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Bovine Seminal Ribonuclease Produced from a Synthetic Gene*

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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.

Bovine seminal ribonuclease (BS-RNase)[1] (EC 3.1.27.5) is an unusual member of the superfamily of secretory ribonucleases (Beintema, 1987). Although the amino acid sequence of BS-RNase is 80% identical to that of bovine pancreatic ribonuclease (RNase A), the quaternary structure and biological properties of BS-RNase have diverged greatly from those of RNase A and other homologous ribonucleases. For example, in contrast to its homologs, BS-RNase is isolated as a dimer in which the subunits are cross-linked by two disulfide bonds. The structure of crystalline dimeric BS-RNase shows that each active site can be composed of residues from different polypeptide chains (Capasso *et al.*, 1983; Mazzarella *et al.*, 1987). Recently, a second dimeric structure was characterized in which the active sites are not composite (Piccoli *et al.*, 1992). Both of these dimeric forms are potent ribonucleases, as is the monomer produced by reducing the two disulfide bonds.

Bovine seminal ribonuclease, like other ribonucleases, catalyzes RNA transphosphorylation and hydrolysis; yet, BS-RNase has several unusual enzymatic properties. For example, the enzyme catalyzes the efficient cleavage of both double-stranded and single-stranded RNAs (Sorrentino *et al.*, 1980). Also, BS-RNase is allosteric during catalysis of the hydrolysis of nucleoside 2′:3′-monophosphates (Piccoli *et al.*, 1988).

Bulls are the only mammals known to produce a seminal ribonuclease. The concentration of BS-RNase in bull seminal plasma has been reported to be as high as 1.5 mg/ml, which corresponds to 3% of total seminal protein (D'Alessio *et al.*, 1991). Although its physiological role is not known, BS-RNase has been shown to have antispermatogenic, antitumor, and immunosuppressive activities (Dostal and Matousek, 1973; Soucek *et al.*, 1986; Tamburrini *et al.*, 1990; D'Alessio *et al.*, 1991; Laccetti *et al.*, 1992).

As a small protein (2 × 13.6 kDa) with unusual properties and a known three-dimensional structure, BS-RNase is an exquisite object for studies that use the techniques of recombinant DNA (Piccoli *et al.*, 1990). To begin to illuminate structure-function relationships within BS-RNase and in comparison to RNase A, a gene that codes for BS-RNase has been designed on criteria based largely on the secondary structure predicted for the corresponding mRNA and on codon usage. This gene has been synthesized and expressed, and the resulting protein has been characterized. This paper reports the heterologous production of active BS-RNase and the isolation of three multimeric forms of this enzyme.

EXPERIMENTAL PROCEDURES

Materials

Plasmid pBluescript II SK(−) was from Stratagene (La Jolla, CA). Expression vector pET17b and *Escherichia coli* strain BL21(DE3)/pLysS 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 Biotechnologies Inc. (New Haven, CT). Terrific broth contained (in 1 liter) Bacto-Tryptone (12 g), Bacto-yeast extract (24 g), glycerol (4 ml), KH_2PO_4 (2.31 g), and K_2HPO_4 (12.54 g). Polycytidylic 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-cyanoethyl 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 Model 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 $\epsilon^{1\%}_{1\,\mathrm{cm}} = 4.65$ at 278 nm for the native enzyme or $\epsilon^{1\%}_{1\,\mathrm{cm}} = 4.40$ at 274 nm for the reduced and denatured protein (Parente and D'Alessio, 1985). Ribonuclease activity was assayed by the method of Kunitz (1946). Free sulfhydryl groups were detected by titration with 5,5′-dithiobis(2-nitrobenzoic acid) (Creighton, 1989).

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[1] The abbreviations used are: BS-RNase, bovine seminal ribonuclease; PAGE, polyacrylamide gel electrophoresis.

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 by washing with aqueous methanol (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: phosphorylase *b* (97.4 kDa unstained and 106 kDa prestained), serum albumin (66.2 and 80.0 kDa), ovalbumin (45.0 and 49.5 kDa), carbonic anhydrase (31.0 and 32.5 kDa), trypsin inhibitor (21.5 and 27.5 kDa), and lysozyme (14.4 and 18.5 kDa). Native PAGE was performed at pH 3.8 as described (Hames, 1990). Zymogram electrophoresis was performed as described (Blank *et al.*, 1982; Kim and Raines, 1993).

