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A STUDY OF THE BONE MORPHOGENETIC PROTEIN DERIVED FROM BOVINE DEMINERALIZED DENTIN MATRIX

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ABSTRACT

We tried to extract and purify bone morphogenetic protein (BMP) from bovine demineralized dentin matrix (DDM).

Crude dentin BMP (d-BMP) was extracted from DDM in 3 sequential steps. Purification of crude d-BMP was carried out by liquid chromatography. The molecular weight and the isoelectric point of the purified d-BMP were determined by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electrofocusing. All fractioned samples were bioassayed in the thigh muscle pouches of AKR strain mice to test their ability to induce new bone formation.

A sequence of 3 extraction steps worked effectively to obtain crude d-BMP. The purified d-BMP was shown to be homogeneous on high performance liquid chromatography (HPLC) and SDS-PAGE. The molecular weight and the pI were 25 kDa and 6.5, respectively. The amino acid compsition was different from that of known bone-derived BMP.

The purified d-BMP induced new bone formation in the thigh muscle pouches. The molecular weight, pI and amino acid composition were different from those of bone-derived BMP.

Key Words: Bone morphogenetic protein (BMP), Bovine dentin matrix, Purification

INTRODUCTION

Urist[1)] established the system of experimental bone induction using 0.6N Hydrochloride (HCl) decalcified bone matrix in 1965. He suggested that these biological phenomena were produced by a protein becoming tightly bound to the collagen of calcified tissue, and termed this substance BMP.[2)]

The solubilization and characterization of BMP have been considered to be very important in the basic research on calcified tissue and its clinical application to bone disease.

Hanamura et al.[3)] and Takaoka et al.[4)] succeeded in extracting a substantial amount of BMP from mouse Dunn osteosarcoma tissue by using 4M guanidine hydrochloride solution (Gdn-HCl). Urist et al.[5)] developed the BMP extraction method from bovine bone matrix using 6M urea solution. These 2 reagents made it easier to establish the biochemical characteristics of BMP. However, the proteins responsible for new bone formation activity remained unknown until the cloning of BMPs in 1988 by Wozney et al.[6)] Now, at least 7 types of BMPs (BMP-2 to BMP-8) have been discovered.

On the other hand, Yeoman et al.[7)] and Bang et al.[8)] proved that the potential for bone induction was present in the DDM derived from rabbit and rat teeth. The first report on BMP

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extraction from rat DDM, published by Butler,[9] described the use of the collagenase digestion method.

In this report, we discuss the purification and characterization of d-BMP derived from bovine teeth.

MATERIALS AND METHODS

1. Preparation of bovine DDM

Fresh anterior teeth from the bovine lower jaw were collected in a slaughter house, the soft tissue and dental pulp were manually removed and evaporated at room temperature after defatting with chloroform:methanol (1:1).

Five Kg (dry weight) of teeth were obtained and pulverized in a Wiley mill at 70°C to about 2 mm^3 cubic in size.

The demineralization procedure was performed for 4 days and involved vigorous stirring as described.[1] The DDM was rinsed thoroughly in deionized water and lyophilized.

2. Preparation of crude d-BMP (Fig. 1)

DDM was extracted by continuous magnetic stirring at 4°C in 10 volumes of 4M Gdn-HCl solution containing 2mM N-ethylmaleimide, 1mM iodoacetic acid, 1mM sodium azide and 1mM phenylmethylsulfonyl fluoride. The extraction was performed 3 times.

All extract solution was centrifuged at 48,000 g for 30 minutes, at 4°C to separate the soluble parts from the undissolved substances. The solution was collected and dialyzed against deionized water to complete precipitation, and then centrifuged for separation into water-soluble and water-insoluble substances; thereafter the water-insoluble substance was lyophilized.

The lyophilized 4M Gdn-HCl soluble, water-insoluble substance was then dissolved in 100 volumes of 0.01M sodium acetate buffer solution (pH 4.8) containing 6M urea.

