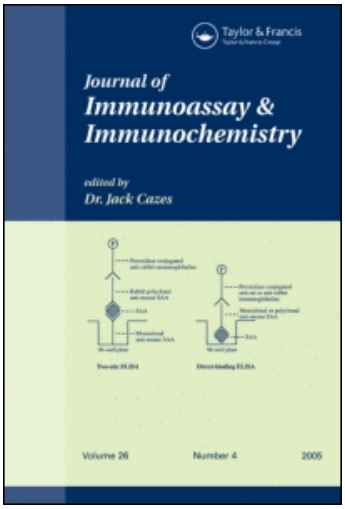

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Extraction, Purification, and Development of an Enzyme-Linked Immunosorbent Assay for Osteocalcin

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Abstract: The present study describes the isolation and purification of osteocalcin (OC) from bovine bones and the development of an enzyme-linked immunosorbent assay (ELISA) for OC as a marker of bone formation, for assessing bone health. Bone proteins were extracted from about 90 g of bovine bone powder using 20% formic acid. The protein extract was fractionated by gel permeation chromatography on Sephadex G-50 column followed by fast protein liquid chromatography (FPLC) on a MONO-Q column. The immunoreactive active fraction was then purified by chromatofocusing, using FPLC on a MONO P column and a single homogeneous band of molecular size of about 5.8 kDa, as judged by Tricine SDS-PAGE following silver staining of the gel, was obtained. It reacted specifically with its antibodies in an ELISA. About 678 μg of purified OC was yielded from about 90 g of bovine bones. The purified OC was subsequently used for the raising antisera, which was used in the development of an indirect ELISA. The developed ELISA has a sensitivity of 2.5–4.0 ng/mL and was used in estimating levels of OC in women of various age groups.

Keywords: Bone formation marker, ELISA for osteocalcin, Osteoporosis

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INTRODUCTION

Osteocalcin (OC), also known as bone γ-carboxyglutamic acid protein (5800 Da), is exclusively produced by osteoblasts, the bone forming cells.[1,2] The serum concentration of OC represents 2% of the total bone proteins and corresponds generally well with the activity of osteoblasts and, thus, reflects the process of bone formation.[3] Circulating OC is associated with changes in the rate of bone turnover in metabolic bone diseases, such as osteoporosis, primary hyper-parathyroidism, hyperthyroidism, Paget's disease, and renal osteodystrophy.[4,5]

Several investigators have taken advantage of the immunological cross-reactivity between bovine OC and human OC and have reported assays for measurement of OC in human samples using antisera raised against bovine OC.[6–9] Most of the immunoassays described for OC are non-competitive sandwich assays that employ two epitope-specific antibodies, one "capture antibody" and other conjugated to a marker.[10,11] The use of two antibodies increases the cost of the test, whereas preparation of specific antibody–enzyme conjugates requires specialized biochemical techniques and the conjugates thus prepared usually lack long-term stability.

The present study reports the development of a simple and sensitive indirect enzyme-linked immunosorbent assay (ELISA) that uses polyclonal antisera against purified bovine OC raised in rabbit, semi purified extract of OC immobilized on microtiter plate, and a goat anti-rabbit antisera coupled to horse radish peroxidase.

EXPERIMENTAL

Purification of OC from Bovine Bones

Bone protein was extracted following the method of Gundberg et al.[12] Briefly, freshly dissected bovine bones, obtained from the local slaughter-house, were cleared of adhering tissues. The marrow particles were removed by splitting of the bones. They were then thoroughly washed with ice-cold Tris protease inhibitor cocktail (TPIC) buffer, pH 7.4, (Tris–HCl 0.020 mmol/L containing Benzamidine HCl 5 mmol/L; 6-aminocaproic acid, 10 mmol/L; p-hydroxymercuribenzoic acid, 0.1 mmol/L; and phenyl-methylsulfonyl fluoride, 0.030 mmol/L). The bone pieces were dipped in liquid nitrogen before pulverization to a fine powder. The powder obtained (90 g) was treated with ice cold TPIC buffer, pH 7.4, for 2–3 hr at 4°C and then treated with 20% formic acid for 24 hr at 4°C. The extract thus obtained was centrifuged at 2000 g for 20 min at 4°C and the supernatant was dialyzed extensively against distilled water using dialysis tubing having an exclusion limit of about 3500 Da (Spectra/Por 3 membrane).

Gel Permeation Chromatography

The dialysate was lyophilized and dissolved in a minimal volume of 20 mmol/L Tris HCl, pH 7.4. It was then fractionated on a Sephadex G-50 column (100 × 1.5 cm). Fractions, 4 mL/pertube, were collected and their absorbance was monitored at 280 nm using a spectrophotometer (UV-160 A, Shimadzu, Japan). The fractions were pooled and designated as per their order of elution. The fractions were checked for their immunoreactivity using an OC specific ELISA kit (Diagnostic Systems Laboratory, USA). Purity of the protein was monitored by Tricine SDSPAGE. The presence of γ-carboxyglutamic acid protein was checked by a diazobenzene sulfonic acid (DBS) reaction analyzed by dot blot. The immunoreactive fractions were dialyzed extensively against distilled water and lyophilized.

