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Isolation of Bovine Angiogenin Using a Placental Ribonuclease Inhibitor Binding Assay†

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Received March 14, 1988

ABSTRACT: Angiogenin, which induces the formation of new blood vessels, was isolated previously from two human sources—HT-29 tumor conditioned media and normal plasma. By use of a newly developed binding assay, a similar protein has now been purified from bovine plasma at levels of 30–80 μg/L. This protein has the structural, enzymatic, and biological characteristics expected for an angiogenin molecule. Its amino acid composition is similar to that of the human protein, and 22 of 31 residues in the amino-terminal sequences are identical, including a block of 11 consecutive residues. Like human angiogenin, the bovine protein binds placental ribonuclease inhibitor, is inactive toward conventional RNase A substrates, and displays selective ribonucleolytic activity toward some rRNAs. In addition, the bovine protein induces angiogenesis in vivo in the chick embryo chorioallantoic membrane assay at levels as low as 44 fmol per egg. Thus, angiogenin is present in bovine sera at levels similar to those observed in man, and its enzymatic and biological activities are identical with those of the human protein.

Human angiogenin is a single-chain, basic protein of 14 100 Da (daltons) which induces blood vessel growth in vivo in doses as low as 35 fmol (Fett et al., 1985). It is present in conditioned media from HT-29 human colon adenocarcinoma cells and also in normal human plasma, where it is recovered at levels of 60–150 μg/L (Shapiro et al., 1987a). The primary sequence of angiogenin shows 35% identity with that of human pancreatic RNase A[1] (Strydom et al., 1985; Kurachi et al., 1985). Although inactive toward commonly used substrates for pancreatic RNases, it does exhibit ribonucleolytic activity toward ribosomal and transfer RNAs (Shapiro et al., 1986, 1987a,b; St. Clair et al., 1987). Both the ribonucleolytic and angiogenic activities are completely inhibited by placental ribonuclease inhibitor (PRI) (Shapiro & Vallee, 1987), which binds tightly to the protein with a K_i below 1 pM.[2]

The isolation of angiogenin from human plasma suggested that sera from other species might also serve as sources of the protein. Bovine plasma was chosen initially because of its ready availability and also because of the potential implications that this material might have for cell culture experiments conducted in bovine serum. Preliminary attempts at purification were unsuccessful, however, due to the lack of a convenient assay. This problem was alleviated by the recent development of a binding assay for angiogenin.[3] We now report the use of this procedure to purify to homogeneity a protein from bovine plasma which, on the basis of its physical, enzymatic, and biological properties, is bovine angiogenin.

Experimental Procedures

Materials. Young calf (1–2 weeks old) and mature bovine (at least 1.5 years old) blood from mixed breeds was obtained from local slaughterhouses. Calf serum (sterile, for cell culture) and fetal calf serum (sterile, for hybridomas) were obtained from Whittaker M. A. Bioproducts. CM-cellulose (grade CM-52) ion-exchange resin was a product of Whatman Ltd. Yeast RNA (highly polymerized) and wheat germ RNA

† This work supported by funds from the Hoechst Co. under agreements with Harvard University. M.D.B. was supported by National Research Service Award H6-06894 from the National Heart, Lung and Blood Institute.
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[1] Abbreviations: RNase(s), ribonuclease(s); RNase A, bovine pancreatic ribonuclease A; PRI, human placental ribonuclease inhibitor; CAM, chorioallantoic membrane; CM 2, salt-eluted fraction from the (carboxymethyl)cellulose column; MS 1, pool of angiogenin-containing fractions from Mono S chromatography in Tris buffer, pH 8.0; MS 2, pool of angiogenin-containing fractions from Mono S chromatography in phosphate buffer, pH 7.0; CM, carboxymethyl; C18, octadecylsilane; HPLC high-performance liquid chromatography; Tris, tris(hydroxymethyl)aminomethane; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; HSA, human serum albumin; TFA, trifluoroacetic acid; poly(C), poly(cytidylic acid); poly(U), poly(uridylic acid); <Glu, pyroglutamic acid.

[2] Frank S. Lee, personal communication.

[3] Manuscript in preparation.

0006-2960/88/0427-6282$01.50/0

were purchased from Calbiochem; 28S and 18S ribosomal RNA (calf liver), poly(U), and poly(C) were from Pharmacia Molecular Biologicals; SEP-PAK C18 cartridges and Millex-HA 0.45-μm filter units were from Millipore Corp.; endoglycosidase F was from New England Nuclear; and N-glycanase was from Genzyme Corp. (Boston, MA).

