Korean Journal of Life Science
Vol. 10. No. 2. 218~227, 2000

Expression, Refolding, and Characterization of the Proteolytic Domain of Human Bone Morphogenetic Protein 1

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Abstract

Bone morphogenetic protein 1 (BMP-1) is part of a complex capable of inducing ectopic bone formation in mammals. Studies on TGF-β1 processing and *Drosophila* dorsal-ventral patterning have focused attention on BMP-1 as important in mediating the biological activity of this bone inducing complex. Herein, the bacterial expression, refolding, purification, and initial characterization of the BMP-1 proteolytic domain (BPD) are described. A semi-quantitative fluorescence-based thin layer chromatography assay was developed to assist in rapidly screening for optimal renaturation conditions. According to a preliminary screen for optimal conditions for the refolding of BPD, a detectable proteolytic activity against a high turnover substrate for astacin, a homologous protease from crayfish was observed. The conditions identified have allowed the expression of sufficient amounts of BPD for the characterization of the protein. Its proteolytic activity exhibits the same cleavage specificity as astacin against seven substrates that were previously synthesized for studying astacin. Furthermore, this activity is inhibited by the metal chelator 1,10-phenanthroline but not by its analogue 1,7-phenanthroline. The collagenase inhibitor Pro-Leu-Gly hydroxamate was found to inhibit both astacin and BPD activity. The results presented in this paper argue that BMP-1 does in fact possess an intrinsic proteolytic activity.

Key words – matrix metalloproteinase; zinc metalloenzyme; fluorescent peptide substrate, metal chelator

Introduction

The study of bone formation is likely to result in an increased understanding of fracture repair, development processes, and a number of exciting clinical applications in treating bone fractures, orthopedic surgery, and osteoporosis. One method of studying bone formation is through the investigation of ectopic bone formation. Ectopic bone formation mirrors the process of endochondral bone formation, the series of biological events through which majority of our bones from cartilage. Factors capable of ectopic bone induction were first discovered by Urist *et al.* fortuitously in 1963 while trying to establish the conditions for remineralization of demineralized bone matrix[23-25]. Separate implantation of demineralized bone matrix or proteins extracted from bone matrix were incapable of inducing bone formation[18]. However, implanting matrix and matrix-extracted proteins together resulted in ectopic bone formation[18]. Bone morphogenetic protein (BMP) was originally defined as a bone-inducing activity xtractable from demineralized bone matrix through the use of chaotropic solvents[24,27]. Using a number of conventional chromatography steps along with the

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in vivo assay, Wang *et al.* purified a 30 kDa factor that was capable of inducing ectopic bone formation[27]. SDS-PAGE revealed that this 30 kDa complex was composed of 16, 18, and 30 kDa components. Wozney *et al.* cloned three human proteins that they termed BMP-1, BMP-2A and BMP-3 using fragments from a trypsin digestion of the 30 kDa complex[28]. Expression of recombinant forms of these BMPs indicated that all three had the ability to induce cartilage *in vivo*. Sequence analysis indicated that BMP-2A and BMP-3 were members of the transforming growth factor-beta (TGF-β) superfamily, while BMP-1 appeared to be a novel proteinase structurally related to the crayfish metalloproteinase, astacin.

The relationship of BMP-1 and astacin remained unclear until 1991, when comparative sequence analysis studies indicated that BMP-1 was a member of a novel family of enzymes that shared as number of common structural motifs[7]. The characterization of the newly named 'astacin family of metalloproteinase' was based on studies with rodent meprin which indicated that crayfish astacin, human BMP-1, the rodent and human meprins, and UVS.2 of *Xenopus laevis*, all contained an astacin-like proteinase domain followed by EGF and CUB domain repeats for most members of family [4]. Despite extensive biochemical and structural studies, the natural substrates of these astacin metalloproteinases are still unknown. More recently, x-ray crystallographic studies led to the proposal that astacins, matrix metalloproteinases, adamalysins (snake venom proteinases) and serralysins (bacterial alkaline proteinases) all belong to a superfamily of structurally related zinc peptidases termed the 'metzincins'[2]. This superfamily classification was based on the presence of a common zinc binding motif (HExxHxxGxxH) and a superimposable methionine-turn beneath the active site metal[21].

