DTT and 0.01 mM EDTA and 100 mM KCl), and eluted at a flow rate of 0.5 ml min^{-1}. The column was attached to a Pharmacia FPLC system with the following components: two P-500 pumps, a P-1 peristaltic pump, a UV-M monitor, a LCC-500 liquid chromatography controller and a Frac-100 fraction collector. Two-minute (1.0 ml) fractions were collected and assayed for IPNS activity.

Ion-exchange (Mono Q HR 5/5) chromatography: Fractions containing IPNS activity from the Superose purification step were pooled, concentrated by ultrafiltration and applied repeatedly in 0.3-ml portions to a Mono Q HR 5/5 column equilibrated with TDEK buffer. After each sample application, the column was washed for 5 min using TDEK buffer and then IPNS was eluted at a flow rate of 0.5 ml min^{-1} using a 30-min linear gradient with TDEK containing 200 mM KCl as the limiting buffer. IPNS-containing fractions were pooled, concentrated by ultrafiltration using a disposable filtration cell (Filtron, Sin-Can Inc, Calgary, Canada) with a molecular weight cut off of 10000 Da, and dialyzed against a 100-fold excess of TDE buffer (TDEK buffer with no KCl) before being stored at −80°C.

SDS-PAGE analysis

Protein samples from various stages of the isolation and purification procedures were analyzed by electrophoresis on 10% polyacrylamide gels containing 0.1% sodium dodecyl sulfate (SDS-PAGE) as described by Blackshear [3]. Chymotrypsinogen A (25000), ovalbumin (45000) and bovine serum albumin (67000) were used as molecular weight markers.

Protein assay

Protein concentrations were estimated by the dye binding method of Bradford [4] as recommended by BioRad (Richmond, USA) using bovine γ globulin as standard.

Isopenicillin N synthase assay

IPNS activity was determined by a reverse-phase high performance liquid chromatography procedure as described previously [9]. One unit of IPNS activity is defined as that amount of enzyme which produces 1 μmol of isopenicillin N min^{-1}.

Results and discussion

Recovery of active IPNS from inclusion bodies

Obtaining active IPNS protein from IB required disaggregation and denaturation of the IB by treatment with 5 M urea, and then removal of the denaturant by passage through a Sephadex G-25 column allowing refolding of the solubilized protein. Active IPNS protein with a specific activity of approximately 80×10^{-3} units mg^{-1} protein was recovered [6]. Purification of this solubilized IPNS material by DEAE-Trisacryl chromatography gave preparations with high specific activity, but the recovery of total IPNS was poor. When the concentration of Tris-HCl in the limiting buffer was increased to 500 mM, more IPNS protein was recovered but it was inactive and resulted in a decrease in the overall specific activity of the purified IPNS. Since the recovered IPNS was essentially pure as judged by SDS-PAGE (Figure 1, lane 2), the refolding process was assumed to have resulted in a mixture of active, properly folded IPNS and inactive, improperly folded IPNS which eluted at slightly higher ionic strength than the properly folded material.

In studies aimed at improving the recovery of active, properly folded IPNS, IB suspensions with an initial protein concentration ranging from 0.2 to 12.1 mg ml^{-1} were treated with denaturant and then renatured either by passing them through a Sephadex G-25 column or by dialysis. The amount of active IPNS recovered increased as the protein concentration was reduced from 12.1 mg ml^{-1} to 1 mg ml^{-1}, and recovery of active IPNS was consistently higher using the dialysis procedure than the gel filtration procedure (data not shown). No further improvement in recovery of active IPNS was noted as protein concentrations were reduced from 1 mg ml^{-1} to 0.2 mg ml^{-1}. At an initial protein concentration of 1 mg ml^{-1}, the amount of activity recovered when IPNS was refolded by dialysis was approximately 50% higher than when refolding was done by the gel filtration method. These results showed that both protein concentration and the method of refolding affect the yield of active IPNS protein.

