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Expression of ribonucleolytic toxin restrictocin in *Escherichia coli*: purification and characterization

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Abstract Restrictocin is a toxin produced by the fungus *Aspergillus restrictus*. The DNA coding for restrictocin was isolated from the host by polymerase chain reaction and cloned into a T7 promoter-based expression vector. The protein was overproduced in *Escherichia coli* and remained insoluble in the cell in the form of inclusion bodies. Recombinant restrictocin was purified in large amounts, by a simple denaturation–renaturation protocol involving a redox system, with typical yields of 45 mg/l of original culture. Restrictocin could be secreted into the bacterial medium using ompA, *pelB* and LTB signal sequences. Among the three signal sequences, ompA was found to be the most efficient in secreting the recombinant protein. The protein secreted into the extracellular medium was properly processed as evident by the amino-terminal sequencing. Recombinant restrictocin was readily purified to homogeneity from either the medium or inclusion bodies by simple chromatographic techniques and was found to be functionally as active as the native fungal protein in inhibiting the eukaryotic translation.

Key words: Ribotoxin; Expression; Recombinant protein; Signal sequence; Secretion

1. Introduction

The ribonucleolytic toxin restrictocin belongs to a class of fungal ribotoxins that are extremely potent inhibitors of eukaryotic protein synthesis [1]. Other members of the ribotoxin family include α-sarcin and mitogillin [1,2]. Ribotoxins cleave a single phosphodiester bond on the 3′ side of G4325 in a universal, 14-nucleotide, purine-rich sequence in the 28S rRNA, thereby rendering eukaryotic ribosomes defective in elongation factor-1 (EF-1)-dependent binding of aminoacyl tRNA, and in EF-2 catalyzed GTP hydrolysis and translocation [3]. There are nearly 7000 nucleotides in mammalian ribosomes; the toxin, however, catalyzes only a single covalent modification that inactivates the ribosomes and is entirely responsible for the toxicity [4]. α-Sarcin has been shown to possess anti-tumor activity [5]. The potent cytotoxic activity of the ribotoxins make them suitable molecules to be used in the construction of immunotoxins for targeted therapy of cancer and other diseases. Though restrictocin can be purified from the filtrate of *A. restrictus*, it is not desirable to handle the fungus in view of the fact that it is thought to be the causative agent of aspergillosis [6]. Earlier gene and cDNA coding for restrictocin have been expressed in *Aspergillus nidulans* and *Saccharomyces cerevisiae*, respectively, to study the role of leader peptide on the survival of the host [7,8]. Ribotoxins, α-sarcin and mitogillin have been expressed earlier using ompA and *pelB* signal sequences but the protein levels obtained were low [9–13]. *Asp f I*, a homologue of mitogillin has also been expressed in *E. coli* but the recombinant protein was 10-fold less active than the native toxin [14]. The aim of the present study was to produce authentic and functionally active ribotoxin restrictocin in large amounts in *E. coli*. The recombinant toxin could be employed in the construction of immunotoxins, and also could be used as a tool to study the eukaryotic translation machinery by investigating the interaction of ribotoxins with ribosomes.

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2. Materials and methods

Enzymes and cell culture reagents were purchased from New England Biolabs and GIBCO-BRL, respectively. Low-melting-point agarose (Sea Plaque) was supplied by FMC Corp. [^{3}H]Leucine was obtained from Amersham. Reagents for in vitro translation assay were from Promega and Pharmacia. All other reagents were of analytical grade and obtained from Sigma. *A. restrictus* strain 34475 was obtained from ATCC.

2.1. Construction of plasmids

DNA coding for restrictocin was amplified by polymerase chain reaction (PCR) using genomic DNA purified from the host, *A. restrictus* as template. Published sequence of *restrictocin* gene was used for designing primers for PCR [7]. Various plasmids constructed for the current study are shown in Fig. 1.