As previously stated, BMP-1 was co-purified with other BMPs that are members of the TGF-β superfamily, and it was proposed that BMP-1 has bone-inducing activity because it catalytically activates latent forms of TGF-β-like BMPs[28]. Concurrent work in *Drosophila* suggests an alternative interaction between BMP-1 and BMP-2. In *Drosophila*, two proteins involved in a dorsal-ventral pattern formation are homologous to BMP-1 and BMP-2. The protein tolloid is homologous to BMP-1 (41% identity)[19] and decapentaplegic (dpp) is homologous to BMP-2 and BMP-4 (75% identity)[17]. dpp acts as a ligand in signal transduction pathways to specify the identity of dorsal ectoderm in the *Drosophila* embryo and also plays a role in gut and appendage development in the *Drosophila* adult[16]. Tolloid is one of several genes that regulate dpp[8,10]. Because the developmental requirement for patterning by tolloid can be bypassed by raising the amount of dpp RNA in embryos, it has been proposed that tolloid acts to modify dpp activity, either by releasing dpp from an inactive complex or by proteolytically activating dpp directly[9]. Recent mutational studies indicated that C-terminal CUB domains and EGF domains function to facilitate tolloid protein and dpp interaction, possibly through the formation of a protein-protein complex in which dpp is subsequently activated by the proteolytic astacin domain of tolloid protein[5,23]. Thus current work in other fields suggest the possible interaction of BMP-1 with other BMP proteins. BMP-1, through it protease homologous region, could be involved in the processing of TGF-β family members such as other BMPs. Recently, Kessler *et al.* found BMP-1 may be multifunctional, affecting the modification of the extracellular matrix (ECM) as such developmental processes as cell differentiation and pattern formation[12]. Biochemical studies by Kessler *et al.* provide strong evidence that BMP-1 and procollagen C-proteinase (PCP) are one and the same enzyme[12]. PCP is the metalloproteinase that cleaves the carboxyl propeptides of type I, II, and III procollagen, the soluble precursor of collagen that self-assembles into the fibrils localized to a variety of ECMs[11].

By studying the protease homologous domain of BMP-1, we may shed further light on the interaction of BMP-1 with other BMPs that may have important implications in other areas as well. Since the proteolytic activity of BMP-1 most likely resides in the astacin homology region of domain A, our initial efforts are directed towards expressing this domain. An *E. coli* expression system was chosen in order to readily obtain sufficient quantities of this domain for its characterization. Our results indicate that the expression of proteolytic domain of BMP-1 (BPD) exhibits astacin-like activity and this proteolytic activity is inhibited by the metal chelator and collagenase inhibitor.

Materials and Methods

Materials

All chemicals were purchased from Sigma. Peptides were prepared using Merrifield's solid-phase peptide synthesis method as described[15]. Astacin was the generous gift from Dr. Walter Stöcker. Bovine trypsin was purchased from Sigma Chemical Co. Carboxypeptidase A was purchased from Sigma and subsequently purified through affinity chromatography[1]. Micropolyamide plates were purchased from Schleicher & Schuell.

Construction of Expression Vector pET-BPD.

