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EXPRESSION OF BOVINE SEMINAL RIBONUCLEASE IN *ESCHERICHIA COLI*

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SUMMARY: Bovine seminal RNAase (BS-RNAase), an unusually dimeric member of the pancreatic-like ribonuclease superfamily, is also a multifunctional biological effector, with antitumor, immunosuppressive, and antispermatogenic activities. We report here the cloning of a semi-synthetic cDNA coding for the protein subunit chain, its expression with a T7 expression system in *Escherichia coli* inclusion bodies, the dimerization of correctly reoxidized monomeric protein, followed by the purification in high yields of the recombinant enzyme, and by its conversion to a protein undistinguishable from BS-RNAase as isolated from seminal vesicles, both in its catalytic activity and in the micro-heterogeneity of its quaternary structure.

Bovine seminal RNAase (BS-RNAase) is an unusually dimeric ribonuclease in the pancreatic RNAase superfamily, with intersubunit disulfide bridges (1 and references cited therein). Recently, it has been reported that in isolated BS-RNAase the majority of its subunits interchange their N-terminal segments, while in the remaining dimers each N-terminal segment folds onto its respective subunit (2). These structural peculiarities are matched by the equally unusual enzymic and biological properties of BS-RNAase: it is allosterically regulated, and is an antitumor, an antispermatogenic, and an immunosuppressive factor (see 1 for a review). Its antitumor action has been recently studied also on epithelial tumor cells, *in vivo* and *in vitro* (3).

Protein engineering can be a very effective approach to studying both the molecular basis of BS-RNAase allosteric properties, and the structural determinants underlying its diverse biological properties. First essential steps in this direction are the cloning of DNA coding for the protein of interest and the definition of a suitable expression system. Here we report a successful procedure that has enabled us to express good yields of recombinant BS-RNAase which is undistinguishable in its structural and catalytic properties from the natural enzyme.

MATERIALS AND METHODS

Materials. BS-RNAase was purified from seminal vesicles as described by Tamburrini *et al.* (1986). The cDNA clone 19-17G3, coding for the sequence 47-124 of BS-RNAase was provided

The abbreviations used are: BS-RNAase, bovine seminal ribonuclease; DTT, dithiothreitol; IPTG, isopropyl-b-D-thiogalacto-pyranoside; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; PCR, polymerase chain reaction.

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by Dr. Adriana Furia (University of Naples); the expression vector pT7-7 by Dr. Gennaro Ciliberto (University of Naples). Plasmid pUC18 and *E. coli* strain JM101 were purchased from Boehringer; *E. coli* strain BL21(DE3)pLysS from AMS Biotechnology; labelled oligonucleotides from Amersham; reagents for PCR from Perkin-Elmer Cetus. The Gene-Clean kit for elution of DNA fragments from agarose gel was obtained from Bio 101. Enzymes, including restrictases, and other reagents for DNA manipulation, were from Promega Biotech. The oligonucleotides for the synthesis of a cDNA coding for residues 1-48 of BS-RNAase subunit sequence (4) were synthesized by Beckman Analytical (Italy).

General Procedures. Bacterial cultures, plasmid purifications and transformations were performed according to Sambrook *et al.* (5). Double-strand DNA was sequenced with the dideoxy method of Sanger *et al.* (6), carried out with a Sequenase Sequencing Kit (U.S.Biochemical Corporation) with deoxynucleotide triphosphates purchased from Pharmacia.

Chromatographic Separations. Cation exchange chromatography on Mono-S columns (Pharmacia) was performed as previously described (7). Gel filtration separations were carried out on an FPLC apparatus (Pharmacia) equipped with HiLoad 26/60 Superdex 75 columns equilibrated with 0.1 M Tris-acetate pH 8.4 containing 0.3 M NaCl, at a flow rate of 0.4 ml/min.

Semi-synthesis of the cDNA Coding for BS-RNAase. Complementary oligonucleotides *RN1/RN4*, *RN2/RN5* and *RN3/RN6* (see Fig.1) were separately annealed in 200 mM Tris-Cl pH 7.4, 20 mM $MgCl_2$, 0.5 M NaCl by 3 min boiling in sealed capillaries. The annealed mixtures were added to a dephosphorylated HinfI-BglII cDNA fragment excised from clone 19-17G3 and coding for the amino acid sequence 49-124 (Fig.1). Ligation was carried out, in the presence of T4 DNA ligase, for 12 h at room temperature. Ligation products, analyzed by 5% polyacrylamide gel electrophoresis under non-denaturing conditions, followed by autoradiography, were found to contain a DNA fragment of the expected size (about 380 bp). Cloning of the fragment into pUC18 vector was achieved by addition to the ligation mixture of pUC18 trimmed with EcoRI and BamHI (compatible with BglII recognition sequence), and dephosphorylated. After 12 h at 15 °C in the presence of freshly added T4 DNA ligase, the ligation mixture was used to transform JM101 competent cells. Selection of recombinant clones containing the coding sequence of BS-RNAase subunit was made by plasmid DNA sequencing. A clone denominated *MC-P-BS* was selected, harboring a recombinant plasmid *pMC-P-BS* that contained the sequence coding for the entire amino acid sequence of the BS-RNAase subunit, inserted between EcoRI and BamHI poly-linker sites.