Anion Exchange Chromatography

The immunoreactive fraction, following gel permeation chromatography, was dissolved in a minimal volume of 10 mmol/L Tris–HCl containing 100 mmol/L NaCl, pH 7.4 (Starting buffer), and equilibrated with the same buffer. This was further fractionated by fast protein liquid chromatography (FPLC) on a MONO-Q column (Amersham Pharmacia, Sweden), pre-equilibrated with starting buffer, and the bound protein was eluted with a linear gradient of 100 mmol–1.0 mol/L NaCl in 10 mmol/L Tris–HCl, pH 7.4 (eluting buffer). An aliquet of 1 mL fractions were collected in order of their elution and their absorbance was monitored at 280 nm using a spectrophotometer. Fractions obtained were checked for their immunoreactivity, and their purity was monitored by Tricine SDS PAGE.

Chromatofocusing

The active peak obtained by anion-exchange chromatography was subjected to chromatofocusing. The protein obtained was dissolved in 25 mmol/L imidazole hydrochloride, pH 7.4, (starting buffer) and equilibrated against the same and was fractionated on an FPLC MONO-P column (HR 5/5, Amersham Pharmacia) pre-equilibrated with starting buffer. The column was washed and the bound fractions were eluted with a pH gradient of Poly-buffer hydrochloride from pH 7.0 to pH 4.0. An aliquot of 1 mL fractions were collected, their absorbance was monitored at 280 nm, and the pH of each tube was checked using pH paper. The peak fractions were extensively dialyzed against distilled water at 4°C for 2 days with 2–3 changes per day. The protein content was monitored and immunoreactivity was checked as described. DBS reaction of the protein was carried out and the purity of the protein was monitored by Tricine SDS PAGE and simultaneously subjected to the DBS reaction.

Characterization of OC

Tricine SDS PAGE

Tricine SDS PAGE was performed by the method described by Schagger and Von Jagow[13] using a vertical slab gel electrophoresis apparatus (Microkin, Techno source, India). Casting of discontinuous polyacrylamide gel consisted of 16.5% polyacrylamide separating or resolving (lower) gel, 10% polyacrylamide spacer (middle) gel, and 4% polyacrylamide stacking (upper) gel. Tricine is substituted as a cathode buffer instead of glycine. This PAGE system can resolve proteins in the range from 1 to 100 kDa. Resolving gel, 16.5%, was prepared in the flask and degassed. The flask was swirled and the contents were poured into glass plates till the required level. 10% spacer gel was added to the mark at 2.5 cm from the top and was left to polymerize together with resolving gel. After polymerization, 4% stacking gel solution was added and allowed to polymerize. After polymerization of the gel, the assembly was filled with cathode and anode buffers, respectively. The protein samples were treated 1 with sample buffer and were heated at 100°C for 10 min in a heating block. Protein samples, 1.5 μg, were loaded into each well. Gel was removed and silver stained.

DBS Reaction

DBS reaction of γ-carboxyglutamic acid was performed by dot blot analysis at each stage of purification, as described by Nishimoto.[14] The proteins, after blotting onto nitrocellulose membrane (Hybond C, Amersham Pharmacia), were stained with DBS, which stains carboxyglutamic-containing proteins red. This method is specific for OC, due to the presence of carboxyglutamic acid; other proteins lack it.

Approximately 5 μg of protein were spotted on the membrane and incubated in the dark with DBS for 2 hr. Only peaks consisting of γ-carboxyglutamic acid (gla) residues or OC were stained red. Bovine serum albumin (BSA) was used as a control, as it does not contain γ-carboxyglutamic acid.

Quantitation of OC by ELISA

The quantity of OC in the protein at each step was confirmed by OC specific ELISA kit (DSL, USA), which measured the immunoreactivity, or to quantitate the amount of OC present in each stage. Approximately 50 ng of protein from each peak was taken for quantitation and the protocol supplied by the manufacturer was followed. The amount of OC purified at each stage is shown in Table 1.

Table 1. Recovery of OC at different stages of purification process

Stage	Amount of protein (mg)	Amount of OC (mg)	Recovery (%)
Crude extract	77.28	8.5	11
Sephadex G-50	19.208	6.146	32
MONO-Q	5.5	3.85	70
MONO-P	0.6918	0.678	98

Immunization of Rabbits

The purified protein was coupled to polyviniylpyrdidone (PVP) as described by Gundberg et al.[12] In brief, OC (1 mg/mL,in 150 mmol/L NaCl), was diluted 1 : 5 (V/V) with 50% (w/v) PVP (Sigma Chemicals, St. Louis, USA), thoroughly mixed, and was left at room temperature overnight. The mixture was later emulsified with Freund's complete adjuvant in the ratio of 1 : 1.