The resultant insoluble fraction was collected, washed in cold water and lyophilized. This fraction was redissolved in 10 volumes of 4M Gdn-HCl. The solution was transferred to a dialyzing tube (M.W. cut off 10,000) and dialyzed against 7 volumes of deionized water, until the final concentration of Gdn-HCl was 0.5M.

The insoluble material remaining after dialysis was collected, washed in cold water and lyophilized in to crude d-BMP.

3. Purification of d-BMP

Purification of the protein fraction was carried out by HPLC using a Superose 12 (HR 10/30, Pharmacia, Sweden) gel column.

The protein sample was dissolved in 4M Gdn-HCl solution, then applied to the column and eluted with the same solution at a flow rate of 1 ml/min.

Positive fraction in bioassay from gel chromatography was applied to HPLC using a Mono Q HR 5/5 prepacked column (Pharmacia, Sweden) equilibrated with a 0.02M Tris-HCl buffer (pH 7.0) containing 6M urea.

The protein sample was dissolved in the same buffer solution and put into the column. The bound fraction was eluted with liner gradient from 0 to 1M NaCl solution (pump P-500 X2 + gradient programmer GP-250, Pharmacia, Sweden). The flow rate was 1 ml/min, and 1 ml per tube was collected.

All chromatograms were monitored at 280 nm and the fractions obtained were dialyzed against deionized water and lyophilized.

4. Bioassay for BMP activity

Five mg of the crude sample and 2 mg of the fractioned sample were implanted in the thigh muscle pouch of AKR mice after being encapsulated in a No. 5 gelatin capsule and sterilized with ethylene oxide gas.

Twenty-one days after implantation, the specimens were harvested and examined for new bone formation using soft X-ray and hematoxylin-eosin stains.

As a control, 5 mg of bovine serum albumin (Sigma) was implanted in the opposite thigh muscle pouch of each mouse.

5. Analytical electrophoresis of BMP fractions

Analytical electrophoresis on sodium dodecylsulfate (SDS) polyacrylamide slab gel (12.5%) was carried out according to Laemmli's method.[10] The molecular weights of the fractions were determined using low molecular standards (BIO-RAD) to be in the range of 92.5 kDa—14.3 kDa. Proteins were stained with 0.044% Coomassie brilliant blue R-250 using Scott's method.[11]

The isoelectric point of the final purified fraction was determined by Wringley's method.[12] A 5% polyacrylamide disc gel (5 × 80 mm) was prepared and Pharmalite (40%, pH 3—9) (Pharmacia) was used to make the pH gradient. After electrofocusing, the gel was stained with 0.1% Coomassie brilliant blue using Righetti's method.[13]

6. Amino acid composition and sequence

The final purified sample was hydrolyzed in 6M HCl at 110°C for 22 hours in an evacuated sealed tube.

The hydrolyzate was analyzed with an automatic amino acid analyzer (JTL-3000; Nippon Denshi).

For sequence analysis, the sample was digested with trypsin, and the tryptic fragments were separated by reverse-phase HPLC (Vydac C4 column developed in 0.1% trifluoroacetic acid to 0.1% trifluoroacetic acid/95% acetonitrile) and applied to a pulsed liquid protein sequencer (Model 473, Applied Biosystems, Foster, CA).

RESULTS

1. Purification of crude d-BMP

About 600 g of DDM were obtained from the 5 kg of bovine teeth.

About 300 mg of crude d-BMP was obtained from 100 g of DDM using a sequence of 3 steps (Fig. 1). According to the results of the bioassay, the crude d-BMP had the following characteristics: soluble in 4M Gdn-HCl, but insoluble in 6M urea (pH 4.8) and in 0.5M Gdn-HCl solutions.

The elution profile of crude d-BMP on the Superose 12 column is shown in Fig. 2. Five main peaks were recognized. The BMP active fraction was rechromatographed on the same column. The elution profile is shown in Fig. 3. The main peak fraction, corresponding to 25 kDa in molecular weight, was precipitated by dialysis.

