N-terminal amino acid sequence and some properties of isopenicillin-N synthetase from *Cephalosporium acremonium*

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Isopenicillin-N synthetase (IPNS) was purified to homogeneity from *Cephalosporium acremonium* C0728. The enzyme existed in two states during purification; an oxidised state with a disulphide linkage and its reduced state. These two forms can be interconverted in the presence or absence of thiol agents, and separated by fast protein liquid chromatography (FPLC) with the strong anion exchange Mono-Q column. The enzyme is a monomer with a molecular mass of 38 kDa and p*I* 5.05. The first 50 amino acid N-terminal sequence of the enzyme was determined. The purified enzyme has an absolute requirement of Fe^{2+} and a 2-electron donor for activity.

Cephalosporium *Isopenicillin-N synthetase* *Amino acid sequence*

1. INTRODUCTION

IPNS catalyses the cyclization of the tripeptide δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine to isopenicillin-N in the presence of oxygen, Fe^{2+} and ascorbate [1]. Formation of isopenicillin-N in this reaction is a step common to the biosynthesis of all penicillin and cephalosporin antibiotics. This enzyme has been partially purified from the β-lactam antibiotic producing microorganisms *Cephalosporium acremonium, Penicillium chrysogenum* and *Streptomyces clavuligerus* [2,3,8]. Here, we report for the first time the appearance of two states of the enzyme due to reduction and formation of an intramolecular disulphide bond, and its N-terminal amino acid sequence of 50 residues. Preliminary characterisation and some properties of the enzyme are also reported.

Abbreviations: IPNS, isopenicillin-N synthetase; FPLC, fast protein liquid chromatography; SDS-PAGE, SDS-polyacrylamide gel electrophoresis

2. MATERIALS AND METHODS

δ-(L-α-Aminoadipyl)-L-cysteinyl-D-valine was prepared according to Baldwin et al. [4]. *C. acremonium* C0728 was a gift from Glaxo, Ulverston, England. Mono Q 5/5 anion exchange and Superose 12 columns were obtained from Pharmacia, Uppsala. All other chemicals were of the highest purity available. Glass distilled water was used throughout.

The growth of *C. acremonium* and initial stages of purification were carried out in a similar procedure according to [2] with several modifications. The phenyl-Sepharose step was substituted by gel filtration with a Sephadex G-75 column because of low recovery of enzyme activity after the hydrophobic chromatography (< 50%). Tris-HCl buffer (50 mM, pH 7.7) was used throughout. In the final stage, the desalted enzyme obtained after DEAE-Sepharose was mixed with 10 mM 2-mercaptoethanol for 10 min at 4°C before 4 mg of the protein sample was loaded onto a Mono Q 5/5 strong

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anion exchange column for FPLC (Pharmacia). IPNS was eluted under a linear gradient of NaCl (80–150 mM) in 20 mM Tris-HCl buffer (pH 8.0). Enzyme activity was assayed in 50 mM Tris-HCl (pH 7.7) according to [2], and the purified enzyme sample was assayed by a couple-enzyme assay method described in [5].

Automated Edman degradation was performed in a Beckman 890C sequencer according to Christie and Gagnon [6], and the amino acid composition analysis was carried out with an LKB 4400 (LKB Biochrom, Sweden) automated amino acid analyser. SDS-PAGE was performed as described by Laemmli [7] on 14% acrylamide gel slabs and isoelectric focusing was carried out as in [2].

3. RESULTS AND DISCUSSION

In the final stage of purification with FPLC, we found that most of the enzyme activity co-ran with 2 major contaminants in an earlier peak before preincubation with thiol reagents. However, after treatment with 2-mercaptoethanol the IPNS was eluted in a later peak and separated from the major contaminants (fig.1). This purified protein was judged to be homogeneous by SDS-PAGE and isoelectric focusing (figs 2,3). It has a molecular mass of 45 kDa by gel filtration analysis and 38 kDa by SDS-PAGE. Isoelectric focusing shows that it has a p*I* of 5.05.