The PCR-amplified fragment of restrictocin was cloned as an *Nde*I–*Eco*RI fragment in an *E. coli* expression vector pVex11 which contains phage T7 promoter and transcription terminator. The resulting plasmid, pRest, contains restrictocin under the control of T7 promoter without any signal sequence. To generate secretion vectors, appropriate restriction sites were created by PCR on both ends of DNA coding for restrictocin and the amplified fragment after digestion cloned into vectors containing the required signal sequence as described below. To make pRest1, where restrictocin DNA is fused to that for the bacterial ompA signal sequence, restrictocin was cloned as an *Nde*I–*Eco*RI fragment in the vector pVex115 which contains T7 promoter and ompA signal sequence. For making pRest2 containing restrictocin fused to the *pelB* signal sequence, DNA for restrictocin was cloned as an *Nhe*I–*Eco*RI fragment into the vector pVNLSPA-AP0216 [15] which contains a T7 promoter and *pelB* signal sequence. To fuse restrictocin to the signal sequence, it was cloned as a *Sac*I–*Hin*dIII fragment in the vector pMMB68 [16] containing tac promoter and LTB signal sequence giving rise to the plasmid pRest3.

2.2. Expression and purification of restrictocin

E. coli strain BL21 (lDE3) was used for expression with all the vectors except pRest3, for which DH5α cells were used. For the purification of restrictocin from inclusion bodies, transformed cells were grown in super broth and induced at an OD_{600} of 2.0, with 1 mM IPTG for 2 h. Inclusion bodies were denatured in guanidine hydrochloride and reduced by dithioerythritol (DTE), followed by renaturation in refolding buffer containing arginine and oxidized glutathione [17]. Renatured material, after dialysis, was loaded on a S-Sepharose column (Pharmacia), equilibrated with 20 mM MES, pH 5.0. In cases where the recombinant protein was secreted, medium or periplasm were loaded onto a S-Sepharose column directly after adjusting the pH to 5.0 using HCl. Restrictocin was eluted with a linear gradient of

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0–1 M NaCl in 20 mM MES, pH 5.0, using an FPLC system (Pharmacia). The S-Sepharose pool was further purified by gel filtration chromatography on a TSK 3000 column (LKB), if indicated.

2.3. Spectroscopic characterization

CD spectra were obtained on a JASCO J710 spectropolarimeter in the far-UV range at 25°C. The proteins were dissolved in 10 mM sodium phosphate buffer, pH 7.0.

2.4. Assay of functional activity of recombinant restrictocin

The activity of restrictocin was evaluated in a rabbit reticulocyte lysate based in vitro translation assay system. Rabbit reticulocyte lysate was prepared and the assay performed as described [18]. Ribonuclease activity of restrictocin was tested on yeast tRNA and synthetic Poly(A) and Poly(G) homopolynucleotides as described [19].

3. Results and discussion

3.1. Construction of plasmids

In order to produce restrictocin by recombinant means, we have cloned a cDNA encoding restrictocin in a T7 promoter-based *E. coli* expression vector (Fig. 1). The sequence of restrictocin in pRest matched perfectly with the published sequence. Restrictocin has also been cloned as fusion with ompA, *pelB* or LTB signal sequence respectively in pRest1, pRest2 and pRest3 (Fig. 1).

3.2. Production and purification of restrictocin

Restrictocin in bacterial cells transformed with pRest, which does not contain a signal sequence, was overexpressed as seen in the total cell pellet and remained in the spheroplast as insoluble inclusion bodies (Fig. 2A). Inclusion bodies were solubalized using guanidine hydrochloride and reduced by adding DTE. After renaturation, in the presence of oxidized glutathione, restrictocin could be purified to homogeneity by successive chromatography on cation exchange and gel filtration columns (Fig. 2A). The protein after the cation exchange chromatography was nearly homogenous as from the subsequent gel filtration column a single homogenous protein peak was obtained. For all further studies, therefore, protein was purified by a single-step purification on a cation exchange column. Although restrictocin can be obtained easily in large amounts from the inclusion bodies, we attempted to secrete restrictocin to compare the activity of recombinant protein refolded from the insoluble aggregates to that of the secreted protein. All three signal sequences used in the present study, directed the overexpressed protein to the medium, albeit, with different efficiencies. In addition, with *pelB* signal sequence