The elution profile of the BMP active fraction from the Superose 12 column on Mono Q HR 5/5 ion exchange column is shown in Fig. 4. One peak of the unbound fraction and 2 peaks of the bound fractions were observed. A fraction eluted by 0.3M NaCl showed BMP activity (Fig. 5).

Finally 3 mg of purified d-BMP was obtained from 100 g of DDM (Table 1).

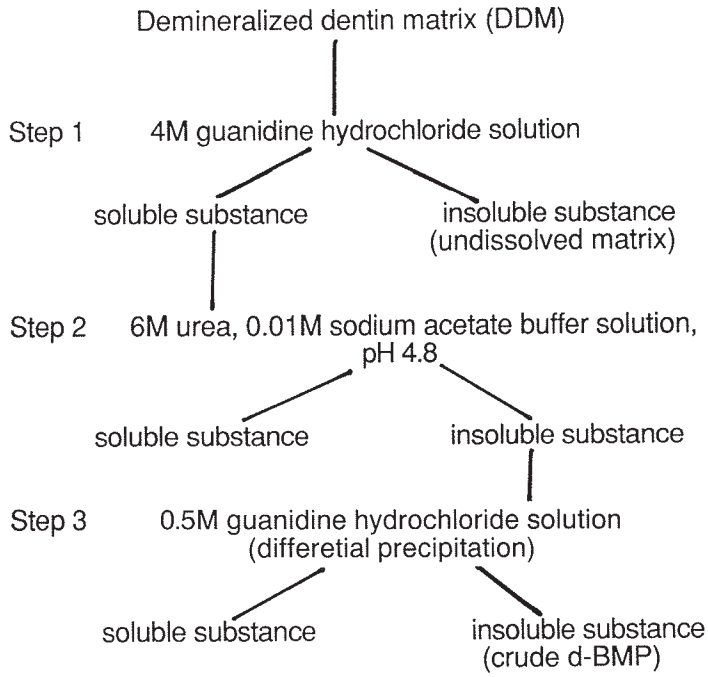

Fig. 1 Sequential steps in the isolation and extraction of dentin BMP (d-BMP) from demineralized bovine dentin matrix (DDM)