Proteins. Human angiogenin was purified from plasma as described by Shapiro et al. (1987a), and the concentration was determined by amino acid analysis. Bovine pancreatic RNase A was obtained from Cooper Biomedical; its concentration was determined spectrophotometrically with $\epsilon_{278} = 9800\ M^{-1}\ cm^{-1}$ (Sela & Anfinsen, 1957). Bovine pancreatic RNase B was purchased from Sigma Chemical Co. and purified with Con A–Sepharose; its concentration was determined with the above ϵ_{278} value. PRI was purified from human placenta by the method of Blackburn (1979) and stored in the affinity column eluting buffer. Its concentration was determined by inhibition of bovine RNase A activity toward yeast RNA. Human serum albumin and chicken egg white lysozyme (grade 1) were from Sigma Chemical Co.

Purification of Bovine Angiogenin. Angiogenin was isolated from bovine plasma (or serum) according to a modification of the procedure described previously for human angiogenin (Shapiro et al., 1987a). Bovine blood (10–40 L) was collected in plastic carboys containing a 12.3% volume of citrate–phosphate–dextrose anticoagulant, which consisted of 26.3 g of sodium citrate dihydrate, 3.27 g of citric acid monohydrate, 25.5 g of dextrose monohydrate, and 2.22 g of sodium phosphate monobasic monohydrate per liter of H_2O. The blood was centrifuged (2500*g*, 30 min) at 4 °C within 24 h, and the plasma was removed and stored frozen at −20 °C. After thawing, the plasma was brought to pH 6.6 with 3 N HCl and passed through Whatman 934-AH glass microfiber filters to clarify; filters were changed repeatedly as needed. The plasma was loaded onto CM-52 cation-exchange resin in a sintered glass funnel, and the resin was washed, transferred to a 2.5 cm diameter column, and eluted as described by Shapiro et al. (1987a). The salt-eluted fraction, which contained the angiogenin, was designated as CM 2.

Several CM columns were performed on a smaller scale, beginning with 0.1–2 L of plasma or serum. In these cases a ratio of 50 mL of resin per liter of starting material was used, and lysozyme (1 mg/0.25 L) was added as "carrier" protein. Anticoagulant solution was also added to serum samples before the pH was adjusted.

The CM 2 was concentrated at 4 °C on an Amicon pressure filtration unit with a YM-5 filter. It was then centrifuged, diluted with 7 parts of 10 mM Tris, pH 8.0, buffer, and applied to a Mono S column (0.5 cm × 5 cm, Pharmacia) equilibrated in 10 mM Tris containing 0.05 M NaCl, pH 8.0. The column was eluted at 0.8 mL/min with an 80-min linear gradient of 0.05–0.40 M NaCl in 10 mM Tris, pH 8.0. Fractions, collected at 1-min intervals, were assayed for both the PRI binding and RNase activities. Those fractions containing significant PRI binding activity but little RNase activity were pooled and subsequently designated Mono S, or MS 1.

For large-scale purifications (10–20 L of plasma), the MS 1 pool was subjected to an additional purification step before being applied to a C18 reversed-phase column. Thus, the MS 1 pool was concentrated as above, diluted 1:3 with H_2O, and applied to a Mono S column previously equilibrated with 90% buffer A (20 mM sodium phosphate, pH 7.0) and 10% buffer B (400 mM sodium phosphate, pH 7.0). Buffers A and B were prepared by titrating Na_2HPO_4 with phosphoric acid to pH 7.0. The column was eluted with a linear gradient from 10 to 50% buffer B over 60 min at 0.8 mL/min; 1-min fractions were collected and assayed. Those fractions containing PRI binding protein(s) but very low RNase activity were pooled and designated MS 2. The MS 2 or MS 1 pools were then applied to a Synchropak RP-P C18 HPLC column (0.46 cm × 25 cm; Synchrom, Inc.) and eluted over 54 min with a linear gradient from 30% to 42% solvent B. Solvent A was 0.1% TFA (v/v), and solvent B was 3:2:2 (v/v) 2-propanol/acetonitrile/water with 0.08% TFA. The flow rate was 0.8 mL/min; fractions were collected at 1-min intervals.