Design of Gene for BS-RNase—A gene that codes for BS-RNase was designed using criteria expected to maximize the production of BS-RNase in *E. coli*. Four computer programs from the University of Wisconsin Genetics Computer Group (Madison, WI) were used to expedite the design process. First, the amino acid sequence of BS-RNase (Suzuki *et al.*, 1987) was reverse-translated into an mRNA sequence by using the BACKTRANSLATE program with the codon frequency file based on highly expressed *E. coli* proteins. Then, the fraction of A/T base pairs near the 5′-terminus of the coding region was maximized so as to minimize the stability of the mRNA secondary structure in this region. Next, the mRNA sequence was adjusted such that the most stable secondary structure predicted by the FOLD program (Zuker and Stiegler, 1981) had the AUG start codon positioned in a loop region and the Shine-Dalgarno sequence relatively exposed. The secondary structure of this sequence was also predicted and examined with the program MFOLD (Zuker, 1989). Finally, restriction endonuclease recognition sites not found in the expression vector pET17b were incorporated by using the MAP/SILENT program. These sites will facilitate future engineering of the gene.

Synthesis of Gene for BS-RNase—A gene that codes for BS-RNase was constructed by a method analogous to that of Guillemette *et al.* (1991). Briefly, 12 overlapping oligonucleotides coding for both strands of the BS-RNase gene were synthesized chemically. The 5′-hydroxyl groups of 10 of the oligonucleotides were phosphorylated with the chemical phosphorylation reagent (2-cyanoethoxy)-2-(2′-*O*-4,4′-dimethoxytrityloxyethylsulfonyl)ethoxy-*N*,*N*-diisopropylaminophosphine (Horn and Urdea, 1986) from Glen Research (Sterling, VA). The oligonucleotides at the two 5′-termini of the gene were not phosphorylated. The individual oligonucleotides were purified by denaturing polyacrylamide gel electrophoresis. The 12 oligonucleotides were annealed and then ligated *in situ* by using T4 ligase. The assembled DNA fragment was inserted into pBluescript II SK(−), which had been previously digested with *Eco*RI and *Bam*HI and gel-purified with the GeneClean II kit from BIO101 (La Jolla, CA), to yield pBSR. The nucleotide sequence of the synthetic gene was determined with the Sequenase version 2.0 kit from United States Biochemical Corp.

Expression of Synthetic Gene in E. coli—Plasmid pBSR was digested with *Nde*I and *Eco*RI. The 460-base pair fragment containing the BS-RNase gene was isolated and inserted into expression vector pET17b, which had been previously digested with *Nde*I and *Eco*RI, to yield plasmid pSR1. Plasmid pSR1 was used to transform *E. coli* strain BL21(DE3)/pLysS, the expression host.

To express the gene for BS-RNase, *E. coli* strain BL21(DE3)/pLysS/pSR1 was grown at 37 °C in terrific broth (0.5 liter) containing ampicillin (100 µg/ml) until the absorbance at 600 nm was 4.0 *A* units. Isopropyl-1-thio-β-D-galactopyranoside was then added to a final concentration of 0.4 mM, and the cells were grown overnight at 37 °C.

Purification of BS-RNase from E. coli—Cells were harvested by centrifugation and then resuspended in 20 mM sodium phosphate buffer, pH 7.0, containing EDTA (10 mM). The resuspended cells were lysed by passage twice through a French press. Insoluble material containing BS-RNase was collected by centrifugation at 12,000 × *g* for 30 min and then washed with the same buffer. The pellet was solubilized by adding 5 ml of 10 mM Tris-HCl buffer, pH 8.0, containing guanidine HCl (6 M) and EDTA (10 mM). Dithiothreitol was then added to a final concentration of 0.1 M. After incubation at room temperature for 3 h, the solution was dialyzed exhaustively *versus* 20 mM acetic acid. The solution was then centrifuged to remove material that had precipitated during dialysis. Active BS-RNase was generated by diluting the dialyzed solution to various protein concentrations in 0.1 M Tris-HOAc buffer, pH 8 (Parente and D'Alessio, 1985). The regeneration solution was incubated in an open container at room temperature to allow for air oxidation. After 24 h, iodoacetamide was added to a final concentration of 10 mM to alkylate any remaining sulfhydryl groups. The appearance of active BS-RNase was monitored by measuring ribonuclease activity and sulfhydryl content and by zymogram electrophoresis.