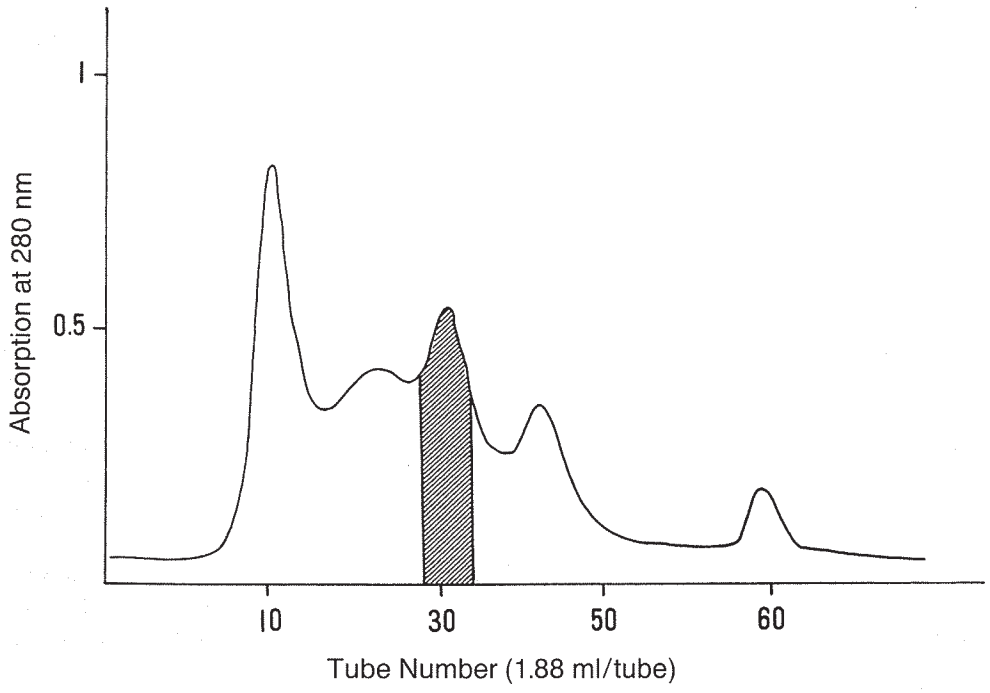

Fig. 2 Elution profile of crude d-BMP on a Superose 12 gel column

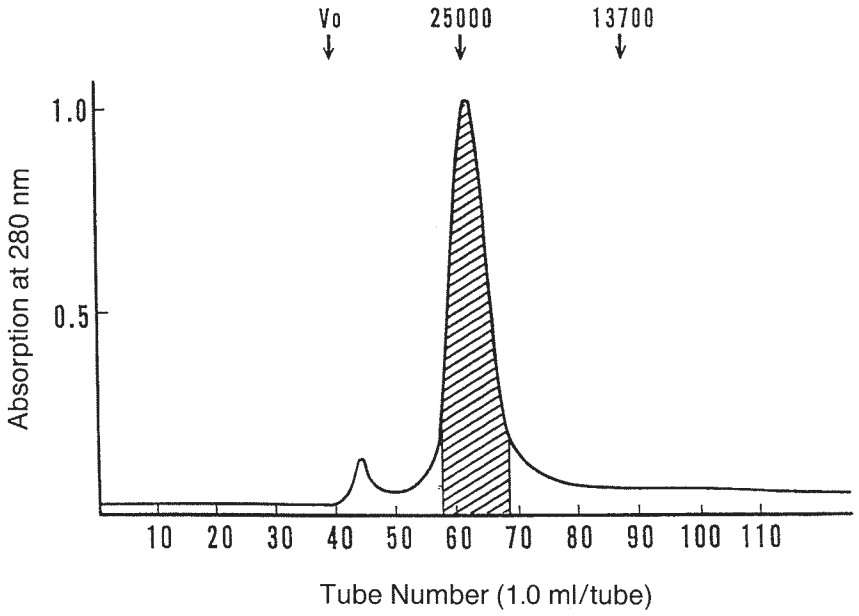

Fig. 3 Elution profile of a d-BMP rerun on a Superose 12 gel column

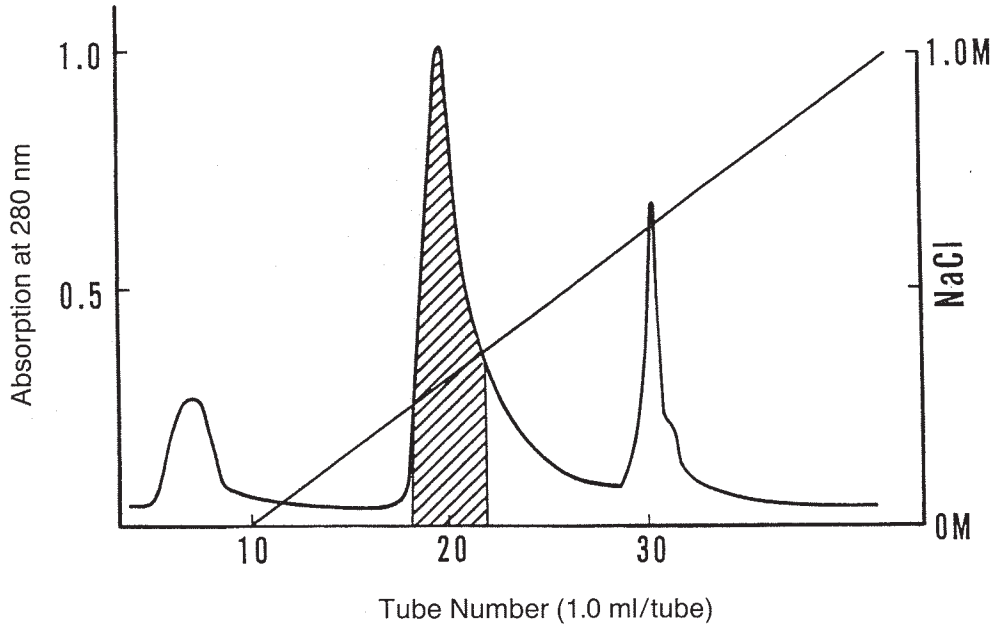