Male rabbits (Belgium White strain weighing approximately 2 kgs) were immunized subcutaneously at multiple sites (60–70) with 100 μg of purified OC coupled with PVP. Test bleeds were collected fortnightly and sera harvested and checked for antibody titer as determined by dot blot. After the peak titer was achieved, the animals were bled and sera were separated. Gamma globulin fractions were obtained (33% saturated ammonium sulfate precipitation) and stored in aliquots at −20°C. The specificity of the antibody raised was determined by Western blot transferred from Tricine SDS PAGE.

Western Blot Analysis

Gel electrophoresis of the crude extract was carried out as described by Schagger and Von Jagow.[13] Gel was transferred onto nitrocellulose membrane (Hybond C, Amersham Pharmacia) overnight at 30 Vs at 4°C as described by Towbin et al.[15] Transfer buffer reagents were prepared with a modification, where Tricine substituted glycine.

Development of Indirect ELISA

The antiserum to OC was reacted with OC standard/sample/OC. The residual antibody was then allowed to react with semi-purified OC (protein obtained following fractionation on MONO-Q) immobilized onto a microtiter ELISA plate, which was later probed with anti-rabbit gamma globulin conjugated to horseradish peroxidase (ARGG-HRP). The concentration of OC in the sample is inversely proportional to enzyme activity in the bound fraction.

Semi-purified OC was immobilized on wells of microtiter ELISA plate (Maxisorp, NUNC, Denmark) by dispensing OC, 125 ng/100 μL of coating buffer (50 mmol/L sodium carbonate–bicarbonate buffer, pH 9.6) and the plate was incubated overnight at 4–8°C. The plate was later washed with saline Tween 20 (150 mmol/L NaCl, containing 0.5 mL/L Tween 20). Antisera, 200 μL, appropriately diluted in immunoassay buffer (100 mmol/L phosphate buffered saline, pH 7.2, containing 10 g/L bovine serum albumin), were dispensed into glass tubes containing 200 μL of standard or sample or buffer. Tubes were then incubated for 2 hrs. at 37°C. The content of the tubes, 100 μL, were transferred to the OC coated microtiter plates and incubated for $1\frac{1}{2}$ hrs at 37°C. The plates were then washed thrice with saline Tween 20 and probed with goat anti-rabbit HRP conjugate (1 : 1000, diluted in immunoassay buffer) and incubated for 1 hr at 37°C. The plate was washed again for three times and enzyme activity was measured in the bound fraction by dispensing 100 μL of 1 : 20 diluted tetramethylbenzidine/hydrogen peroxide (TMB/H_2O_2) solution (Bangalore Genie, India) or 100 μL of substrate chromogen solution (1.5 μL 30% H_2O_2 and 10 μL 6 mg/L TMB in 1 mL 0.1 mol/L phosphate buffer, pH 6.0) per well and the color reaction was stopped by addition of 50 μL of 4 N sulfuric acid. The plates were then read on an ELISA plate reader at 450 nm (μQuant, Biotek Instruments Inc., Germany).

All reagents used were standard high quality chemicals, from Qualigens (Qualigens, India Limited), Merck (Darmstadt, Germany), or Sigma Chemicals (St. Louis, USA).

RESULTS

Purification of OC

Bovine bone proteins were extracted using a protocol described earlier.[12] The extraction of about 90 g of finely pulverized bovine bones with 20% formic acid yielded about 77 mg of crude protein, which was used for subsequent purification.

Gel Permeation Chromatography

The extract obtained was subjected to gel permeation chromatography. Figure 1 shows the elution profile of the gel permeation chromatography of bone protein extract on Sephadex G-50, which resolved it into three fractions. Fraction II showed immunoreactivity with antibody to OC in an ELISA, which was further fractionated by ion exchange chromatography on a MONO-Q column. Purities of these fractions were checked by Tricine SDS PAGE. The presence of γ-carboxyglutamic acid, which is the marker

