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# EXTRACTION OF OSTEOCALCIN FROM FOSSIL BONES AND TEETH

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Osteocalcin (also called 'bone Gla-protein') was detected in fossil bovid bones ranging from 12 000 years to 13 million years old and in rodent teeth 30 million years old. Both the antigenic activity and the protein-bound Gla-residues have remained intact. The protein is indistinguishable from recent bovine osteocalcin when analyzed by HPLC using ion exchange and size exclusion columns. If sufficient amounts can be extracted and an adequate purification procedure is established, this would be the first time that amino acid sequences in a protein from fossil bones may be determined. Such sequence data could offer a new aproach to the phylogenetic study of extinct taxa.


In recent decades, molecular biology has provided new information about the phylogenetic relationships between organisms [1,2]. Whereas the traditional morphological approach to phylogeny is based on comparison of characters from extinct species with those from living ones, molecular biology has been confined almost exclusively to modern forms. The few exceptions are studies of mummified or frozen soft tissue remains [3,4]. The usual remains of extinct vertebrate taxa, fossil bones and teeth, are not known to contain macromolecules of evolutionary interest.

Osteocalcin (also designated as bone Gla-protein [5,6]), is a protein, very tightly bound to the mineral phase (hydroxyapatite) of bone. The osteocalcin of most species contains 49 amino acid residues, three of which are gammacarboxyglutamic acid (Gla). The Gla-residues have a high affinity for $Ca^{2+}$ and are located in such a way that strong cooperativity occurs in the binding of osteocalcin to crystals of calcium phosphate and hydroxyapatite

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Abbreviations used: y, year; my, million years

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[7]. The primary structure of osteocalcin has been determined for diverse taxa [5] and the function of osteocalcin (especially in relation to a number of bone diseases) is now a subject of investigations in many different institutes.

During the preparation of osteocalcin from fresh human and bovine bones it was found that prolonged heating of the bones for several days at 120 $^{o}$C reduced neither the antigenic activity of the protein nor its Gla-content [8]. Other well known properties of osteocalcin, which seem to remain unaffected by heat treatment are its ability to inhibit calcium phosphate precipitation from a supersaturated solution [9] and - after thermal decarboxylation - to serve as a substrate for bovine liver vitamin K-dependent carboxylase [8]. This exceptional stability of osteocalcin has lead us to investigate its presence in fossil bones and teeth.

## MATERIALS AND METHODS

Chemicals and buffers. Buffer A: 1 M EDTA, 50 mM Tris/HCl, pH 8.0, 0.1 M NaCl, 30 mM benzamidine, 100 000 IU trasylol and 0.02% (w/v) soybean trypsin inhibitor. Buffer B: 0.1 M NaCl, 50 mM Tris/HCl, pH 7.4. All chemicals were of the highest purity commercially available. QAE Sephadex was purchased from Pharmacia (Sweden). Polyclonal, monospecific antibodies against rat osteocalcin were a kind gift of Dr. I. Gorter-de Vries (University of Brussels). Purified bovine osteocalcin was prepared as described earlier [8] and polyclonal antibodies against it were raised in rabbits.

Fossil samples. All bone and teeth samples were from geologically well-documented sites (see Table I). The sample weights varied from 1-1000 g.

Crude bone extracts. The various bone and teeth samples were carefully cleaned and dried, and subsequently crushed and powdered mechanically using a Retsch centrifugal mill ZM-1 equipped with an 8 µm pore seize sieve. Small fragments (< 10 g) were powdered with a freezer/mill (Spex 6700). A known dry weight of bone powder was mixed with buffer A (2 ml/g bone) and the slurry was transferred into a dialysis bag (Spectrapor, MW cut of 3500) and dialyzed against the same buffer for 7 days with changes of the buffer every 24 h. EDTA was removed from the samples by a subsequent dialysis against buffer B for 3 days with 3 changes of the buffer. Insoluble material was removed by centrifugation (10 min at 4000 g). The remaining supernatant was designated as crude bone extract.

Osteocalcin detection. Osteocalcin antigen of bovid origin was measured routinely using a commercial radioimmuno assay (RIA) kit (INCSTAR, USA). In some cases we also used an ELISA (enzyme-linked immuno sorbent assay) in which polyvinlychloride microplates (Titertek, Flow Laboratories) were coated with the osteocalcin-containing samples and subsequently with ovalbumin, incubated with anti-osteocalcin antibodies and with a horse-radish peroxidase-linked second antibody.

