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Purification, Primary Structure, and Antimicrobial Activities of Bovine Apolipoprotein A-II[1]

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We purified an antimicrobial protein of 76 residues, denoted bovine antimicrobial protein-1 (BAMP-1), from fetal calf serum using hydrophobic chromatography, gel filtration, and reverse-phase high-performance liquid chromatography. The amino acid sequence of BAMP-1 was similar to that of human apolipoprotein A-II (apo A-II), a major component of high-density lipoprotein (HDL), and the amino acid composition was almost identical to that of a previously reported candidate for bovine apo A-II. BAMP-1 was recovered from the post-HDL fraction, but not from the HDL fraction of the serum and was associated with a small amount of triglycerides (5%, w/w). These results suggest that BAMP-1 is the bovine homologue of apo A-II and is present in almost free form in serum. BAMP-1 showed a weak growth-inhibitory activity against *Escherichia coli* and yeasts tested in phosphate-buffered saline (PBS).

Key words: amphipathic helix, antimicrobial activity, apolipoprotein A-II, HDL, membrane.

Recently in our laboratory, mouse serum was found to exhibit antifungal activity against the yeast *Saccharomyces cerevisiae*. Similar activity was also found in fetal calf serum (FCS) and was probably protein-mediated, as the activity disappeared upon heating of the serum. In mammals, many antimicrobial proteins and peptides of diverse nature have been isolated from leukocytes (*1*), small intestine (*2, 3*), skin (*4*), parotid secretion (*5*), seminal plasma (*6*), and tracheal mucosa (*7*). These proteins or peptides are considered to play important roles in innate immunity. Although it has been reported that mammalian sera have some inhibitory activity against Gram-positive and negative bacteria (*8*), little is yet known about serum antifungal proteins. We purified and characterized an antifungal protein denoted bovine antimicrobial protein-1 (BAMP-1) from FCS, and found that it showed a high degree of homology to apo A-II from several other species (*9-11*).

Apo A-II is a second major protein of HDL. The main function of apo A-II is to modulate the lipid binding and LCAT activities of HDL by promoting the dissociation of apo A-I from HDL (*12*). Additionally, human apo A-II has been shown to inhibit a tissue factor (coagulation factor III) in the activation of coagulation factor X by factor VIIa (*13*), to inhibit the polymerization of complement protein C9 (*14*) and to stimulate placental lactogen release from human placental tissue (*15*). Here, we report the primary structure of bovine apo A-II and its potential to interact with microorganisms.

MATERIALS AND METHODS

Materials—Fetal calf, newborn calf, and adult bovine sera were purchased from Sigma Chemical (St. Louis). Pyroglutamate aminopeptidase, *Achromobacter* protease I, and endoproteinase Asp-N were purchased from Takara Shuzo (Tokyo).

Microorganisms and Culture Media—*Saccharomyces cerevisiae* A364A (Yeast Genetic Stock Center, CA), *Candida albicans* ATCC 10259 (American Type Culture Collection, Rockville, MD), *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* IFO 12732 (Institute for Fermentation, Osaka) were used in the present study. Growth media consisted of YPD medium (2% peptone, 1% yeast extract, 2% glucose) for *S. cerevisiae*, Sabouraud medium (2% glucose, 1% peptone) for *C. albicans*, LB medium (1% peptone, 0.5% yeast extract, 1% NaCl) for *E. coli*, and 1% peptone, 0.2% yeast extract, and 0.1% $MgSO_4$ for *S. aureus*.

Antimicrobial Assay—To detect antifungal activity in fractions of the eluate from column chromatography, 10 μl aliquots were spread on thin agar plates (0.7×0.7 cm) containing YPD medium and 10 cells of *S. cerevisiae* were transferred to the plate using a micromanipulator (Narishige, MN151). After incubation for 18 h at 30°C the

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Abbreviations: FCS, fetal calf serum; apo A-I, apolipoprotein A-I; apo A-II, apolipoprotein A-II; HDL, high-density lipoprotein; RP-HPLC, reverse-phase high-performance liquid chromatography; CFU, colony-forming unit.