Both Mono S and C18 chromatographies were performed with a Waters Associates system together with a Hewlett-Packard 3390A integrator. Column effluents were monitored at either 214 or 254 nm. Fractions were collected in 5-mL polypropylene chemtubes (Bio-Rad).

Samples to be tested for ribonucleolytic activity against 28S and 18S rRNA were subjected to rechromatography on the C18 column with the same gradient. Reinjection improved separation from trace amounts of a plasma RNase.

Detection of Angiogenin. Angiogenin was detected in chromatographic fractions according to a procedure described in detail elsewhere.[3] Briefly, the procedure employs two assays. The first assay detects binding to PRI, whether by angiogenin, ribonucleases, or other molecules. The results are expressed in terms of RNase A equivalents, i.e., the concentration in micrograms per milliliter of a solution of RNase A which would be required to bind the same amount of inhibitor. The second assay detects RNase activity toward yeast RNA, and activities are also expressed in terms of RNase A equivalents. The presence of angiogenin in a given fraction is indicated by a high value of RNase A equivalents in the PRI binding assay with a relatively low value in the RNase assay.

The concentration of angiogenin in a sample which is devoid of contaminating RNases can be determined by multiplying the RNase equivalents from the binding assay by 0.97 to account for the different molecular weights of angiogenin and RNase A.

RNase Assays. RNase activity toward yeast RNA was measured by a modification of the precipitation method of Blackburn et al. (1977) as described elsewhere.[3] Activity toward poly(C) and poly(U) was measured by a modification of the method of Zimmerman and Sandeen (1965) as described by Shapiro et al. (1986). RNase activity toward wheat germ RNA was determined by the precipitation assay of Blank and Dekker (1981) with 4–6-h incubations at 37 °C, as described by Shapiro et al. (1986).

All water and buffer solutions used in the assay were passed through Sep-Pak C18 cartridges to remove trace amounts of RNase activity.

Biological Assays. Angiogenesis was assessed according to the chick embryo chorioallantoic membrane (CAM) method of Knighton et al. (1977) as described (Fett et al., 1985). Fifteen eggs were employed in each set of assays at a given concentration. The eggs were read after 68 (±2) h. Average probabilities were calculated as described (Shapiro et al., 1987a). A significance level of ≤5% has to be attained for a sample to be considered active.

Cleavage of 28S and 18S rRNA. Ribonucleolytic activity toward 28S and 18S rRNA was determined as described (Shapiro et al., 1986) with calf liver rRNA as substrate.

Cleavage of Reticulocyte Ribosomes and Inhibition of in Vitro Protein Synthesis. The effects of bovine and human angiogenin on intact ribosomes and on protein translation in rabbit reticulocyte lysate were determined as described by St. Clair et al. (1987).

Table I: Purification of Angiogenin from Bovine Plasma

purification step	recovery (mg) protein[a]	angiogenin[b]
bovine plasma (20 L)	7.2×10^5	*c*
CM 2	53	*c*
MS 1	8.6	1.5
MS 2	0.71	0.72
C18 HPLC	0.56	0.60

[a] Determined by amino acid analysis. [b] Estimated from PRI binding assays of chromatographic fractions. [c] Not measured due to the presence of plasma RNases.

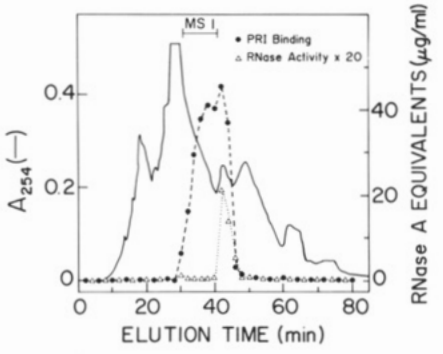

FIGURE 1: Mono S cation-exchange chromatography of the CM 2 fraction from 5 L of bovine plasma. The CM 2 containing approximately 18 mg of protein was concentrated to 2 mL, diluted to 16 mL with 10 mM Tris, pH 8.0, and applied to the column. Elution was accomplished with a linear gradient, 0.05–0.40 M NaCl in 10 mM Tris, pH 8.0, over 80 min. Fractions were assayed for both PRI binding and RNase activities.