Other assays. Gla was detected by HPLC after alkaline hydrolyzis of the samples, as described by Kuwada and Katayama [10]. Protein concentrations were determined as described by Sedmak and Grossberg [11].

## RESULTS

Bovid bones of varying ages and from different sources were powdered and extracted with buffer A. The osteocalcin content of the crude extracts was

TABLE I
Description of bone and teeth samples

| Sample nr. | Species | Bone | Site | Period | Collection |
|---|---|---|---|---|---|
| 1 | Bos taurus | tibia | Slaughterhouse Maastricht The Netherlands | recent | |
| 2 | Myotragus balearicus | tibia | Son Muleta, Mallorca Spain | Late Pleistocene | IVAU |
| 3 | Bison sp. | metatarsus | North Sea, The Netherlands | Late Pleistocene | IVAU |
| 4 | Bison sp. | tibia | Swanscombe, lower gravel, United Kingdom | Middle Pleistocene | BMNH |
| 5 | Bubalus palaeokerabau | radius | Trinil, Java, | Early Pleistocene | RMNH |
| 6 | Bovidae | metatarsus | Chinji, Pakistan | Miocene | IVAU |
| 7 | Caprotragoides sehlini | metacarpus | Manchones I Spain | Miocene | IVAU |
| 8 | Theridomorpha | incisors | Olalla, Teruel Spain | Oligocene | RMNH |

Abbreviations used are: IVAU, Institue of Earh Sciences, Utrecht, The Netherlands; RMNH, Rijksmuseum van Geologie, Mineralogie en Natuurlijke Historie, Leiden, The Netherlands; BMNH, British Museum, National History, London, United Kingdom.

measured by RIA. As is shown in Table II, the osteocalcin content of the fossil material varied considerably, but in all cases its presence could be clearly established. The osteocalcin present in the various crude bone extracts could be recovered quantitatively after incubating the various samples with pre-swollen QAE Sephadex (0.1 ml of slurry per ml) while rotating the tubes end over end. After removal of the supernatant and washing the Sephadex with buffer B, all osteocalcin could be eluted from the Sephadex with 1 M NaCl in buffer B. The results shown in Table II were verified with an ELISA using immuno-purified polyconal antibodies against purified bovine osteocalcin. Also

TABLE II
Osteocalcin content and estimated age of fossil samples

| Sample | Estimated age | Detection method | Osteocalcin content (μg/g bone) |
|---|---|---|---|
| 1 | 0 y | RIA | 26.8 |
| 2 | 12 000 y | RIA | 2.4 |
| 3 | 40 000 y | RIA | 4.4 |
| 4 | 350 000 y | RIA | 0.18 |
| 5 | 1 my | RIA | 0.13 |
| 6 | 13 my | RIA | 0.03 |
| 7 | 13 my | RIA | 5.0 |
| 8 | 30 my | ELISA | 0.8 |

The sample numbers correspond to those described in Table I.

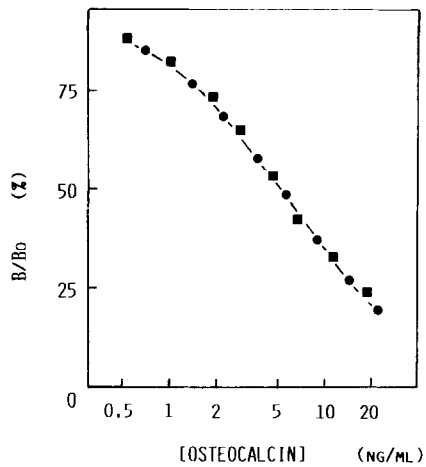


Fig. 1. Radioimmuno assay of osteocalcin. A dose/response curve of purified osteocalcin from recent bovine bone (●-●) was compared with that of the crude bone extract from sample 7 (■-■). B/Bo represents the percentage of label precipitated as compared to the sample containing $^{125}$I-labeled tracer only.

with this technique osteocalcin was detected in the samples (data not shown) and the amounts were comparable with those determined with the RIA. Because teeth are a more dense mineralization, are more often preserved and are better determinable than bone fragments, we have also tried to detect osteocalin in fossil teeth. These rodent incisors (see Table I, sample 8) were treated in the same way as the bone samples. Because no commercial RIA kit for rat osteocalcin is available, the teeth extract was only analyzed by ELISA, using polyclonal antibodies against recent rat osteocalcin. The amount of osteocalcin detected in this way was 0.8 ug per g of mineral (Table II, sample 8).