Purification of Multimeric Forms of BS-RNase from E. coli—The solution of oxidized enzyme was concentrated by using a Centriprep-10 concentrator (Amicon Corp.). The resulting concentrate (10 ml) was loaded onto a column (100 cm × 5 cm^2) of Sephadex G-75 superfine resin that had been equilibrated with 20 mM sodium phosphate buffer, pH 7.0. The column was eluted at a rate of 10 ml/h with the same buffer, and fractions (6.4 ml) were collected. The fractions were assayed for A_{280} and ribonuclease activity. The apparent molecular mass of BS-RNase was determined by comparing its elution volume with that of proteins of known molecular mass, including aggregates produced by lyophilization of RNase A from 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 (41.0 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, 1964).

NH$_2$-terminal Sequence Analysis—Protein to be sequenced was separated by electrophoresis on an SDS-polyacrylamide gel and then transferred to a polyvinylidene difluoride membrane (Bio-Rad) by using a Mini-Trans-Blot Cell (Bio-Rad). NH$_2$-terminal sequence was determined by using an Applied Biosystems Model 470A Protein Sequencer at the Biotechnology Center, University of Wisconsin (Madison, WI).

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 (Tamburrini *et al.*, 1986).

RESULTS

Design and Construction of Synthetic BS-RNase Gene—A gene was designed to code for BS-RNase and to maximize translational initiation and protein elongation in *E. coli*. To facilitate the initiation of translation, the nucleotide sequence of the gene was adjusted to expose the start codon and Shine-Dalgarno sequence of its transcript in vector pSR1. The structure of lowest free energy predicted for this transcript by the program FOLD ($\Delta G^0 = -45.9$ kcal/mol) is shown in Fig. 1. In this structure as well as in the lowest free energy structure predicted by the program MFOLD ($\Delta G^0 = -38.3$ kcal/mol), the start codon and 2 bases of the Shine-Dalgarno 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 Preuβ *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 produced constituted ~15% of the total *E. coli* protein or ~100 mg of BS-RNase from the 0.5-liter culture. BS-RNase appeared to form inclusion bodies, which facilitated its purification. The insoluble fraction was dissolved in 6 M guanidine HCl and reduced with dithiothreitol. The resulting solution of denatured and reduced protein was dialyzed *versus* dilute acid to remove guanidine HCl and dithiothreitol while inhibiting the formation of disulfide bonds. BS-RNase remained soluble during dialysis, but most other proteins precipitated and were hence easily removed by centrifugation.

Generation of Ribonuclease Activity by Air Oxidation—Ribonuclease activity was generated by air oxidation. The catalytic activity of ribonuclease generated after 14 h of air oxidation was independent of protein concentration if the protein concentration was <0.7 mg/ml. Less active ribonuclease was generated at protein concentrations >0.7 mg/ml, presumably due to protein aggregation. The concentration of active