Fig. 4 Elution profile of d-BMP on a Mono Q HR5/5 ion exchange column

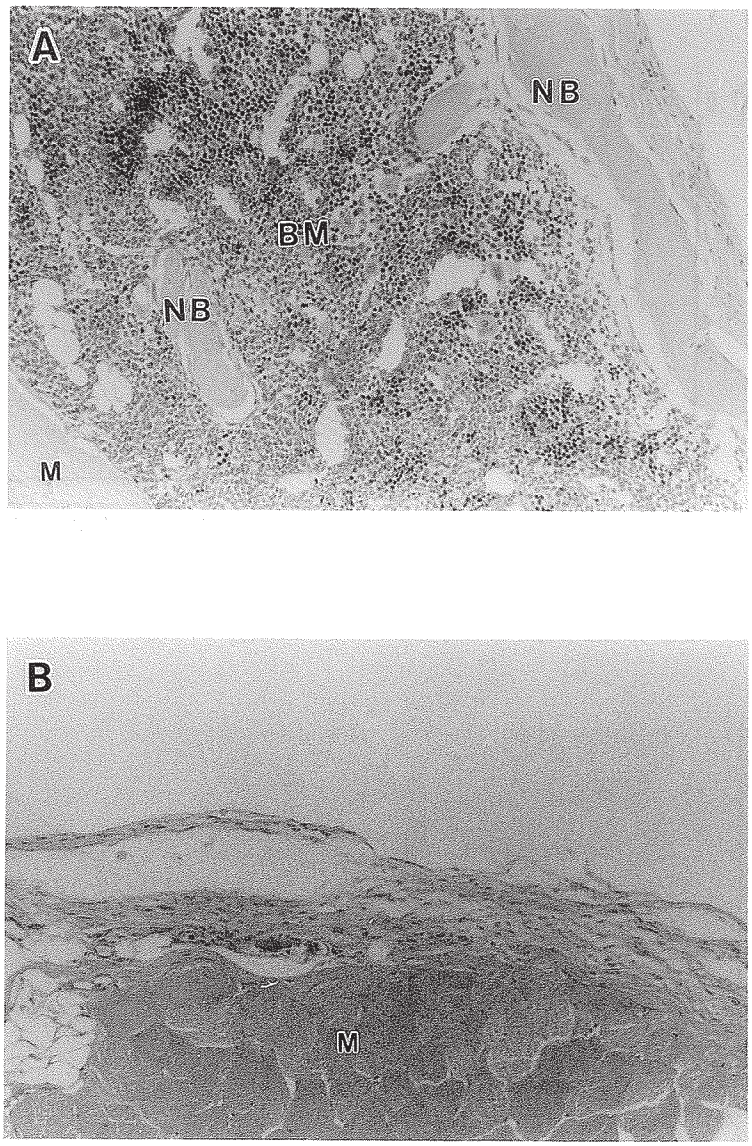

Fig. 5 Microscopic photographs of d-BMP implantation
A: Matured lamellar new bone (NB) with bone marrow (BM) is evident.
B: Only muscle (M) can be seen in the control.

