Bovine seminal ribonuclease (BS-RNase), a homolog of bovine pancreatic ribonuclease (RNase A), is isolated as a dimer in which the subunits are cross-linked by two disulfide bonds. In addition to this anomalous quaternary structure, the enzyme has extraordinary biological properties, such as antispermatogenic, antitumor, and immunosuppressive activities. The molecular bases for these properties are well-suited for exploration with the techniques of recombinant DNA. Accordingly, a gene encoding BS-RNase was designed based on criteria expected to maximize the translational efficiency of its mRNA in Escherichia coli. This gene was constructed from 12 synthetic oligonucleotides and expressed with the phage T7 system. The protein thus produced was insoluble and accumulated under optimal conditions to 15% of total cellular protein or 200 mg/liter of culture. Ribonuclease activity was generated by air oxidation of the reduced and denatured protein. Three forms of active BS-RNase were isolated by gel filtration chromatography: the well-characterized dimer and monomer and a previously uncharacterized form that migrated as a trimer. The ribonuclease activities of all three forms were equivalent to or higher than that of dimeric BS-RNase isolated from bull seminal plasma.

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**EXPERIMENTAL PROCEDURES**

**Materials**

Plasmid pBluescript II SK(−) was from Stratagene (La Jolla, CA). Expression vector pET17b and Escherichia coli strain BL21(DE3)/pLyS were from Novagen. E. coli strain JM109 was from Promega Biotec. Reagents for DNA synthesis were from Applied Biosystems Inc. (Foster City, CA), except for acetonitrile, which was from Baxter Healthcare (McGaw Park, IL). Restriction endonucleases and T4 DNA ligase were from Promega Biotec. Bacto-Tryptone and Bacto-yeast extract were from Difco. Ampicillin (sodium salt) was from International Biotechnol-igies Inc. (New Haven, CT). Terrific broth contained (in 1 liter) BactoTryptone (12 g), Bacto-yeast extract (24 g), glycerol (4 ml), KH₂PO₄ (2.31 g), and K₂HPO₄ (12.54 g). Polycytoic acid was from The Midland Certified Reagent Co. (Midland, TX). RNase A (type III-A) was from Sigma. All other chemicals and reagents were of commercial reagent grade or better and were used without further purification.

**Methods**

DNA oligonucleotides were synthesized on an Applied Biosystems Model 392 DNA/RNA synthesizer by using the β-cyanethyl phosphoramidite method (Sinha et al., 1984). Other manipulations of DNA were performed as described (Ausubel et al., 1989). Ultraviolet and visible absorbance measurements were made on a Cary 3 spectrophotometer equipped with a Cary temperature controller. Protein concentration was determined with the protein assay kit from Bio-Rad. The concentration of purified BS-RNase was determined by using an absorption coefficient of ε⁺⁺⁺ = 4.65 at 278 nm for the native enzyme or ε⁺⁺⁺ = 4.40 at 274 nm for the reduced and denatured protein (Parente and D’Alessio, 1985). Ribonuclease activity was assayed by the method of Kurtz (1948). Free sulfhydryl groups were detected by titration with 5,5'-dithiobis(2-nitrobenzoic acid) (Creighton, 1989).
Polyacrylamide gel electrophoresis (PAGE) was performed in the presence of SDS (0.1%, w/v) according to Ausubel et al. (1989). Gels were fixed and stained with aqueous methyl blue (40%, v/v) containing acetic acid (10%, v/v) and Coomassie Brilliant Blue (0.1%, w/v). The following molecular mass standards were from Bio-Rad: phosphorolysine b (19.4 kDa), RNase A (13.7 kDa), RNase A dimer (27.4 kDa), RNase A trimer (41.9 kDa), ovalbumin (43.0 kDa), RNase A tetramer (54.7 kDa), and bovine serum albumin (67.0 kDa). The molecular mass of each multimer of BS-RNase was calculated by assuming that the logarithm of its molecular mass was inversely proportional to its elution volume minus the column void volume (Whitaker, 1963; Andrews, 1984).