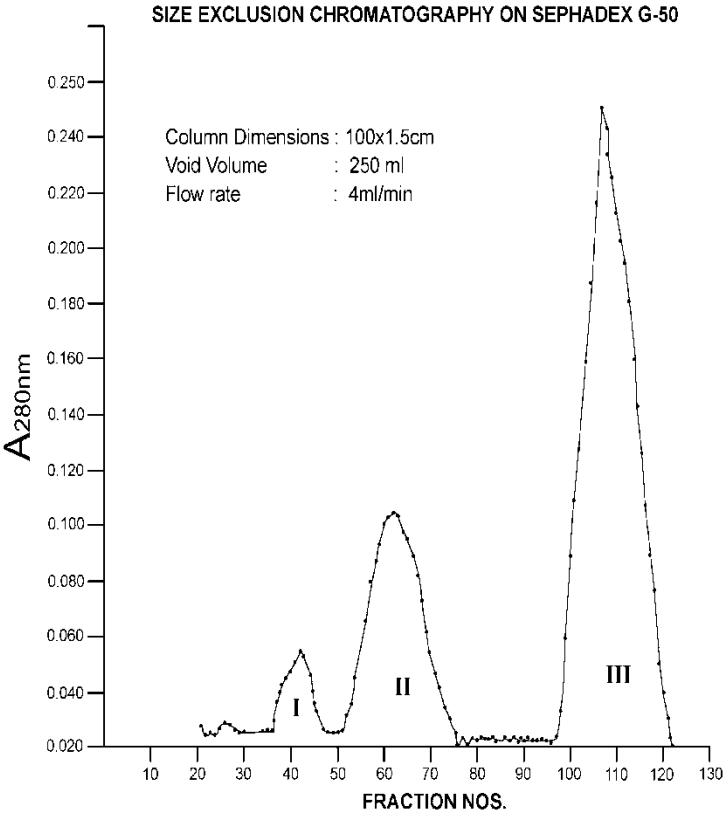

Figure 1. Elution profile of bone protein extract (77.28 g) fractionated on a Sephadex G-50 column. Three different protein peaks (I, II, and III) were observed.

for OC, was confirmed by its specific reaction with DBS by dot blot analysis, as described by Koyama et al.[10] and fraction II showed the presence of this protein in the region below 6.5 kDa when calibrated with Rainbow low molecular weight protein markers (Amersham Pharmacia, Sweden).

Anion Exchange Chromatography

Fraction II was further fractionated by anion exchange chromatography by FPLC on a MONO-Q column which resolved it into two fractions (Figure 2). Fraction I showed immunoreactivity with specific anti-OC antibodies in an ELISA, as well as reactivity for the presence of γ-carboxyglutamic acid by dot blotting, thus suggesting the presence of OC in this fraction.

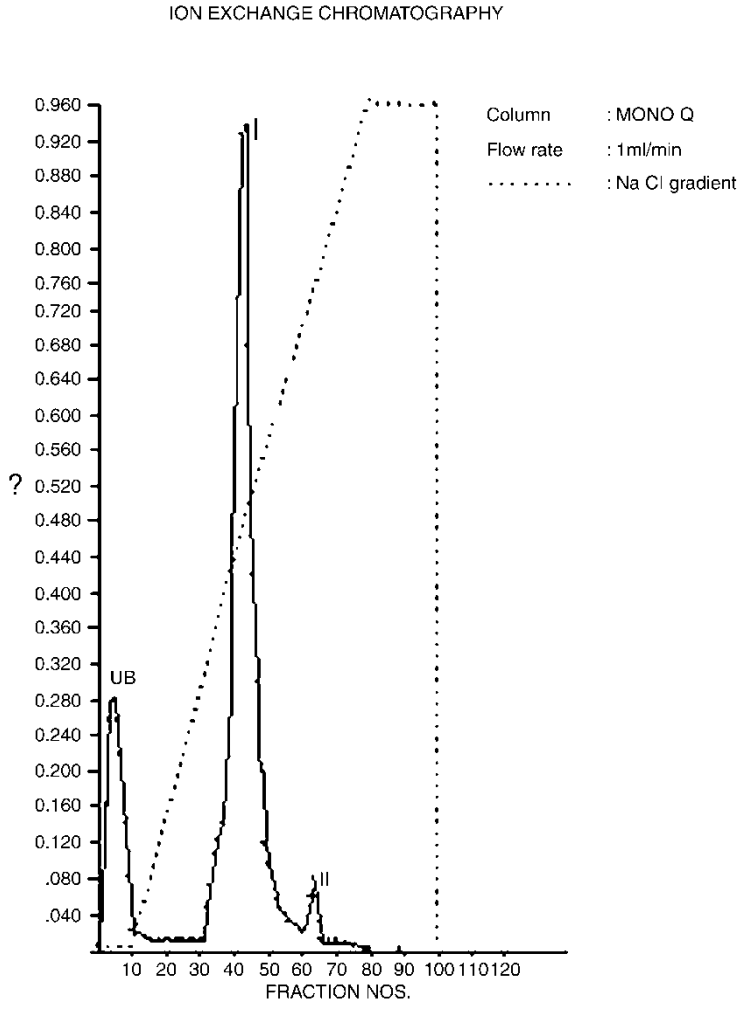

Figure 2. Elution profile of purified protein (Fraction II obtained after fractionating on Sephadex G-50) on FPLC MONO-Q. Fraction was eluted with 100 mmol/L–1.0 mol/L gradient in 10 mmol/L Tris-HCL, pH 7.4, and two distinct peaks (I, II), along with unbound (UB) peak were observed.

