

# Identification of Peptide Fragments Generated by Digestion of Bovine and Human Osteocalcin with the Lysosomal Proteinases Cathepsin B, D, L, H, and S

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## ABSTRACT

We have determined the primary cleavage sites in the bone Gla protein (BGP; osteocalcin) for several of the proteases that could act on the protein during bone resorption and turnover, cathepsins B, D, L, H, and S. The time course of BGP digestion by each cathepsin was first determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis. We then incubated human and bovine BGP with each cathepsin for a sufficient time to reduce the level of intact protein by at least 20-fold, isolated the major cleavage peptides, and identified each by N-terminal sequence analysis and by amino acid analysis. Our results show that BGP has relatively few cathepsin-sensitive sites and that these sites are located at the N and C terminus of the 49-residue protein. Cathepsins B, L, H, and S readily cleave BGP at the G<sub>7</sub>-A<sub>8</sub> bond; cathepsin L also cleaves at R<sub>43</sub>-R<sub>44</sub>; cathepsin B also cleaves at R<sub>44</sub>-F<sub>45</sub>; and cathepsin D cleaves only at A<sub>41</sub>-Y<sub>42</sub>. The immunoreactivity of the major peptides generated by cathepsin cleavage was evaluated using the original radioimmunoassay developed for the detection of BGP in human serum. The BGP 8-49 fragment cross-reacts identically with native BGP, while the 8-43 and the 1-44 fragments require 20- to 40-fold higher concentrations to achieve the same level of displacement as the native protein. The 1-41 and 8-41 fragments are unable to significantly displace the labeled native BGP tracer at any concentration tested. These results demonstrate the utility of peptides generated by cathepsin digestion in the mapping of the antigenic epitopes recognized by a given BGP immunoassay. (*J Bone Miner Res* 1997;12:447-455)

## INTRODUCTION

**B**ONE GLA PROTEIN (BGP or osteocalcin), a small protein of 6 kD, accounts for 15% of the noncollagenous protein found in bone.<sup>(1)</sup> BGP is found predominantly in the calcified bone matrix, but nanomolar concentrations also can be detected in serum. The circulating BGP represents a portion of the newly synthesized protein that is not absorbed into the bone, but rather released directly into the bloodstream.<sup>(2)</sup> Serum BGP is a commonly used biochemical marker for the measurement of bone formation.

A lot is known about BGP's structure and biosynthesis, but little is known about its turnover and degradation.<sup>(3-5)</sup> Intact plasma BGP is derived from de novo biosynthesis by osteoblasts<sup>(6)</sup> and is not released from bone matrix by resorptive processes.<sup>(2)</sup> Therefore, BGP fragments must be released during osteoclastic bone resorption. Interestingly,

there are unexplained discrepancies in the reported serum BGP concentrations in the same blood samples as determined by radioimmunoassays (RIAs) using different antibodies. It is believed that these differences are caused by varying immunoreactivity of BGP fragments.<sup>(7-9)</sup> We suggest that cysteine proteinases are responsible for the degradation of BGP and other noncollagenous proteins during osteoclastic bone resorption. Recent research provides evidence to support the hypothesis that cysteine proteinases play a key role in osteoclastic bone resorption.

Cysteine proteinases are among the most active proteinases in the body. Most of them are localized in lysosomes, such as cathepsin L, B, H, C, and S. They have a broad substrate specificity and exhibit endo- and/or exopeptidase activity. Cathepsin B, L, and S have collagenolytic activity and cleave the nonhelical telopeptide extensions of the collagen at acidic pH. This allows depolymerization and

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solubilization of the collagen molecules. Cathepsins L, B, and H are inactivated irreversibly above pH 7 and have  $M_r$ 's in the range of 24,000 to 30,000.<sup>(10)</sup> The procathepsins and cathepsin S are stable above pH 7.<sup>(11)</sup>