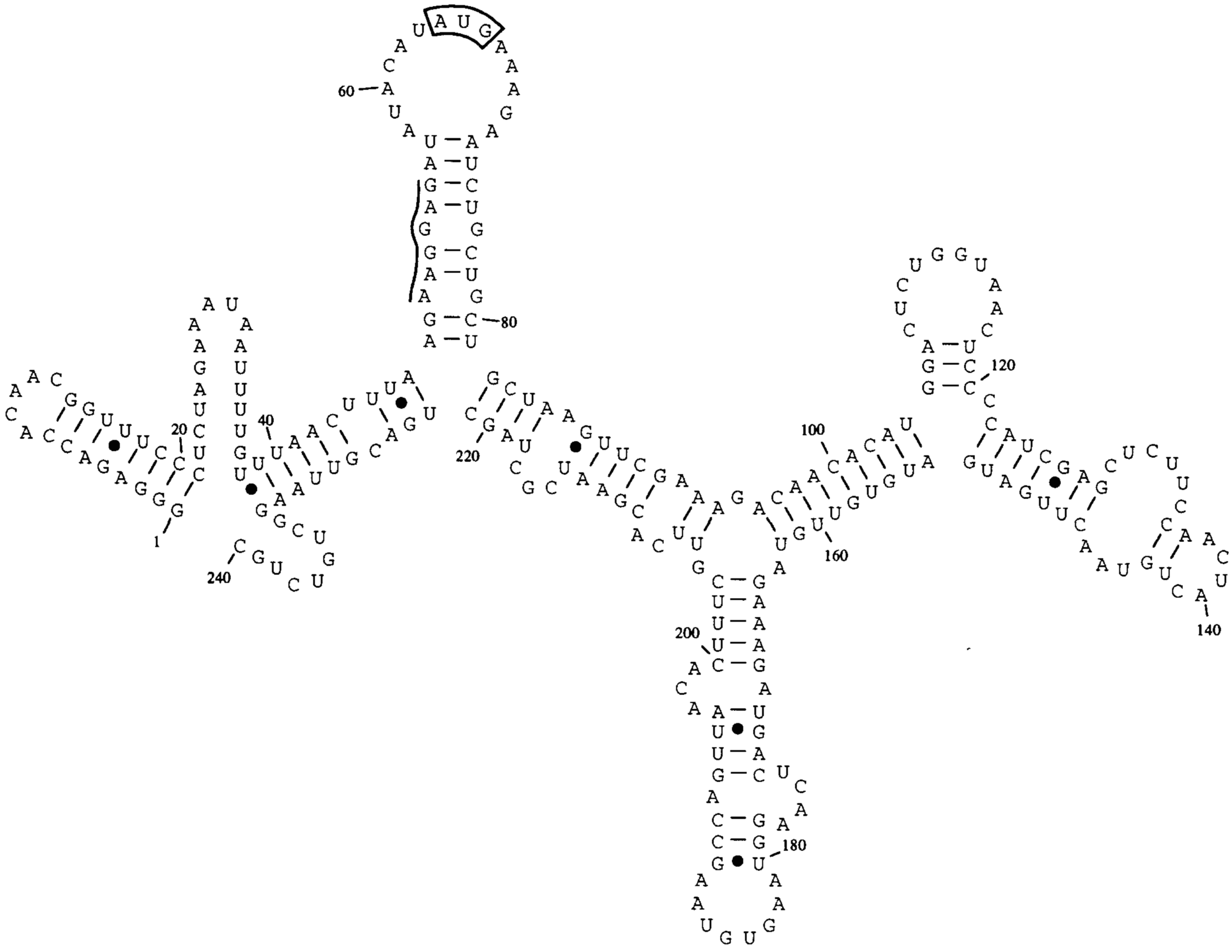

FIG. 1. **Predicted secondary structure of mRNA transcribed from pSR1.** The secondary structure of lowest free energy ($\Delta G^0 = -45.9$ kcal/mol) was predicted by the program FOLD (Zuker and Stiegler, 1981) and drawn by the program LOOPVIEWER (Don Gilbert, Indiana University, Bloomington, IN). The Shine-Dalgarno sequence is *side-lined*, and the start codon is *boxed*.

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 NH_2 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 monomerized 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-Dalgarno 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

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  (BamHI)NdeI                          Csp45I
  5'GATCCCATATGAAAGAATCTGCTGCTGCTAAGTTCGAAAGACAACACATGGACTCTGGTAACTCCCCATCG
      3'GGTATACTTTCTTAGACGACGACGATTCAAGCTTTCTGTTGTGTACCTGAGACCATTGAGGGGTAGC
            Met_-1LysGluSerAlaAlaAlaLysPheGluArgGlnHisMetAspSerGlyAsnSerProSer_20
SacI
AGCTCTTCCAACTACTGTAACTTGATGATGTGTTGTAGAAAGATGACTCAAGGTAAGTGTAAGCCAGTTAACACT
TCGAGAAGGTTGATGACATTGAACTACTACACAACATCTTTCTACTGAGTTCCATTCACATTCGGTCAATTGTGA
SerSerSerAsnTyrCysAsnLeuMetMetCysCysArgLysMetThrGlnGlyLysCysLysProValAsnThr_45
               NheI                 PstI
TTCGTTCACGAATCGCTAGCTGACGTTAAGGCTGTCTGCAGCCAAAAGAAGGTTACTTGTAAGAACGGTCAAACC
AAGCAAGTGCTTAGCGATCGACTGCAATTCCGACAGACGTCGGTTTTCTTCCAATGAACATTCTTGCCAGTTTGG
PheValHisGluSerLeuAlaAspValLysAlaValCysSerGlnLysLysValThrCysLysAsnGlyGlnThr_70
                                                 AgeI                MunI
AACTGTTACCAATCTAAGTCCACCATGAGAATCACTGACTGTAGAGAAACCGGTTCCTCTAAGTACCCCAATTGT
TTGACAATGGTTAGATTCAGGTGGTACTCTTAGTGACTGACATCTCTTTGGCCAAGGAGATTCATGGGGTTAACA
AsnCysTyrGlnSerLysSerThrMetArgIleThrAspCysArgGluThrGlySerSerLysTyrProAsnCys_95