The plasmid containing the entire BMP-1 cDNA, pSP64, was provided by Dr. E. Wang (Genetics Institute, Cambridge, MA). Two oligomers were synthesized to generate a PCR product containing the astacin homologous region of BMP-1 (amino acid residues 121 to 321). The 5′ primer, 5′-CCCGCATATGGCGGCGACGTCCCGA-3′ was designed to place a Nde I site immediately prior to this region. This restriction site inserts an initiating methionine in front of the first amino acid of the homology region (Ala^{121}). The 3′ primer 5′-GCGGATCCAGGGTCTCTCC-TCAGGC-3′ is complementary to nucleotides 1002-1018 of pSP64 except for a substitution of a T for an A at position 1008 which changes the TGT codon for Cys^{322} to a TGA termination codon. This primer also contains an additional Bam HI site to the termination codon. These primers were used to amplify the desired region of the pSP64 using PCR under standard conditions (denature at 92°C for 3 min, anneal at 55°C for 2 min, synthesis at 72°C for 3 min and then denature at 92°C for 1 min 15 sec, anneal at 55°C for 2 min, synthesis at 72°C for 3 min; 30 cycles and extra synthesis at 72°C for 7 min). The PCR product was electrophoresed on a 1% NuSieve GTG agarose gel (FMC Corp.). The 0.6 kbp band was excised and purified with a Wizard Prep Kit from Promega. The purified DNA was digested with Bam HI and Nde I and ligated into a Bam HI/Nde I digested pET-11a (Novagen). Plasmid containing the insert was sequenced using Sequenase system (USBiochemicals, Cleveland, OH) after transformation into the competent NovaBlue strain of *E. coli*. pET-BPD was transformed into a competent strain of BL21 (DE3) *E. coli* and plated onto ampicillin plates to select for transformants. The BL21 (DE3) host strain carries a chromosomal T7 polymerase gene under a lacUV5 control which allows expression when induced with IPTG.

BPD Expression and Inclusion Body Isolation

8 L of LB medium containing ampicillin (50 μg/ml) was inoculated with 100 mL of overnight culture of *E. coli* BL21(DE3)/pET-BPD in the same medium. Cells were grown at 37°C until mid-log phase (OD_{600}=0.8 - 1.0) before induction by adding IPTG to a final concentration of 0.2 mM. Cells were harvested 3-4 hours after induction and kept at -70°C until use.

Refolding and Purification of BPD

The cells (45g of wet weight) grown at 37°C were resuspended in 250 mL of 20 mM Tris-HCl buffer, pH 7.6, 10% sucrose, 10 mM EDTA, 200 mM NaCl, 0.5 mM

PMSF, and incubated with 0.1 mg/mL of lysozyme for 45 min on ice. For efficient sonication, the mixture was divided into 60 mL portions and subjected to 4 rounds of sonication (15 pulses each round) using a Branson 450 sonifier (Duty cycle 70%, Output 7). Sonicated portions were pooled and centrifuged at 12,000 rpm for 10 min. After discarding the supernatant, the pellet was washed twice with 180 mL of 20 mM Tris, 10% sucrose followed by a single wash with 180 mL of deionized water. The pellet was thoroughly drained of remaining liquid and solubilized with 30 mL of 8 M deionized urea, 20 mM Hepes, pH 7.6, and 15 mM β-mercaptoethanol. The mixture was centrifuged at 12,000 rpm for 20 min. The supernatant was adjusted to 6 M urea, 20 mM Hepes at pH 7.6. At this stage, the denatured BMP-1 showed > 90% purity. After it was concentrated, denatured BPD (3 - 5 mg/ml protein) was diluted to 0.1 - 0.5 M urea in the presence of PEG-3350 to refold the protein. The refolding mixture was centrifuged at 12,000 rpm for 10 min. The solution containing the soluble BPD was concentrated and applied to DEAE-Sephacel column and eluted with 20 mM Hepes, pH 7.5, and 1 M NaCl. The fractions showing high activity were collected and pooled. The BPD was then applied to phenyl-Sepharose in the presence of 10 mM Hepes, pH 7.5 and 1 M ammonium sulfate and eluted from the column with a decreasing gradient of ammonium sulfate. The purity of refolded BPD was examined by SDS-PAGE.

