Effective expression and purification of recombinant onconase, an antitumor protein

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Abstract Several members of the RNase A superfamily are endowed with antitumor activity, showing selective cytotoxicity toward several tumor cell lines. One of these is onconase, the smallest member of the RNase A superfamily, which is at present undergoing phase III clinical trials. We report here the expression of recombinant onconase in *Escherichia coli* inclusion bodies, the correct processing of the protein, followed by its purification in high yields. The recombinant protein has biological and catalytic properties identical to those of the natural enzyme.

Key words: Ribonuclease; Onconase; Antitumor; Recombinant expression

1. Introduction

Members of the RNase A superfamily are endowed with antitumor activity, showing selective cytotoxicity toward several tumor cell lines. Among these are bovine seminal ribonuclease (BS-RNase) from bull semen, and onconase (ONC) from oocytes of *Rana pipiens* [1]. These proteins have been extensively studied because of their potential as antitumor drugs, and onconase has reached phase III clinical trials [2].

Therapeutic use of onconase as an antitumor drug would greatly benefit from the availability of the biologically active recombinant protein produced in good yields. A mutant recombinant onconase has been obtained by Boix et al. [3], with leucine replacing methionine at position 23 ((M23L)-ONC). However, the mutant showed a reduced antitumor action compared to that of native onconase [3,4], and the yield of the protein was not clearly stated.

More recently, Leland et al. [5] have described an *Escherichia coli* system for the expression and secretion of mature recombinant onconase, identical to the natural protein. Surprisingly, the protein was found to be processed in the cytoplasm and found to segregate in inclusion bodies. After extraction, refolding and purification, its catalytic activity on poly(C), and its cytotoxicity on K562 cells were found to be comparable to those of native onconase. Yields of 5–50 mg/l culture were given, and the protein was further characterized.

Here we report an effective expression and purification system in high yields of recombinant onconase. The protein has been characterized in its cytotoxic activity and found to be identical to the natural protein when assayed on myelogenous leukemia cells K562, and on malignant, SV40-transformed SVT2. Moreover, also its catalytic properties are identical to those reported for the natural enzyme [3].

2. Materials and methods

2.1. Materials

Onconase purified from oocytes of *R. pipiens* was kindly provided by Dr. Kuslima Shogen (Alfacell Corp., USA). Plasmid pET22b(+) and *E. coli* strain BL21(DE3) were from AMS Biotechnology. *E. coli* strain JM101 was purchased from Boehringer. RNase A (Type XIIA) was purchased from Sigma-Aldrich, Milano, Italy; labeled oligonucleotides from Amersham. The Wizard DNA purification kit for elution of DNA fragments from agarose gel, enzymes and other reagents for DNA manipulation were from Promega Biotech.

2.2. General procedures

Bacterial cultures, plasmid purifications and transformations were performed according to Sambrook et al. [6]. Double-strand DNA was sequenced with the dideoxy method of Sanger et al. [7], carried out with a Sequenase version II Kit (Amersham) with deoxynucleotide triphosphates purchased from Pharmacia.

2.3. Recombinant DNA methodologies

The cDNA encoding Met(−1)(Q1E)-ONC cloned between *Nde*1 and *Bam*H1 sites of vector pET11d, kindly provided by Dr. R.J. Youle (National Institute of Neurological Disorders and Stroke, NIH), was subcloned into vector pET22b(+) between the same restriction sites. Oligonucleotide mediated site directed mutagenesis according to Kunkel [8] was used to restore the glutamine residue at position 1, and yield the cDNA encoding recombinant onconase. This latter cDNA was further mutated to replace methionine residue at position 23, yielding the coding sequence for mutant (M23L)-ONC. Protein expression was carried out after transformation of *E. coli* strain BL21(DE3) with the appropriate plasmid. Cells were grown to about 3.5–4 OD at 600 nm in TB medium. Expression was induced by IPTG at a final concentration of 0.4 mM and cells were collected after overnight induction.