Chromatofocusing

Fraction I obtained by anion exchange chromatography was subjected to chromatofocusing by FPLC on a MONO-P column and eluted with Polybuffer 74 in the pH gradient of 7.0–4.0. The fraction eluted at pH 4.0 showed immunoreactivity, suggesting its isoelectric point in the vicinity of 4.0 (Figure 3).

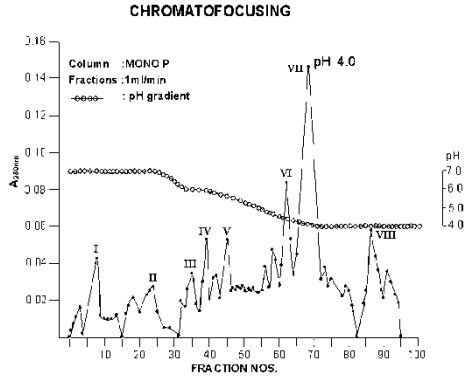

Figure 3. Chromatofocusing by FPLC on a MONO-P column of protein obtained after fractionating on a MONO-Q column. It was eluted with poly buffer 74 in the pH gradient 7.0–4.0 and the OC was eluted in peak VII at pH 4.0.

Subsequent dot blot analysis of this fraction resulted in reaction with DBS, which shows the presence of γ-carboxyglutamic acid. Tricine SDS-PAGE showed a single band with a molecular size of about 5.8kDa.

Characterization of OC

Tricine SDS PAGE

The Tricine 16.5% SDS PAGE system was used to check the purity of OC, as described. Immunoreactive fractions obtained at each stage of purification were checked for purity by Tricine gel electrophoresis. A 1.5 μg of protein were loaded into each well and calibrated with rainbow low molecular weight protein markers (2.5–45.0 kDa, Amersham Pharmacia). Figure 4 depicts the homogeneity of the proteins separated at each stage of purification. The molecular size of the purified homogeneous band of OC was estimated to be of about 5.7 kDa (Figure 5).

DBS Reaction

Figure 6 depicts the DBS reaction of γ-carboxyglutamic acid performed by dot blot analysis, as described. The proteins, after blotting onto a nitrocellulose membrane, stained the proteins red.

Quantitation of OC by ELISA

The quantity of OC in the protein purified at each stage is shown in Table 1.

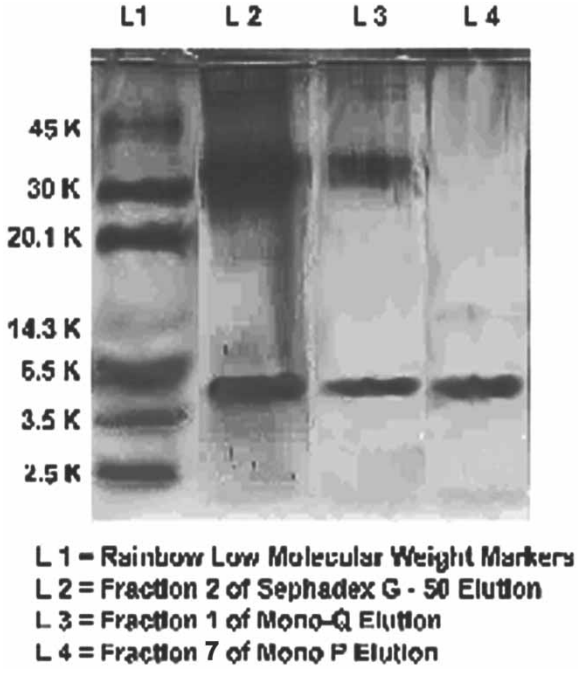

Figure 4. The SDS PAGE analyses of active fractions after silver staining on 16.5% Tricine gel electrophoresis. *Lane 1:* rainbow low molecular weight protein markers (2.5–45 kDa); *Lane 2:* Sephadex G-50; *Lane 3:* ion-exchange chromatography; *Lane 4:* Chromatofocusing.

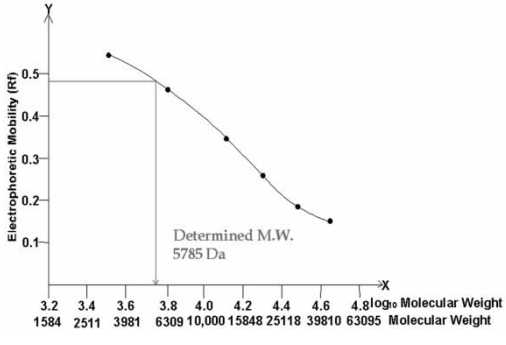

Figure 5. Determination of molecular weight of the purified protein by electrophoretic mobility (Rf). The electrophoretic mobility on an arithmetic scale (*Y*-axis), molecular weight ($\log_{10}$) (*X*-axis) scale. The molecular weight of the protein as calculated is 5785 Da.