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numbers of colonies on the plates were counted under a microscope. The antimicrobial activity of purified BAMP-1 was tested as previously described (*16*). Briefly, 5×10^4 colony-forming units (CFU) of cells collected during exponential growth were incubated for 2 h at 37°C in 50 μl of phosphate-buffered saline (PBS) in the presence or absence of BAMP-1. The cells were then plated in duplicate on agar plates and the number of CFU was determined following incubation at 30°C.

Purification of BAMP-1—NaCl was added to 1 liter of fetal calf serum (FCS) to a final concentration of 1 M, and the solution was applied to a butyl-Toyopearl 650M column (2.5×10 cm, Tosoh, Tokyo) pre-equilibrated with 1 M NaCl. Proteins were eluted stepwise with 200 ml of each of 1 M and 0.15 M NaCl and H_2O. The H_2O fractions containing the antimicrobial activity were pooled and filtered through a TSK gel G3000SW column (2.15×60 cm, Tosoh) equilibrated with 10 mM sodium phosphate (pH 7) containing 0.1 M NaCl. The proteins were eluted with the same solution at a flow rate of 4 ml/min. The molecular size of the antimicrobial protein was estimated using thyroglobulin (670 kDa), gamma globulin (158 kDa), ovalbumin (44 kDa), equine myoglobin (17 kDa), and cyanocobalamin (1.35 kDa) as markers. The fractions with the activity were pooled and analyzed by means of reverse-phase high-performance liquid chromatography (RP-HPLC) using a C4 column 214TP510 (1×25 cm, Vydac) connected to a Waters HPLC system. The proteins were eluted with a linear gradient of 32-56% solution B [0.1% (v/v) trifluoroacetic acid in acetonitrile] in solution A (0.1% trifluoroacetic acid in H_2O) at a flow rate of 3 ml/min. Elution profiles of proteins were monitored spectrophotometrically at 280 nm.

Determination of Amino Acid Sequence—Purified protein (10 nmol) was lyophilized and dissolved in 200 μl of 50 mM sodium phosphate, 1 mM sodium EDTA, and 10 mM dithiothreitol, pH 7.2. The mixture was incubated with 4 mU of pyroglutamate aminopeptidase for 8 h at 37°C to remove a pyroglutamate residue at the amino terminus. The deblocked protein was again purified using RP-HPLC with a C18 column 218TP54 (0.46×25 cm, Vydac) and digested with either *Achromobacter* protease I or endoproteinase Asp-N. Peptide fragments were purified by RP-HPLC using a C18 column with a 50 ml, 0 to 56% gradient of acetonitrile at a flow rate of 1 ml/min. The amino acid sequence of isolated fragments was determined by pulsed-liquid Edman degradation using a Model 473A protein sequencer (Perkin-Elmer Applied Biosystems Division, Foster, CA). The C-terminal amino acid sequence was determined by amino acid analysis of residues released by carboxypeptidase Y hydrolysis as previously described (*17*).

Purification of Apolipoproteins from HDL—HDL was isolated from FCS by precipitation with dextran sulfate and further purified by ultracentrifugation (*18*). After delipidation of HDL, apo A-I and A-II were separated by gel filtration and RP-HPLC as described previously (*19*).

Mass Spectrometry and Other Analytical Procedures—Molecular masses of native BAMP-1 and N-terminal deblocked BAMP-1 were determined using a Kratos Kompact MALDI 2 mass spectrometer. Cholesterol, triglycerides, and phospholipids were quantified using appropriate kits from Wako Pure Chemical Industries (Osaka). Protein concentrations were determined by the method of Lowry *et al.* (*20*).