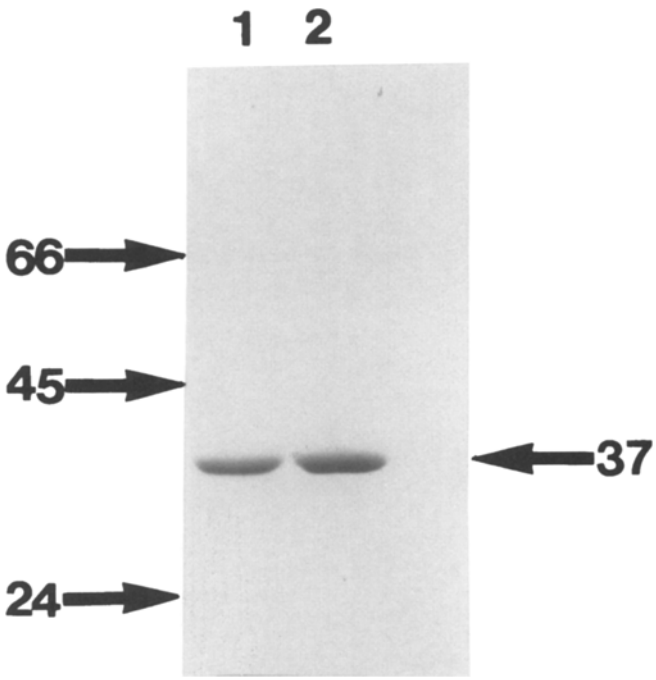

Figure 1 SDS-PAGE analysis of samples from the purification of solubilized IPNS. Lane 1 contained 10 μg of protein from the particulate fraction (after solubilization). Lane 2 contained 15 μg of protein from the concentrated fractions of DEAE-Trisacryl ion-exchange chromatography. The positions of molecular weight marker proteins, bovine serum albumin (66000), ovalbumin (45000) and trypsinogen (24000) are indicated with arrows.

Proteins tend to fold in characterized intermediate stages called 'molten globule intermediates' [7,14]. Under favorable conditions, molten globule intermediates follow a constructive folding pathway leading to fully functional structures. However, under unfavorable, non-physiological conditions such as high concentrations of protein, these intermediates are trapped, leading to IB formation. It is likely that in the gel filtration procedure, the high localized concentrations of unfolded protein together with concomitant rapid removal of urea, facilitated aggregate formation rather than proper folding. In the dialysis procedure, the more gradual removal of urea enhanced the recovery of active protein.

As a result of the various refolding studies, a 120% improvement was observed in the activity of IPNS in crude solubilized preparations, from an initial specific activity of 80×10^{-3} units mg^{-1} protein for protein refolded by gel filtration chromatography to a final value of 172×10^{-3} units mg^{-1} protein for protein refolded by dialysis at optimum protein concentrations. Based on these studies, the dialysis method was chosen for future studies using an initial protein concentration of about 1 mg ml^{-1}.

Production of soluble IPNS

Assay of IPNS activity in crude extracts prepared from cultures grown at different temperatures after induction of *pcbC* gene expression, indicated that as the temperature was lowered, soluble IPNS activity gradually increased (Figure 2). This temperature-dependent solubility was most dramatic when expression at 20°C was compared with that at

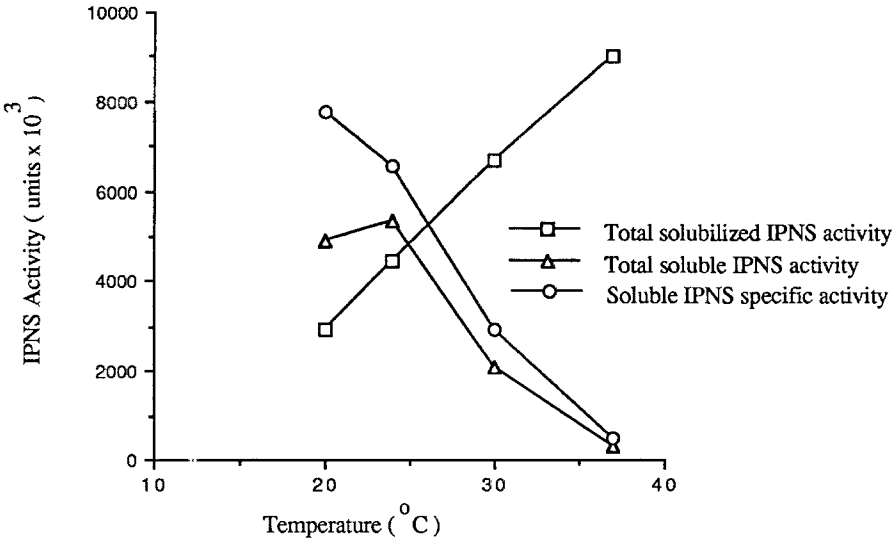

Figure 2 Effect of growth temperature on the solubility of IPNS. Cultures incubated at four different temperatures after induction were used to prepare cell extracts and solubilized IB preparations. Specific activities are expressed per 100 mg of protein to enable comparison on a single axis.