Mutagenesis of 5' EcoRI Site of pMC-P-BS cDNA. The cDNA coding for BS-RNAase was excised from p*MC-P-BS* with EcoRI and HindIII, whose recognition sequence is located downstream the BamH1 site. The fragment, separated by agarose gel electrophoresis, was eluted and subjected to site-directed mutagenesis to convert its EcoRI 5' ending into a NdeI site for proper frame positioning into pT7-7 vector. The mutagenesis was carried out by a PCR procedure using appropriate synthetic oligonucleotides. The DNA fragment resulting from PCR amplification (402 bp) was isolated by agarose gel electrophoresis, eluted, and digested with NdeI and SalI restriction endonucleases. The digestion product was purified by electrophoresis, ligated with the pT7-7 vector previously cut with NdeI and SalI, and used to transform JM101 competent cells. Selection of a recombinant clone containing the BS-RNAase coding sequence was achieved by direct sequencing of recombinant clones. 5 different clones (*T-MC-P-BS*-1 to 5) were selected, cultured in liquid Luria-Bertani medium, containing ampicillin, and stored at -80 °C in 20% glycerol.

Other Methods. RNAase activity on yeast RNA was assayed with the method of Kunitz (8). Sulfhydryl groups were determined with dithio-nitrobenzoate (9). SDS-PAGE was carried out according to Laemmli (10). Protein sequence determinations were performed on an Applied Biosystems sequencer mod. 473A, connected on-line with an HPLC apparatus for identification of phenylthiohydantoin derivatives.

RESULTS AND DISCUSSION

Construction of the Expression Vector and Expression of Recombinant BS-RNAase. A cDNA coding for BS-RNAase was constructed as illustrated in Fig.1. A HinfI/BglII digest of the available cDNA clone 19-17G3 (11), coding for residues 49 through 124 of BS-RNAase subunit, was ligated to a 148 bp synthetic DNA fragment coding for the missing upstream sequence 1-48. This was drawn from the amino acid sequence of the protein (4) with the

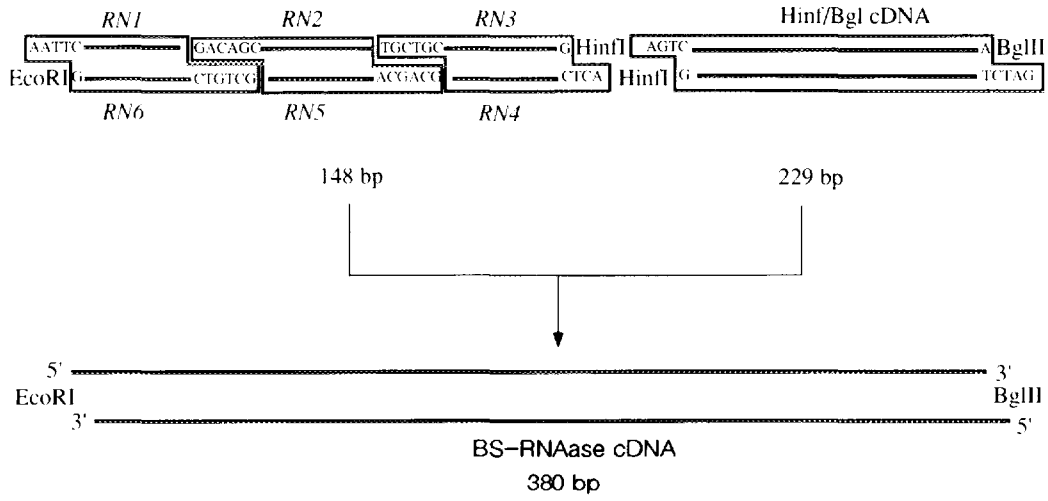

Fig.1. Strategy for the construction of a cDNA coding for BS-RNAase subunit chain.

aid of a computer program (12) designed for: (i) including the maximum number of unique restriction sites, useful for future cassette mutagenesis, and (ii) privileging *Escherichia coli* preferred codons.