RESULTS

Purification of an Antimicrobial Protein from FCS—We began to purify serum antimicrobial protein from FCS using *S. cerevisiae* as a test organism. FCS was first applied to a column containing a hydrophobic absorbent pre-equilibrated with 1 M NaCl, and proteins with antimicrobial activity were eluted stepwise with H_2O (Fig. 1a). A weak antifungal activity was found in the flow-through fraction from a hydrophobic column, suggesting that FCS may contain different kinds of antifungal protein. Antimicrobial activity was detected in the fraction corresponding to a molecular size of approximately 24 kDa on gel filtration through a G3000SW column (Fig. 1b). A 24 kDa antimicrobial protein was finally purified by RP-HPLC (Fig. 1c). The

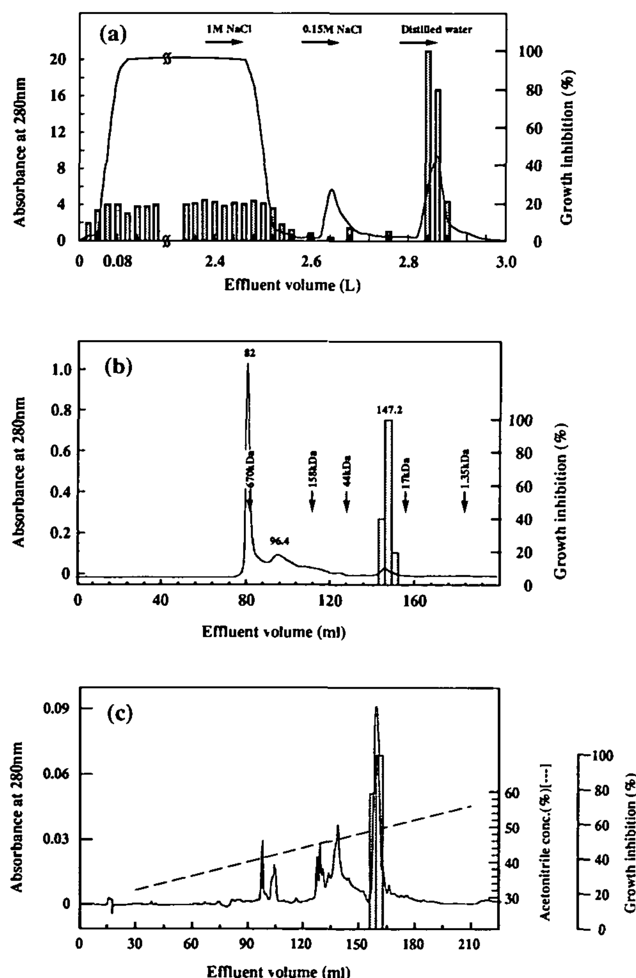

Fig. 1. **Purification of antimicrobial protein.** (a) Chromatography of fetal calf serum using a column containing hydrophobic absorbent. Proteins were eluted stepwise with 200 ml of each of 1 M and 0.15 M NaCl and H_2O. (b) Gel filtration of active fraction (H_2O fraction from panel a) through a TSK gel G3000SW column. (c) Reverse-phase HPLC of active fraction from gel filtrate. A dashed line indicates the concentration of acetonitrile. —, absorbance at 280 nm. Columns indicate anti-*S. cerevisiae* activity. Growth inhibition (%) was calculated from number of colonies to total cells ratio.

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protein was almost homogeneous on SDS-polyacrylamide gel electrophoresis (Fig. 2, lane 5) and was named BAMP-1. Approximately 6 mg of BAMP-1 was obtained from one liter of FCS, which contained about 100 g of protein.

Amino Acid Sequence of BAMP-1—To determine the amino acid sequence of BAMP-1, the purified protein was analyzed by means of sequential Edman degradation. However, no N-terminal amino acid signal could be obtained, suggesting that the terminal was blocked. When BAMP-1 was treated with pyroglutamate aminopeptidase, the signal appeared, indicating that the N-terminal was blocked by pyroglutamate. The N-terminal deblocked BAMP-1 was digested with either *Achromobacter* protease I or endoproteinase Asp-N. The digests were fractionated into 6 peptides each by RP-HPLC (Fig. 3, upper) and named AP-1 to 6 and D-1 to 6, respectively. Amino acid sequencing of the fractionated peptides was attempted using an automated protein sequencer. However, sequencing of the overlapping fragments D-5 and D-3 failed due to termination of sequencing reactions around residues 53-55 (Fig. 3, lower). As the sequence of the N-terminal region of deblocked BAMP-1 showed a high degree of homology to apo A-II, BAMP-1 is probably associated with lipids that interfere with sequencing. Measurement of the lipid content of purified BAMP-1 revealed that the protein is associated with 5% triglycerides by weight. Other lipids such as cholesterol and phospholipids were not detected (<0.2%). BAMP-1 was delipidated with ethanol/ether (*21*), and the proteolytic digests were fractionated again by RP-HPLC (Fig. 3, upper). New fragments D-7 and AP-7 eluted last in the gradient, suggesting that these fragments were also associated with lipids. The sequencing of BAMP-1 was completed using these fragments (Fig. 3).

