Purification and Partial Amino Acid Sequence of Osteogenin, a Protein Initiating Bone Differentiation

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Osteogenin was purified from bovine bone matrix and its activity monitored by an in vivo bone induction assay. The purification method utilized extraction of the bone-inducing activity with 6 M urea, followed by chromatography on heparin-Sepharose, hydroxyapatite, and Sephacryl S-200. Active fractions were further purified by preparative sodium dodecyl sulfate gel electrophoresis without reduction. Osteogenin activity was localized in a zone between 30 and 40 kDa. The amino acid sequences of a number of tryptic peptides of the gel-eluted material were determined. Reduction and alkylation of purified osteogenin in 7 M guanidine hydrochloride resulted in the total loss of biological activity. Sodium dodecyl sulfate gel electrophoresis under reducing conditions revealed a broad band with an apparent molecular mass of 22 kDa.

It is well known that bone has a remarkable potential for repair. However, the biochemical and cellular mechanisms underlying bone repair are not understood. The presence of factors in bone which initiate endochondral bone formation has been amply demonstrated by implantation of demineralized bone matrix in extraskeletal sites (1-4). The sequential developmental cascade in response to implantation of demineralized matrix consists of the following major steps: 1) chemotaxis and attachment of mesenchymal cells to the matrix; 2) proliferation of progenitor cells; and 3) differentiation resulting in the formation of cartilage, bone, and hematopoietic marrow (4, 5). The bone-inductive protein that initiates this cascade, osteogenin, was recently isolated from bovine bone matrix by heparin affinity chromatography (6). This report describes an improved purification method for osteogenin as well as the amino acid sequence of a number of tryptic peptides obtained from this protein.

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MATERIALS AND METHODS

Partial Purification—For the purification of osteogenin, 5- to 10-kg lots of dehydrated diaphyseal bovine bone matrix powder (particle size 74-420 μm, American Biomaterials) were demineralized at room temperature in 0.5 M HCl (seven extractions of 4 volumes each) (2). The acid-demineralized matrix was extracted with 20 volumes of 6 M urea, 50 mM Tris-HCl, pH 7.4, containing 1 M NaCl, 100 mM L-cysteine, 50 mM benzamide HCl, and 0.5 mM phenylmethylsulfonfyl fluoride at room temperature for 16 h (7). The extract was concentrated, exchanged with 6 M urea to reduce the salt concentration, and loaded onto a 2-liter hydroxyapatite (Pharmacia LKB Biotechnology Inc.) column. The column was washed and eluted as described (6). The 100 mM sodium phosphate eluate was loaded directly onto a 0.5 liter heparin-Sepharose (Pharmacia LKB) column, which was washed and eluted as described (6). The 0.5 M NaCl eluate was concentrated and loaded onto tandem Sephacryl S-200 gel filtration columns (2.6 × 100 cm each), equilibrated with 4 M guanidine chloride (GdmCl), 50 mM Tris-HCl, pH 7.4. The material was eluted with the same buffer at a flow rate of 36 ml/h; 20-ml fractions were collected and assayed for biological activity.

Gel Elution—Active fractions (25, 26, and 27) from the S-200 column were concentrated and equilibrated first with 6 M urea, 50 mM Tris-HCl, pH 7.4, and then with SDS sample buffer (0.05 M Tris-HCl, pH 6.8, 10% glycerol, 1% SDS with 6 M urea) (3). Fraction 25 was then applied to a 12.5% acrylamide gel (8 cm × 7 cm × 1.5 mm) prepared according to Laemmli (8). The separating gel was cast 1 day in advance and subjected to pre-electrophoresis for 30 min at 100 V. Following this, the gel was incubated in Aurodye Forte (Janssen, Scherler & Schuell, BA 85) over the gel, and putting two pieces of Whatman 3MM paper (cut to the same size as the gel and presoaked in PBS), a stack of dry paper towels, and a weight over this assembly. Transfer by capillary action was allowed to proceed for 10 min, after which the blot was washed in PBS supplemented with 0.3% Tween 20 at 37 °C for 15 min with three changes of buffer, washed extensively with distilled water, and incubated for 1 h in Aurodye Forte (Janssen, Life Sciences Products) with constant agitation. The molecular weight markers on the stained blot were then aligned with the prestained standards on the unstained gel and used as a template for gel slicing. The 2 × 2-mm slices were electroeluted at room temperature in 50 mM ammonium bicarbonate, 0.1% SDS for 5 h using a Bio-Rad (model 422) electroeluter at a constant current of 8 mA/glass tube. At the end of the electroelution, the polarity of the electrodes was reversed for 1 min in order to minimize losses of protein on the dialysis membrane. The eluates were filtered through an Acrordel 13 (Gelman) filter and bioassayed for bone-inductive activity. Fractions 26 and 27 were separated on 16 cm × 20 cm × 0.75-mm SDS gels and electroeluted using radiolabeled gel-eluted material from fraction 25 as a marker (10).