Cathepsin L is described as the most powerful lysosomal enzyme with endopeptidase activity; cathepsins S and T share with cathepsin L the properties of a high activity on collagen and the lack or a minimum of exopeptidase activity. In contrast, cathepsins B and H have a moderate endopeptidase activity and more exopeptidase activity: specifically, cathepsin H has aminopeptidase activity and cathepsin B has peptidyl dipeptidase activity.<sup>(12)</sup> The lysosomal cathepsins L, B, C, H, G, and D are localized in bone tissue, predominantly in osteoclasts.<sup>(13-15)</sup>

This study has been undertaken to compare the degradation of BGP by the cysteine proteinases cathepsins L, B, H, S, and the aspartic proteinase cathepsin D. Our work was designed to answer the questions: Which larger BGP fragments are produced *in vitro* by different lysosomal proteinases, and is the polyclonal rabbit antibody against bovine BGP immunoreactive to these fragments? The present work is the first to report the structure of BGP fragments produced by a physiologically important group of enzymes located in osteoclasts.

## MATERIALS AND METHODS

### Chemicals

Optima grade methanol and high performance liquid chromatography (HPLC) grade acetonitrile were purchased from Fisher Scientific (Tustin, CA, U.S.A.). HPLC grade trifluoroacetic acid (TFA) was purchased from Pierce Chemical (Rockford, IL, U.S.A.). Electrophoresis grade chemicals were from Bio-Rad (Hercules, CA, U.S.A.), pre-cast polyacrylamide gels were from Novex (San Diego, CA, U.S.A.), and protein sequencer reagents were obtained from the Applied Biosystems division of Perkin-Elmer (Foster City, CA, U.S.A.). Goat antirabbit precipitating antiserum for RIAs was purchased from Chemicon International (Temecula, CA, U.S.A.). All other chemicals used were analytical reagent grade or better.

### Enzymes

The rat cathepsins L, B, H, S, and D were a gift of Heidrun Kirschke (Martin Luther University, Halle Wittenberg, Germany). The pure enzymes were prepared, and their activities were determined by active site titration as previously described.<sup>(10,11)</sup>

### Bovine-BGP

Bovine noncollagenous matrix proteins were extracted by demineralization with formic acid as described.<sup>(16)</sup> BGP was purified over a  $1 \times 160$  cm Sephacryl S-100 HR size exclusion column (Pharmacia Biotechnology, Alameda, CA, U.S.A.) which was equilibrated with 6 M guanidine HCl, 0.1 M Tris-HCl, pH 9.0, buffer. The chromatographic peak corresponding to the bovine bone BGP was pooled and characterized by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

### Human BGP

The cleaned, rinsed, and ground bone samples were prepared<sup>(17)</sup> and extracted with formic acid as described.<sup>(16)</sup> Human BGP was purified over a  $1 \times 160$  cm Sephacryl S-100 HR size exclusion column that was equilibrated with 6 M guanidine HCl, 0.1 M Tris-HCl, pH 9.0, buffer, and the chromatographic peak corresponding to human bone BGP was pooled, loaded onto a Vydac  $C_{18}$  reserve-phase HPLC column, and eluted from the column with a 2 h linear gradient from 0.1% trifluoroacetic acid in water to 0.1% trifluoroacetic acid in 60% acetonitrile at a flow rate of 1 ml/minute. The purity of the BGP peak fractions was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

### Enzymatic degradation

The cysteine proteinases, cathepsin L, B, H, and S, were initially activated in 0.1 M buffer (acetate buffer pH 5.5 for cathepsin L and B; phosphate buffer pH 6.5 for cathepsin H and S) with 1 mM EDTA and 1 mM DTT for 5 minutes at room temperature. The aspartic proteinase cathepsin D does not need activation. The enzymatic degradation for each of the enzymes in buffer was performed at 37°C (Table 1). The reaction was stopped by the addition of *L-trans*-Epoxy succinyl-leucylamido(4-guanidino)butane (E-64) (CalBiochem, La Jolla, CA, U.S.A.) (cysteine proteinases, cathepsins L, B, H, and S) or by freezing (aspartic proteinase, cathepsin D). E-64 is a highly specific, irreversible inhibitor of cysteine proteinases with an effective concentration of 1–10  $\mu$ M.<sup>(18)</sup>

