Characterization of Mineral-binding 40-kDa Glycoprotein Extracted from Young Adult Rabbit Alveolar Bone

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Abstract

A forty-kilodalton (40-kDa) protein was extracted from alveolar bone of young adult rabbit with 0.5 M EDTA after extraction with 4 M GuHCl, and purified by gel-filtration, anion-exchange and hydroxyapatite columns using a high-pressure liquid chromatography system under denaturing conditions.

The purified 40-kDa protein was not susceptible to bacterial collagenase and thrombin, but was cleaved by cyanogen bromide. The protein was stained blue with Stains-all. Among various lectins, concanavalin A and lentil lectin agglutinin bound to this protein, but peanut agglutinin, *Ricinus communis* agglutinin, phytohemagglutinin-E and wheatgerm lectin agglutinin did not. Lectin binding assays showed that the protein is a glycoprotein containing large amounts of mannose and/or glucose residues, but is not a fragment of proteoglycan. The amino acid composition of the protein shows a characteristically high content of acidic amino acids. Therefore, the mineral-binding 40-kDa glycoprotein is considered to be osteonectin/secreted protein acidic and rich in cysteine (SPARC), in terms of similarities to bovine and porcine osteonectins with regard to molecular weight and contents of glycoses and amino acids.

Introduction

Several bone proteins have been isolated from mineralized matrix of bone, and their characteristics, especially those related to calcification and matrix formation, have been determined; small proteoglycans^[1,2], bone sialoprotein^[3], osteopontin^[4,5], osteocalcin^[6,7], small collagenous proteins^[8-10] and osteonectin^[11-13] have received special attention.

Among these, osteonectin was first characterized by TERMINE et al.^[11], who

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demonstrated that it was a phosphorylated glycoprotein binding to both hydroxyapatite and collagen, and accounting for about 25% of all non-collagenous proteins in fetal bovine bone. With regard to the *in vitro* biosynthesis of osteonectin, OTSUKA et al.^[14] reported that osteonectin was produced by fetal porcine calvarial cells, and KUWATA et al.^[15] identified pre-osteonectin produced by cell-free translation of fetal porcine calvarial mRNA. Subsequently, DOMENICUCCI et al.^[12] determined the amino acid sequence of osteonectin extracted from fetal porcine calvaria.

Recently, it was reported that osteonectin from adult bovine bone inhibited hydroxyapatite crystal formation more strongly than osteocalcin^[16], although it bound to a partially purified type I collagen preparation, as described by TERMINE et al.^[11].

Concerning osteonectin in alveolar bones, EDA^[13] reported that osteonectin extracted from rabbit mandibles was able to bind to both hydroxyapatite and gelatin, and determined its amino acid composition. However, its other properties are still unclear.

In this study, we purified a mineral-binding 40-kDa glycoprotein from alveolar bone of young adult rabbit and clarified its characteristics; the protein was treated with bacterial collagenase, thrombin and cyanogen bromide, and its lectin-binding abilities and amino acid composition were determined. The data obtained indicated that this 40-kDa glycoprotein was rabbit alveolar bone osteonectin.

Materials and Methods

Extraction Procedures

Bone proteins of rabbit (3 months old) mandibular alveolar bone were extracted according to the three-step procedure described by OHMORI et al.^[17]. The alveolar bone blocks were carefully cleaned to avoid contamination with surrounding soft tissues, and frozen in liquid nitrogen. Then, the bone blocks were broken with a mortar and pestle into small pieces (less than 8 mm³).

Fifty grams of the fragmented bone was washed overnight with ice-cold phosphate-buffered saline solution (PBS), pH 7.4, deficient in Ca²⁺ and supplemented with the following protease inhibitors (PIs): 1 mM phenylmethylsulfonyl fluoride, 5 mM benzamidinium hydrochloride, 10 mM ϵ -amino caproic acid and 5 mM N-ethylmaleimide. First, the bone fragments were washed with 2 liters of 4 M guanidine hydrochloride solution (GuHCl) containing the PIs with constant stirring to remove organic materials bound to the non-mineralized phase of the fragments; the pH value of the solution was adjusted to 7.4 with 50 mM Tris-HCl buffer. The fragments were rinsed for 5 days by changing the GuHCl solution every day, and the supernatant solution was collected by centrifugation for recovery of organic materials; this fraction was termed the G₁-extract.

