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Isolation and Characterization of Angiogenin-1 and a Novel Protein Designated Lactogenin from Bovine Milk

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This paper reports the isolation and characterization from bovine milk of two proteins: angiogenin-1, a recently discovered angiogenin, and lactogenin, a novel protein. Both proteins were adsorbed on and eluted closely from CM-Sepharose and Mono S. Lactogenin possessed a molecular weight (17 kDa) slightly higher than that of angiogenin-1 (15 kDa). Lactogenin had a higher ribonucleolytic (RNase) activity than angiogenin-1 towards yeast transfer RNA (tRNA). The Km values estimated for the RNase activities of angiogenin-1 and lactogenin were 51 μM and 40 μM respectively. Both were specific for poly C. The optimal pH for the RNase activities of angiogenin-1 and lactogenin was 7.75 and 7.5 respectively. Comparison of the amino acid sequences of cyanogen bromide fragments and the pyroglutaminase-treated N-terminal fragment of lactogenin with the sequence of bovine liver RNase (RNase BL4) revealed identity in residues 3-22, 24, 26-27, 37, 41-44, 46-50, 54, 56, 63, 72-80, and 83. Considerable similarity to the N-terminal sequence of angiogenin-2 was also noted. Both lactogenin and angiogenin-1 inhibited cell-free translation in a rabbit reticulocyte lysate system with an IC_{50} below 100 nM. © 1999 Academic Press

Key Words: **bovine milk; angiogenin; lactogenin.**

Angiogenin, a protein with a molecular weight of 14 kDa and the ability to stimulate blood vessel formation in the standard assays for angiogenesis such as the chick chorioallanotic membrane and rabbit cornea assays (1, 2), has been isolated from human carcinoma cells (1), human plasma (3), rabbit, pig and mouse sera (4), and bovine serum and milk (5).

In athymic mice the growth of human colon adenocarcinoma (HT-29) cells is reliant on angiogenin production (6), and antiangiogenin monoclonal antibodies inhibit tumor growth (7, 8). The data suggest that angiogenin has a role to play in the early stages of tumorigenesis.

Angiogenin exhibits homology to the ribonuclease (RNase) superfamily of proteins which comprises pancreatic RNases and other RNases. The weak RNase activity of angiogenin (9) is essential to its angiogenin activity. Both activities undergo a dramatic decline when angiogenin binds to placental RNase inhibitor (3).

The isolation of angiogenin-1 from bovine serum and milk has been reported (10, 11). Strydom *et al.* (5) described the purification and primary structure of angiogenin-2 from the same sources. Angiogenin-2 is also a member of the RNase superfamily although its RNase activity is very low.

We report herein the isolation of angiogenin-1 from bovine milk. Instead of angiogenin-2, another novel protein which we designated lactogenin was detected. Lactogenin resembled bovine liver ribonuclease (RNase BL-4) more closely than bovine angiogenin-2 (12).

MATERIALS AND METHODS

Materials. Fresh unsterilized bovine milk was obtained from a local dairy farm. CM-Sepharose CL-6B and Mono S ion-exchange resin were products of Pharmacia Biotech Ltd. Pyroglutamate aminopeptidase, lysozyme, ribonuclease A, placental ribonuclease inhibitor, yeast RNA, poly U, poly G and poly C were purchased from Sigma Chemical Company, St. Louis, MO.