2.4. Cell cultures

Human chronic myelogenous leukemia cells K562, malignant, SV40-transformed SVT2 fibroblasts and the parental non-transformed Balb/C 3T3 line were obtained from ATCC (Richmond, VA, USA), and cultured in 24-well plates at a density of 50×10^3/ml of Dulbecco's modified Eagle's medium, supplemented with glutamine, penicillin, streptomycin, and 10% fetal calf serum. The RNases being tested were added to the cells before plating, and cells were counted after time intervals as indicated, allowing at least three to four rounds of

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Abbreviations: ONC, onconase; (M23L)-ONC, mutant of onconase with a leucine residue at position 23 replacing methionine; BS-RNase, bovine seminal ribonuclease; RNase A, bovine pancreatic ribonuclease; poly(U), polyuridilic acid; DTT, dithiothreitol; IPTG, isopropyl-β-D-thiogalacto-pyranoside; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; CNBr, cyanogen bromide; ES-MS, electrospray mass spectrometry; TB, terrific broth; AAP, *Aeromonas proteolytica* aminopeptidase

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replication in the control cultures. Because of their morphology, viable K562 cells were counted with the trypan blue exclusion test.

2.5. Other methods

Protein sequence determinations were performed on an Applied Biosystems sequenator (model 473A), connected on-line with a high performance liquid chromatography apparatus for identification of phenylthiohydantoins. The electrospray mass spectrometric (ES-MS) analyses were performed using an Api-100 triple quadrupole mass spectrometer (Perkin Elmer) equipped with an electrospray ion source, at CEINGE Biotecnologie Avanzate (Naples, Italy). RNase activity on yeast RNA was assayed with the method of Kunitz [9], or by the precipitation assay [10]. SDS-PAGE was carried out according to Laemmli [11].

3. Results and discussion

3.1. Expression and purification of recombinant onconase and of mutein (M23L)-ONC

Recombinant onconase and its variant (M23L)-ONC were expressed according to the procedure described in Section 2. An SDS-PAGE analysis of induced and non-induced cells extracted in SDS containing 2-mercaptoethanol showed that in both cultures a protein of about 11 kDa was produced only in the IPTG-induced cells (Fig. 1). These proteins were tentatively identified with onconase and (M23L)-ONC, respectively. Yields were of about 100–120 mg of protein/l of bacterial culture, on the basis of a densitometric scanning of the electrophoretic profile. SDS-PAGE analysis after sonication of a cell pellet revealed (data not shown) that proteins of interest were present only in the insoluble fraction, hence presumably contained in cell inclusion bodies.

For preparative purposes, cells from 1 l culture were suspended in 10 ml of buffer A (50 mM Tris-acetate, pH 8.4, containing 10 mM EDTA), and sonicated (6×1 min cycle). The suspension was then centrifuged at 17 400×*g* for 60 min at 4°C. The cell pellet was freed from membrane proteins by two washes in 0.1 M Tris-acetate, pH 8.4, containing 10 mM EDTA, 2% Triton X-100 and 2 M urea, followed by repeated washes in 0.1 M Tris pH 8.4, containing 10 mM EDTA to eliminate traces of Triton and urea. This procedure eliminated several contaminant proteins and cellular debris entrapped in inclusion body pellets.

Inclusion bodies were then dissolved in 0.1 M Tris-acetate pH 8.4, 10 mM EDTA, 6 M guanidine-Cl and 25 mM DTT, purged with N_2, and incubated at 37°C for 3 h. The protein solution was dialyzed overnight versus 0.1 M acetic acid at 4°C. Any insoluble material was removed by centrifugation and the supernatant, containing the RNases in the completely reduced form, was lyophilized.