Subsequently, the bone fragments were rinsed with 2 liters of PBS-PI/day for 3 days, and then organic materials in the mineral phase of the bones were extracted with 2 liters of 0.5 M ethylenediaminetetraacetic acid (EDTA) containing PIs for 10 days, changing the solution five times in 2 days; the pH was adjusted to 7.4 with

Tris-HCl buffer. The supernatant fraction containing the proteins from the mineral phase was recovered by centrifugation; this fraction was termed the E-extract.

The demineralized collagenous residues of the bones were again rinsed with 2 liters of PBS-PI/day for 3 days, and the proteins were extracted with the above GuHCl solution in the same way. The supernatant containing the proteins in the matrix phase was recovered by centrifugation, and termed the G₂-extract.

Each extract was concentrated 200-fold by ultrafiltration on a Diaflo membrane (PM-10, Amicon Corp., Lexington, MA, USA), and dialyzed exhaustively against 0.1 M ammonium bicarbonate solution containing 0.005%(v/v) Brij 35 (Sigma Chemical Co., St. Louis, MO, USA). Then, the solution of each extract was freeze-dried for use.

SDS-Polyacrylamide Gel Electrophoresis

SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using 5-20% gradient cross-linked polyacrylamide gels with a discontinuous Tris/glycine buffer system as described by LAEMMLI^[18]; minislab gradient gels 0.75 mm thick were made with a Mighty Small II (Hoefer Scientific Instruments, San Francisco, CA, USA). The freeze-dried preparations described above were dissolved in 10-15 μ l of sample buffer for SDS-PAGE consisting of 1% SDS, 2 M urea and bromophenol blue marker, which was heated at 90°C for 5 min before application to the slab-gels. For SDS-PAGE under reducing conditions, 15 mg of dithiothreitol was added to 1 ml of the sample buffer before heating. The electrophoresis was carried out at a constant voltage of 150 V for 60 min. After the electrophoresis, the gel was stained with 0.2% (w/v) silver nitrate^[19] or 0.025% (w/v) Stains-all^[20].

Purification Procedures

Approximately 200 mg of the freeze-dried E-extract from 50 g of the bone blocks (wet weight) was dissolved in 2 ml of 4 M GuHCl (pH 7.4), and centrifuged at 10,000 $\times$ g for 30 min. The supernatant fraction was applied to a Sepharose CL-6B column (2.6 $\times$ 90 cm, Pharmacia Fine Chemicals, Uppsala, Sweden); elution was performed at a flow rate of 10 ml/h, and 2-ml fractions were collected. The fractions containing proteins of 35 to 45 kDa were separately pooled, desalted with a PD-10 column (1.5 $\times$ 5 cm, Pharmacia Fine Chemicals) and freeze-dried for the next step.

The freeze-dried material was dissolved with 10 mM Tris-HCl buffer containing 7 M urea (pH 7.4), and applied to an analytical column (0.78 $\times$ 7.5 cm, Shinwa Kako Co., Kyoto, Japan) filled with DEAE-Sepharose resin (Pharmacia Fine Chemicals). The proteins were eluted with a linear gradient of NaCl (0 to 1.0 M) at a flow rate of 1.0 ml/min using a high-pressure liquid chromatography (HPLC) system (Shimadzu Corp., Kyoto, Japan), and 1.0-ml fractions were collected. The fractions containing the 40-kDa protein were pooled, desalted using a PD-10 column and freeze-dried for the following step.