Purification of angiogenin-1 and lactogenin from bovine milk. Acid whey (1 litre) was prepared by the procedure previously described for bovine milk (13). Globulin fraction was removed from acid whey by precipitation with 1.8 M ammonium sulphate (AS) and centrifugation at 10,000 × g, 20 min, at 4°C. The 1.8 M AS whey obtained was brought to 3.6 M with AS. Precipitate (3.6 M AS-PPT) was prepared by centrifugation at 10,000 × g, 20 min at 4°C. After dialysis against deionized water, it was loaded onto a CM-Sepharose cation-exchange column (1.5 × 18 cm) at pH 7.7. The starting buffer (50 mM sodium phosphate, pH 7.7) was passed through the resin until the absorbance at 280 nm decreased to <0.05. The adsorbed proteins were then eluted with a linear gradient of NaCl formed by

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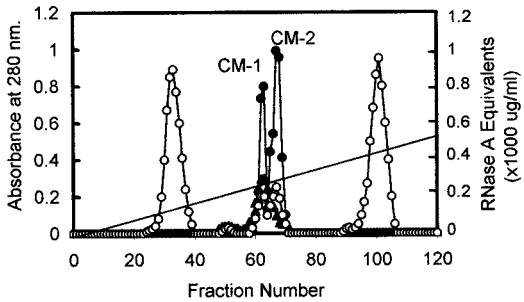

FIG. 1. Purification of angiogenin-1 (CM-2) and lactogenin (CM-1) on a CM-Sepharose column (1.5 × 18 cm). A 142 ml portion (701 mg protein) of dialyzed 3.6 M ammonium sulfate precipitate was applied on the column in 50 mM PO_4 bufer (pH 7.7). The column was then washed with the PO_4 buffer. Adsorbed proteins were eluted with a linear gradient (250 ml in each chamber) of NaCl from 0 to 500 mM in the same PO_4 buffer, pH 7.7. The eluate was analyzed by absorbance at 280 nm (○), PRI binding assay (●), and activity towards yeast RNA (▲) (which are plotted as RNase A equivalents). RNase activity is shown amplified 10-fold. The light solid line drawn across the chromatogram refers to a linear salt gradient (0 M NaCl in fraction 1 and 0.5 M NaCl in fraction 120).

250 ml of pH 7.7, 50 mM phosphate buffer in one chamber and 250 ml of the same buffer containing 500 mM NaCl in another chamber. Fractions with placental ribonuclease inhibitor (PRI) binding activity, pooled from several runs on CM-Sepharose, were dialyzed against deionized water and lyophilized.

Lyophilized fractions were reconstituted in 50 mM phosphate buffer (pH 7.7), clarified by centrifugation at 12,000 rpm for 5 min, and applied to Mono S HR 5/5 (1 ml). The Mono S column was eluted with a 20-min linear gradient from 0 to 500 mM NaCl in 50 mM phosphate buffer (pH 7.7) at a flow rate of 1 ml/min. Column effluents was monitored at 280 nm. A fraction was collected every minute.

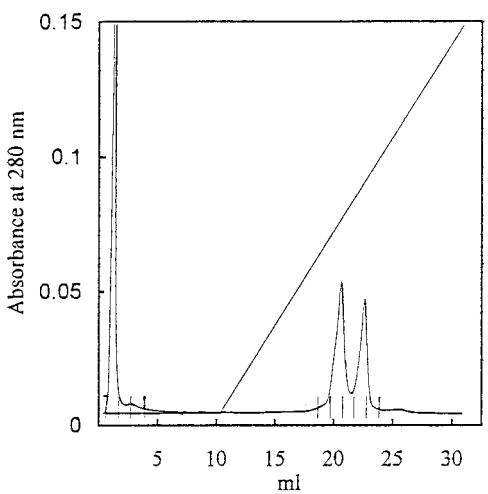

FIG. 2. Rechromatography of PRI binding fraction from CM-Sepharose on a Mono S FPLC column. Fractions with PRI binding activity pooled from several runs on CM-Sepharose were lyophilized. The sample was dissolved in 50 mM PO_4 buffer (pH 7.7) and applied on a Mono S column (HR 5/5, 1 ml). After unadsorbed proteins had come off the column, adsorbed proteins were eluted with a 20-min linear gradient from 0 to 500 mM NaCl in the same buffer at a flow rate of 1.0 ml/min.