The final product, cloned into pUC18 between EcoRI and BamH1 sites, was subsequently transferred to the pT7-7 vector, a convenient system for the controlled expression of genes coding for toxic products (13, 14). For the latter cloning, the EcoRI 5'-end of the DNA fragment was first mutagenized to create a NdeI site, necessary for proper positioning of the cDNA in the pT7-7 vector. The correct sequence was amplified by PCR, isolated by agarose gel electrophoresis and inserted into the pT7-7 vector. DNA from clone *T-sBS-1* was used to transform BL21(DE3)pLysS cells that express the T7 RNA polymerase under the IPTG inducible lacUV 5 promoter (13). Fresh recombinant colonies were used to inoculate liquid cultures, then IPTG was added to 0.4 mM final concentration and bacterial growth was continued for 3 h. An SDS-PAGE analysis of induced and non-induced cells extracted in SDS containing 2-mercaptoethanol showed that a protein of about 14 kDa, comigrating with BS-RNAase subunit, was produced only in the IPTG-induced cells (see Fig. 2A). This protein was tentatively identified with the BS-RNAase

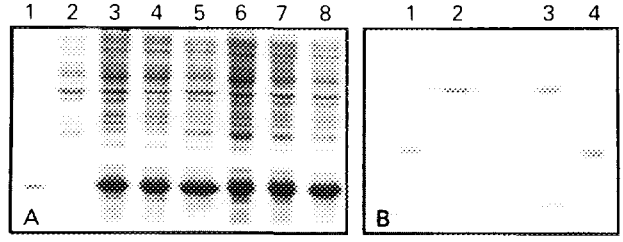

Fig.2. SDS-PAGE electrophoresis in 15% polyacrylamide gels; **A**, carried out under reducing conditions on cell lysates from non-induced (lane 2) and induced (lanes 3 to 8) *Escherichia coli* clones harboring plasmid p*T-MC-P-BS*; in lane 1 BS-RNAase purified from seminal vesicles, run as a control. **B**, electrophoresis of rBS-RNAase (lanes 1 and 2) and of BS-RNAase purified from seminal vesicles (lanes 3 and 4), carried out under reducing (lanes 1 and 4) and non-reducing conditions (lanes 2 and 3).

subunit. Yields were of about 10 mg of protein/ml of bacterial culture, on the basis of a densitometric scanning of the electrophoretic profile. SDS-PAGE analysis after sonication of a cell pellet (data not shown) revealed that the protein of interest was present only in the insoluble fraction, hence presumably contained in cell inclusion bodies.

Preparation and Properties of Recombinant BS-RNAase. Unless otherwise stated, the following steps were performed at 4 °C. Pellets of BL21(DE3)pLysS cells from a 500 ml culture, transformed with DNA from clone *T-sBS*-1 and induced with IPTG, as were suspended in 20 ml of 50 mM Tris-Cl pH 8.0 (60 mg of wet cells/ml of buffer), and disrupted by sonication. The insoluble fraction was suspended in 90% formic acid, then 10-fold diluted with 0.1 M ammonium acetate pH 5, and extracted by sonication. After centrifugation, the soluble fraction was collected, while the insoluble fraction was resuspended as above and sonicated again. The resulting soluble fraction was combined with the first fraction, concentrated by ultrafiltration, lyophilized and stored at -20 °C until utilized.

The lyophilized material (5 mg/ml) was disolved in 0.1 M Tris-Cl, pH 8.4, containing 5 M guanidine-Cl, 2 mM Na_2EDTA, and 5 mM DTT. After 3 h at 37 °C, the protein mixture was desalted by gel filtration on a Sephadex G25 column in 0.1 M ammonium acetate, 0.1 M NaCl, pH 5.

For renaturation, the procedure of Smith et al. (15) was followed. The protein solution was first diluted to 0.3 mg/ml of 0.1 M Tris-Cl pH 8.4, and purged with N_2; then oxidized and reduced glutathione were added to final concentrations of 0.6 and 3 mM, respectively. Maximal renaturation was achieved after 24 h at room temperature, as judged by recovery of RNAase activity. Gel filtration on HiLoad Superdex 75 was carried out after removal of insoluble material by centrifugation. More than 85% of the protein coeluted with monomeric BS-RNAase, with a specific activity of about 50% that of BS-RNAase monomers (15).

Dimeric BS-RNAase was obtained by concentration of renatured monomers, removal of the glutathione moieties by selective reduction with DTT (16), and dialysis for 24 h at room temperature against 0.1 M Tris-Cl, pH 8.4. The resulting protein, freed of any unreacted monomers by gel filtration on a HiLoad Superdex 75 column, was purified on a Mono-S column, equilibrated in 0.1 M Tris-Cl pH 8.4 containing 0.1 M NaCl, washed with the same buffer containing 0.2 M NaCl, and eluted by increasing the NaCl concentration. The eluted protein was dialyzed and lyophilized.