TLC Assays

Enzyme activity was measured using the heptapeptide fluorescent substrates. Substrate concentrations were determined spectrophotometrically by the absorbance of the danyl group (ε_{340} = 4,300 M^{-1} cm^{-1}). Reactions were performed at 1 x 10^{-4} M of substrate, 20 mM Hepes, pH 7.5, in an assay volume of 10 μl at 37°C. 0.5 or 1 $\mu\ell$ aliquots were spotted on micropolyamide sheets. The sheet was briefly dried with a blow-dryer before being developed in 0.3 N HCl. The dansylated substrate and product were visualized under a long-wave ultraviolet lamp.

HPLC Assays

Assays were conducted in 20 mM Hepes, pH 7.5 at room temperature. A 95 mL aliquot of substrate (final concentration of 2×10^{-4} M) was incubated with 5 mL of enzyme solution (final concentration of 1×10^{-7} M). The reaction was stopped by the addition of 5 mL of glacial acetic acid. The samples were then analyzed on a Waters Associates Liquid chromatography system. Peak areas were determined at 225 or 260 nm with a Hewlett Packard 3380A integrator. Waters NovaPak C_{18} (3.9×150 mm) reversed-phase column was used and samples were eluted with a linear acetonitrile gradient (20 - 60%) in 0.1% TFA at a flow rate of 1.5 mL/min. The concentration of products at a given time, P_t, was determined according to the equation: $P_t = (P_a + P_b) / (P_a + P_b + S_a) \times S_o$ where P_a, P_b and S_a are the integrated areas of product A and B and substrate peaks, respectively, and S_o is the initial substrate concentration[3]. Enzyme concentrations were determined by amino acid analysis or calculated from measurements of the optical density at 280 nm using a molar absorptivity constant of ε_{280} = 42,800 M^{-1} cm^{-1}. The cleavage site was identified by amino acid analysis of the product A and B.

Inhibition Studies

Various protease inhibitors were used to examine the inhibiting ability for proteolytic activity of the refolded BPD. Inhibitors of final concentration of 0.5 to 5 mM were pre-incubated with the enzyme of 1×10^{-7} M for 1 hour. The reaction mixture was then assayed under the condition of 20 mM Hepes, pH 7.5, and 2×10^{-4} M Dns-LKRAPWV substrate at room temperature. The hydrolysis of the substrate in the absence of inhibitors was examined using TLC and HPLC method and compared with in the presence of inhibitors.

Results and Discussion

Expression and Subcellular Localization of Recombinant BPD in E. coli

Expression of BPD was assessed through SDS-PAGE (Fig. 1). Upon induction, a strong band at 25 - 26 kDa appears which corresponds to the predicted size of BPD (lane 2). The expression level of BPD was approximately 1% of total cellular protein. When the cells were grown at 37°C, BPD was present in the insoluble form. Although most overexpressed recombinant proteins are accumulated as inclusion bodies, some of the recombinant protein may also be present in soluble form within cytoplasm. Usually, soluble proteins are correctly folded and active. However, the soluble fraction did not contain any activity toward our high-turnover astacin substrate. Because it is not easy to make the insoluble form, various efforts to make an soluble form were done. Regardless of the time of cell growth, the concentration of IPTG, and growth temperature, the BPD was still remained in insoluble form.