Amino Acid and Sequence Analyses. Amino acid analysis was performed as described (Strydom et al., 1985) according to the picotag derivatization method (Bidlingmeyer et al., 1984). Samples for cysteine analysis were oxidized with performic acid (Moore, 1963) prior to hydrolysis. Samples for tryptophan analysis were hydrolyzed in 4 N methanesulfonic acid containing 0.2% tryptamine (Pierce Chemical Co.) and then neutralized with NaOH before derivatization.

Automated Edman degradation was performed on 2 nmol of bovine angiogenin by use of a Beckman 890 C sequencer with 0.1 M quadrol coupling buffer and Beckman program 121078 as described (Strydom et al., 1985).

Gel Electrophoresis of Proteins. SDS–PAGE was performed on 15% polyacrylamide gels with 5% stacking gels as described by Laemmli (1970). Gels for photography were stained with Coomassie Blue by conventional procedures. Low molecular weight standards were from Bethesda Research Laboratories.

RESULTS

Isolation Procedures. In the first step of the purification, bovine plasma is applied to CM-52 cation-exchange resin; the vast majority of the plasma protein passes through unbound (Table I). The remaining protein is then eluted with 1 M NaCl and designated CM 2. After being concentrated and desalted, this material is applied to a Mono S column and eluted with a salt gradient. Fractions are assayed for both PRI binding and RNase activities (Figure 1) and typically reveal a broad peak of PRI binding activity partially separated from a plasma RNase. Fractions containing significant levels of PRI binding activity and little or no RNase activity are pooled and designated MS 1.

For most preparations the MS 1 fraction is applied directly to a C18 reversed-phase column, the final step in the purification. For the large-scale preparations (10 L of plasma or more), however, the MS 1 is first chromatographed on the Mono S column under new conditions to remove the high molecular weight contaminants. In these cases it is concentrated, applied to the Mono S column, and eluted with a sodium phosphate gradient at pH 7.0. The PRI binding activity elutes in a relatively sharp peak with little contaminating RNase activity (not shown); appropriate fractions are pooled and designated MS 2.

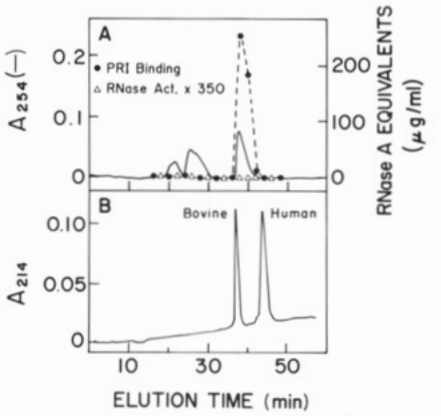

FIGURE 2: (A) Final step in the purification of bovine angiogenin. The MS 2 fraction (0.71 mg of protein) from 20 L of bovine plasma was chromatographed on a Synchropak RP-P C18 column as described under Experimental Procedures. Even-numbered fractions were assayed for PRI binding and RNase activities. (B) C18 HPLC of a mixture of purified bovine angiogenin (5 μg) and human angiogenin (8 μg). Chromatographic conditions were the same as in (A), except that column effluent was monitored at 214 nm.

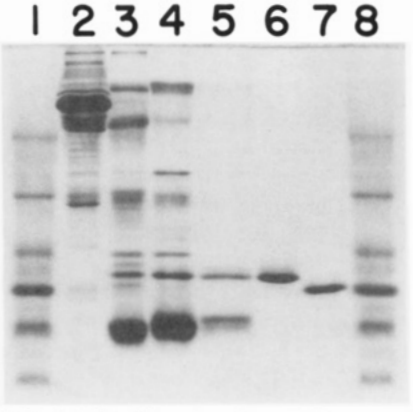

FIGURE 3: SDS–PAGE of fractions from different steps in the purification of bovine angiogenin from 20 L of plasma. Lane 2 contained bovine plasma; lane 3, the CM 2 fraction; lane 4, the MS 1 fraction; lane 5, the MS 2 fraction; lane 6, C18 HPLC purified bovine angiogenin; lane 7, human angiogenin; lanes 1 and 8, standards with molecular weights shown at the left ($\times 10^{-3}$).