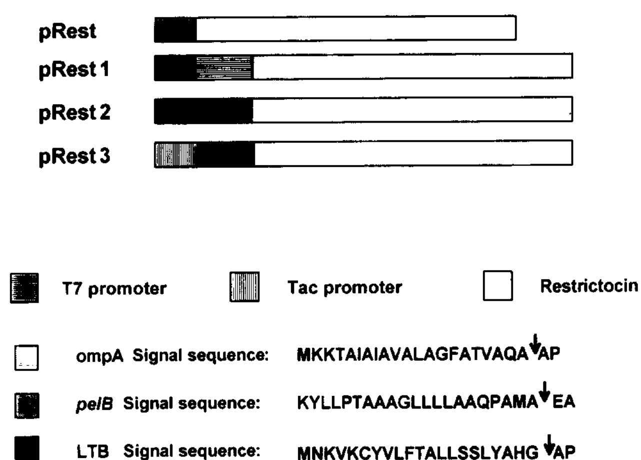

Fig. 1. Schematic representation of different constructs of restrictocin. In pRest1, pRest2 and pRest3 restrictocin has been cloned behind ompA, *pelB* and LTB signal sequence, respectively. pRest does not contain any signal sequence.

restrictocin was also present in the periplasmic space. The secretion of restrictocin into the medium with LTB signal sequence was much lower as compared to that using ompA and *pelB* signal sequences as shown on a quantitative western blot (Fig. 2B). Restrictocin was purified to homogeneity from the medium and periplasm on a single cation exchange column. The comparative yields of purified protein from different sources are shown in Table 1. The yield was highest when restrictocin was purified from the inclusion bodies, i.e., 45 mg/l. Among the various signal sequences used, ompA was found to be most efficient in secreting restrictocin and 4 mg of pure protein was obtained from a liter of culture. In the present investigation, the yields with ompA signal sequence are higher whereas those with *pelB* signal sequence are the same as reported by others [9,13].

3.3. Characterization of recombinant restrictocin

3.3.1. Amino terminal sequencing

Purified restrictocin from different sources was sequenced to check the authenticity of the protein. The amino-terminal sequence of restrictocin from the inclusion bodies was found to be ATWTCINQQLNPKTNKWEDK which matched perfectly with the published protein sequence [2]. The N-terminal sequences of soluble restrictocin purified from the culture medium of cells transformed with the plasmids with ompA,

Table 1
Yield and activity of recombinant restrictocin produced in *E. coli*

Toxin	Signal sequence	Yield	Activity	
(source)		(mg/l)	ID50 (pM)	% Native
Native Restrictocin	–	7.0	35	100
rRestrictocin (IB)	None	45.0	35	100
rRestrictocin (M)	ompA	4.0	35	100
rRestrictocin (M)	*pelB*	0.4	35	100
rRestrictocin (P)	*pelB*	1.0	350	10
rRestrictocin (M)	LTB	0.3	35	100
Ricin A chain	–	–	33	–

Toxins were tested for their protein synthesis inhibitory activity in a cell-free translation assay, containing rabbit reticulocyte lysate. Rabbit reticulocyte lysate was prepared and the assay performed as described [18]. Serial dilutions of toxin were tested over a range of 0.05–500 ng/ml. Incorporation of [^{3}H]leucine was measured as a function of toxin concentration. ID_{50} is the concentration of toxin required to inhibit protein synthesis by 50% as compared to a control where no toxin was added. Activities of recombinant restrictocin from different sources were compared. M, medium; IB, inclusion bodies; P, periplasm.

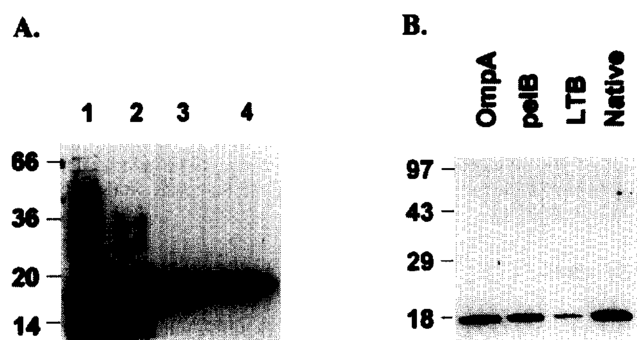

Fig. 2. Purification of recombinant restrictocin. (A) pRest was used to transform BL21(λDE3). A 12% SDS-polyacrylamide gel was run and stained with Coomassie Blue. Lane 1, total cell pellet; lane 2, inclusion bodies; lane 3, protein after S-Sepharose column; lane 4, purified protein after gel filtration. (B) An immunoblot of a 12% SDS-polyacrylamide gel where equal volumes of culture supernatants were loaded from different cultures grown to equal absorbence. Restrictocin was detected using polyclonal anti-restrictocin antibodies raised in rabbit. Molecular weight markers are shown $\times 10^{-3}$ Da.