The freeze-dried material was dissolved in 500 μ l of 10 mM Tris-HCl buffer containing 7 M urea and 10 mM phosphate (pH 7.4): the buffer consisted of 19% NaH₂PO₄ and 81% Na₂HPO₄. The sample solution was applied to an analytical hydroxyapatite column (0.75 $\times$ 5 cm, Tonen Corp., Tokyo, Japan) equilibrated with the above buffer. After applying the sample solution at a flow rate of 0.2 ml/

min, the column was washed with 19 ml of the buffer at a flow rate of 0.5 ml/min, and the proteins were eluted with a linear gradient of 10 to 500 mM phosphate at a flow rate of 0.5 ml/min using a HPLC system; 1.0-ml fractions were collected. The fractions containing the 40-kDa protein were pooled, desalted with a PD-10 column and freeze-dried for final purification.

The freeze-dried material containing the 40-kDa protein was dissolved in 500 μ l of 50 mM Tris-HCl, pH 7.4, and applied to an analytical column (0.5×5 cm, Pharmacia Fine Chemicals) of Mono Q resin. The protein was eluted with a linear gradient of 0 to 1.0 M NaCl at a flow rate of 1.0 ml/min using the HPLC system, and 1.0-ml fractions were collected. The purity of the protein preparation was assessed by staining slab-gels with silver or Stains-all to visualize the protein bands. The fractions containing the proteins of interest were desalted using the PD-10 column to determine some of their characteristics.

Digestion with Bacterial Collagenase

The purified 40-kDa protein was incubated with highly purified bacterial collagenase (22.5 unit/0.084 mg, Advance Biofactures Co., Linbrook, NY, USA); the enzyme was used at one tenth of the protein concentration. The digestion was performed at 37°C for 60 min in 20 μ l of 50 mM Tris-HCl, pH 7.4, supplemented with 5 mM CaCl_2 and 5 mM N-ethylmaleimide.

Digestion with Thrombin

The purified protein was treated with thrombin (100 unit/0.05 mg, Sigma Chemical Co.), using the same enzyme-protein concentration described above. The digestion was performed at 37°C for 60 min in 20 μ l of 10 mM Tris-HCl buffer, pH 8.0, supplemented with 10 mM CaCl_2 .

Cleavage with Cyanogen Bromide

The purified protein was dissolved in 100 μ l of 70% (v/v) formic acid, and flushed with nitrogen for 1 min. Approximately 1 mg of cyanogen bromide crystals was added to 5 μ g of the protein. The contents were briefly flushed again with nitrogen before sealing the tube, and incubated at 27°C for 4 h. The solution was then diluted 40-fold with water, and freeze-dried. The sample was dissolved in water and again freeze-dried to remove residual cyanogen bromide.

Western Blotting

The binding abilities of several HRP-conjugated lectins to 500 ng of the 40-kDa protein were determined using the following lectins: concanavalin A (Con A), lentil lectin agglutinin (LCA), peanut agglutinin (PNA), *Ricinus communis* agglutinin (RCA), phytohemagglutinin-E (PHA) and wheatgerm lectin agglutinin (WGA).

Immunotransfer analysis was performed on a Multiphor II Novo Blot system (LKB, Uppsala, Sweden) using a nitrocellulose membrane (Bio-Rad Laboratories, Richmond, CA, USA) with a continuous buffer system composed of 39 mM glycine, 48 mM Tris, 0.0375% SDS and 20% (v/v) methanol. Electrophoresis was carried out at a constant current of 0.8 mA/cm² of gel for 60 to 90 min. After the transfer, the excess protein-binding sites on the membrane were blocked with 3% bovine serum albumin (BSA; Inter Gen, Kankakee, IL, USA) dissolved in 10 mM Tris-buffered saline containing 145 mM NaCl, pH 7.4, (TBS), at 4°C for 18 h. The

membrane was then washed three times with TBS containing 0.05% Tween-20 (TBS-Tween). The sheets were incubated at 22°C for 60 min with each HRP-conjugated lectin diluted 1 : 200 with TBS containing 3% BSA. The sheets were washed three times with TBS-Tween, and once with TBS. The membrane was then incubated with TBS containing 0.06% 4-chloro-1-naphthol, and 0.04% H₂O₂.

Amino Acid Analysis

The purified 40-kDa protein was hydrolyzed with 6.0 N HCl at 110°C for 22 h in a nitrogen-flushed tube. The amino acid composition of the protein was determined using a Shimadzu amino acid analyzer (column: Shim-pak ISC-07/S1504 Na, Shimadzu Corp.).