Amino Acid Sequence Analysis—The gel-eluted fractions with in vivo biological activity were pooled, acetone-precipitated, and digested with trypsin (Worthington) in 0.1 ml of ammonium bicarbonate. Two 0.5-μl aliquots of trypsin were added during the 18-h incubation at 37 °C. Digestion was terminated by the addition of 0.1 ml of 6 M GdmCl, 20 mM DTT, 50 mM Tris-HCl, pH 7.5. Tryptic peptides were separated by reverse-phase chromatography, and the sequence of each peptide was determined using an automated gas-phase sequenator (ABI, 470A).

Reduction of Osteogenin—To determine whether the in vivo biological activity was sensitive to reduction, partially purified samples after S-200 filtration were dialyzed under nitrogen against 4 M guanidine chloride (GdmCl), 7 M GdmCl in 50 mM Tris-HCl, pH 8.5, with or without 10 mM DTT.

The abbreviations used are: GdmCl, guanidinium chloride; SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline; DTT, dithiothreitol; HPLC, high pressure liquid chromatography.

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Iodoacetamide was added to a final concentration of 50 mM, and the pH was readjusted to 8.5. The samples were again purged with nitrogen and kept for 2 h in the dark at 4°C. Following incubation aliquots of the nonreduced control and the reduced/alkylated sample were reconstituted directly for bioassay. The bulk of the sample was purified and tested for bone induction by reconstituting a portion of rat demineralized bone matrix (11). Typically, either 2-20 mg of the urea extract, 0.2-2.0 mg of the hydroxypatite fraction, 20-200 µg of the heparin-Sepharose fraction, 2-20 µg of the S-200 fraction, or 0.1-1 µg of the final gel-eluted fraction were used in each reconstitution. One milligram of chondroitin 6-sulfate, sodium salt (Seikagaku Kogyo Co., Japan), and 500 µg of acid-soluble type I rat tail tendon collagen were added to each sample as carriers (11). The samples were mixed and left for 1 h at room temperature before the proteins were precipitated with absolute ethanol overnight. The samples were then centrifuged at 12,000 rpm (Beckman Microfuge) for 15 min. The supernatants were discarded, and the pellets were washed three times with 85% ethanol, dried, and implanted subcutaneously into male Long-Evans rats (28-35 days old) at bilateral sites located over the ventral thorax. Each animal received two implants, and fractions were assayed in quadruplicate. The day of implantation was designated as day 0, and the implants were removed on day 10. They were cleared of adherent tissue, weighed, and homogenized in 2 ml of ice-cold 3 mM sodium bicarbonate containing 0.15 M NaCl. The homogenate was centrifuged at 4,500 × g for 30 min. Alkaline phosphatase activity of the supernatant and calcium content of the acid-soluble fraction of the pellet were used as quantitative parameters for new bone formation (12). Implants were also examined by histology.