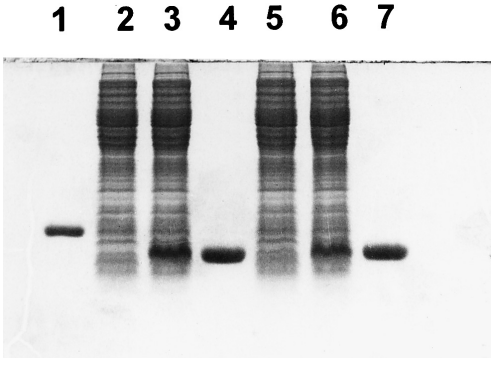

Fig. 1. SDS-PAGE electrophoresis in 15% polyacrylamide gel under reducing conditions of: cell lysates from non-induced (lane 2), and induced (lane 3) *E. coli* clones expressing r-ONC; cell lysates from non-induced (lane 5), and induced (lane 6) *E. coli* clones expressing (M23L)-ONC. In lanes 4 and 7 are shown r-ONC and (M23L)-ONC, respectively, purified after affinity chromatography on Blue-Agarose. In lane 1 RNase A, run as a control.

In vitro renaturation of recombinant onconase and (M23L)-ONC was carried out as described [12]. The refolded proteins, with an N-terminal methionine as determined by Edman sequencing, had a low catalytic activity as reported by Boix et al. [3]. Removal of the N-terminal residue was attempted using *Aeromonas proteolytica* aminopeptidase (AAP) [13], which proved to be useful in the removal of Met-1 from other RNases such as angiogenin [14] and BS-RNase [15]. The attempt met with no success. A likely explanation for this result can be drawn from the three-dimensional structure of onconase. The protein has an N-terminal region shorter than those of homologous RNases, and closer to its main body [16]. Thus steric hindrance could explain the inability of AAP to cleave the N-terminal residue. On the basis of this hypothesis we tried to remove Met-1 from the unfolded proteins prior to folding. In order to maintain onconase and (M23L)-ONC in the unfolded conformation suitable to the activity of AAP, the lyophilized reduced and denatured proteins were dissolved at 2 mg/ml in 0.2 M Tris-acetate pH 9.3, containing 10 mM EDTA, 6 M guanidine-Cl. Cystamine was added at 0.2 M final concentration, and the reaction mixture was incubated at 37°C for 20 h to promote the formation of mixed disulfides between cysteine residues of the proteins and cysteamine. It should be noted that cystamine was chosen instead of other disulfide small molecules such as oxidized glutathione, because of its higher solubility and its positive charge, which could improve the solubility of the polypeptide chain. The samples were then dialyzed versus 0.1 M acetic acid and lyophilized. Lyophilized material was dissolved in 4 M guanidine-Cl, and diluted ten times in 0.2 M potassium phosphate pH 7.2, containing 0.5 mM $ZnSO_4$. AAP was added to the solution in a molar ratio 1:2000, and the mixture incubated at 37°C. The reaction was followed by Edman sequencing. The N-terminal sequence of the untreated recombinant onconase was found, as expected, to be NH_2-Met-Gln-Asp-. After 10 h of incubation only a small fraction of protein presented an N-terminal Met, while in an even smaller fraction N-terminal Gln was detected. After 15 h of incubation the protein was found to be totally resistant to the Edman reaction. These findings indicate that the removal of the N-terminal Met by AAP from the recombinant protein was complete, and that upon the removal the exposed Gln completely cyclized into pyroglutamate. This result was confirmed by the data from an electrospray mass spectrometry analysis which yielded an MW of 12 430.13 ± 0.83 Da, corresponding to processed onconase, with a pyroglutamyl residue, and coupled to eight molecules of cysteamine (calculated MW = 12 429.6).