**RESULTS**

Design and Construction of Synthetic BS-RNase Gene—A gene was designed to code for BS-RNase to maximize translational initiation and protein elongation in E. coli. To facilitate the initiation of translation, the nucleotide sequence of the gene was designed to expose the start codon and Shine-Dalgalno sequence of its transcript in vector pSR1. The structure of lowest free energy predicted for this transcript by the program FOLD ($\Delta G^\circ = -45.9$ kcal/mol) is shown in Fig. 1. In this structure as well as in the lowest free energy structure predicted by the program MFOLD ($\Delta G^\circ = -33.8$ kcal/mol), the start codon and 2 bases of the Shine-Dalgalno sequence were free of base pairing. To facilitate protein elongation, the codons used were those found in highly expressed genes of E. coli. To make future mutagenesis more convenient, seven unique restriction endonuclease recognition sites were incorporated by considering codon redundancy. The synthetic gene was constructed from 12 overlapping oligonucleotides as shown in Fig. 2. (The cDNA sequence of the natural precursor protein of BS-RNase was reported by Preuss et al. 1990.) The nucleotide sequence of the synthetic gene was found to be as expected.

Expression and Purification—E. coli strain BL21(DE3)/pLYSs containing pSR1 was grown to late log phase. Expression of the BS-RNase gene was then induced by the addition of isopropyl-1-thio-β-D-galactopyranoside. The amount of BS-RNase produced by induced cells was not dependent on the precise time of induction during late log phase. BS-RNase thus generated at protein concentrations $>0.7$ mg/ml was lyophilized from an aqueous acetic acid (50%, v/v) (Crestfield et al., 1962). The molecular mass standards were RNase A monomer (13.7 kDa), RNase A dimer (27.4 kDa), RNase A trimer (41.9 kDa), ovalbumin (43.0 kDa), RNase A tetramer (54.7 kDa), and bovine serum albumin (67.0 kDa). The molecular mass of each multimer of BS-RNase was calculated by assuming that the logarithm of its molecular mass was inversely proportional to its elution volume minus the column void volume (Whitaker, 1963; Andrews, 1984).

Purification of Native BS-RNase from Bull Seminal Plasma—Bull seminal plasma was obtained from American Breeders Service (De Forest, WI). BS-RNase was purified by Mono-S cation-exchange and Superdex-75 gel filtration fast protein liquid chromatography (Pharmacia, Uppsala) as described (Tamburini et al., 1986).
multimeric forms of BS-RNase increased with time as shown in Fig. 3. Free sulfhydryl groups disappeared faster than activity was generated (data not shown), as has been observed with RNase A (Anfinsen et al., 1961). After incubation for 24 h at a protein concentration of 0.7 mg/ml, active dimeric BS-RNase had accumulated to ~40% of total protein. Sequence analysis indicated that dimeric BS-RNase had a methionine residue at its NH₂ terminus.

Purification of Active BS-RNase—Three homogeneous forms of BS-RNase were isolated by gel filtration chromatography. The migrations of these forms are shown in Fig. 4, and their catalytic activities and apparent molecular masses are given in Table I. The catalytic activity of the peak II protein was similar to that of native BS-RNase purified from seminal plasma and contained a protein that co-migrated during nonreducing SDS-PAGE with dimeric BS-RNase from seminal plasma as shown in Fig. 5. Although the peak II fraction showed the same gel filtration behavior 2 weeks after isolation, this fraction appeared to contain a noncovalent dimer that dissociated during nonreducing SDS-PAGE (Fig. 5) as well as a covalent dimer that dissociated only during reducing SDS-PAGE (data not shown). The peak III protein was the most active catalyst, which is consistent with a previous report that artificially monomimerized BS-RNase was more active than the native dimer (Tamburrini et al., 1989), and co-migrated during SDS-PAGE with monomeric BS-RNase from seminal plasma (data not shown). The peak I protein was as active a catalyst as the dimeric enzyme from seminal plasma, but showed a lower mobility during nonreducing SDS-PAGE. Under reducing conditions, the proteins from peaks I–III co-migrated with monomeric BS-RNase from bull seminal plasma, as expected (data not shown). Native PAGE performed at pH 3.8 revealed that these three fractions were homogeneous and that only the peak II protein co-migrated with dimeric BS-RNase from bull seminal plasma (data not shown).

DISCUSSION

The T7 expression system has been used to produce many proteins in E. coli (Studier et al., 1990). The high selectivity of T7 RNA polymerase for the T7 promoter coupled with the high chain elongation rate of this polymerase results in the accumulation of a large number of transcripts; yet, an abundance of transcripts does not necessarily translate into an abundance of protein because of either poor translational initiation or protein elongation (Gold, 1990). The secondary structure of mRNA has been postulated to play an important role in the initiation of translation (Iserentant and Fiers, 1980). In particular, the accessibility of the start codon and Shine-Dalgarino sequence is believed to be critical for maximal expression (Gheysen et al., 1982; Hermes and Knowles, 1987; Li et al., 1991). The secondary structure of the mRNA transcribed from the synthetic BS-RNase gene was designed to allow for such access. The codons of the synthetic gene were also chosen for efficient protein
Bovine Seminal Ribonuclease from a Synthetic Gene