Results

Proteins present in rabbit alveolar bone were sequentially extracted, initially with 4 M GuHCl, then with 0.5 M EDTA, followed by 4 M GuHCl solution; the extracts obtained were termed G₁-extract, E-extract and G₂-extract, respectively. The proteins in each extract were then analyzed by SDS-PAGE using Stains-all (Fig. 1). Among the three extracts, the E-extract contained a limited number of clearly resolved proteins migrating at positions corresponding to molecular masses of 80~67 kDa and 55~40 kDa, with some faint bands below the 40-kDa region. A predominant protein (arrowhead in Fig. 1) in the E-extract was purified by column chromatography using an automated HPLC system.

Fig. 1 SDS-PAGE analysis of proteins extracted from alveolar bone of young adult rabbit. SDS-PAGE (5-20% gradient cross-linked gel) was performed under reducing conditions to separate the proteins, which were then stained with Stains-all. Mr values indicate molecular weight marker proteins. G1, proteins extracted with 4 M GuHCl (G1-extract); E, proteins extracted with 0.5 M EDTA (E-extract); G2, proteins extracted with 4 M GuHCl a second time (G2-extract).

Proteins in the E-extract were first fractionated by gel filtration on Sepharose CL-6B in the presence of 4 M GuHCl. An elution profile of the CL-6B run is shown in Fig. 2. Proteins in every third fraction of the run were monitored by SDS-PAGE under reducing conditions, and the fractions containing 45~35-kDa proteins were pooled (bar in Fig. 2). The pooled material was then applied to a DEAE-Sepharose column, and eluted using a linear gradient of NaCl (0~1.0 M)

Fig. 2 Sepharose CL-6B column chromatography of E-extract

Approximately 50 mg of the E-extract was dissolved in 2.0 ml of 4 M GuHCl in 50 mM Tris-HCl, pH 7.4, and the solution clarified by centrifugation. The solution was applied to a Sepharose CL-6B column (2.6×90 cm), which was eluted at 10 ml/h, and 2.0-ml fractions were collected. Protein concentrations in the eluate were monitored by absorbance at 280 nm. Fractions containing 35~45-kDa proteins were pooled (bar), desalted on PD-10, and then freeze-dried.

in 7 M urea. The proteins were separated into three symmetrical peaks as illustrated in Fig. 3, except for the flowthrough fraction. According to the gel staining after SDS-PAGE, a 40-kDa protein showed the highest peak eluting with 250 mM NaCl, and the fractions containing this protein were pooled (bar in Fig. 3). The pooled material was suspended in 10 mM phosphate with 7 M urea, and applied to a hydroxyapatite column. The protein was separated into two peaks, a small unbound peak and a large peak bound to the hydroxyapatite, as shown in Fig. 4; the 40-kDa protein was in the bound peak (bar in Fig. 4). After combining the fractions containing the bound peak, the pooled material was applied to a Mono Q column in the presence of 7 M urea, and each fraction was determined by SDS-PAGE. The 40-kDa protein was purified to apparent homogeneity with a sharp symmetrical peak (bar in Fig. 5).

The purified 40-kDa protein was stained strongly with silver (Fig. 6, lane 1), and also stained blue with Stains-all (Fig. 6, lane 8) after SDS-PAGE under reducing conditions. Under non-reducing conditions, however, the protein band was stained weakly with silver; the band migrated to the same position as that

Fig. 3 DEAE-Sepharose column chromatography

The freeze-dried material pooled from the CL-6B run was dissolved in 500 μ l of 50 mM Tris-HCl, pH 7.4, containing 7 M urea and applied to a DEAE-Sepharose column (0.78 $\times$ 7.5 cm). The proteins were eluted with a linear gradient of NaCl (0-1.0 M), and the eluate was collected in 1.0-ml fractions; the column was run at 1.0 ml/min. Protein concentrations in the eluate were monitored by absorbance at 230 nm. Fractions containing the 40-kDa protein were pooled (bar), desalted on PD-10, and then freeze-dried.