To exclude the possibility that unknown processes during the long geologic history of our fossil material would give rise to false-positive results in the various assays, we have performed two control experiments. First, we have compared the behaviour of serial dilutions of the various bone extracts with that of purified bovine osteocalcin. A typical experiment is shown in fig. 1 in which we have plotted the dose/response curves in the RIA for extracts from recent bovine bone as well as from 13 my old bone (sample 7). We have tested samples 2,3,4 and 5 in a similar way and, in all cases, parallel dilution curves were obtained (data not shown) . The amounts of osteocalcin present in sample 6 was to small to include it in this experiment. A second proof of the authenticity of osteocalcin in the fossil bones was obtained by partly purifying the protein in the extracts from samples 4 and 5. Approximately 50 g of bone were extracted for each sample and the crude EDTA extracts were dialyzed against buffer B, adsorbed to QAE Sephadex (10 ml of slurry) and eluted batchwise with 1 M NaCl. The eluate was fractionated on a Mono Q ion exchange column using a fast protein liquid chromatography (FPLC, Pharmacia) system and

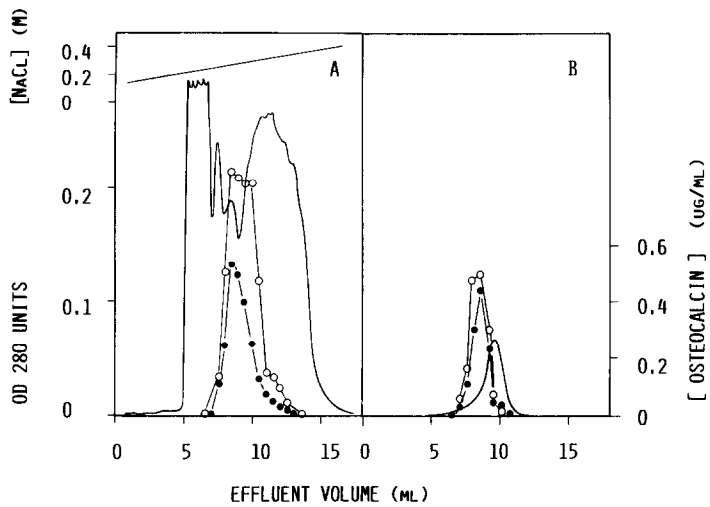


Fig. 2. Fractionation of bone extracts by HPLC. The solid line is the extinction profile obtained after chromatography of the samples. The osteocalcin antigen in the various fractions of sample 4(●-●) and sample 1 (O-O, recent bone) were determined by RIA. A: Three ml of bone extract were loaded on a Mono Q anion exchange column (Pharmacia) and eluted with a linear gradient from 0.1 M NaCl to 1 M NaCl in 20 mM Tris/HCl, pH 7.4. B: The peak fractions shown in A were pooled, concentrated and applied to a TSK-125 size exclusion column in buffer B.

the various fractions were tested for the presence of osteocalcin (fig. 2A). The immunoreactive peak was pooled and fractionated further on a TSK-125 size exclusion column. Immunoreactive osteocalcin eluted as a single peak in a position similar to that of recent osteocalcin (fig. 2B). The data displayed in fig. 2 are those for sample 4. Similar results were obtained with sample 5. None of the extractions yielded purified, homogenous osteocalcin, mainly because of the presence of dark colored material which was distributed uniformly over the various fractions and which partly coeluted with osteocalcin.

In the next set of experiments we investigated the preservation of protein-bound Gla-residues in fossil bones and teeth. The samples used were the same ones listed in Table I. No free Gla could be detected before hydrolysis of the samples. However, protein-bound Gla-residues were 2-4 fold more abundant than calculated from the RIA results. A typical example of the Gla analysis is shown in fig. 3 in which we have compared the presence of Gla in recent and in 13 my old bone (samples 1 and 7). The discrepancy between the observed protein-bound Gla concentration and that expected from the concentration of the osteocalcin antigen, may be caused by the presence of a second Gla-containing protein in the bone extracts (e.g. the matrix Gla-protein [12]) or by a loss of antigenic determinants due to a partial degradation of osteocalcin during the aging of the bones.