### Purification of the BGP fragments

The enzymatic digest was injected onto a Vydac  $C_{18}$  reverse-phase HPLC column (4.6 mm id  $\times$  25 cm length) and eluted from the column with a 2 h linear gradient from 0.1% trifluoroacetic acid in water to 0.1% trifluoroacetic acid in 60% acetonitrile at a flow rate of 1 ml/minute. The protein peak fractions were dried and dissolved in 5 mM HCl for characterization.

### Characterization of the BGP fragments

**Electrophoresis:** SDS-PAGE was performed under reducing conditions as described<sup>(19)</sup> using precast 18% polyacrylamide gels run at a constant 150 V. Protein bands were visualized by staining the gel for 60 minutes with 0.2% Coomassie Blue R-250, 10% trichloroacetic acid, and 10% 5-sulfosalicylic acid and destained overnight in 5% methanol, 7.5% glacial acetic acid in water.

**N-terminal protein sequencing:** Aliquots of the HPLC fractions were subjected to automated Edman degradation using an Applied Biosystems Model 470-A gas phase sequencer equipped with a Model 120 on-line HPLC using the 03RPTH program provided by the manufacturer.<sup>(20)</sup> Data reduction was accomplished with a Perkin Elmer 7500 computer equipped with Chrom 3 software.

**Amino acid analysis:** Proteins and peptides were hydrolyzed in 6 N HCl for 24 h at 110°C. Dried hydrolysates were derivatized with 9-fluorenylmethyl chloroformate and ana-

TABLE 1. CONDITIONS FOR BGP DEGRADATION BY CATHEPSINS L, B, H, S, AND D

Degradation conditions	Cath. L	Cath. B	Cath. H	Cath. S	Cath. D
Buffer	0.1 M acetate buffer	0.1 M acetate buffer	0.1 M phosphate buffer	0.1 M phosphate buffer	0.1 M acetate buffer
pH	5.5	5.5	6.5	6.5	5.5*
[b-BGP], mg/ml	2	2	2	2	2
[E], $\mu$ g/ml	0.75	6.8	14.9	4.5	20.0
[b-BGP]/[E], mg/mg	2700/1	300/1	130/1	500/1	100/1
Time required for >95% degradation of BGP, minutes	30	30	120	30	240

\* Not optimal conditions for this E.

lyzed using a Perkin Elmer Series 4 HPLC equipped with a Varian Amino Tag C<sub>18</sub> column (4.6 mm  $\times$  15 cm) and a Kratos model FS970 fluorescence detector. The concentration of the fragments was determined by including norvaline as an internal standard.

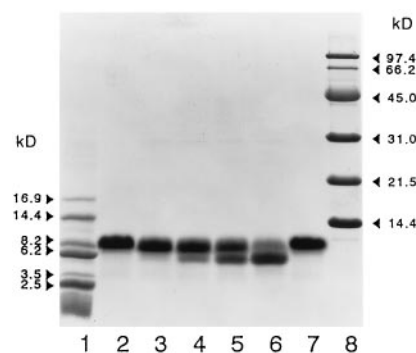
#### Radioimmunoassay

Bovine BGP, human BGP, and BGP fragments were measured using the radioimmunoassay that has been described elsewhere.<sup>(21)</sup>