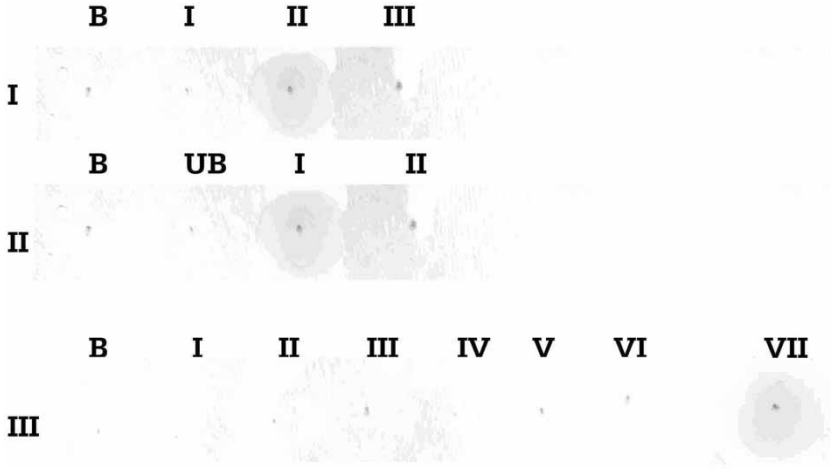

Figure 6. DBS reaction by dot blot analysis of the fractions obtained at each stage of purification. (1) Sephadex G-50 column chromatography; (2) Ion-exchange chromatography; (3) chromatofocusing. [B BSA (control); UB, Unbound fraction]. The roman numbers indicate the fractions at each stage of purification.

Production of Antibodies

Polyclonal antibodies were raised in rabbits (BW strain) as per the protocol described by Gundberg et al.[12] The titer of the antibody was determined by dot blot analysis and the specificity of the antibody raised was confirmed by Western blot analysis.

Western Blot Analysis

Figure 7 depicts the Western blot analysis of the antisera raised in rabbits. In brief, SDS PAGE of the crude protein was run as described. After electrophoresis, the proteins were transferred from gels to the nitrocellulose membranes by applying an electric field of 30 V at 4°C overnight. The membrane was cut to the size of the gel, prewetted in water, equilibrated in transfer buffer (*Tris*–base 0.3%, Tricine 1.44%, methanol 20% in distilled water) for 10 min, and then placed over gel. The air bubbles were then carefully removed with the help of a glass rod. Filter papers and sponge were placed in the gel transfer unit (Bio-Rad). The gel transfer took place overnight at 4°C at 30 V. The membrane was removed and placed in the blocking buffer (10 mmol/L PBS containing 0.1% Tween 20 and 3% BSA) for 1 hr at room temperature. The membranes were then incubated with the primary antibody to OC raised in rabbits at a dilution of 1:1000 in 10 mmol/L PBS at room temperature for

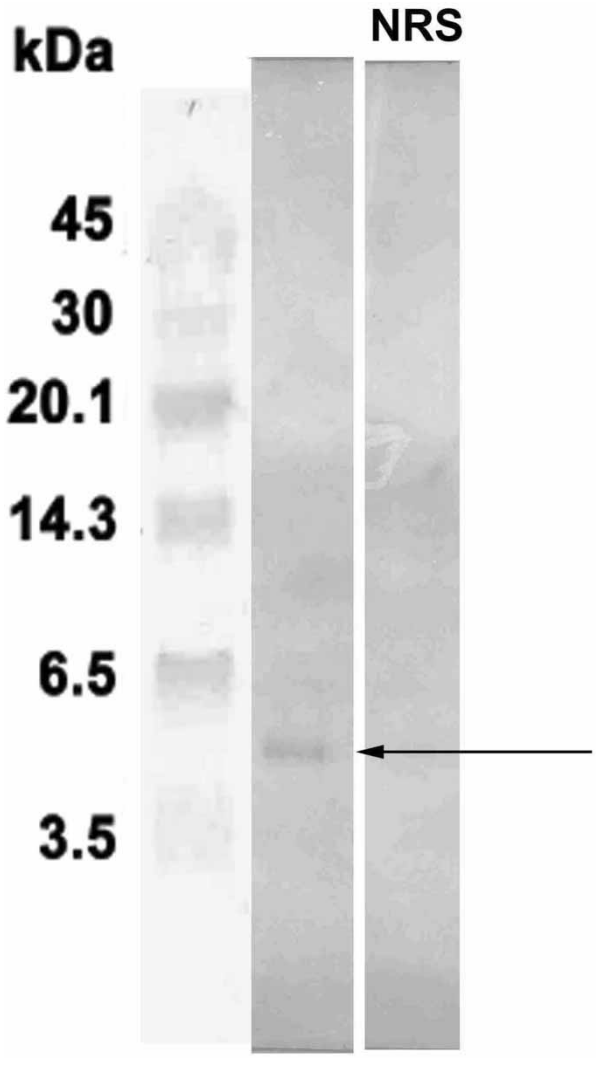

Figure 7. Western blot analysis of antisera raised against OC. *Lane 1:* Rainbow low molecular weight protein markers (2.5–45 kDa) *Lane 2:* recognition of OC antisera in crude extract. *Lane 3:* Pre-immune sera (NRS).