GCTTACAAGACCACTCAAGTTGAAAAGCACATCATTGTTGCTTGTGGTGGTAAGCCATCCGTTCCAGTTCACTTC
CGAATGTTCTGGTGAGTTCAACTTTTCGTGTAGTAACAACGAACACCACCATTCGGTAGGCAAGGTCAAGTGAAG
AlaTyrLysThrThrGlnValGluLysHisIleIleValAlaCysGlyGlyLysProSerValProValHisPhe_120
                (EcoRI)
GACGCTTCTGTTTAATG 3'
CTGCGAAGACAAATTACTTAA 5'
AspAlaSerVal_124
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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.

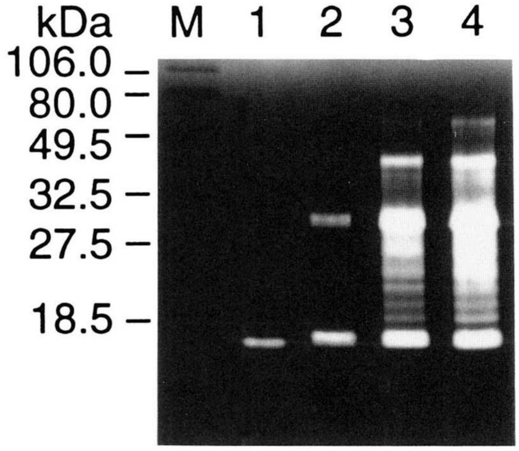

FIG. 3. **Zymogram showing time course of generation of activity during oxidation of BS-RNase from *E. coli*.** BS-RNase activity was generated by air oxidation of reduced and denatured protein. Protein samples (100 ng) after various times of oxidation were separated by electrophoresis on nonreducing SDS-polyacrylamide gel containing poly(C). Ribonuclease activity left a clear band after staining with toluidine blue O. *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.

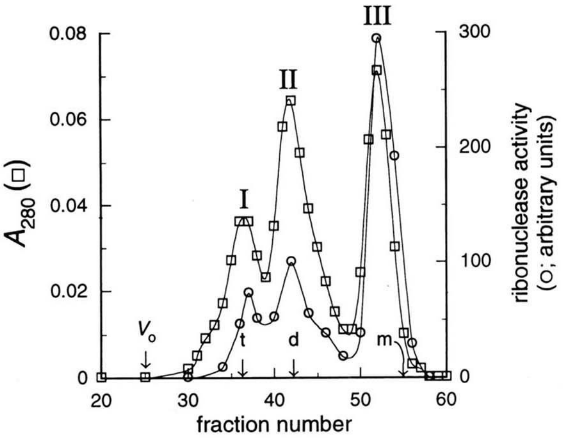

FIG. 4. **Gel filtration of oxidized BS-RNase from *E. coli*.** BS-RNase was regenerated by air oxidation and subjected to gel filtration on Sephadex G-75 resin. Absorbance (□) and ribonuclease activity (○) of fractions are shown. The column void volume (V_0), three peak fractions (*peaks I–III*), and the predicted migration for a monomer (*m*), dimer (*d*), and trimer (*t*) of BS-RNase are indicated.

elongation by considering the codons common in highly expressed genes of *E. coli*.

BS-RNase produced in *E. coli* by the T7 RNA polymerase expression system appears to form inclusion bodies, which here has several advantages. For example, the heterologous production of active ribonucleases is thought to be toxic to *E. coli* (Nambiar *et al.*, 1987; Hartley, 1989). BS-RNase in inclusion bodies is likely to be inactive. Another advantage is that isolation of inclusion bodies after cell lysis results in a substantial purification of BS-RNase (Fig. 5). This purification is of particular utility in separating endogenous *E. coli* ribonucleases, such as RNases I and II (Cannistraro and Kennell, 1991), from BS-RNase. Fortunately, these *E. coli* enzymes remain soluble and are therefore easily separable from insoluble inclusion bodies containing BS-RNase.