Reversed-phase chromatography of the MS 1 or MS 2 pools using a 2-propanol/acetonitrile/water gradient typically reveals several peaks (Figure 2A), only one of which displays PRI binding activity. Fractions across the peak have no detectable RNase activity, even when assayed at much higher concentrations than those used for the binding assays. When the peak fractions are pooled and an aliquot is rechromatographed on the C18 column on an analytical scale, a single symmetrical peak is observed with a retention time of 37 min, approximately 7 min earlier than human angiogenin (Figure 2B). SDS–PAGE of the pooled material reveals a single band (Figure 3, lane 6).

The yield from most purifications is 30–80 μg of the bovine angiogenin per liter of plasma or serum (Table I), higher yields being obtained when the Mono S column in phosphate buffer (to give MS 2) is omitted. The age of the donor animals does not appear to affect the yield; recoveries are essentially the

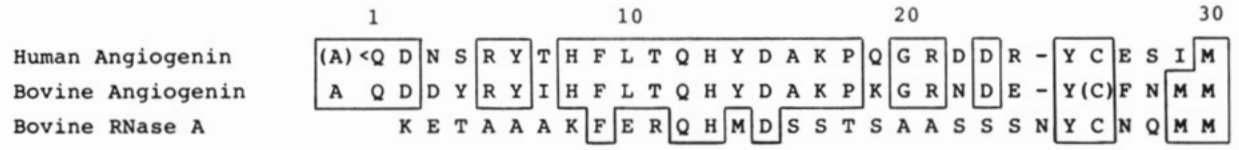

FIGURE 4: Comparison of the amino-terminal sequences of human angiogenin (Strydom et al., 1985), bovine angiogenin, and bovine pancreatic RNase A (Smyth et al., 1963). The alignment is numbered according to the human protein. The gap at 24 is arbitrarily placed and could occur anywhere from 16 through 24. The Ala at position −1 in the human sequence is from the gene sequence (Kurachi et al., 1987), and the Cys at position 26 in bovine angiogenin is inferred from the human sequence. <Glu is pyroglutamic acid.

same for plasma from young calves (1–2 weeks), calves (1–6 months), or mature animals (>1.5 years).

The bovine protein was also isolated from fetal bovine serum. It is indistinguishable from that obtained from adult animals on the basis of molecular weight, amino acid composition, and amino-terminal sequence (data not shown). The yields from fetal serum are lower, however, and average approximately 30 μg/L.

In some purifications from serum a second form of bovine angiogenin was observed which eluted 1.5 min later on the reversed-phase column. The amino-terminal sequences of the two forms were identical, as were the molecular weights. Amino acid compositions were the same within experimental error, except that the second form appeared to contain one less His residue. Since the second protein was never detected in plasma, a plausible explanation might involve limited cleavage at the carboxyl terminal by proteases generated during blood coagulation.

Physicochemical Characterization. The molecular weight of the bovine protein is 16 200 ± 400 by SDS–PAGE (Figure 3), which is greater than the 14 400 ± 400 value obtained for human angiogenin. The molecular weight is not affected by the presence or absence of 2-mercaptoethanol or by treatment with N-glycanase or endoglycosidase F, enzymes which deglycosylated pancreatic RNase B (Tarentino et al., 1985; Hirani et al., 1987) in a series of control experiments. Cation-exchange HPLC suggests that the bovine protein is very basic, although less so than human angiogenin. The purified protein elutes from the Mono S column (Tris buffer, pH 8.0, gradient as indicated under Experimental Procedures) with a retention time of ~53 min, which is greater than the ~39-min retention time of lysozyme (p*I* 10.5) but less than the ~71 min for human angiogenin (data not shown).

Table II shows the amino acid composition; calculations are based on a total of 123 residues per mole. Of all the amino acids, only Ser and Asp (+Asn) differ by more than two residues per mole from the composition of human angiogenin. Compared to one Met and one Trp residue in human angiogenin, the bovine protein contains two Met residues but no Trp. Human angiogenin contains six cysteines which form three disulfide bonds; all six Cys residues appear to be present in the bovine protein.

Amino-Terminal Sequence. Edman degradation of the native bovine protein allowed assignment of 30 of the first 31 residues at the amino terminus (Figure 4). The Cys in position 26 (numbering as in human angiogenin) was inferred from the human sequence; the lack of an identifiable peak at this cycle is consistent with the presence of a disulfide bond, as occurs in human angiogenin (Strydom et al., 1985).

RNase A Type Activity. The purified bovine protein had no detectable activity toward wheat germ RNA, poly(C), or poly(U), which are conventional RNase A substrates. Under the assay conditions employed, any activity greater than 1×10^{-5} that of bovine RNase A would have been detected.