BAMP-1 consists of 76 amino acid residues and has a calculated molecular weight of 8,549. By mass spectrometry, the molecular mass of BAMP-1 was determined to be 8,545 Da, which is consistent with the predicted molecular weight. Furthermore, the molecular mass of N-terminal deblocked BAMP-1 decreased by 106 Da, which corresponds almost exactly to pyroglutamic acid (111 Da). Searches of the SwissProt and PIR protein data banks revealed that the homology between BAMP-1 and human (*9*), rhesus (*10*), and mouse apo A-II proteins (*11*), is 66, 71, and 59%, respectively (Fig. 4). Although the amino acid compositions of several kinds of apolipoproteins in bovine HDL have already been reported (*22, 23*), this is the first report of the amino acid sequence of bovine apo A-II. The amino acid composition of BAMP-1 is almost identical to that of D2, a candidate for bovine apo A-II (*22, 23*). These results suggest that BAMP-1 is the bovine homologue of apo A-II.

Characterization of BAMP-1 in Serum—Apo A-II is the second major component of fetal calf HDL (Fig. 2, lane 6), which is a very small lipid and protein complex composed of 50% apolipoproteins and 50% lipid by weight (*22-24*). Therefore, we examined the possibility that BAMP-1 was apo A-II that had been artificially dissociated from HDL. HDL and post-HDL fractions were isolated from 100 ml of

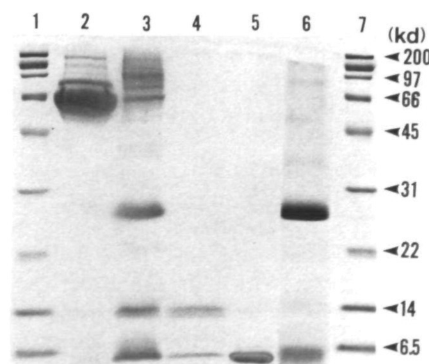
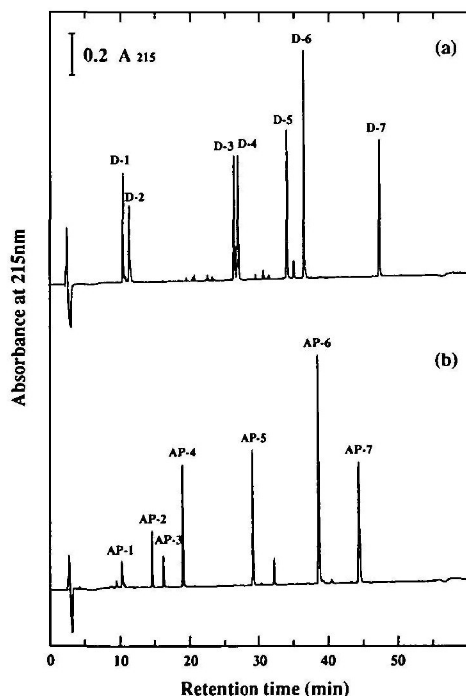


Fig. 2. **SDS-polyacrylamide gel electrophoresis of purified antimicrobial protein.** Protein samples, 6 μg protein from FCS (lane 2), 4 μg protein from active fraction from butyl-Toyopearl chromatography (lane 3), 1 μg protein from active fraction from gel filtration through a G3000SW column (lane 4), 2 μg of protein purified by RP-HPLC (lane 5), 4 μg of apoHDL protein (lane 6), and molecular weight markers (broad range, Bio-Rad) (lanes 1 and 7), were resolved in 16% SDS-polyacrylamide gel and stained with Coomassie Blue.