TABLE 1

Recovery of Protein and PRI Binding Activity during Purification of Angiogenin-1 and Lactogenin from Bovine Milk

Purification step	Protein (mg)	PRI binding activity (units)	Specific activity (units/mg)
Acid Whey	7673	1.46×10^6	190.7
3.6 M AS-PPT*	701	3.25×10^5	463.2
CM-Sepharose			
Lactogenin	3.96	1.77×10^4	4469.7
Angiogenin-1	5.96	3.91×10^4	6560.4
Mono S			
Lactogenin	0.73	2.37×10^4	32,466
Angiogenin-1	0.79	2.55×10^4	32,278

* Whey protein precipitated by 3.6 M ammonium sulfate.

Detection of angiogenin-1 and lactogenin in chromatographic fractions. The presence of angiogenin-1 and lactogenin was monitored in chromatographic fractions with a joint PRI-binding/RNase activity assay (10). A constant amount of PRI was mixed with an aliquot of each fraction, calculated to be in excess over angiogenin and PRI-binding RNase. A known amount of RNase A was introduced, and a fraction bound tenaciously to the remaining PRI and was inhibited. The solution was assayed for the residual RNase A activity using a slightly modified precipitation method of Blackburn *et al.* (14). The result was compared with that from a direct RNase assay of the column fraction. A high value in the first and a low value in the second assay is consistent with the presence of angiogenin.

Enzymatic assays on the purified angiogenin-1 and lactogenin. Activity towards yeast tRNA was determined by using a precipitation assay (3) with angiogenin-1 or lactogenin concentrations of 80-680 nM. Activity towards poly C, poly G, poly U and poly A was determined with a modification of the method of Shapiro *et al.* (9).

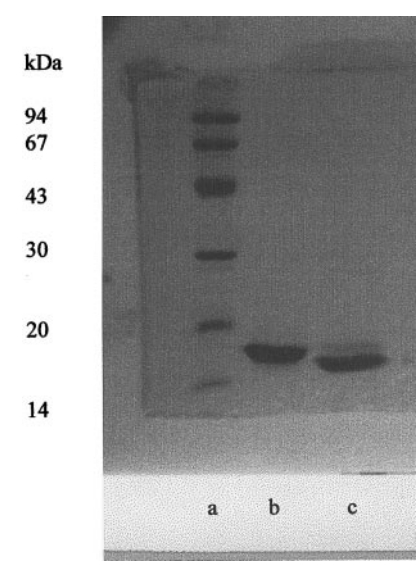

FIG. 3. SDS-PAGE of pooled fractions after Mono-S FPLC. Lane a: molecular weight markers. Lane b: lactogenin. Lane c: angiogenin-1.

TABLE 2

Inhibition of Cell-Free Translation

Protein	Concentration (nM)	Inhibition (%)
Lactogenin	118.0	96.6
	11.8	41.2
	1.2	15.0
Angiogenin-1	118.0	90.5
	11.8	23.8
	1.2	15.9

The effect of pH on activities of bovine angiogenin-1 and lactogenin was tested by using the following buffers with pH values at 37°C: Acetate, pH 4.0, 4.5, 5.0 and 5.5; Mes, pH 5.5, 6.0, 6.5 and 7.0; Hepes, pH 7.0, 7.5, 8.0 and 8.5; Tris, pH 8.5, 9.0 and 9.5.

Angiogenin-1 or lactogenin (0.067 μg/μl) in Tris-HCl buffer (pH 7.5) was incubated at 37°C for 25 min with tRNA concentrations ranging from 0.33 to 6.67 mg/ml followed by determination of the production of acid-soluble substances (3). Data were analysed for Km by the statistical method described by Cleland (15). Km values of substrate were corrected for tRNA content.

Inhibition of in vitro protein synthesis. The effects of bovine angiogenin-1 and lactogenin on protein translation in a rabbit reticulocyte lysate were determined as described (16).