Fig. 4 Hydroxyapatite column chromatography

The freeze-dried material pooled from the DEAE column was dissolved in 500 μ l of a starting buffer (50 mM Tris-HCl, pH 7.4, containing 10 mM phosphate and 7 M urea), and applied to a hydroxyapatite column (0.75 $\times$ 5 cm) at 0.2 ml/min. The column was washed with the starting buffer at 0.5 ml/min, and the proteins were eluted with a linear gradient of 10-500 mM PO₄ at the same flow rate, 1.0-ml fractions being collected. Protein concentrations were monitored by absorbance at 230 nm. Fractions containing the 40-kDa protein were pooled (bar), desalted on PD-10, and then freeze-dried.

Fig. 5 Mono Q column chromatography

The freeze-dried material pooled from the hydroxyapatite column was dissolved in 500 μ l of 50 mM Tris-HCl, pH 7.4, containing 7 M urea and applied to a Mono Q column (0.5 $\times$ 5.5 cm). Proteins were eluted with a linear gradient of NaCl (0-1.0 M) and 1.0-ml fractions were collected; the column was run at 1.0 ml/min. Protein concentrations were monitored by absorbance at 230 nm. Fractions containing the 40-kDa protein were pooled (bar), desalted on PD-10, and then freeze-dried to determine some of the characteristics of the 40-kDa protein.

Fig. 6 Characterization of the purified 40-kDa protein

The purified 40-kDa protein was applied to a gradient gel (5-20%) on a minislab system under reducing (lanes 1-6 and 8) or non-reducing (lane 7) conditions. The SDS-polyacrylamide gel was stained with silver (lanes 1-7) or Stains-all (lane 8). Lane 1, the 40-kDa protein stained with silver under reducing conditions; Lane 2, the protein treated with bacterial collagenase; Lane 3, bacterial collagenase; Lane 4, the protein treated with thrombin; Lane 5, thrombin; Lane 6, the protein cleaved with cyanogen bromide; Lane 7, the protein stained with silver under non-reducing conditions; Lane 8, the protein stained blue with Stains-all.

under reducing conditions.

The 40-kDa protein was incubated with bacterial collagenase or thrombin at 37°C for 60 min, but was not susceptible to these enzymes (Fig. 6, lanes 2 and 4).

Upon incubation with cyanogen bromide at 27°C for 4 h, the protein was completely cleaved (Fig. 6, lane 6).

Lectin-binding assays carried out by Western blotting showed that Con A and LCA bound to this protein, whereas PNA, RCA, PHA and WGA did not (Fig. 7).

Fig. 7 Western blotting using several HRP-conjugated lectins

The 40-kDa protein was transferred to nitrocellulose membrane, and incubated with various HRP-conjugated lectins. AB, the 40-kDa protein stained with amido black; CoA, concanavalin A; LCA, lentil lectin agglutinin; PNA, peanut agglutinin; RCA, *Ricinus communis* agglutinin; PHA, phytohemagglutinin; WGA, wheatgerm lectin agglutinin.

Table 1 Amino acid composition of 40-kDa protein

Amino acid	Composition (residues/1000residues)			
	Young adult-rabbit alveolar bone 40-kDa glycoprotein	Fetal-porcine calvaria osteonectin*	Fetal-bovine long bone osteonectin**	Murine endoderm SPARC protein***
Hydroxyproline	0	0	0	—
Aspartate	126	124	130	116
Threonine	52	49	59	60
Serine	35	26	42	25
Glutamate	189	179	148	158
Proline	69	66	72	25
Glycine	63	52	65	56
Alanine	54	53	58	49
Cystine	11	35	6	49
Valine	76	65	69	70
Methionine	5	11	11	18
Isoleucine	34	38	37	42
Leucine	91	93	90	84
Tyrosine	12	26	24	24
Phenylalanine	45	44	44	42
Histidine	41	54	44	42
Lysine	64	64	71	67
Arginine	31	31	28	28
Tryptophan	0	10	ND	10

* DOMENICUCCI et al.^[12], ** TERMINE et al.^[11], *** MASON et al.⁽²¹⁾

ND; not detected

The amino acid composition of this protein is shown in Table 1. The protein possessed a high content of acidic amino acids or their amines (32%), with leucine (9%) and valine (8%), but contained little methionine, cysteine and tyrosine; hydroxyproline and tryptophan were not detected.