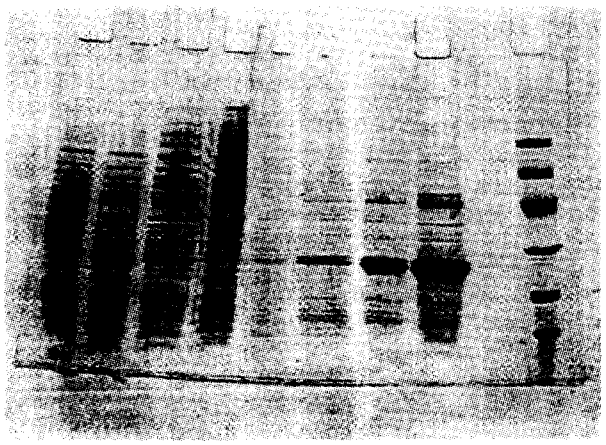

Fig. 1. Expression, Localization, and solubilization of BPD. lane 1, uninduced *E.coli* containing BPD; lane 2, induced *E.coli* containing BPD; lane 3, total cell extract; lane 4, soluble fraction of cell extract; lane 5-8, 2, 4, 6, and 8 M urea solubilized proteins of insoluble fraction; lane 9, molecular weight markers (14.4 kDa, 20.1 kDa, 30 kDa, 45 kDa, 67 kDa, 95 kDa)

Denaturation and Refolding of BPD

Since very little BPD is present in the soluble fraction, the majority of expressed BPD must be present within the inclusion body pellet. To solubilize these aggregates, chaotropic agents such as urea and guanidine hydrochloride are often used. Since the ionic nature of guanidine hydrochloride would interfere with gel electrophoresis and TLC assay, urea was selected as the solubilizing agent. he pellet was solubilized with 2, 4, 6, and 8 M urea. Fig. 1 shows that as the urea concentration increases, the amount of solubilized protein also increases. Typically, it is desirable to begin refolding with homogeneous protein as the presence of other proteins may interfere with renaturation[13]. After washing with 2 - 3 M urea, the solubilized protein was quite homogeneous. N-terminal sequencing of this sample yielded the expected sequence AATSRPERVWPDG corresponding to the first 13 amino acids of BPD. 8 M urea was subsequently used to solubilize BPD from inclusion body pellets. This solution was adjusted to 6 M urea/20 mM Hepes for subsequent refolding experiments.

Dialysis represents one of the simplest methods of removing denaturant to allow refolding to occur. 2.5 mg/mL BPD in 8 M urea was dialyzed three times against 200-fold excess of 20 mM Hepes, pH 7.5. No appreciable activity above a negative control was seen (data not shown). Co-solvents such as polyethylene glycol (PEG) have been shown to enhance the amount of active protein recoverable upon refolding through the prevention of aggregation. Refolding using PEG involves the rapid dilution of denatured protein in urea followed by a variable incubation time to allow refolding to occur. Variables such as PEG concentration, protein concentration, denaturant concentration can be controlled to search for optimal renaturation conditions. The experiments for all conditions performed in triplicate, varied one variable while keeping all others constant. After 24 hours of refolding at 37°C or room temperature, a small aliquots of refolded solution from each condition were withdrawn

and assayed for activity using TLC. One condition in each experiment was arbitrarily chosen a standard and the fluorescence intensity from its astacin-specific cleavage (Dns-LKR) spot was set to 1.00. The fluorescence intensity of the other conditions were measured relative to the arbitrary standard in each replica. The relative fluorescence intensities for each condition were averaged. We were able to obtain optimal active BPD with PEG/BMP molar ratio of 20:1 to 40:1. Cleland *et al.* have proposed that at low PEG/protein molar ratios, PEG interacts with an early folding intermediate to prevent aggregation, and the addition of more PEG increases the inhibition of aggregation[6]. The optimal final urea concentration was determined by rapidly diluting denatured BPD in 6 M urea/20 mM Hepes. The final protein concentration was approximately 9.5×10^{-7} M in all samples. Lower final urea concentrations resulted in greater activity. Protein concentration during refolding can be critical, especially when the correctly folded protein contains disulfides. Optimal redox conditions allow the shuffling of protein disulfide bonds and promote the formation of native disulfide bonds. Under dilute conditions, the formation of intramolecular bonds is favored over intermolecular bonds. The proper folding is favored as the final protein concentration decreases. Low molecular weight thiols such as glutathione have been used during *in vivo* folding reactions to allow the shuffling of disulfide bonds[26]. Redox conditions are varied by changing the ratio of the reduced (GSH) to oxidized (GSSG) forms of glutathione to find conditions promoting the formation of correct disulfide bonds. Denatured BPD was rapidly diluted to 1.9×10^{-7} M BPD/0.1 M urea/20 mM Hepes/PEG 20 : 1 while varying the concentration ratio of GSH to GSSG. Refolding was enhanced under oxidizing conditions, and reducing conditions did not seem conductive to increasing refolding yield.