Table 1 Yield of d-BMP at a given purification step

Purification steps	Yield (mg)	(%)
DDM	100,000	100
differential precipitation	300	0.3
Superose 12	20	0.02
Mono Q	3	0.003

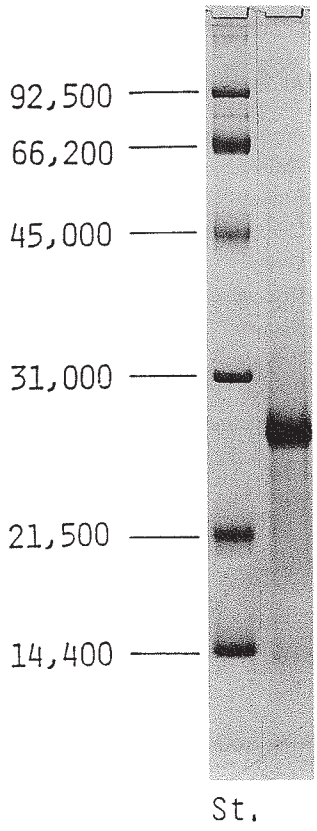

Fig. 6 Electrophoretic pattern of d-BMP on SDS-PAGE

2. SDS-PAGE

The crude BMP had numerous dense stained bands ranging from 100 kDa to 12 kDa. The relationship between BMP activity and molecular weight was not clear.

After 2 sessions of Superose12 gel chromatography, the BMP active fraction showed a single major band, corresponding to 25 kDa in molecular weight, and many other bands were faintly stained.

Mono Q HR 5/5 ion exchange chromatography of the further purified fraction showed that the component of 25 kDa became clearer and other components almost disappeared (Fig. 6).

The fraction of 25 kDa was thought to be homogeneous on SDS-PAGE.

The isoelectric point of purified d-BMP was 6.5 as determined by electrofocusing (data not shown).

3. Amino acid analysis

The amino acid composition of the purified d-BMP is shown in Table 2. This protein had relatively high amounts of Tyrosine (Tyr), Glutamic acid (Glu), Serine (Ser) and Glycine (Gly) and a low amount of Lysine (Lys).

Table 3 shows the amino acid sequences that were obtained from 5 tryptic fragments. The N-terminal sequence of d-BMP tryptic fragments has no homology to other reported BMP.

Table 2 Amino acid composition of d-BMP

Amino acid	mole %
Aspartic acid	14.3
Threonine	4.4
Serine	8.7
Glutamic acid	8.9
Proline	6.9
Glycine	7.8
Alanine	7.5
Valine	2.5
Cystine	1.5
Methionine	1.7
Isoleucine	2.2
Leucine	5.9
Tyrosine	10.2
Phenylalanine	4.1
Histidine	3.5
Lysine	2.7
Arginine	7.2

Table 3 Amino acid sequences of d-BMP tryptic fragments

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                          10                  20                  30
Fr. 1 :  V K P G N Y I L K V S V N P S Y L V P E S D Y S N N V V R G E I
                    40
         R Y T G H H A Y A S G C W I  (46)

                          10                  20                  30
Fr. 2 :  Y E H P R P G S R Y R P G Y G T G Y F Q Y G L P D L V P D P
                                                                (30)

                          10
Fr. 3 :  F S H Y D L L D A S T Q R R V A E  (17)

                          10
Fr. 4 :  N Q G T S D F L P S R P R Y  (14)

Fr. 5 :  Y R G D V R D Y D  (9)
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DISCUSSION

The solution of 4M Gdn-HCl has been proved effective when extracting BMP from the bone matrix of various species and osteosarcoma tissue. However, a lot of non-BMP substances, such as collagen and proteoglycan, were also extractable in 4M Gdn-HCl.

To remove non-BMP from 4M Gdn-HCl soluble substances, Hanamura et al.[14] employed the Cesium Chloride (CsCl) density gradient centrifugation method and the Amicon thin channel ultrafiltration method, while Takaoka et al.[15] employed the ethanol fractionation method and Urist et al.[16] employed the 1M or 1.5M Gdn-HCl differential precipitation and ultrafiltration method.