## RESULTS

### Digestion of bovine BGP by cathepsins D, H, S, B, and L

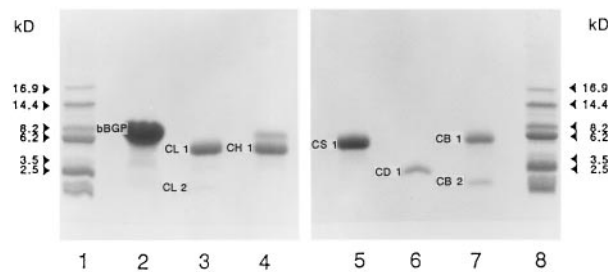
To establish the minimum conditions of enzyme concentration and digestion time required to reduce the amount of intact BGP to undetectable levels, the time course of each cathepsin digest of BGP was evaluated using SDS-PAGE. The results of one such kinetic analysis for the digestion of bovine BGP with cathepsin H are shown in Fig. 1. As can be seen, bovine BGP is progressively converted to a smaller fragment with increasing digestion time. Similar results were also obtained with cathepsins D, L, B, and S (data not shown). The final conditions chosen to achieve complete digestion of bovine BGP are shown in Table 1. Figure 2 shows that using these conditions with each of the five cathepsins did indeed result in the complete conversion of BGP to one or more major fragments. Extending the digestion times using the assay conditions in Table 1 to 2- to 5-fold longer did not significantly change the intensity of the cathepsin CH1 fragment, the cathepsin CS1 fragment, or the cathepsin CD1 fragment, which demonstrates that in each instance the largest cathepsin fragment is relatively resistant to further digestion by the respective cathepsin. In contrast, extending the digestion times to 2- to 5-fold longer caused a progressive decrease in the cathepsin CL1 band and a corresponding increase in the CL2 band and caused a progressive decrease in the cathepsin CB1 band and a



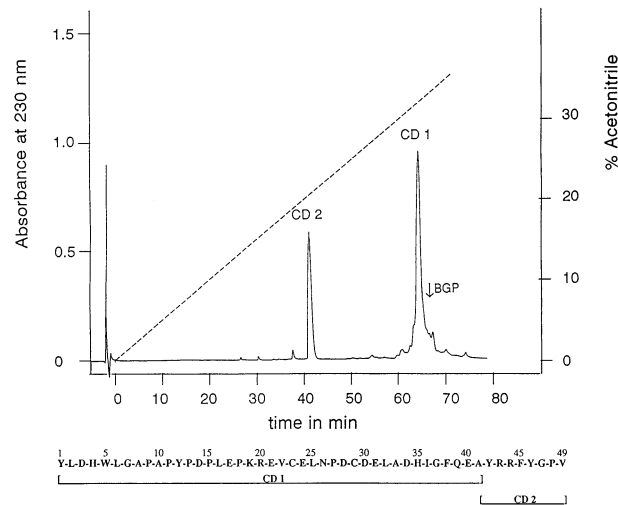
**FIG. 1.** SDS-PAGE of bovine BGP after digestion with cathepsin H for different times. Bovine BGP was treated with cathepsin H as described in Materials and Methods. At  $t = 5$  minutes (lane 3), 15 minutes (lane 4), 30 minutes (lane 5), and 60 minutes (lane 6) aliquots were removed and the reaction was stopped by the addition of the cathepsin inhibitor E64. The samples were electrophoresed on an 18% SDS-polyacrylamide gel and stained with Coomassie blue. Lane 2 is a bovine BGP control sample from  $t = 0$ , and lane 7 is the same untreated sample after 60 minutes of incubation without enzyme addition. Lanes 1 and 8 are molecular weight markers (see Materials and Methods).

corresponding increase in the CB2 band. These results indicate that the cathepsin CL2 band could be derived from CL1 by a subsequent cathepsin L cleavage, and that the cathepsin CB2 band could be derived from CB1 by a subsequent cathepsin B cleavage.