Degradation of 28S and 18S rRNA. Human angiogenin displays endonucleolytic activity toward 28S and 18S rRNA (Shapiro et al., 1986). It cleaves the rRNA to form characteristic products of 100–500 nucleotides. Thus, the bovine protein was incubated with 28S and 18S rRNA, and the cleavage products were analyzed by agarose gel electrophoresis. The cleavage pattern generated is nearly identical with that of human angiogenin (Figure 5), indicating that the two

Table II: Amino Acid Composition of Bovine Angiogenin

amino acid	residues/mol	
	bovine angiogenin (±SD)[a]	human angiogenin[b]
Asp	17.31 ± 0.65	15
Glu	10.41 ± 0.30	10
Ser	5.39 ± 0.23	9
Gly	9.30 ± 0.31	8
His	5.71 ± 0.13	6
Arg	12.10 ± 0.28	13
Thr	6.12 ± 0.16	7
Ala	4.46 ± 0.40	5
Pro	7.54 ± 0.28	8
Tyr	5.22 ± 0.20	4
Val	3.61 ± 0.42	5
Met	2.10 ± 0.10	1
Ile	8.61 ± 0.57	7
Leu	4.41 ± 0.21	6
Phe	5.98 ± 0.10	5
Lys	8.66 ± 0.49	7
Cys	5.71	6
Trp	0.21	1

[a] Average of 11 different analyses, except for Cys and Trp, which were from duplicate analyses from a single preparation. Cys was determined as cysteic acid; Trp was determined after hydrolysis in methanesulfonic acid in the presence of tryptamine. [b] From sequence (Strydom et al., 1985).

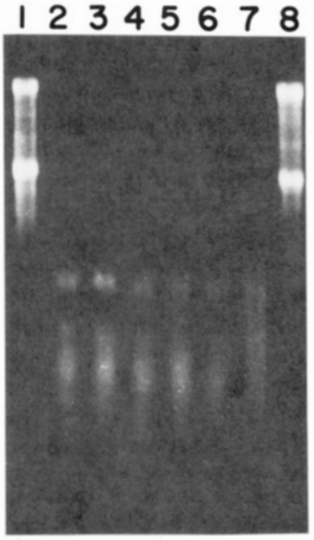

FIGURE 5: Degradation of 28S and 18S rRNA by bovine and human angiogenins. Samples were incubated with calf liver 28S and 18S rRNA at 37 °C for 30 min and after being quenched were electrophoresed under denaturing conditions on a 1.1% agarose gel. RNA was visualized with ethidium bromide. Lanes 1 and 8, 28S and 18S rRNA controls; lanes 2 and 3, samples containing human angiogenin at 33 and 22 μg/mL, respectively; lanes 4–7, samples containing bovine angiogenin at 73, 58, 44, and 29 μg/mL, respectively.

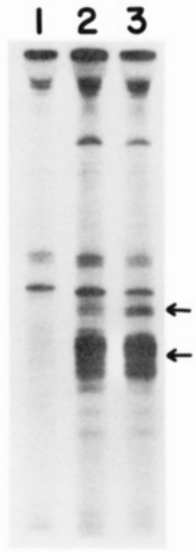

FIGURE 6: Effects of bovine (lane 2) and human (lane 3) angiogenin on reticulocyte RNA. The doublet and the group of five to seven prominent bands generated by the angiogenins are indicated by arrows. Bovine or human angiogenin was incubated at 0.59 μg/mL in nuclease-treated rabbit reticulocyte lysate for 15 min at 30 °C. After the reaction was quenched with PRI, the rRNA was labeled at the 5′ end with [^{32}P]ATP and electrophoresed on a 10% 7 M urea/polyacrylamide gel. RNA was visualized by autoradiography. Lane 1 is a water control.

proteins exhibit the same selectivity toward 28S and 18S rRNA. On the basis of the angiogenin concentrations required for cleavage, in this system the human protein is approximately 3-fold more active than the bovine.

Effects of Bovine Angiogenin on Reticulocyte Ribosomes and Protein Synthesis. Human angiogenin is a potent inhibitor of cell-free protein synthesis (St. Clair et al., 1987). When incubated with reticulocyte lysate, it completely abolishes the uptake of [^{35}S]Met into newly synthesized proteins. The inhibition is apparently due to specific ribonucleolytic activity of angiogenin toward the reticulocyte ribosome, as electrophoretic analysis of RNA from the reaction mixture reveals a reproducible pattern of the limited cleavage products.