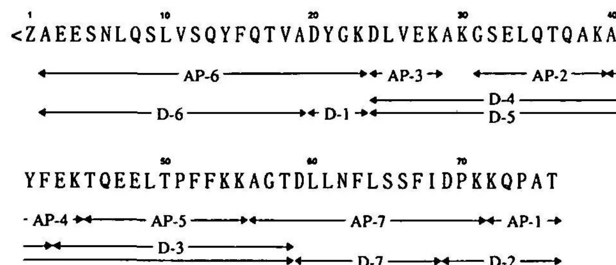


Fig. 3. **Reverse-phase chromatography of proteolytic peptides derived from BAMP-1 (upper).** N-Terminal deblocked and delipidated BAMP-1 was digested with endoproteinase Asp-N (a) and *Achromobacter* protease I (b). RP-HPLC was carried out using a C18 column (0.46×25 cm). **Amino acid sequence of BAMP-1 (lower).** Arrows indicate fragments isolated from RP-HPLC of proteolytic peptides with endoproteinase Asp-N (with D-) and with *Achromobacter* protease I (with AP-1). <Z, pyroglutamic acid.

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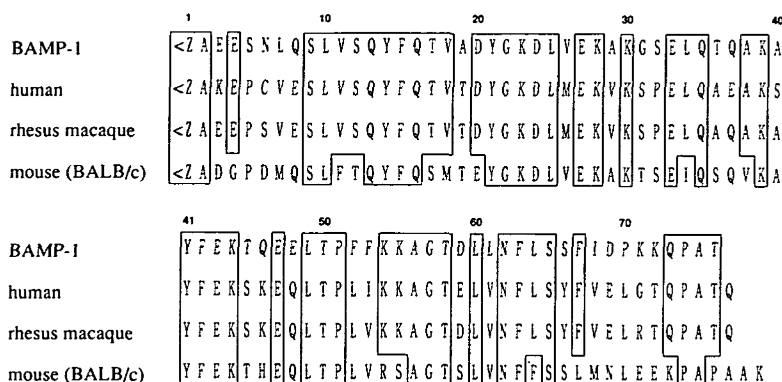

Fig. 4. **Amino acid homology between BAMP-1 and apolipoprotein A-II of several species.** The sequence alignment is indexed to highly conserved residues, which are boxed. <Z, pyroglutamic acid.

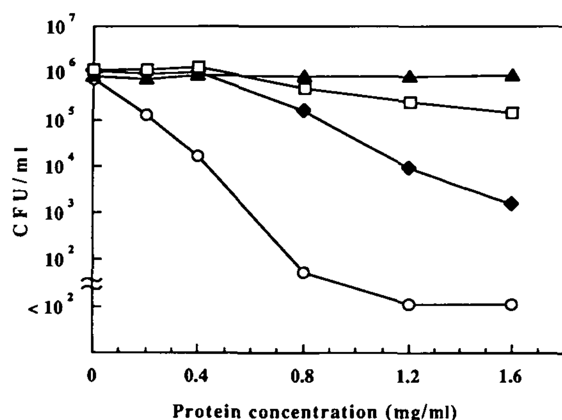

Fig. 5. **Antimicrobial activity of BAMP-1 against *E. coli* (○), *S. aureus* (▲), *S. cerevisiae* (◆), and *C. albicans* (□).** Exponentially growing cells were collected and 5×10^4 CFU were incubated for 2 h at 37°C with various concentrations of BAMP-1 in 50 μl of PBS.

FCS treated with dextran sulfate (*18*) and separately analyzed using column chromatography under the same conditions used for the purification of BAMP-1. HDL was identified *via* determination of cholesterol concentration. Only a small proportion of HDL was eluted with H_2O from the butyl-Toyopearl column and the eluate was further filtered through a G3000SW column. We could not detect any antifungal activity in the peak at 147.2 ml, which corresponded to the elution position of BAMP-1 (Fig. 1b). Instead, BAMP-1 was recovered from the post-HDL fraction of the serum and the isolated BAMP-1 was associated with a small amount of triglycerides (5%, w/w). These results suggested that BAMP-1 is present in almost completely free form in serum.