37°C. SDS-PAGE analysis of the soluble crude extract and the insoluble IB pellet material agreed with the activity data (Figure 3). It was estimated by SDS-PAGE that nearly 70% of the IPNS protein was present in the soluble form at 20°C while more than 90% of the IPNS was present in IB at 37°C. Although total soluble IPNS activity observed at 20°C was low compared to the total solubilized IPNS obtained at 37°C, this could be explained by the fact that growth slowed considerably after cultures were transferred to 20°C. At the time of harvest, expression cultures incubated at 37°C had an OD_{600} of approximately 4, while the OD_{600} of expression cultures incubated at 20°C was around 2.5.

Expression of heterologous genes in *E. coli* at low temperature can favor protein production in a soluble form although not all proteins respond in this way [21]. In general, the stronger the promoter used to drive expression, the greater the likelihood that the protein will be produced in an insoluble, inactive form. Expression of the *pcbC* gene from *Aspergillus nidulans* using the λP_L promoter resulted in IB formation (10–15% of total protein) while expression using the *lac* promoter resulted in soluble IPNS formation (40% of total soluble protein) [2]. The T7 promoter is much stronger than the *lac* promoter, and the T7 RNA polymerase is very processive for transcription. *pcbC* genes from *S. clavuligerus*, *Streptomyces jumonjinensis* and

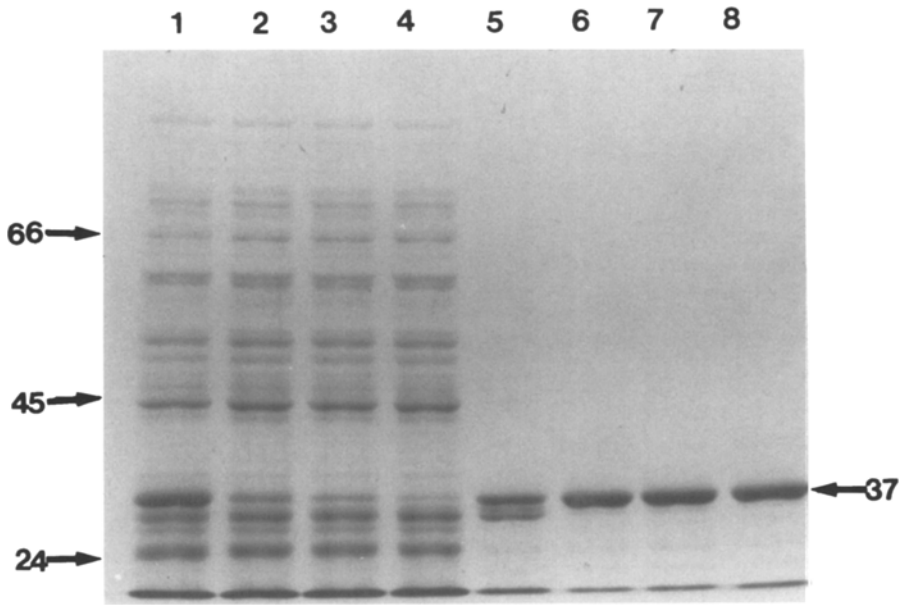

Figure 3 SDS-PAGE analysis of IPNS preparations from *E. coli* carrying pMDOP7VW after production at different temperatures. Lanes 1 through 4 contained 30 μg of protein from the soluble fraction of cell extracts derived from cultures incubated at 20, 35, 30, or 37°C. Lanes 5 through 8 contained 15 μg of protein from the particulate fraction (after solubilization) from cultures incubated at 20, 25, 30, and 37°C. The positions of molecular weight marker proteins bovine serum albumin (66000), ovalbumin (45000) and trypsinogen (24000) are indicated with arrows.

Flavobacterium spp all result in IB formation when expressed using the T7 promoter [6,15]. In this study, growth at low temperatures may slow the rate of transcription from the strong T7 promoter, leading to production of IPNS in a soluble form.