17395

(BamHI) NdeI

\[ 5' \text{GATCCCATATGAAAGAATCTGCTGCTGCTAAGTTCGAAAGACAACATGGACTCTGGT} \]

\[ 3' \text{GGTATACTTTCTTAGACGACGACGATTCAAGCTTTCTGTTGTGTACCTGAGACCATTGAGGGGTAGC} \]

Met, Lys, Glu, Ser, Ala, Ala, Lys, Phe, Glu, Arg, Gln, His, Met, Asp, Ser, Gly, Asn, Ser, Pro, Ser

SacI

\[ \text{AGCTTCGACGAGTACTGTAGTTGAGAAAGATGACTCTGCAAGTGAATAGTGAAGCCGTTAACACT} \]

TCA GAAAGTTGATGACATTGAACTTACACACACAACTTTTCTCTGACTTACACTCAGGTGACATTTGGA

Ser, Ser, Ser, Asn, Tyr, Cys, Asn, Leu, Met, Met, Cys, Cys, Arg, Lys, Met, Thr, Gln, Gln, Gly, Lys

NheI

\[ \text{GTTGTTCAAGCGACGTGACGTTAAGGCTGTCTGCAGCCA} \]

\[ \text{Ala, Tyr, Lys, Thr, Thr, Gln, Val, Glu, Lys, His, Ile, Ile, Val, Ala, Cys, Gly, Gly, Lys, Pro, Ser, Val, Pro, Val, His, Phe} \]

\( \text{(EcoRI)} \)

\[ \text{CGATTGTTCAAGCGACGTGACGTTAAGGCTGTCTGCAGCCA} \]

\[ \text{Ala, Tyr, Lys, Thr, Thr, Gln, Val, Glu, Lys, His, Ile, Ile, Val, Ala, Cys, Gly, Gly, Lys, Gly, Pro, Ser, Val, Pro, Val, His, Phe} \]

Fig. 2. DNA sequence of synthetic BS-RNase gene. The individual oligonucleotides used to construct the synthetic gene are enclosed by contiguous white or black backgrounds. Restriction enzyme recognition sites not found in expression vector pET17b are indicated.

Fig. 3. Zymogram showing time course of generation of activity during oxidation of BS-RNase from \( E. \) coli. BS-RNase activity was generated by air oxidation of reduced and denatured protein. Protein samples (100 ng) after various times of oxidation were separated by electrophoresis on nonreducing SDS-polyacrylamide gel containing poly(C). Ribonuclease activity left a clear band after staining with toluidine blue. Lane M, prestained molecular mass markers (in kilodaltons); lane 1, 0 h of air oxidation; lane 2, 4 h; lane 3, 17 h; lane 4, 40 h.

Fig. 4. Gel filtration of oxidized BS-RNase from \( E. \) coli. BS-RNase was regenerated by air oxidation and subjected to gel filtration on Sephadex G-75 resin. Absorbance (a) and ribonuclease activity (b) of fractions are shown. The column void volume (V_0), three peak fractions (peaks I-III), and the predicted migration for a monomer (m), dimer (d), and trimer (t) of BS-RNase are indicated.
forms have been produced by refolding and reoxidizing BS-RNase that had been reduced and denatured. For example, Smith et al. (1978) were unable to regenerate a significant amount of dimer from oxidation by glutathione. In contrast, Parente and D’Alessio (1985) reported that a substantial amount (40%) of dimer was regenerated by air oxidation of the NH2-terminal methionine residue, the dimer produced by oxidation by glutathione. In contrast, Smith et al. (1986) were unable to regenerate a significant amount of dimer from oxidation by seminal plasma. We have now under study.

Acknowledgment—We thank Dr. S. P. Lorton (American Breeders Service) for the kind donation of bull seminal plasma.

Note Added in Proof—The protein from peak I has been determined to be a cross-linked dimer in which one subunit is folded properly and the other subunit is folded improperly. This finding will be elaborated on elsewhere.

REFERENCES


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**TABLE I**

<table>
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<th>Source</th>
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<td>Bull seminal plasma</td>
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</table>

* Assayed by the method of Kunitz (1946).
** Calculated from Fig. 4 as described in text.
*** If peaks I–III correspond to a trimer, dimer, and monomer, respectively.
**** Not determined.