SHORT COMMUNICATION

A simple method to survey proteins which affect *in vitro* mineralization

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Introduction

Biological mineralization is controlled by various proteins. Among these, the best characterized group are the matrix proteins of tooth and bone. Although the major matrix component of dentin and bone is type I collagen, minor acidic proteins are thought to be important effectors of mineralization. These acidic matrix proteins include bone Gla protein (BGP)/osteocalcin¹⁾, osteonectin²⁾, bone proteoglycans³⁾, bone sialoprotein (BSP)⁴⁾, and dentin phosphophoryn^{5,6)}. These proteins bind large amounts of calcium ions⁶⁾ causing a change in protein conformation⁷⁾. They have an affinity to hydroxyapatite^{8,9)} and affect *in vitro* mineralization¹⁰⁾.

Solution systems of *in vitro* mineralization have been successfully employed in evaluating the effects of matrix proteins on mineralization¹¹⁾, however, the major drawback of this system is that it requires relatively large amounts of protein. An alternative system of *in vitro* mineralization is the gel system in which

calcium and phosphate ions diffuse through a gel matrix. Both single-diffusion¹²⁾ and double-diffusion¹⁰⁾ systems were used, and have the advantage that gel systems require only small amounts of protein. Moreover, this method mimics the biological processes, in which calcium and phosphate ions are supplied only by diffusion through the matrix.

This paper presents gel systems which use electrophoretic gels as the diffusion media. This simple method enables a comparison of the effects of various proteins on *in vitro* mineralization.

Materials and Methods

The extraction of noncollagenous protein from bone and the purification of BGP and osteonectin were carried out as in our previous reports^{8,13)}. Gamma-carboxy glutamic acid residues of BGP were decarboxylated by heat treatment as described by Poser and Price¹⁴⁾.

Method 1 : Samples were subjected to SDS-polyacrylamide-gel electrophoresis. This method is also applicable to native poly-

crylamide-gel electrophoresis and isoelectric focusing. The gels were then fixed in 10% sulfosalicylic acid for 30 min. The gels were rinsed with distilled water and put in 15 mM sodium phosphate, 165 mM KCl, 10 mM HEPES, pH 7.4 for 1 h. After briefly rinsing in water, they were transferred to 25 mM CaCl_2 , 165 mM KCl, 10 mM HEPES, pH 7.4 and left overnight. A white precipitate of calcium phosphate developed in the gels, and was observed either against a black background or by staining with 0.1% alizarin red S. To examine the effect of native proteins native electrophoresis may be used instead of SDS gels and the step of fixation can be omitted.

Method 2 : After electrophoresis, the gels were put in 50 mM HEPES, pH 7.4 for 10 min. Several sheets of filter paper (0.8–1.0 mm thick) were dipped in either 10 mM CaCl_2 , 165 mM KCl, 10 mM HEPES, pH 7.4, or 10 mM sodium phosphate, 165 mM KCl, 10 mM HEPES, pH 7.4. The gel was sandwiched between the layers of filter paper containing calcium ions and the filter paper containing phosphate ions. The sandwich was set in a semidry electrotransfer apparatus (Atto Inc., Tokyo) so that the side containing calcium ions faced the anode. A current of 1 mA/cm² was applied on the apparatus for 5 min. The calcium phosphate precipitates were then visualized as described above.

Results and Discussion

Precipitation of calcium phosphate in the gels occurred using both method 1 and method 2. The precipitates were observed as an opaque-white zone against a black background, or were stained pink by alizarin red. When cross sections of the gels were examined, the precipitates were found in a central layer of the gels.

Proteins in the electrophoretic gels affected

Fig. 1 SDS electrophoretic analysis of calcium phosphate precipitation.

(A) The bovine bone extracts were analysed by SDS gel electrophoresis. The gel was stained with Coomassie Brilliant Blue and several bands of proteins were observed. (B) After calcium phosphate precipitation by natural diffusion or electric diffusion, the gel was stained with Alizarin Red. Calcification was obviously inhibited by the bands corresponding to Osteonectin (ON), the 30 kDa protein and BGP.

the precipitation of calcium phosphate. When guanidine/HCl bone extract (after HCl decalcification) was separated in 15% gels and subjected to calcium phosphate precipitation using method 1, several transparent bands were observed (Fig. 1). These bands indicate an inhibition of calcium phosphate precipitation. When compared with the protein staining, the bands were identified as individual proteins. The most prominent inhibitory band was identified as BGP. Other minor inhibitory bands were osteonectin and an unidentified protein with an apparent molecular weight of 30,000. An apparent acceleration of the precipitation was observed in the high-molecular-weight area, where diffuse bands of proteoglycans were present. Purified BGP and osteonectin had inhibitory effects on the precipitate formation in the gel

Fig. 2 Effects of purified bone proteins on calcium phosphate precipitation.

(A) stained with Stains All. (B) stained with Coomassie Brilliant Blue. (C) stained with Arizalin Red after calcium phosphate precipitation. Calcification was enhanced at the level of the band corresponding to proteoglycan II (PG II), but inhibited at the bands corresponding to Osteonectin (ON) and BGP. A sharp inhibitory band was observed within a broader Stained band at the level of the 30 KD protein.

(Fig. 2). Method 2 which used the electric current produced the similar results to method 1, however the background precipitation was higher in method 2 than in method 1.

In solution systems, BGP and osteonectin are known to have inhibitory effects on *in vitro* mineralization^{1,15}. The results indicate that the inhibitory activity in bone mineralization is caused by BGP. Decarboxylation of BGP reduced the inhibitory activity (Fig. 3) as reported by Poser¹⁴ and Price. This gel system has shown to be a rapid way to evaluate the effect of proteins on *in vitro* mineralization. The reliability of the gel system is indicated by the reproduction of previous results obtained by solution systems. The gel system seems to reflect the physiological conditions of mineralization, as Ca^{2+} and PO_4^{3-} are supplied by diffusion into the gel, rather than being supplied freely as in the solution system.

Fig. 3 Comparison between the effects on calcification of BGP and of decarboxylated BGP (decarb. BGP).

(A) stained with Coomassie Brilliant Blue. (B) stained with Arizalin Red. Calcification was inhibited at the site of the BGP band, but not at the band of decarboxylated BGP. BGP was decarboxylated by heating *in vacuo* at 110°C for three hours after lyophilizing in a 0.05 M HCl solution.

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