Renaturation of the derivatized proteins was achieved by diluting samples in 0.1 M potassium phosphate pH 7.8, 10 mM EDTA, followed by addition of a redox mixture containing 3 mM cysteamine and 0.6 mM cystamine. Maximal renaturation was obtained after 12 h at room temperature, as judged by recovery of the RNase activity, measured by the precipitation assay [10] using yeast RNA as a substrate. Renatured proteins were further purified by a single affinity chromatographic step on Blue-Agarose. Samples were loaded on the

column (15×170 mm) in 50 mM sodium acetate pH 5.0; elution was performed with an NaCl gradient (0 to 1 M, 400 ml), at 10 ml/h. The proteins recovered from Blue-Agarose were found to be homogeneous, as judged by SDS-PAGE (Fig. 1).

Molecular weights of recombinant proteins were determined by ES-MS. The molecular mass of r-ONC was found to be 11 821.53 ± 1.52 Da, in good agreement with that expected for onconase with < Glu-1 (11 820.54 Da). The molecular weight of mutant (M23L)-ONC was found to be 11 802.63 ± 1.15 Da, i.e. that expected for mutant (M23L)-ONC with < Glu-1 (calculated MW = 11 801.6).

The final yield for both recombinant onconase and the mutant (M23L)-ONC was about 40 mg per liter of culture.

3.2. Catalytic activity

Onconase prefers yeast RNA and poly(U), as judged by its specific activity values. Moreover, on both substrates it is slightly more active at pH 5.5 than at pH 7.5 [3]. Table 1 reports the activity on yeast RNA at pH 5.5 for RNase A, onconase, recombinant onconase and (M23L)-ONC. r-Onconase prepared with the procedure described in the present paper is catalytically undistinguishable from the natural protein. Moreover, r-ONC is about 230-fold less active than RNase A in 0.2 M sodium acetate, in good agreement with previous data [3]. When the same experiments were carried out at lower ionic strength, in 0.1 M sodium acetate, pH 5.5, r-ONC was found to be only 27-fold less active than RNase A. As Boix et al. [3] have reported that onconase catalytic activity is not influenced by ionic strength between 0.1 M and 0.2 M NaCl at pH 7.5, we decided to explore the effect of ionic strength at pH 5.5. In Fig. 2 RNase activity of the three is plotted as a function of NaCl concentration. While RNase A activity increases with ionic strength, onconase activity sharply decreases between 0.1 M (50 mM sodium acetate plus NaCl 50 mM) and 0.25 M (50 mM sodium acetate plus 200 mM NaCl) salt concentration. It should also be noted that a comparison of the data in Table 1, with those reported in Fig. 2 shows that NaCl and sodium acetate have a similar inhibitory effect on onconase and (M23L)-ONC. In fact, both proteins have the same activity in 0.2 M sodium acetate and in 0.05 M sodium acetate containing 0.15 M NaCl.

Furthermore, as shown in Table 1 and in Fig. 2, catalytic activity on yeast RNA of mutant (M23L)-ONC is surprisingly high, compared to that of onconase, and is less sensitive to ionic strength. In fact, it is 5-fold more active than the wild-type enzyme at low ionic strength, and 15-fold more active at high ionic strength. These data are also to be compared with those reported by Boix. (M23L)-ONC was found to be only 1.7-fold more active than n-ONC on poly(U), at pH 5.5 in 0.2 M sodium acetate [3]. In our opinion this difference might be due either to different substrate specificity of ONC and (M23L)-ONC for RNA and poly(U), or to the different procedures for removing the N-terminal methionine from the recombinant proteins (see below).

Table 1
Catalytic activity on yeast RNA of onconase (n-ONC), recombinant onconase (r-ONC), and (M23L)-ONC, at different sodium acetate concentrations

	[Na acetate]		
	0.05 M	0.1 M	0.2 M
	Specific activity[a]		
RNase A	**100 ± 3**	288 ± 20	352 ± 17
r-ONC	9.1 ± 0.6	10.9 ± 0.2	1.7 ± 0.1
n-ONC	9.2 ± 0.16	11.0 ± 0.2	1.7 ± 0.2
(M23L)-ONC	50.6 ± 2.8	54.9 ± 1.9	22.2 ± 2.8

[a]Relative to RNase A specific activity at 25°C in 0.05 M sodium acetate pH 5.5 taken as **100**. The activity of RNase A ranged between 99 and 110 Kunitz units/mg of protein.