1 hr. Pre-immune sera were used as negative control. The membranes were then washed with 10 mmol/L PBS containing 0.1% Tween 20 twice for 5 min. The membranes were then incubated with goat ARGG-HRP secondary antibody for 1 hr at room temperature at a dilution of 1 : 2000 in 10 mmol/L PBS, followed by three washes of 10 mmol/L PBS containing

0.1% Tween 20. The membranes were then detected using DAB as a signal system (8 mg DAB, 10 μL of 30% hydrogen peroxide in 10 mL distilled water).

Cross Reactivity of OC Antisera

The cross reactivity of the antisera was indirectly checked by estimating OC in 30 samples obtained from women in varying age groups by the developed ELISA employing the generated antisera and by commercial kit. The estimates obtained by both the ELISAs correlated well, with a correlation coefficient of $r = 0.983$ (Figure 8).

Development of ELISA for OC

The optimum dilution of antibody (1 : 2000) and conjugate (1 : 2000, goat ARGG-HRP) was used on the basis of checkerboard titration. All determinations were performed in duplicate.

The purified protein was used as standard and calibrated against standard OC supplied with the commercial kit. Calibrated standard OC was prepared in immunoassay buffer and stored in aliquots at -20°C. Working assay standards (range: 2.5–160 ng/mL) were prepared from the stock standard prior to use in the assay.

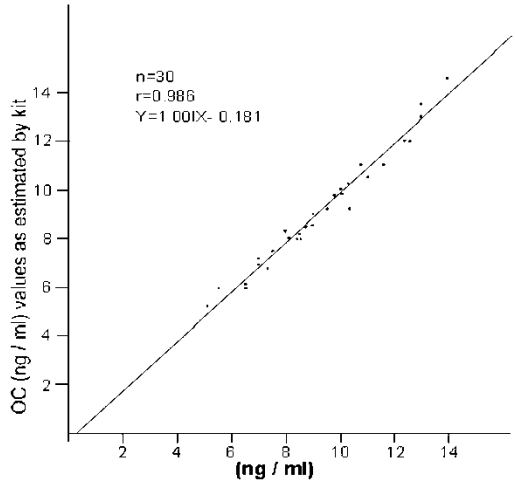

Figure 8. Comparison of estimates of 30 samples by the developed ELISA (*X*-axis) and by commercial kit (*Y*-axis).

Validation of ELISA for OC

Characteristics of the Assays

The composite standard curve ($n = 6$) of the ELISA for OC is shown in Figure 9. The sensitivity of the assay ranged between 2.5 and 4 ng/mL. To evaluate the recovery, various concentrations of OC were added to standard pooled serum sample obtained from four women OC. The recovery of OC by the ELISA in these spiked samples ranged between 95% and 104% (Table 2).

The OC antiserum raised was specific as judged by the agreement between estimates of 30 samples by the developed ELISA and by a commercial kit. The specificity of the assay was determined by the parallelism between the sample dilution curve and standard curve, which indicated the immunochemical similarity between the standard curve and test preparations, and absence of matrix effect (Figure 10).

The precision of the assay was assigned on the basis of the variation in the estimates of OC in two quality control pools with different concentrations. The intra- and inter-assay coefficient of variation (CV) of the quality control pools were 7–11% and 12–15%, respectively.

OC estimated by the developed ELISA was compared with a commercially available kit in the fasting serum samples collected in the morning around 9.00–10.00 am.

The sample estimates obtained by both methods compared well (correlation coefficient, $r = 0.983$) (Figure 8).

DISCUSSION

OC is the most abundant non-collagenous protein synthesized by the osteoblasts. The higher levels of OC in serum reflect increased osteoblastic activity or bone remodeling. Moreover, utility of OC as a bone formation marker has been well documented.[1,2,16–18]

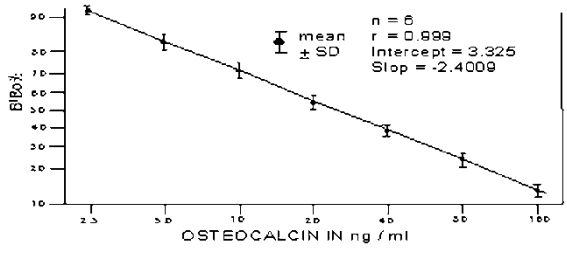

Figure 9. Composite standard curve of ELISA for OC.