The observation of 2, chromatographically distinct, forms of the same enzyme was probably due to the reduction of an intramolecular disulphide bond since both forms have the same molecular mass (≅45 kDa) when determined by FPLC-Superose gel filtration analysis against molecular mass markers. The reduced form of the enzyme was converted back to the oxidised form during storage at 4°C as judged by the elution profile on FPLC. When IPNS freshly prepared in the re-

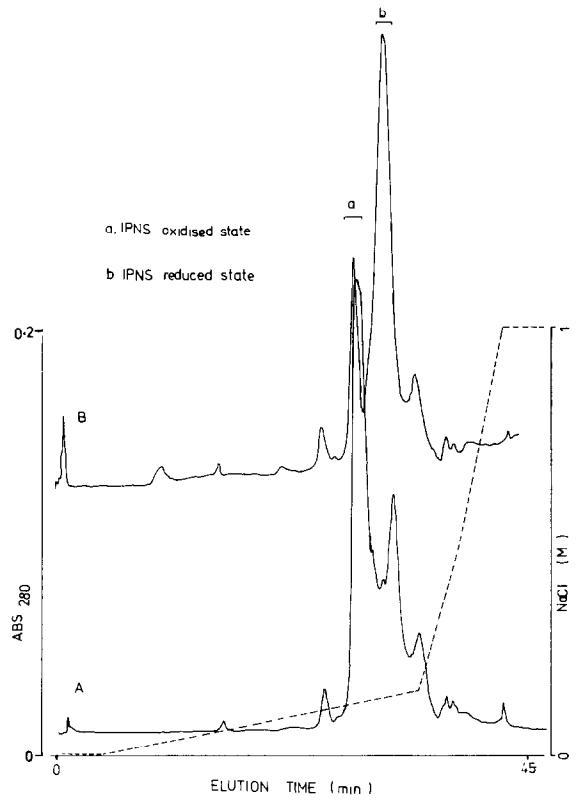

Fig.1. FPLC of IPNS: (A) before treatment with 10 mM mercaptoethanol and (B) after treatment with 10 mM mercaptoethanol. (——) Absorbance at 280 nM, (– – –) NaCl gradient. For details see text.

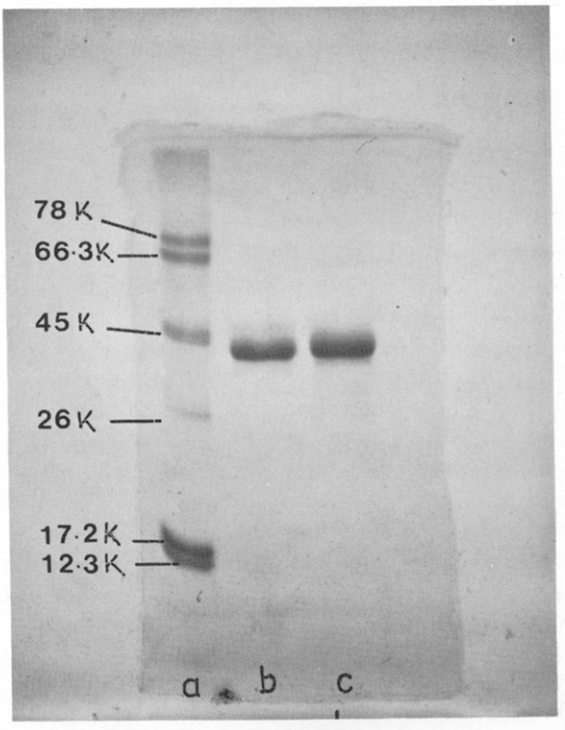

Fig.2. SDS-PAGE of IPNS; (a) molecular mass markers, (b) oxidised form and (c) reduced form. The oxidised form was obtained by FPLC of the reduced form which had been left at 4°C overnight.