Protein concentration. Protein content was measured using a dye-binding assay (protein assay kit, Bio-Rad Co., Ltd) or using absorbance at 280 nm with bovine serum albumin as the standard.

Gel electrophoresis of proteins. SDS-PAGE was performed on 12% polyacrylamide gels as described by Laemmli (17). The proteins were stained with Coomassie brilliant blue R-250 (CBB).

Cyanogen bromide digestion. Lactogenin (5 mg) was incubated with 0.1% BrCN in 8 ml of 80% formic acid at room temperature for 24 hr.

Sequence analyses. Angiogenin-1, lactogenin, lactogenin pretreated with pyroglutamylpeptidase and the peptide derived from lactogenin were subjected to automated Edman degradation using an HP G1000A protein sequencer and an HP 1000 HPLC system.

RESULTS

Angiogenin-1 and lactogenin were adsorbed on CM-Sepharose and eluted as two adjacent peaks (CM-1 and CM-2 respectively) when a linear NaCl concentration gradient was applied (Fig. 1). Both proteins were adsorbed on Mono S (Fig. 2). Table 1 presents the increase in specific activity of lactogenin and angiogenin-1 as purification proceeded (Table 1). Lactogenin and angiogenin-1 exhibited a molecular weight of 17 kDa and 15 kDa respectively as estimated by SDS-polyacrylamide gel electrophoresis (Fig. 3). Lactogenin exhibited a more potent (about 4-fold higher) RNase activity than angiogenin-1 on yeast tRNA. The RNase activity of both proteins was dose-dependent when tested up to 0.68 μM. The pH optimum of this RNase activity was 7.5 for lactogenin and 7.75 for angiogenin-1. The RNase activity of lactogenin and angiogenin-1 was also dependent on the amount of yeast tRNA added; maximal activity was attained at an RNA concentration of 3 mg/ml or above. The Km values were 51 μM and 40 μM, respectively. Both lactogenin and angiogenin-1 were potent towards poly C, and exhibited slight activity towards poly U, minimal activity towards poly A and undetectable activity towards poly G. The activities of lactogenin toward poly C, poly U and poly A were respectively 9.28, 0.55