Table 2. Data on validation of ELISA for OC ($n = 6$)

Specificity: slope of dose–response curves		Sensitivity (ng/mL)	Accuracy: recovery of the added analyte (range)	Precision: coefficient of variation of two pools		Comparison with RIA correlation coefficient (r)
Standard	Sample			Intra %	Inter %	
−2.400	−2.378	2.5–4	95–104	7–11	12–15	0.983

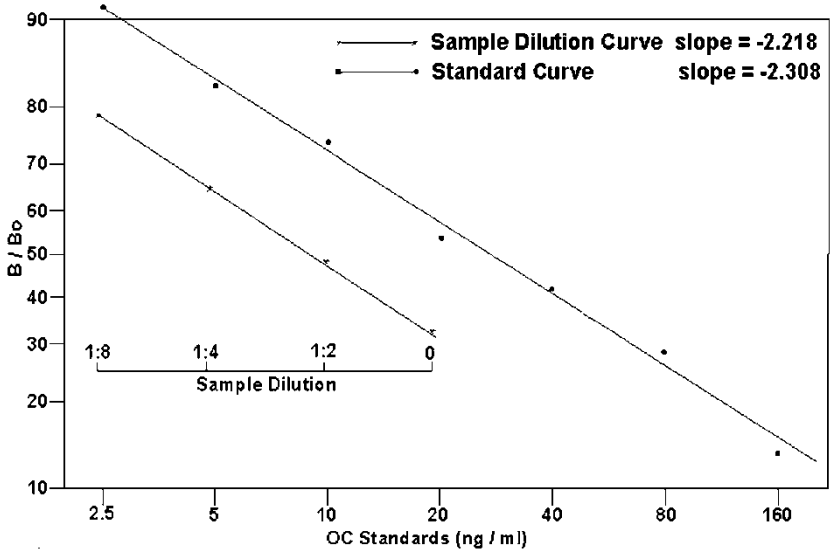

Figure 10. Parallelism obtained between standard curve and sample inhibition curve using an OC ELISA.

Commercial test kits available for OC detection are based on RIA, ELISA, or IRMA formats for the detection of total OC.[19] The kits available for OC are expensive, so our aim was, therefore, to purify and develop an in-house ELISA for OC.

Gla containing protein or OC has been isolated and purified from various species and a high degree of amino acid sequence homology has been reported.[6] Most of the commercial assays report the use of either bovine OC or synthetic OC. In view of the sequence homology between species, we purified OC from bovine bones extracted by gel permeation chromatography, ion exchange chromatography, and chromatofocusing.

The femur was chosen, as the diaphyseal bone is a richer source of OC than the metaphyseal bone. A fine bone powder of particle size below 100 μm is essential for extraction of OC from hydroxyapatite with high yield. However, this could not be achieved in the present study due to non-availability of a suitable grinder. Moreover, the acid extraction employed often leaves 10–25% of the protein behind, due to precipitation during the process of extraction or during dialysis. The extract was subjected to size exclusion chromatography on a Sephadex G-50 column; this step yielded 19.208 mg (32%) of immunoreactive protein. Further purification of OC was achieved by anion-exchange chromatography on a MONO–Q column. This step yielded 5.5 mg (72%) of immunoreactive protein. The final step of purification of OC was carried out by chromatofocusing (98%). The protein

was eluted on the basis of its isoelectric point. OC eluted as a major peak at pH 4.0. To characterize the protein at various stages of purification, purity of OC was checked by Tricine SDS PAGE (Figure 4), identified by its reactivity with DBS in dot blot analysis and its immunoreactivity by ELISA. The estimated molecular size of purified OC was found to be about 5.7 kDa (Figure 5).

Furthermore, quantitation of OC at each stage of purification was performed using an OC specific ELISA kit. We could purify 0.678 mg of OC from 90 g of bovine bones, whereas reports have documented up to 1 mg from 100 g of bovine bone. Most of the earlier studies have reported the immunoreactivity of the purified protein, but have not documented the purity of OC. Although the yield was less (0.678 mg) when compared with the reported yield of purification, the purified OC obtained in the present study was highly homogeneous, as seen by silver staining of SDSPAGE gel, which is considered to be the most sensitive method of staining.

The developed ELISA appears to be highly specific and sensitive and fulfils the validatory criteria described for immunoassays. The reagents are simple to prepare and the developed ELISA has the desired sensitivity to detect OC in pre- and post-menopausal women.

CONCLUSION

The highly purified OC, to the extent of about 0.7 mg from 90 g of bovine bone extract, with a purity of about 98%, was achieved. The purification process described was found to be reproducible and yielded similar amounts of purified OC during subsequent batches of purification. The purified protein was used in generation of polyclonal antisera and subsequent development of an in-house sensitive and specific ELISA for estimation of OC in pre- and post-menopausal women.

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