### Results and Discussion

#### Purification

Table I summarizes the results of the purification of osteogenin. After acid demineralization of 5 kg of bovine diaphyseal bone powder approximately 1 kg of demineralized matrix was obtained. Extraction with 6 M urea, 1 M NaCl, 50 mM Tris-HCl, pH 7.4, yielded about 18 g of protein. After hydroxyapatite and heparin-Sepharose affinity chromatography, the active fraction contained 119 mg of protein. As is shown in Table I, there is an increase in total activity after hydroxyapatite and heparin-Sepharose chromatography, in comparison to the crude urea extract. This increase has been observed previously and suggests the possible removal of endogenous inhibitors of osteogenic activity (6). Gel filtration of the active heparin-Sepharose fraction on Sephacryl S-200 is shown in Fig. 1. Bone-inductive activity was found in a single peak. The protein profiles of the S-200 fractions were analyzed by SDS gel electrophoresis under nonreducing conditions and silver stained.

![Sephacryl S-200 gel filtration](image-url)

**Fig. 1. Sephacryl S-200 gel filtration.** The bioactive heparin-Sepharose fractions were equilibrated with 4.0 M guanidine hydrochloride, 50 mM Tris-HCl, pH 7.4, concentrated, and loaded on a Sephacryl S-200 column. The column was eluted with the same buffer, and the absorbance at 280 nm is shown (top). V₀ is the void volume. The arrows indicate the position of elution of molecular mass standards (Sigma): 1, ovalbumin (43 kDa); 2, chymotrypsinogen (25 kDa). The fractions were bioassayed, and the calcium content of the implants, an index of bone formation, was determined. Calcium values are depicted in the inset of the figure. The fractions were analyzed by 15% SDS gel electrophoresis under nonreducing conditions and silver stained (bottom).

Previous work had established the utility of heparin affinity chromatography for the isolation of osteogenin activity (6). In that work, a 22-kDa protein band was found on SDS gel electrophoresis of 12,400-fold purified material. Localization of activity in the gel was not established, however. In the present experiments, employing 5-15-kg lots of starting material, we demonstrate that the major activity was confined to a narrow region from 28 to 32 kDa (data not shown). The apparent low yields may be due to surface adsorption and possible inactivation of osteogenin activity during electrophoresis from SDS gels. In more recent experiments, electrodosmotic elution after preparative SDS gel electrophoresis (13) of the bioactive S-200 fractions yielded substantially higher recoveries of osteogenin (7-17%).

**Table I: Purification of osteogenin**

| Purification step | Protein | Total activity | Osteogenin specific activity | Purification |
|-------------------|---------|----------------|-----------------------------|--------------|
| Urea extract      | 18,000  | 140            | 0.008                       | 1            |
| Hydroxyapatite    | 1,400   | 1,500          | 1.1                         | 130          |
| Heparin-Sepharose | 119     | 9,000          | 76                          | 9,500        |
| S-200             | 6.3     | 2,600          | 419                         | 52,000       |
| Gel elution       | 0.02    | 60             | 3,000                       | 380,000      |

* Specific activity of osteogenin was expressed as units of alkaline phosphatase or as micrograms of calcium/mg of protein used for reconstitution in the bioassay.
The major advantage of the current urea extraction method over the previously described GdmCl extract is that it circumvents the time-consuming buffer exchange from guanidine to urea. It is also noteworthy that fewer contaminating bands were observed on SDS gel electrophoresis of material isolated by urea extraction.

Analysis of Reduced Osteogenin—A gel-eluted active fraction was radiolabeled with \(^{125}\)I (IODO-GEN procedure (14)). SDS gel electrophoresis of this labeled material showed coincidence of radioactivity and protein visualized by silver staining (data not shown). SDS gel electrophoresis under nonreducing and reducing conditions of radiolabeled protein is shown in Fig. 2. Without reduction, the gel showed a broad band at 35 kDa; following reduction with 20 mM DTT, 1% \(\beta\)-mercaptoethanol (15) the gel showed a broad band centered at 22 kDa. Incomplete reduction was observed when only 10 or 20 mM DTT was used (Fig. 2). Reduction of osteogenin with 10 mM DTT in 7 M GdmCl followed by alkylation resulted in a total loss of biological activity, while alkylation without reduction or the same reduction and alkylation in 6 M urea had no effect on the biological activity. These findings and the gel results described above demonstrate that stringent conditions are required to fully reduce osteogenin.