Purification of BPD

Refolded BPD exhibited catalytic activity towards the dansylated peptide substrate Dns-LKRAPWV. Cleavage mainly occurred between R and A to produce Dns-LKR as was observed for astacin. However, the cleavage was also observed between K and R. Dns-LK and Dns-L products were produced when the enzyme was incubated with substrate in a long time. This phenomenon explains that trypsin-like proteases or some exopeptidases are contaminated during refolding step because trypsin cleaves the peptide between K and R[20]. Otherwise, BPD has a weak exopeptidase activity. The trypsin-like or exopeptidase activity of refolded BPD was eliminated after DEAE-Sephacel and phenyl-Sepharose chromatography. Therefore, it is thought that refolded proteins contained other trypsin-like protease and exopeptidases as well as BPD and separated during the purification by column chromatography. SDS-PAGE analysis of purified BPD showed a single band (data not shown).

Substrate Specificity of BPD

One of the synthetic substrates developed for astacin, Dns-LKRAPWV has been chosen for this study based on its high turnover. The predicted cleavage sites by astacin and other proteases are shown in Fig. 2 based on their known specificities[20]. To establish the identity of hydrolyzed products, TLC and HPLC method was used. In

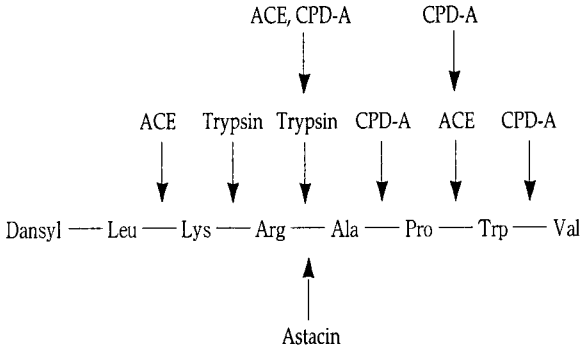

Fig. 2. Substrate Cleavage Sites for Dns-LKRAPWV.
The fluorescent peptide substrate Dns-LKRAPWV is cleaved at a number of sites by various proteases. ACE, angiotension converting enzyme; CPD-A, carboxypeptidase A. Figure adopted from ref. 15.

TLC assay, the fluorescent peptide Dns-LKR, Dns-LK, and other substrates are examined to compare the spots migrated from baseline. HPLC analysis gave a different retention time of product (Dns-peptide and tetrapeptide) and substrate. The cleaved sites by BPD were identified and compared with astacin (Table 1). All the substrates were cleaved at the R/K/N-A bond. The cleavage pattern for fluorescent heptapeptide substrates suggests that BPD cleaves each of these substrates at the same site as astacin. Of the single amino acid replacement designed to test the specificity of astacin, the substitution of leucine for alanine at the scissile bond has the most dramatic effect. Astacin cleaves the Arg-Ala (R-A) bond with a k_{cat}/K_m of 1.4×10^6 M^{-1} s^{-1} when Ala was in the P_1' position. However, this enzyme shows only a slight degree of hydrolysis even at 1000 times more enzyme and a 100 times greater period of incubation when Ala was replaced by Leu[20]. BPD also shows the same pattern with astacin for Dns-LKR*L*PWV hydrolysis.