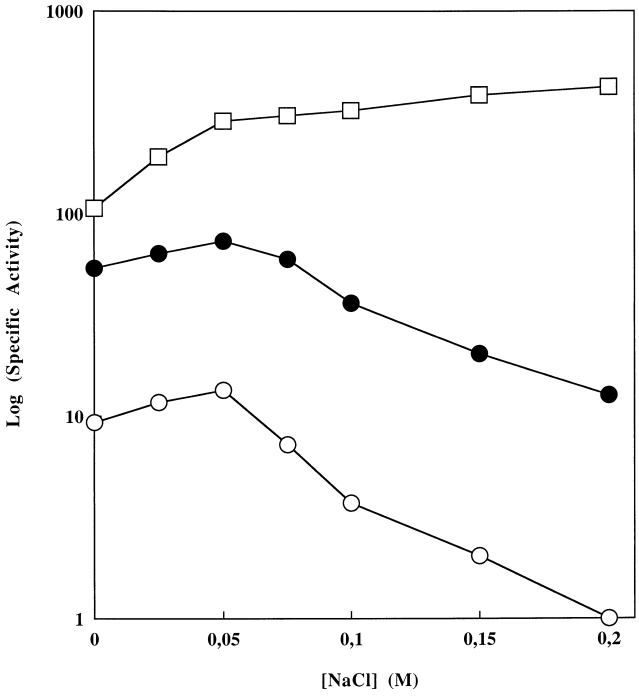

Fig. 2. Effect of NaCl on the catalytic activity on yeast RNA of r-ONC (○), (M23L)-ONC (●), and RNase A (□). All assays were performed in 0.05 M sodium acetate, pH 5.5, in the presence of the indicated NaCl concentration.

The effect of the mutation M23L in increasing the catalytic activity of onconase is quite surprising as Met and Leu are considered very similar residues for hydrophobicity, size and α-helix preference. Moreover, Met-23 is in the hydrophobic core of the protein [17] and apparently does not directly contribute to the structure of the active site. Preliminary calorimetric studies (F. Catanzano et al., manuscript in preparation), show that onconase is an exceptionally stable enzyme with a very high melting temperature at pH 6, whereas (M23L)-ONC has a lower T_m under the same conditions. Several examples are available [18] of mutations which decrease thermostability and increase catalytic activity. Thus, the hypothesis can be advanced that replacement of leucine for methionine at position 23 of onconase decreases the stability of the protein, hence increasing its flexibility and, as a consequence, its catalytic activity. However, further physicochemical studies and onconase mutants are required to test this hypothesis.

3.3. Antitumor activity

The antitumor activity of onconase, recombinant onconase and (M23L)-ONC was assayed on two very different tumor cell lines, SVT2 malignant fibroblasts, extensively used in the case of other ribonucleases [19,20], and K562 erythroleukemic