TABLE 3

Comparison of Lactogenin, Including Lactogenin Fragments Obtained by BrCN Cleavage, with Bovine RNase BL4 (12) and Angiogenins (5)

```
                                  10                  20                  30
RNase BL4          E D R M Y Q R F L R Q H V D P D E T G G N D S Y C N L M M Q
Angiogenin               Y I H F L T Q H Y D A K P K G R N D E Y C F N M M K N
Angiogenin-2       Q N D A Y R G F L R K H Y D P S P T G H D D R Y C N T M M E
PYR:                 G R M Y Q R F L R Q H V D P D
BrCN-L3:                 Y Q R F L R Q H V D P D E T G G N D H Y L N L S R R N

                                  40                  50                  60
RNase BL4          R R K M T S H Q C K R F N T F I H E D L W N I R S I C S T T N I Q
BrCN-L3:           I Q C P R E Q C R N C N
BrCN-L1:                 N R H E G V R F N T D I H E D L T N R R P I
Angiogenin-2       R R N M T R P  C K D T N T F I H G N S D D I R A V C D D R N G E

                             70                  80                  90
RNase BL4          C K N G Q M N C H E G V V R V T D C R E T G S S R A P N C R Y
BrCN-L2:                   D E H E G V V R V T D K T E E G
Angiogenin-2       P Y R N G L R R S R S P F Q V T T C R H R G G S P R P P C R Y

                        100                 110                 120
RNase BL4          R A K A S T R R V V I A C E G N P E V P V H F D K
Angiogenin-2       R A F R A N R V I V I R C R D G F P I H L E E N F I P P R P
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Note. The alignment is numbered according to the sequence of bovine RNase BL4. PYR: sequence of pyroglutaminase-treated bovine lactogenin. BrCN-L1, L2 and L3: peptides derived from a cyanogen bromide digest of lactogenin.

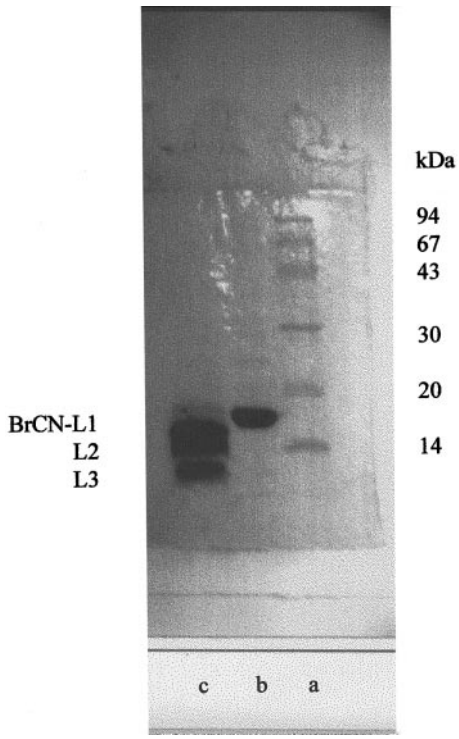

FIG. 4. SDS-PAGE of the fragments produced from lactogenin by BrCN cleavage. Lane a: molecular weight markers. Lane b: lactogenin. Lane c: lactogenin fragments.

and 0.1 μ/mg while the corresponding activities of angiogenin-1 were 1.53, 0.38 and 0.06. The two proteins inhibited cell-free translation in a rabbit reticulocyte lysate system with similar potencies (Table 2).

The N-terminal amino acid sequence of angiogenin-1 isolated in this study was similar to that reported earlier (Table 3). Lactogenin was subjected to cyanogen bromide cleavage followed by SDS-polyacrylamide gel electrophoresis to separate the resulting fragments (Fig. 4). Lactogenin was also subjected to digestion with pyroglutaminase and N-terminal amino acid sequencing. Alignment of this N-terminal sequence of lactogenin (PYR), the sequences of its CNBr fragments BrCN-L1, BrCN-L2 and BrCNL-4, the sequence of the alkaline RNase BL-4 from bovine liver, and the sequence of angiogenin-2 is presented in Fig. 4. Similarity of lactogenin to RNase BL-4 is noted.

DISCUSSION

The present investigation corroborates the presence of angiogenin-1 in bovine milk (5). Interestingly, a protein which closely resembled angiogenin-2 in chromatographic behavior turned out to be a novel protein which we designated as lactogenin. Although lactogenin resembled angiogenin-2 at positions 4-25, the similarity of the former protein to the alkaline RNase BL-4 from bovine liver was much more extensive. Lactogenin exhibited a larger molecular weight and a higher RNase activity towards yeast tRNA and poly C when compared with angiogenin-1. Both proteins demonstrated the same base specificity, similar optimal pH regarding RNase activity towards yeast tRNA, and similar potencies in inhibiting cell-free translation.

It is noteworthy that lactogenin, similar to angiogenin-1 and angiogenin-2, could be isolated from bovine milk with the help of two assays simultaneously: an assay for RNase activity and an assay for the ability to bind placental RNase inhibitor. Lactogenin and angiogenin-1 were purified to the same specific activity in the present investigation. Based on the aforementioned properties, it is very likely that lactogenin represents yet another angiogenin. The finding of lactogenin instead of angiogenin-2 in the present investigation may be due to the different varieties of cattle used.

RNase BL4 belongs to the category of secretory RNases because the characteristic features of nonsecretory RNases include the presence of the hexapeptide from the 17th to the 22nd residues (18). Likewise lactogenin can be regarded as a secretory RNase. Its presence in milk also bears this out. RNase BL4 and lactogenin are both specific towards pyrimidine bases, with higher activity towards poly C than poly U (12).

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