Antimicrobial Activity of BAMP-1—Purified BAMP-1 affected the viability of the Gram-negative bacterium *E. coli* and the yeasts *S. cerevisiae* and *C. albicans*, but not that of the Gram-positive bacterium *S. aureus* (Fig. 5). It was most effective on *E. coli* and least effective on *C. albicans*.

To assess the antimicrobial activity of BAMP-1, we tested whether apo A-I and apo A-II derived from HDL showed this activity. Antimicrobial activity of apo A-II was similar to that of BAMP-1, whereas apo A-I and HDL did not show any antimicrobial activity at up to 2 mg/ml in PBS (data not shown).

DISCUSSION

We have purified the antimicrobial protein BAMP-1 from FCS using *S. cerevisiae* as a test organism and showed that this protein is most likely the bovine homologue of apo A-II, which is present in almost completely free form in serum. The apparent molecular mass of BAMP-1 estimated on a G3000SW column (24 kDa) was higher than that calculated from its amino acid sequence (8,549 Da), probably because helical apolipoproteins are in an asymmetric form in aqueous solution and tend to form aggregates (*25*). We could not determine what proportion of the whole antimicrobial activity of serum is accounted for by BAMP-1, because the antimicrobial activity of BAMP-1 is too weak to allow quantitative determination using zone assay (*26*) or liquid culture assay (for example Ref. *27*).

Recently, a small amount of a lipoprotein particle that contained only apo A-II (Lp A-II) was identified in human plasma by using immunoaffinity column chromatography (*28, 29*). The lipid-to-protein ratio of purified BAMP-1 was much lower (0.05) than that of human Lp A-II (2.2). Thus, because a hydrophobic absorbent and organic solvents were used during purification, the lipid content of BAMP-1 may have been reduced. Since the sample prior to RP-HPLC still contained a small amount of triglycerides, most cholesterol and phospholipids may have been lost during chromatography on butyl-Toyopearl 650M. However, it seems more likely that the amount of lipid associated with native BAMP-1 is small, as BAMP-1 was not precipitated by dextran sulfate. Furthermore, in the case of direct application of FCS to a G3000SW column, antimicrobial activity was detected in a low molecular weight region between 10 and 24 kDa. These findings suggest that BAMP-1 is present in almost completely free form in serum. Although apo A-II associated with a small amount of triglycerides (BAMP-1) was shown to possess antimicrobial activity, it may not show bactericidal activity *in vivo* since the effective bactericidal concentration *in vitro* was much higher than the content of BAMP-1 in serum calculated from the recovery rate (6 μg/ml).

Secondary structure analysis according to the method of Chou and Fasman (*30*) predicts that BAMP-1 can form an amphipathic helix between residues 37–57 and a β-sheet between residues 8–18. The amphipathic helix has been shown to play an important role in the interaction of peptides and proteins with lipids (*31*). Lipid-associating

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amphipathic helices have been grouped into seven classes on the basis of the size and charge distribution of their hydrophilic domain (A, H, L, G, K, C, and M; *32*). Class A amphipathic helices, which are present in plasma apolipoproteins, can stabilize membrane bilayers, whereas class L amphipathic helices, which are present in lytic antimicrobial peptides or proteins such as magainin 1, 2, and seminalplasmin, can disrupt membrane structure (*33, 34*). Amphipathic helices in apolipoproteins are further grouped into four classes (A_1, A_2, G^*, and Y). Edmundson wheel representation of BAMP-1 shows that its amphipathic helix is similar to that of class Y. The class Y motif is predicted to have lower affinity to phospholipid (*35*). This may partly explain why BAMP-1 was isolated as a nearly free form. From these findings, we speculate that the amphipathic helix of BAMP-1 acts as binding domain to the membrane of microorganisms and another region of BAMP-1 acts as a toxic domain. To substantiate this hypothesis, we are currently synthesizing peptides covering various regions of BAMP-1. The analysis of the BAMP-1 antimicrobial domain may contribute to the development of therapeutic drugs which target the membrane of microorganisms.

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