Our previous report[17] suggested that non-BMP substances containing collagen were soluble in a 0.01M sodium acetate buffer solution (pH 4.8) containing 6M urea, and BMP was precipitated during dilution from 4M to 0.5M Gdn-HCl concentration.

We applied this method to extract d-BMP from DDM. It proved to be very effective with respect to d-BMP extraction. The solubility of d-BMP was the same as that of bone-derived BMP.

Our results showed that 0.3% crude d-BMP was solubilized and 0.003% purified d-BMP was obtained from 100 g of DDM.

The molecular weight of the BMP active fraction was 25 kDa following HPLC both Superose 12 column chromatography and SDS-PAGE. Even 1 mg of the 25 KDa component could induce the growth of new bone in mouse thigh muscle. This suggested that the 25 kDa protein might be d-BMP itself.

There have been many reports on the molecular weight of BMP, suggesting values of 17−18 kDa,[5] 17.5 kDa,[17] 17 kDa[18] and 22 kDa[23] from decalcified bovine bone, 21.5 kDa[21] from decalcified rabbit bone, 18.5 kDa[16] from decalcified human bone and 23 kDa[20] and 25 kDa[21] from decalcified rabbit dentin. The molecular weight of dentin-derived BMP was somewhat larger than BMP derived from other sources. While the structure of BMP was unknown, there might be a partially common structure among BMP and d-BMP because both of them induced new bone formation in thigh muscle pouches in the same time period after implantation.

The pI of d-BMP was estimated as about 6.5 by electrofocusing gel electrophoresis. Other reports said that the pI of BMP was 5.0 ± 0.2[5] and 5−6[17] from decalcified bovine bone, 5.5[20] from rabbit DDM and 9.2[15] from mouse Dunn osteosarcoma. Bessho et al.[22] reported that human dentin-matrix-derived BMP was 20.0 kDa in molecular weight with a pI value of 8.8, determined by isoelectric focusing.

Amino acid analysis showed that d-BMP had the composition of an acidic polypeptide and included higher amounts of Aspartic acid (Asp), Tyr, Glu, Ser and Gly and lower amounts of Lys. This result coincided with other reports on the amino acid composition of bovine bone,[5] human bone[16] and rabbit dentin[20] (except for the Tyr content) and also resembled the composition of human dentin[22] except for Gly. BMP from mouse osteosarcoma tissue included higher contents of Glu, Gly and Ala.[15]

The amino acid sequences of d-BMP, obtained from tryptic fragments, differ from previously reported BMP sequences[6] and other proteins[22,23] which have a similar bone inductive capability. This fact suggests that d-BMP might be another BMP, while also having a similar ability to induce new bone in vivo.

Unlike bone tissue, tooth tissue can not be remolded. The d-BMP may be produced by odontoblast, cementoblast or ameloblast, and secreted to the matrix the tooth.

The role of d-BMP is not known; d-BMP may participate in secondary dentin formation, or in the regeneration of the alveolar process of the mandible in marginal periodontitis, or in the calcification of the tooth during the embryonal process.

CONCLUSION

The d-BMP from bovine DDM was extracted and purified. Its biochemical characteristics were as follows:

1. The solubility of d-BMP was the same as that of bone-derived BMP i.e. soluble in 4M Gdn-HCl solution, insoluble in both 0.5M Gdn-HCl solution, and 0.01M sodium acetate buffer solution (pH 4.8) containing 6M urea, and insoluble in water.
2. The molecular weight of the purified d-BMP following gel filtration and SDS-PAGE was 25 kDa.
3. The isoelectric point of the purified d-BMP was 6.5.
4. Amino acid analysis showed that the purified d-BMP contained relatively higher amounts of Asp, Tyr, Glu and Ser and a lower amount of Lys. The N-terminal sequence of d-BMP tryptic fragments had no homology to other reported proteins.

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