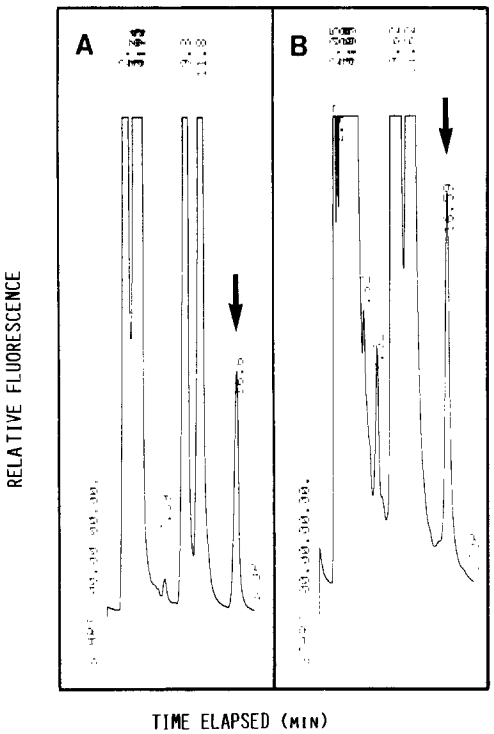


Fig. 3. Gla-detection in crude bone extracts. Samples were hydrolyzed under alkaline conditions and assayed for Gla (arrow) by HPLC analysis. A: extract of recent bone (sample 1). B: extract of 13 my old bone (sample 7).

## DISCUSSION

It has been shown by Huq et al. [13] that osteocalcin antigen has remained preserved in moa (Pachyornis elephantopus) bone approximately 3600 and 7400 years old. In the present paper we report that osteocalcin antigen as well as its Gla-residues remain detectable in bovid bones even after 13 my. The osteocalcin extracted from the fossil bones was detectable by both RIA and ELISA. Moreover, on both ion exchange and size exclusion columns, the protein eluted at a position which is characteristic for recent bovine osteocalcin. These results strongly indicate that the osteocalcin molecule has remained intact for millions of years.

The amount of osteocalcin which could be extracted from the various bone samples varied substantially and probably reflects differences in the soil conditions the bones have experienced (humidity, pH, fossilisation, etc.). In all cases the osteocalcin content of the fossil bones was considerably lower than that in recent bone. No strict relation was found between the osteocalcin content and the age of the bone samples. Therefore, it seems likely that osteocalcin will be found in much older bones, provided that they were preserved under optimal conditions. In this respect, it should be mentioned that

all data in this paper are expressed as mg osteocalcin per g of bone (dry weight). The bone samples contained varying amounts of insoluble material, however, and we do not know to what degree the bones contained contaminating cements (phosphates, silicates, carbonates). If the amounts of extracted osteocalcin are expressed per gram of EDTA-soluble material, the figures are generally substantially higher, but again it is unknown to what extent the contaminating cements are dissolved in EDTA. Neither do we know if part of the insoluble material originates from the bone itself and if in that case osteocalcin is washed off from the debris. Therefore, an accurate quantification of osteocalcin relative to the bone matrix is not possible from the data presented here. This does not affect the impact of our observations, however.

The striking stability of osteocalcin during millions of years may lead to a new approach to paleontology: if the extracted osteocalcin may be purified with a reasonable recovery, fragments of 1-10 g of bone or teeth may contain sufficient amounts of extractable antigen to sequence the entire molecule (usually 49 aminoacid residues). This could be of help in determining the relation between extinct taxa on other than morphological characters. However, the differentiation will probably be only at rather high taxonomic levels, because osteocalcin seems to be a very conservative molecule. For example, the amino acid sequence of the cow [5], an artiodactyl, differs from that of the horse, a perissodactyl (Chang and Sandberg, unpublished data), by very little more than it differs from other artiodactyls, such as the goat [5].

Obviously the sequencing of osteocalcin requires a quick procedure for the complete purification of the protein from the crude bone extracts but this turned out to be a serious problem. Because HPLC did not yield a homogenous preparation, other purification procedures (e.g. based on immuno-adsorption and elution) will have to be used. The development of such procedures is one of the topics of our future investigations.

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