As shown in Fig. 2B, SDS-PAGE revealed that the recombinant protein was homogeneous, and with the same molecular size as BS-RNAase; when denatured in the presence of 2-mercaptoethanol, its molecular size halved, just as occurs for BS-RNAase after reductive cleavage of the intersubunit disulfides. The final yields of dimeric BS-RNAase were of about 8 mg/l of original cell culture.

Sequence analyses of the first 19 residues of the recombinant protein indicated (data not shown) that the N-terminal residue was methionine, followed by the first 18 residues of BS-RNAase. However, the uncorrect processing of the coding sequence had no consequence on the catalytic activity of rBS-RNAase which, when tested on RNA as a substrate, was found to have a specific activity identical to that of the natural enzyme, i.e. about 40 Kunitz units/mg of protein. An identical uncorrect processing with no effect on catalytic ability was obtained also when

recombinant bovine pancreatic RNAase A (17) and bovine angiogenin (18), both homologous to BS-RNAase, were expressed in *Escherichia coli*.

When freshly refolded rBS-RNAase was selectively reduced at the intersubunit disulfides by a limited excess of DTT, and analyzed by gel-filtration on Sephadex G-75, only monomers were detected (data not shown). The natural protein, subjected to the same treatment, yields 1/3 dissociated subunits and 2/3 non-covalent dimers (2, 16).

This result indicated that rBS-RNAase, obtained as described above by association of refolded subunits, was made up entirely of M=M dimers, the dimeric form which upon reduction dissociates fully into monomers, as the dimers are made up of subunits which do not interchange their N-terminal segments. This was not surprising, since it has been ascertained (2) that when unfolded BS-RNAase is renatured, it refolds only into the form with no interchange of N-terminal ends between subunits. It has also been shown that the M=M dimers can spontaneously transform into the MxM form (with interchange of N-terminal ends between subunits) until an equilibrium is reached when the ratio MxM to M=M is equal to 2, i.e. the ratio found in BS-RNAase isolated from seminal vesicles (see above).

A final step was thus added in the preparation of natural-like recombinant BS-RNAase, consisting in the incubation of refolded rBS-RNAase at 37 °C in 0.1 M Tris-Cl at pH 7.3 for 96 h. After the incubation, rBS-RNAase was found to contain, upon selective cleavage of the intersubunit disulfides, about 2/3 of non-covalent dimers and 1/3 of monomers, just like the natural protein (data not shown).

As shown in Fig. 3, rBS-RNAase, analyzed by ion exchange chromatography on Mono S columns, eluted with a salt gradient (7), separated with the typical three-peak pattern of natural BS-RNAase. This is produced by the characteristic isoenzymic composition of the protein, made up of α_2, $\alpha\beta$ and β_2 isoenzymes, produced by the progressive transformation of β subunits into α subunits through selective deamidation at Asn^{67} (19). This finding indicates that after refolding rBS-RNAase spontaneously deamidates in solution in the same fashion as the natural enzyme when it is released in the seminal vesicle secretion (20).

Concluding Remarks. The procedure described for the production of recombinant BS-RNAase in high yields is easy and effective. The recombinant protein is undistinguishable in its catalytic activity and even in the micro-heterogeneity of its quaternary structure, from the protein isolated from seminal vesicles. It should be noted that few successful procedures have been described in

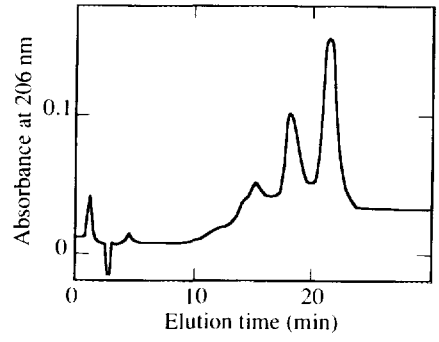

Fig.3. Isoenzymic pattern of purified rBS-RNAase eluted with a salt gradient from a Mono S column. The conditions are described under Materials and Methods.

the literature for the expression in yields higher than 0.5 mg/ L of cell culture of recombinant mammalian RNAases (18, 21, 22). These procedures have proved to be inadequate for expressing BS-RNAase. This may be likely due to the unique dimeric structure of BS-RNAase, and to the presence in the dimer of intersubunit disulfides. It could also be related to a higher RNAase toxicity of the seminal enzyme with respect to angiogenin and also to RNAase A (1). Certainly, the T7 expression system (13) has been confirmed here to be an effective system for expressing toxic proteins.

While this manuscript was in preparation, we became aware that Shultz and Baldwin (17) satisfactorily used the same system for the expression of bovine pancreatic RNAase A, albeit with 5- to 20-fold lower yields. This may be explained by differences in the procedures used for recovering the RNAase chains from the inclusion bodies, and for refolding. In particular, in the procedure described here, the reduction step (carried out with DTT) was separated, by removing excess reagents and by-products, from the renaturation step.

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