The air oxidation of reduced and denatured protein was followed by zymogram electrophoresis (Fig. 3), which provides both the catalytic activity and molecular mass of ribonucleases. At least five minor bands appear between monomer and dimer (Fig. 3, *lanes 3* and *4*). These bands are not visible on a polyacrylamide gel stained with Coomassie Brilliant Blue (data not shown). We suspect that these bands (<5% of total protein) are mixed disulfides between peptides and monomeric BS-RNase. (Zymogram electrophoresis is performed on a denaturing but nonreducing gel.) Multimers seen above the dimer region (Fig. 3, *lanes 3* and *4*) may also appear during gel filtration chromatography (Fig. 4, shoulder on *peak I*), but were not further characterized.

Bovine seminal ribonuclease isolated from seminal plasma is a dimer (Fig. 5, *lane 8*). Nevertheless, different multimeric

TABLE I

Catalytic activity and apparent molecular mass of BS-RNase isolated from E. coli or bull seminal plasma

Source	Activity[a]	Apparent molecular mass[b]	Molecular mass[c]
	units/mg	*kDa*	*kDa*
E. coli peak I	26	40	40.8
E. coli peak II	21	29	27.2
E. coli peak III	53	16	13.6
Bull seminal plasma	22	ND[d]	ND

[a] Assayed by the method of Kunitz (1946).

[b] Calculated from Fig. 4 as described in text.

[c] If peaks I–III correspond to a trimer, dimer, and monomer, respectively.

[d] Not determined.

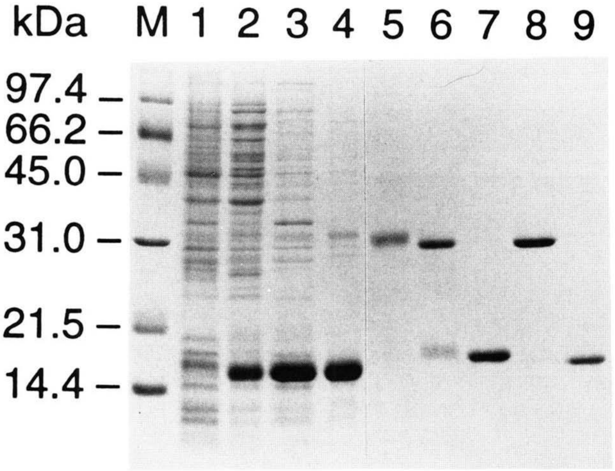

FIG. 5. **SDS-polyacrylamide gel showing purification of BS-RNase from *E. coli*.** Recombinant BS-RNase was purified from *E. coli* strain BL21(DE3)/pLysS/pSR1 and compared to native BS-RNase purified from bull seminal plasma (*lane 8*) and RNase A (*lane 9*). Proteins were separated by electrophoresis on an SDS-polyacrylamide gel under reducing (*lanes 1–4*) or nonreducing (*lanes 5–9*) conditions. *Lane M*, unstained molecular mass markers (in kilodaltons); *lane 1*, total cellular protein from BL21(DE3)/pLysS/pSR1 before induction; *lane 2*, total cellular protein after induction by isopropyl-1-thio-β-D-galactopyranoside; *lane 3*, insoluble fraction collected by centrifugation of cell lysate; *lane 4*, reduced and denatured protein after dialysis in 20 mM acetic acid; *lane 5*, peak I fraction from chromatography on Sephadex G-75; *lane 6*, peak II fraction; *lane 7*, peak III fraction.

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 reduced and denatured protein from seminal plasma. We have generated a similar fraction of dimer by air oxidation of the protein from *E. coli*. Furthermore, despite the presence of an NH_2-terminal methionine residue, the dimer produced by *E. coli* is as active a catalyst as that isolated from seminal plasma. The structure of crystalline BS-RNase indicates that the NH_2 terminus of each subunit is on the surface of the protein, far removed from the active site (Capasso *et al.*, 1983). The presence of an NH_2-terminal methionine residue therefore should not (and apparently does not) affect its ability to cleave single-stranded RNA.

We have also observed that another form of active BS-RNase, in addition to the monomer and dimer reported by previous workers, is generated by air oxidation. The molecular mass of this protein is estimated by gel filtration chromatography to be 40 kDa, which is consistent with its being a BS-RNase trimer. Since this putative trimer migrated only slightly slower than the BS-RNase dimer on a nonreducing SDS-polyacrylamide gel, the structure of the denatured form of this protein must be relatively compact. The properties of this apparent trimer are 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.

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