Purification of IPNS

Purification of solubilized IPNS: Solubilized IPNS was purified by chromatography on DEAE Trisacryl in Tris-HCl buffer, using conditions similar to those described for purification of native IPNS from *S. clavuligerus* [10]. Optimal recovery with maximum purity was obtained using a gradient varying from 0.2 to 0.3 M Tris-HCl (pH 8.0) with 250 ml each of starting and limiting buffer, and a column with dimensions of 1.6 × 30 cm. Under these elution conditions, 73% of the IPNS activity was recovered with a specific activity of 240.6 × 10^{-3} units mg^{-1} protein (Table 1).

IB formation provides an advantage during purification of recombinant proteins. Isolation of IB pellets after repeated sonication and washing to remove associated cell debris typically gives rise to material that is more than 50% recombinant protein. In our studies, based on the intensity of the IPNS protein band compared to the contaminants on SDS-PAGE gels, it appeared that IB were more than 90% pure prior to solubilization (Figure 1, lane 1). However, IB formation may not be desirable if the expressed protein fails to refold to give active material following solubilization. In the case of the *S. jumonjinensis pcbC* gene, although expression levels were good (more than 50% of total cell protein), the recovery of active IPNS protein was only 3–4% due to inefficient refolding [15]. In the present study, the yield of solubilized IPNS was estimated to be 250 mg of IPNS protein per L of culture when expressed at 37°C, which is much higher than our earlier reported estimate [6].

Purification of soluble IPNS: Production of IPNS in a soluble form eliminates the need for refolding. The low temperature procedure was particularly suitable for this purpose, because it allowed the same expression system and genetic background to be employed for production of both solubilized and soluble IPNS, making it easier to compare the two forms of IPNS. This study is the first report of high-level expression of a *pcbC* gene from a *Streptomyces* spp to yield soluble IPNS protein. We estimate that the level of production of soluble IPNS at 20°C was around 20% of the total soluble protein.

The purification protocol used by Jensen *et al* [10] was simplified for the purification of soluble IPNS. The steps involved precipitation with ammonium sulfate, ion-exchange chromatography using DEAE-Trisacryl, size-exclusion chromatography using Superose 12 and ion exchange chromatography using Mono Q. The IPNS specific activity of the crude extract obtained after expression at 20°C was 22.9 × 10^{-3} units per mg protein which is 15-fold higher than the activity seen in *S. clavuligerus* crude cell extracts. As a result of this higher starting specific activity, only a 10-fold purification was required to attain a near homogeneous preparation, and milligrams of IPNS protein could readily be obtained. DEAE-Trisacryl anion exchange chromatography removed the majority of contaminating proteins. Concentrated material from the ion exchange column was then passed through a Superose 12 column to remove several contaminating proteins larger than the IPNS protein. However, the IPNS material obtained from the Superose 12 step still contained low molecular weight contaminants which required chromatography on a Mono Q column to obtain homogeneous IPNS material. The results are summarized in Table 2, and SDS-PAGE analysis of samples from the various purification stages is shown in Figure 4.

Characterization of IPNS

The similarities between the specific activities of purified solubilized IPNS (241 × 10^{-3} units per mg protein) and soluble IPNS (244 × 10^{-3} units per mg protein) indicated that major differences between the two IPNS preparations were unlikely. Comparison of the conformation of the solubilized and soluble IPNS by far- and near-ultraviolet circular dichroism (CD) studies also indicated that they are very similar (data not shown). Therefore, either form should be equally suitable for characterization studies, making it possible to use the more readily purified solubilized IPNS. Crystallization studies of recombinant, solubilized human hemoglobin showed this material to be identical to native hemoglobin in its structure [16] indicating that the solubilization process did not introduce any observable or detrimental defects in the structure of the recombinant protein.