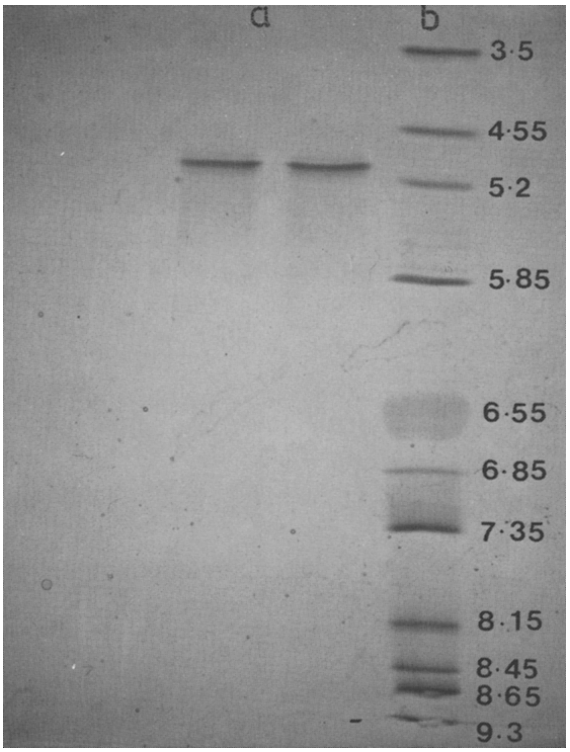

Fig.3. Isoelectric focusing of (a) IPNS and (b) Pharmacia protein p*I* markers in the range p*I* 3–10.

duced form was titrated with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) it gave 2 equivalents of -SH groups under native conditions and a value of 2.6 in the presence of 6 M guanidinium chloride. The oxidised form of the enzyme obtained by FPLC after storage at 4°C overnight in air did not show a titratable thiol group under non-denaturing conditions, and gave result of 0.8 equivalent of -SH when titrated in the presence of 6 M guanidinium chloride. This further substantiates the view that an easily accessible intramolecular disulphide bond is the difference between these two forms of the enzyme.

Table 1

Effect of some common 2-electron donors on IPNS activity

2-electron donors	Relative activity (%)
None	0
Ascorbic acid	100
D-Isoascorbic acid	120
Alloxan	0
Dihydroxyfumaric acid	25
2,4,5-Triamino-6-hydroxy-pyrimidine	0

All were tested at 1 mM with 2.73×10^{-2} IU of enzyme by bioassay

Table 2

Amino acid composition of IPNS (mol/mol enzyme, M_r = 38000)

Amino Acids	A	B
Cys	–	3
Asp	36	36
Thr	15	14
Ser	15	17
Glu	31	32
Pro	23	22
Gly	28	19
Ala	29	26
Val	22	21
Met	4	4
Ile	15	16
Leu	24	25
Tyr	15	8
Phe	15	15
His	8	10
Lys	20	19
Arg	15	19

(A) Results from acid hydrolysis alone and (B) results obtained after performic acid oxidation and then acid hydrolysis

The purified IPNS has an absorption maximum at 278 nm with a shoulder at 290 nm. The absorbance ratio of 280/260 nm is 1.98. It has no absorption in the spectral region 320–900 nm. This enzyme has an absolute requirement of Fe^{2+} for activity. It also requires ascorbate for activity. Dihydroxyfumarate could replace ascorbate with 25% efficiency at the same concentration. Other common 2-electron donors have no effect when tested under the same conditions (table 1). The specific activity of the purified IPNS obtained here was 2.73 IU/mg protein when determined by bioassay. The purified enzyme is stable at −70°C in freeze-dried form and unstable when kept at 4°C in 20 mM Tris-HCl buffer (pH 8.0).

The results of the amino acid composition analysis of the homogeneous IPNS with and without performic acid oxidative pretreatment are

Table 3

N-terminal amino acid sequence of IPNS from *Cephalosporium acremonium* C0728

Ser-Val-Pro-Val-Pro-Val-Ala-Asn-Val-Pro-__-Ile-Asp-Val-Ser-Pro-Leu-Phe-Gly-Asp-Thr-Lys-Glu-Lys-__-Leu-Glu-Val-Ala-__-Ala-Ile-Asp-Ala-Ala-Ser-__-__-Thr-Gly-Phe-Phe-Tyr-Ala-Val-Asn-__-Gly-Val-Asp

shown in table 2. The first 50 N-terminal amino acid sequence is shown in table 3. The purified IPNS and its amino acid sequence reported here has been supplied to other groups for raising monoclonal antibodies and cloning of the IPNS gene.

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