Amino Acid Sequencing—Several attempts to obtain amino acid sequences directly from gel-eluted material were unsuccessful (data not shown), suggesting that the amino terminus of osteogenin may be blocked. Therefore, active gel-eluted material from Sephacryl S-200 fractions 25–27 was digested with trypsin and the tryptic peptides separated by reverse-phase HPLC (Fig. 3). Table II shows the amino acid sequences that were obtained. Four peaks gave unique sequences and three gave mixtures. The unique sequence from peaks 26 and 28 was used to resolve the mixture sequences. The same sequence was found in different peaks from a number of tryptic fragments, perhaps due to incomplete digestion of the sample. Computer-assisted searches of a protein data base (17) showed that these sequences do not match any known proteins. We do note, however, some homology of peptide 15 with inhibin-\(\alpha\) (18) and decapentaplegic gene product (19), two members of the transforming growth factor-\(\beta\) family. Transforming growth factor-\(\beta\) either purified from human platelets (6) or expressed by recombinant techniques\(^2\) was not

\(\)\(^\text{2} F. P. Luyten, N. S. Cunningham, S. Ma, N. Muthukumaran, R. G. Hammonds, W. B. Nevins, W. I. Wood, and A. H. Reddi, unpublished data.

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**Fig. 2.** Preparative SDS gel electrophoresis and elution fraction 25 from the Sephacryl S-200 column was electrophoresed on a 12.5% polyacrylamide gel with prestained standards. The gel was sliced, electroeluted, and bioassayed. The results demonstrate that activity was confined to the region between 30 and 40 kDa (top). The gel-eluted fraction was radiolabeled by the IODO-GEN method (14) and analyzed by SDS gel electrophoresis under nonreducing (lane a) and reducing conditions (10 mM DTT, lane b; 20 mM DTT, lane c; 20 mM DTT with 1% \(\beta\)-mercaptoethanol, lane d), (bottom).

**Fig. 3.** Reverse-phase HPLC of tryptic peptides. A trypsin digest of gel-eluted osteogenin was chromatographed on a Synchropak RP4-4000 column (100 × 2.1 mm). The column was equilibrated in 0.08% trifluoroacetic acid, 8% acetonitrile, and eluted with a linear gradient to 0.093% trifluoroacetic acid, 40% acetonitrile in 60 min. The numbered peaks were sequenced (Table II). Peaks 25a and 25b were sequenced together.

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**Table II**

| Peak | Sequence | Initial yield pmol |
|------|----------|--------------------|
| 15   | SFDAYY(C)SGA(C)Q | 34                |
| 17   | SFDAYYXSQA(C)Q  | 12                 |
| 24-1 | AVGVVPQIEPXXVPEKM | 36                |
| 24-2 | VDFADI      | 10                 |
| 25-1 | AVGVVPXIEP(C)VPE | 10                |
| 25-2 | VDFADLXW     | 3                  |
| 26-1 | AVGVVPQIEP(C)VPEDM | 10                |
| 26-2 | QWIEPNNAXYLVKVDFA | 3                  |
| 27   | AVGVVPQIEPE  | 6                  |
| 28   | AVGVVPQIEPXXVPEK | 11                |
ostegenic in this bioassay. In conclusion, osteogenin was purified more than 300,000-fold, and amino acid sequences of tryptic peptides of active highly purified protein were determined. Final proof that the protein we have characterized here is in fact the osteogenin activity will require molecular cloning and expression of sufficient recombinant material to demonstrate bone formation in vivo.

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Note Added in Proof—Since the submission of this manuscript, amino acid sequences of four bone morphogenetic proteins (BMPs) were reported (20). The amino acid sequences reported here for osteogenin show considerable homology to BMP-3. However, unlike recombinant BMP-3 which only induces cartilage, purified native osteogenin initiates both cartilage and bone formation in vivo.

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