However N-terminal sequence analysis of the solubilized and soluble IPNS suggested that the initiator methionine was not removed in the case of the soluble IPNS while the solubilized IPNS contained correctly processed IPNS. This interpretation was based on the observation that no sequence information was obtained for the soluble IPNS preparation, suggesting that the N-terminus was blocked. However, blocked N-termini can result from other non-biological modifications occurring during the purification process, and so it is not possible to say unequivocally that the N-terminal methionine is still present. It was also not clear whether the solubilized IPNS preparation might contain some non-processed IPNS, since the sequence analysis procedure was not quantitative. In general, it is observed that the N-terminal residues are not properly processed and not removed in proteins which are produced at high level in *E. coli*. However, since the specific activities for both

Table 1 Purification of solubilized IPNS

Purification step	IPNS activity (units × 10^3)	Protein (mg)	IPNS specific activity (units × 10^3 per mg protein)	Recovery (%)
Crude extract	2943	16.1	182.7	100
DEAE-Trisacryl eluate	2139	8.9	240.6	72.6

Table 2 Purification of soluble IPNS

Purification step	IPNS activity (units × 10^3)	Protein (mg)	IPNS specific activity (units × 10^3 per mg protein)	Recovery (%)
Crude extract	4856	211.2	22.9	100
$(NH_4)_2SO_4$ precipitate	2377	102.0	23.3	48.9
DEAE-Trisacryl eluate	2070	12.9	160.3	42.6
Superose 12 eluate	1664	7.5	221.5	34.3
Mono Q eluate	1201	4.9	243.9	24.8

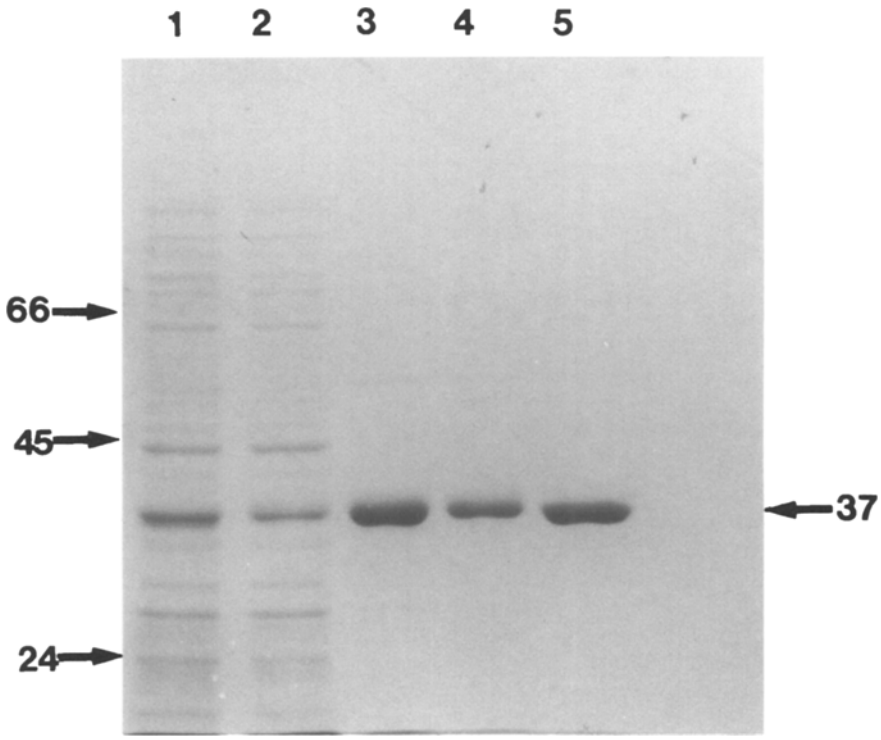

Figure 4 SDS-PAGE analysis of samples from the purification of soluble IPNS purification. Lane 1 contained 50 μg of protein from the crude extract. Lane 2 contained 30 μg of protein from the $(NH_4)_2SO_4$ fractional precipitation step. Lane 3 contained 20 μg of protein from the concentrated fraction of DEAE-Trisacryl ion exchange chromatography. Lane 4 contained 10 μg of protein from the concentrated fraction of Superose 12 gel filtration chromatography. Lane 5 contained 15 μg of protein from the Mono Q ion-exchange chromatography. The positions of molecular weight marker proteins bovine serum albumin (66000), ovalbumin (45000) and trypsinogen (24000) are indicated with arrows.

soluble and solubilized recombinant IPNS were as high or even higher than the activity of native IPNS from *S. clavuligerus* (204 × 10^{-3} units per mg protein), the N-terminal methionine residues, if present, did not decrease the activity of the recombinant IPNS. Similar findings have been reported from other studies on high level production of IPNS [1].

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