Discussion

A sequential procedure for extraction of bone proteins, employing 4 M GuHCl followed by 4 M GuHCl with 0.5 M EDTA, has frequently been used to isolate mineral-binding proteins^[1,3,6,11]. However, as this method extracts all of the materials present in both the mineral and matrix phases, the present study utilized a three-step extraction procedure to characterize mineral-binding proteins in the second extract, as described by DOMENICUCCI *et al.*^[12]. This involved GuHCl (G₁-extract), followed by EDTA without GuHCl (E-extract), and then GuHCl (G₂-extract).

Rabbit alveolar bone proteins were sequentially extracted using this method, and analyzed by SDS-PAGE with staining of the protein bands using silver or Stains-all. The E-extract contained some distinct proteins, giving major bands at 80~67 kDa, 55~40 kDa and 14.4 kDa, which were strongly stained blue with Stains-all. We focused on a mineral-binding 40-kDa protein positive with Stains-all, and purified it using four kinds of column.

Proteins in the E-extract were first fractionated by Sepharose CL-6B column chromatography in the presence of 4 M GuHCl. The eluate containing 45~35-kDa proteins was pooled to purify the mineral-binding 40-kDa protein by HPLC; the columns utilized were DEAE-Sepharose, hydroxyapatite and Mono Q columns in the presence of 7 M urea.

EDA^[13] had shown previously that osteonectin in rabbit alveolar bone was eluted with 61 mM phosphate on hydroxyapatite (Bio-Rad Laboratories, Richmond, CA, USA) column chromatography. However, we found that the mineral-binding 40-kDa protein was eluted with 210 mM phosphate on a hydroxyapatite column (Tonen Corp.). This difference may have been due to the type of hydroxyapatite packed in the column.

The mineral-binding 40-kDa protein was purified to apparent homogeneity in the sharp symmetrical peak on final purification by Mono Q column chromatography. The protein was strongly stained with silver upon gel-staining after SDS-PAGE under reducing conditions. Under non-reducing conditions, however, the protein staining was weak in comparison with that under reducing conditions. These results suggested that the protein was a glycoprotein. Also, like bovine^[11] and porcine^[12] osteonectins, the protein was stained blue with Stains-all, suggesting that it was rich in acidic groups and/or phosphate.

Since MAENO *et al.*^[10] showed previously that a 28-kDa protein in the E-extract of rabbit alveolar bone was digested by bacterial collagenase, producing a 19-kDa fragment, and NAGATA *et al.*^[5] showed that a 44-kDa osteopontin synthesized by rat bone cells was digested with thrombin, producing a 26~28-kDa fragment, the 40-kDa glycoprotein was incubated with bacterial collagenase or thrombin, and the molecular weight of the product was determined by SDS-

PAGE. It was found that the protein was not susceptible to either enzyme, indicating that it was neither a collagenous protein nor osteopontin.

TERMINE et al.^[11] showed that calf osteonectin contains sialic acid, glucosamine and galactosamine as carbohydrate side chains. Therefore, to determine the type of carbohydrate in the 40-kDa glycoprotein, the binding of lectins was analyzed by Western blotting. Con A and LCA bound to the protein, but PNA, RCA, PHA and WGA did not. These results suggest that the protein has mannose and/or glucose residues as carbohydrate chains, and that it is not a proteoglycan fragment. Furthermore, the protein was cleaved completely with cyanogen bromide, suggesting that it contains methionine residues.

The amino acid composition of the protein showed a characteristically high content of acidic amino acids and/or their amines, together with leucine, proline, lysine and glycine residues similar to the composition of calf^[11] and porcine^[12] osteonectin and murine SPARC protein^[21].

These results suggest that the present mineral-binding 40-kDa glycoprotein extracted from alveolar bone of young adult rabbit is the same protein as osteonectin/SPARC proteins from other tissues.

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