Bovine angiogenin was tested for its effect on in vitro protein synthesis in nuclease-treated rabbit reticulocyte lysate. When present at 0.59 μg/mL, both it and human angiogenin completely inhibit the uptake of [^{35}S]methionine into newly synthesized protein (data not shown). Urea/polyacrylamide gel electrophoresis of RNA from the reaction mixtures shows that the bovine and human proteins generate nearly identical cleavage products (Figure 6). Both give rise to a doublet and a group of five to seven prominent bands which run below the 5S rRNA fragment.

Biological Characterization. The bovine protein was tested for angiogenic activity in the chick embryo CAM assay. Statistical analysis of the data (Table III) indicates the induction of an angiogenic response at doses as low as 0.62 ng per egg. In comparison, human tumor derived angiogenin is active down to 0.5 ng per egg (Fett et al., 1985).

Table III: Angiogenic Activity of Bovine Angiogenin in CAM Assays[a]

ng	sets of assays	average significance (%)	−SEM	+SEM
50	3	≪0.1	≪0.1	≪0.1
25	3	≪0.1	0.1	≪0.1
10	4	≪0.1	<0.1	≪0.1
5	5	0.2	0.6	<0.1
2.5	4	0.3	0.7	<0.1
1.3	5	0.4	0.8	0.2
0.62	2	<0.1	0.1	<0.1
0.31	3	13.2	24.9	6.0
0.16	2	13.4	*b*	*b*

[a] Average percent significance and standard error of the mean (SEM) values were calculated as described (Shapiro et al., 1987a). Each set of assays contained 15 eggs; angiogenesis was assessed 68 ± 2 h after sample implantation. A significance level of ≤5% has to be attained for a sample to be considered active. [b] Since at this concentration the data were identical for each set of CAM assays, the limits on the means cannot be calculated.

Discussion

Angiogenin was isolated originally from human tumor conditioned media using the CAM assay to monitor angiogenic activity. Later, it was detected in human plasma on the basis of its chromatographic behavior. The protein elutes well separated from other peaks on both Mono S cation-exchange and C18 reversed-phase columns. The relative abundance of angiogenin in human plasma and the straightforward purification pointed to a convenient means of obtaining the protein from other species. Initial attempts at its identification in bovine plasma on the basis of chromatographic behavior or molecular weight by SDS–PAGE, however, were not successful. The bovine protein evidently eluted differently in both HPLC systems from the human, and no simple means of detecting it was available. Existing assays for angiogenin were poorly suited for screening chromatographic fractions; on the basis of angiogenic or ribonucleolytic activities, they were either nonquantitative, or time consuming, or required highly purified samples. The finding that PRI was a potent inhibitor of angiogenin (Shapiro & Vallee, 1987) led to the development of a binding assay for the protein which was rapid and convenient and which greatly facilitated the purification.

The assay is based on the capacity of angiogenin to bind to a known quantity of PRI in the assay mixture and thus prevent the inhibition of some fraction of an added amount of bovine RNase A, which is then measured by standard methods. Since RNases can also bind to PRI, the binding assay is used in parallel with a conventional RNase assay to differentiate the proteins. Thus, a significant level of PRI binding activity with little or no corresponding RNase activity is indicative of angiogenin, whereas activity in both assays is representative of an RNase, or a mixture of angiogenin and RNase.

These procedures were applied to fractions from Mono S chromatography of bovine plasma CM 2 in an attempt to identify an angiogenin-like protein. The assays typically revealed a broad peak of PRI binding activity centered at 40 min in the gradient (Figure 1). RNase assays indicated that the latter portion of the peak was in fact a plasma RNase. The leading portion, however, had no detectable RNase activity and was therefore consistent with it being angiogenin. Appropriate fractions at this step were pooled and designated MS 1; analysis by SDS–PAGE revealed the presence of several proteins, two of which had molecular weights in the range of 14000–18000 (Figure 3, lane 4). The MS 1 fraction was then subjected to chromatography over a C18 reversed-phase column, with or without an intervening Mono S column in sodium phosphate buffer (Experimental Procedures). Several peaks having absorbance at 280 nm were observed from the C18 column, only one of which displayed PRI binding activity; the same peak had no detectable RNase activity. Fractions across the peak were pooled and shown by SDS–PAGE to contain a single protein of M_r 16200 (Figures 2A and 3). This material was subjected to further characterization.