Inhibition by Metal Chelators and Collagenase Inhibitor

The metal chelator 1,10-phenanthroline (OP), at millimolar concentrations, has been shown to reversibly inhibit astacin in a time dependent manner through the removal of its catalytic zinc atom[22]. However, the nonchelating analogue, 1,7-phenanthroline (MP) exhibits only slight instananeous inhibition towards astacin that is not time dependent. Peptide hydroxamates have been found to inhibit human collagenases by binding to the active site groove and coordinating the catalytic zinc atom [14]. Pro-Leu-Gly hydroxamate (PLG-NHOH) was found to mimic the substrate residues P_1 - P_3 (the three substrate residues on the N-terminal side of the cleavage site) and to coordinate the catalytic metal with two of its hydroxamic acid oxygens. PLG-NHOH, the affinity ligand used for the purification of collagenase, was also examined.

Table 1. Substrate specificity of Astacin and BPD[a]

	P_3	P_2	P_1		P_1'	P_2'	P_3'	P_4'
Dns -	L	K	R	↓	A	P	W	V
Dns -	L	K	K	↓	A	P	W	V
Dns -	G	K	R	↓	A	P	L	V
Dns -	G	K	N	↓	A	P	L	V
Dns -	G	P	R	↓	A	P	L	V
Dns -	A	A	R	↓	A	P	L	V

[a]cleavage site was identified by HPLC method
↓; Cleavage site

Fig. 3 shows the inhibition pattern of OP, MP, and PLG-NHOH for astacin. 2.7×10^{-7} M astacin was pre-incubated with no inhibitor (lane 2), 2.5 mM OP (lane 3), 2.5 mM MP (lane 4), 0.3 mM and 5.9 mM PLG-NHOH (lane 5, 6) before the addition of substrate to initiate the assay. Assays were spotted onto TLC plates five minutes after incubation at room temperature. Lane 1 shows a negative control in which water was added in place of astacin. As expected, OP and PLG-NHOH inhibited the hydrolytic activity of astacin while MP did not inhibit

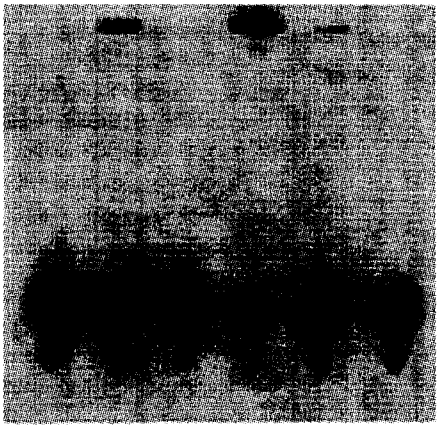

Fig. 3. Inhibition of astacin by OP, MP and PLG-NHOH.
lane 1, no enzyme; lane 2, added astacin; enzyme pre-incubated with 2.5 mM OP (lane 3), 2.5 mM MP (lane 4), 0.3 mM PLG-NHOH (lane 5), and 5.9 mM PLG-NHOH (lane 6).
The enzyme solution was pre-incubated at 37°C for one hour in the presence of inhibitors before the addition of substrate Dns-LKRAPWV. The reaction mixtures were spotted on TLC plates after five minutes incubation.

the activity by showing the Dns-LKR spot. Based on its sequence homology to astacin, BPD is expected to display a similar behavior toward these substrates. Refolded BPD was separately incubated in 20 mM Hepes with no inhibitor (lane 2), 2.5 mM OP (lane 3), 2.5 mM MP (lane 4), 0.3 mM and 5.9 mM PLG-NHOH (lane 5, 6) before the addition of substrate to start the reaction (Fig. 4). After one hour incubation with each inhibitor, OP and PLG-NHOH inhibited BPD activity while MP had no effect. These results suggest that BPD is a metalloenzyme responsible for the proteolytic activity present in refolded samples.