pelB and LTB signal sequences were ahmATWTCIN, eAsWTCINQQ, and ApWTCINQQL, respectively. The N-terminal amino acid sequence of restrictocin purified from the periplasm of cells transformed with pRest2 was same as the N-terminus for protein purified from the medium of the same culture. The N-terminal sequence analysis confirmed that the processing of the mature protein was correct, and occurred at the engineered sites shown in Fig. 1. The changes from the native restrictocin N-terminus found in the first few amino acids of secreted restrictocin are shown in lower case, and were expected because of the inclusion of enzyme recognition sites for cloning purposes.

3.3.2. Spectroscopic characterization of purified proteins

The far-UV CD spectra of purified restrictocin from the fungal host culture medium, secreted using the ompA signal sequence, and isolated from the bacterial inclusion bodies have been compared in Fig. 3. The secondary structure values obtained from CD measurements are shown in Table 2. The values for recombinant protein isolated from inclusion bodies and native restrictocin are in good agreement with those reported for α-sarcin, a known homologue of restrictocin [20], and the values obtained for restrictocin from Garnier method of secondary structure prediction [21]. The CD spectral characteristics of the native protein and that isolated from the inclusion bodies were similar indicating that the recombinant restrictocin purified from the inclusion bodies was conformationally close to the native protein produced by the fungal host.

Table 2
Secondary structure analysis of restrictocin

	Native	Inclusion bodies	Secretory	Predicted[a]
Helical	21.1	18.9	13.9	18.7
β sheet	20.5	13.9	32.9	12.0
Turn	25.0	24.9	15.4	28.1
Random coil	33.3	41.8	39.3	40.9

The secondary structure analyses from the CD measurements were performed by using Yang reference parameters [25]
[a]Secondary structure prediction by the method of Garnier [21].

3.3.3. In vitro ribonucleolytic activity of recombinant restrictocin

The recombinant restrictocin was tested in a cell-free system using rabbit reticulocyte lysate, for its inhibitory activity towards protein synthesis. Recombinant restrictocin purified from inclusion bodies was found to be as potent as the native toxin secreted by the host and inhibited translation of globin mRNA with an ID_{50} of 35 pM (Table 1). Although protein from soluble sources had a modified N-terminus containing either a few extra amino acids or changes, enzymatic activity was same as that of the native protein, indicating that a few additions or changes at the amino terminus are well tolerated and do not affect the enzymatic activity of the protein. The protein purified from the insoluble inclusion bodies contains similar activity to that of the native or secreted recombinant restrictocin, implying that using a protocol involving a redox system protein refolded correctly and attained its native conformation. Activity of the recombinant protein was similar to ricin A chain, another ribosomal inactivating protein that acts on the same intracellular target. Recombinant restrictocin purified from periplasm was found to be 10-fold less active than restrictocin purified from the medium or inclusion bodies (Table 1). This loss of activity could be due to improper folding as has been shown for some other recombinant proteins secreted into the periplasm [22,23]. Recombinant restrictocin also showed ribonuclease activity when synthetic homopolynucleotides were used as substrates. As shown in Fig. 4, the toxin caused extensive digestion of Poly(A) and moderate