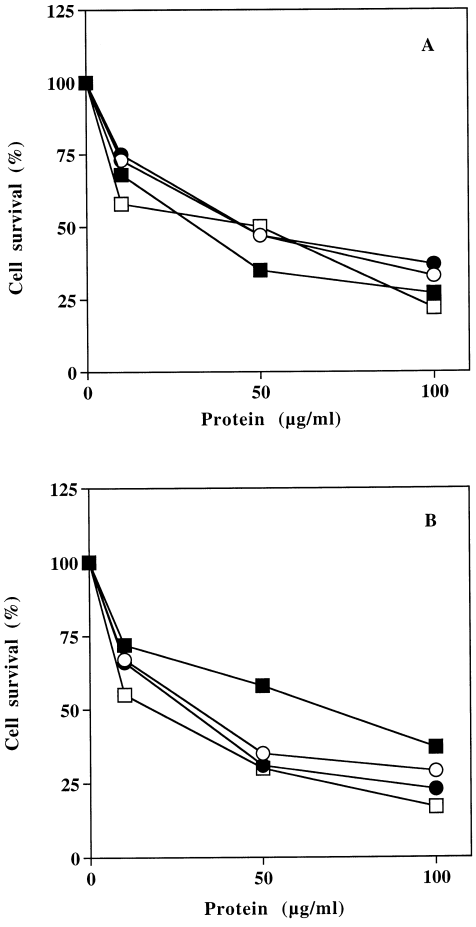

Fig. 3. Dose-response curves of (A) malignant fibroblasts (SVT2), and (B) human chronic myelogenous leukemia cells (K562) grown in the presence of (□) onconase; (○) r-ONC; (●) (M23L)-ONC; (■) BS-RNase.

cells, used in the case of recombinant onconase [5]. Recombinant onconase and (M23L)-ONC were also compared to BS-RNase, a mammalian antitumor RNase.

On SVT2 cells onconase and recombinant onconase showed very similar activity, confirming the identity of the properties of the recombinant protein with those of the natural one. The same biological activity was displayed by the mutant (M23L)-ONC and by BS-RNase (Fig. 3A). On the other hand, on K562 cells, onconase, recombinant onconase, and (M23L)-ONC were more active than BS-RNase. The IC_{50} (the protein concentration for 50% growth inhibition) was higher (about 25 μg/ml) for onconase and recombinant onconase than that reported by Leland et al. (6 μg/ml) [5]. However, it should be taken into account that Leland et al. determined the IC_{50} measuring ^{3}H-timidine incorporation, a more sensitive test than the measure of cell survival.

3.4. Concluding remarks

The availability of a recombinant protein, identical to its cognate natural counterpart in chemical and biological properties, is a powerful tool for the study of the molecule itself. Moreover, in the case that the protein has biologically relevant properties, as it is the case of onconase, endowed with a potent antitumor action, the recombinant production of the protein gives an easier and very rich source for its use as a potential drug.

The expression system described in the present paper is such a source, because it allows the reproducible purification of relatively large amounts (more than 40 mg/l of bacterial culture) of recombinant onconase with an antitumor activity undistinguishable from that of the natural protein. Moreover, with the procedure reported here a processed mature protein is obtained which does not suffer from the reduced antitumor activity shown by the recombinant molecule prepared with previous procedures [3]. This difference might be attributed to damages to the protein structure caused by the treatment (CNBr in HCl) used by Boix et al. for removing the N-terminal methionine from the recombinant protein, although the mutant had the expected molecular mass.

Our data confirm and expand the observation that the mutein (M23L)-ONC is catalytically more active than native and recombinant onconase. This data, together with the preliminary reports on the lower thermal stability of the mutant, can lead to the hypothesis that replacement of leucine for methionine at position 23 of onconase increases its flexibility and, as a consequence, its catalytic activity.

The data presented in the present paper shed also light on the relationships between catalytic activity and antitumor action in onconase. The mutein (M23L)-ONC is a better catalyst than onconase but not a better antitumor agent. It has been widely demonstrated that catalytic activity is one of the factors determining the antitumor activity of a ribonuclease [1]. Recently [21], a hypothesis has been advanced on the 'mosaic' nature of the selective cytotoxicity of ribonucleases, which depends on several extra- and intra-cellular steps, such as binding at the cell surface, internalization, progression to the cytosol, RNA degradation and resistance to the cytosolic RNase inhibitor. Thus, on the basis of the mosaic hypothesis it should not be surprising that catalytic activity and antitumor action do not parallel in the case of (M23L)-ONC. This would indicate, at least for SVT2 and K562 cells, that the intrinsic catalytic efficiency may not be the limiting step in the cytotoxic action of a ribonuclease.

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