Recent finding of Kessler *et al.* indicated that recombinant BMP-1 (rBMP-1) is indeed a protease, but that its substrate is procollagen, not TGF-β. The cleavages had occurred between Ala and Asp of COOH-propeptide subunit of procollagen[12]. It has been indicated that the C-terminal CUB and EGF domains function to interact between tolloid and dpp in *Drosophila* which is homologue of BMP-1 and BMP-2 in human[5,23]. Therefore, the astacin-like activity of BPD shown in our study is likely due to the lack of the other domain of BMP-1. It is assumed that the different substrate specificity between BPD and rBMP-1 may be due to the different tertiary structure between two proteins. Truncated BMP-1 which has only proteolytic domain (BPD) may not function properly as a complete BMP-1. Since rBMP-1 is available, the detailed studies of proteolytic ability for rBMP-1 and BPD will promise to further our knowledge of the structural basis of each domain of BMP-1 as well as the biological role of BMP-1 in morphogenesis.

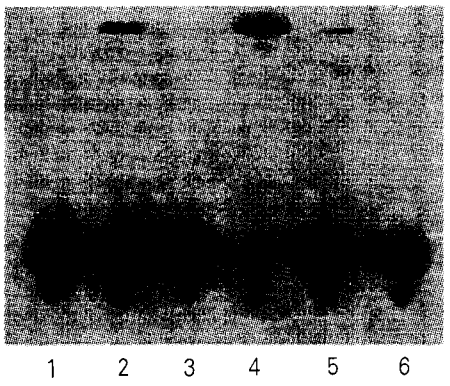

Fig. 4. Inhibition of BPD by OP, MP and PLG-NHOH.
lane 1, no enzyme; lane 2, added BPD; enzyme pre-incubated with 2.5 mM OP (lane 3), 2.5 mM MP (lane 4), 0.3 mM PLG-NHOH (lane 5), and 5.9 mM PLG-NHOH (lane 6).
The enzyme solution was pre-incubated at 37°C for one hour in the presence of inhibitors before the addition of substrate Dns-LKRAPWV. The reaction mixtures were spotted on TLC plates after five minutes incubation.

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초록 : 뼈형성 단백질(Bone Morphogenetic Protein 1)의 단백질 분해 부위의 발현 및 특성 연구

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Bone morphogenetic proteins (BMPs)은 포유동물의 뼈를 형성하는데 관여하는 단백질로서 알려져 있다. 대부분의 BMP들은 TGF-β family에 속하는 것으로 알려져 있으나, 그 중의 하나인 bone morphogenetic protein 1 (BMP-1)은 BMP-2, BMP-3, BMP-4와 비교했을 때 구분되는 염기서열을 가지고 있는 astacin family에 속하는 효소단백질로서 생각되고 있다. 이 단백질의 효소로서의 기능을 조사하기 위하여 BMP-1의 proteolytic domain (BPD)을 코딩하는 유전자가 PCR방법에 의해 클로닝되고 *E. coli*에서 발현되었다. 발현된 BPD는 *E. coli*에서 불용성한 형태로 존재하였고 이 단백질을 활성화시키기 위하여 denaturing agent인 urea로 용해시키고 난 후, 여러조건 하에서 refolding하였다. Refolding되는 정도는 가재(crayfish)의 단백질 분해효소인 astacin에 대한 기질을 이용하여 검사하였다. 활성화된 BPD는 astacin에 대한 여러 합성펩타이드 기질에 대하여 높은 반응성을 보였으며, HPLC로 분석한 결과 astacin과 같은 작용부위를 가지는 것으로 나타났다. 또한, BPD가 astacin처럼 metalloprotease의 저해제인 ortho-phenanthroline (OP)와 collagenase의 저해제인 Pro-Leu-Gly-NHOH에 의해 저해되었다. 이상의 결과로서 BMP-1은 단백질 분해능을 가지는 효소로서 작용하는 것으로 생각되며 astacin family에 속하는 단백질임을 알 수 있었다.