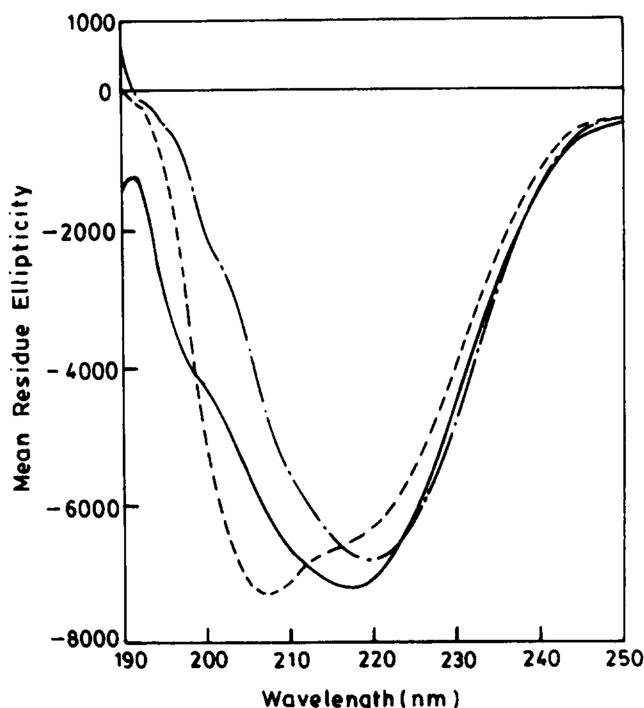

Fig. 3. CD spectra of restrictocin purified from different sources. Far-UV spectra of restrictocin purified from fungal culture medium (– · –), bacterial culture medium (- - -), and bacterial inclusion bodies (——). Mean residue ellipticity is expressed as degree cm^{-1} $dmol^{-1}$.

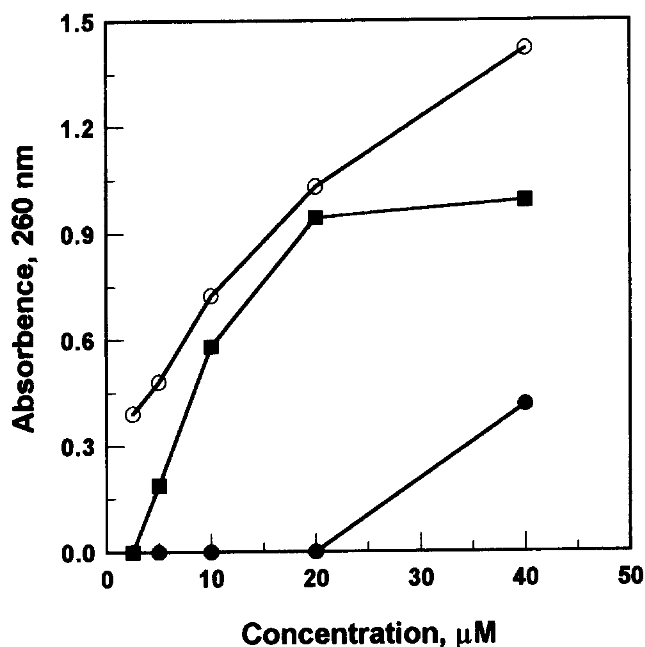

Fig. 4. Ribonuclease activity of recombinant restrictocin. Activity was measured on yeast tRNA (○), and synthetic polyribonucleotides Poly(A) (■) and Poly(G) (●). Yeast tRNA (40 μg) or homopolymers (40 nmol) were incubated for 60 min, at 40°C in 50 mM Tris·HCl, pH 7.3, with different concentrations of the toxin. Undigested RNA was precipitated on cold with perchloric acid and uranyl acetate. Precipitated material was removed by centrifugation and the absorbence of the supernatant was read at 260 nm.

hydrolysis of Poly(G). Restrictocin caused degradation of yeast tRNA in a dose-dependent manner (Fig. 4). Since restrictocin unlike RNaseA is a specific ribonuclease, a more pronounced inhibitory activity in a cell-free protein synthesis assay confirms specific recognition of the target ribosomal RNA by the recombinant proteins. In addition, its preferential activity on Poly(A) is in agreement with the earlier report on α-sarcin [24].

Earlier *Asp f I*, a homologue of restrictocin, has been renatured and purified from insoluble source; however, the recombinant protein was found to be 10-fold less active than the native toxin [14]. This reduction in enzymatic activity has been attributed to misfolding of the recombinant toxin due to the presence of a histidine fusion peptide at the amino terminus of the recombinant toxin used in the purification of the recombinant *Asp f I* [14]. In the present study, the recombinant restrictocin purified from the insoluble inclusion bodies has an authentic amino terminus and contains full enzymatic activity.

In conclusion, we have shown that the ribotoxin restrictocin could be over-produced in *E. coli* and purified to homogeneity by a single-step purification protocol. Using the protocol employed, large amounts of functional recombinant protein could be obtained from the inclusion bodies. Properly processed and functional restrictocin is secreted with the help of ompA, *pelB* and LTB signal sequences, ompA being the most efficient in secretion. Small modifications at the amino terminus of restrictocin do not affect its enzymatic activity.

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