The protein was found to bind PRI in a 1:1 molar ratio, assuming a molecular weight similar to that of human ang-

iogenin. No significant activity toward the RNase A substrates wheat germ RNA, poly(U), and poly(C) was detected. The single-chain polypeptide has an M_r of 16 200 ± 400 (Figure 3) by SDS–PAGE and does not contain an Asn-linked oligosaccharide. The amino acid composition is very similar to that of human angiogenin (Table II). Both proteins have six cysteine residues as contrasted with the eight found for virtually all RNases; in addition, they contain only one or two Met residues, compared to three to four in RNases.

The amino-terminal sequence of the bovine protein is 71% identical with that of human angiogenin (Figure 4). In comparison, human and bovine pancreatic RNases show 67% identity in this region. The bovine protein extends one residue beyond the human protein, beginning with Ala rather than <Glu, and thus it is not blocked. The Ala residue is actually conserved in the postulated leader sequence of human angiogenin, according to the gene sequence (Kurachi et al., 1985). Pancreatic RNases contain a half-cystine and an active site His residue in the amino-terminal region, both of which are present in human angiogenin. These residues are likewise conserved in the bovine protein, in positions 26 and 13 (human numbering), respectively. Strikingly, a stretch of 11 consecutive residues surrounding His-13 is completely conserved in the angiogenin molecules; only six consecutive residues are conserved here in human and bovine RNases. This strong homology is consistent with recent evidence from angiogenin/RNase A hybrid experiments which points to an important role for the N-terminal region in determining angiogenin's substrate selectivity (Harper et al., 1988).

Purification yields of the bovine protein are similar to but slightly lower than those obtained from human plasma derived angiogenin. The yields were the same within experimental error for sera from both calves and mature cattle. The protein is also present in fetal serum at 30–50% of the postnatal levels. Heat treatment of fetal and calf sera at 60 °C for 30 min, as is commonly used in cell culture, does not affect the yields. Therefore, the protein is present in all types of bovine sera commonly used to maintain cell lines, and media consisting of as little as 1% fetal bovine serum contain potentially significant levels of angiogenin. Although the effects of angiogenin in cell culture are not known, its presence or absence may alter the growth and/or functioning of various cell types, either through direct effects or by modulation of the effects of other cytokines. Investigations in this area are currently under way.

Human angiogenin can be characterized by its singular ribonucleolytic activities toward rRNA substrates (Shapiro et al., 1986; St. Clair et al., 1987). The protein isolated from bovine plasma was therefore tested in the same assay systems. In all cases it was found to give responses identical with those of the human protein. Thus, it catalyzes the limited degradation of 28S and 18S rRNA, yielding relatively stable products of 100–500 nucleotides (Figure 5). It also cleaves rabbit reticulocyte rRNA selectively to give a defined pattern of cleavage products indistinguishable from that of human angiogenin (Figure 6), and this treatment renders the reticulocyte system incapable of maintaining protein synthesis. Finally, the bovine protein was found to induce angiogenesis in the CAM assay (Table III). Statistical analyses reveal that a positive response was obtained with as little as 0.6 ng per egg, which is strikingly similar to the value of 0.5 ng per egg demonstrated by human angiogenin. The data confirm that the protein isolated from bovine plasma is indeed bovine angiogenin. Thus, the present results demonstrate conclusively that angiogenin is present in the plasma of species other than man, and at comparable levels. Enzymatic and biological activities previously established for human angiogenin are completely conserved in the bovine protein and are, therefore, likely relevent to the physiological mechanism of the protein. The complete amino acid sequence of bovine angiogenin is currently being determined, and it is expected that the resulting data will provide information concerning specific regions of angiogenin which are essential to its activity.

ACKNOWLEDGMENTS

We thank Dr. D. J. Strydom for performing the Edman degradation and amino acid analyses, Dr. R. Shapiro for supplying PRI and highly purified human angiogenin, and Dr. D. K. St. Clair for extensive help with in vitro protein translation and reticulocyte rRNA degradation assays. The excellent technical assistance of N. Nguyen is gratefully acknowledged.

Registry No. RNase A, 9001-99-4; nuclease inhibitor, 39369-21-6.

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