To identify the sites of cleavage in bovine BGP produced by each cathepsin, portions of the respective digests whose electrophoresis is shown in Fig. 2 were subsequently resolved by HPLC using a C<sub>18</sub> reverse phase column. As seen in Fig. 3, two major peptides could be resolved from the cathepsin D digest of bovine BGP. Fragment CD1 has an N-terminal amino acid sequence identical with intact BGP and an amino acid composition consistent with a 1–41 sequence. Fragment CD2 has a sequence and composition corresponding to residues 42–49 in bovine BGP. We there-



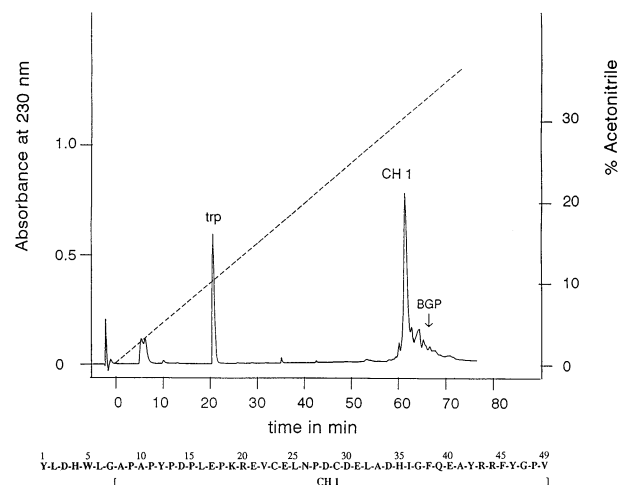
**FIG. 2.** SDS-PAGE of bovine BGP after digestion with cathepsin L (lane 3), cathepsin H (lane 4), cathepsin S (lane 5), cathepsin D (lane 6), and cathepsin B (lane 7). Bovine BGP was digested with cathepsins as indicated in Table 1; aliquots were electrophoresed on an 18% SDS-polyacrylamide gel and stained with Coomassie blue. Lane 2 is an untreated bovine BGP sample; lanes 1 and 8 are molecular weight markers.



**FIG. 3.** Reverse phase HPLC separation of peptides generated by digestion of bovine BGP with cathepsin D. Bovine BGP was digested with cathepsin D, and the resulting digest was injected onto a Vydac C18 reverse phase column to purify cathepsin D-generated peptides (see Materials and Methods for details). Aliquots of the peaks indicated were subjected to N-terminal protein sequencing and amino acid analysis. The identity of a given peptide and its location within native BGP is shown at the bottom of the figure.

fore conclude that there is only one initial cleavage site for cathepsin D in bovine BGP, and this lies between Ala 41 and Tyr 42 (Table 2).

The HPLC chromatogram for the cathepsin H digest of bovine BGP, shown in Fig. 4, revealed the presence of a single major peptide whose sequence and composition correspond to residues 8–49 in bovine BGP. The only other major  $A_{230}$ -absorbing component recovered from this chromatogram is free tryptophan. Since the only tryptophan in bovine BGP lies at position 5 in the sequence, free tryptophan must have arisen from the N-terminal region of BGP. One possible explanation for the presence of both free tryptophan and the 8–49 peptide in the digest is that the

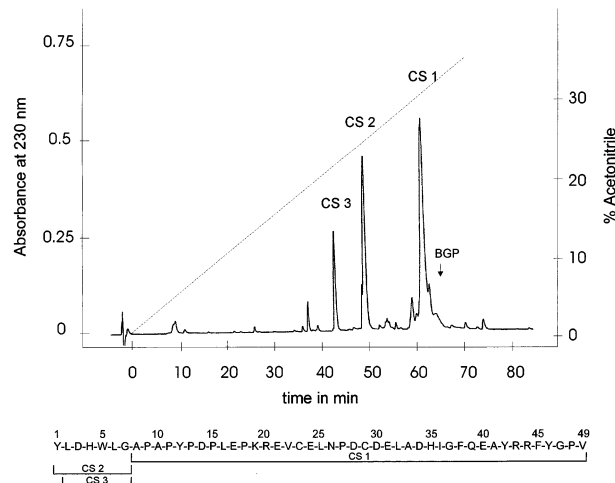


**FIG. 4.** Reverse phase HPLC separation of peptides generated by digestion of bovine BGP with cathepsin H. (For details, refer to Fig. 3).

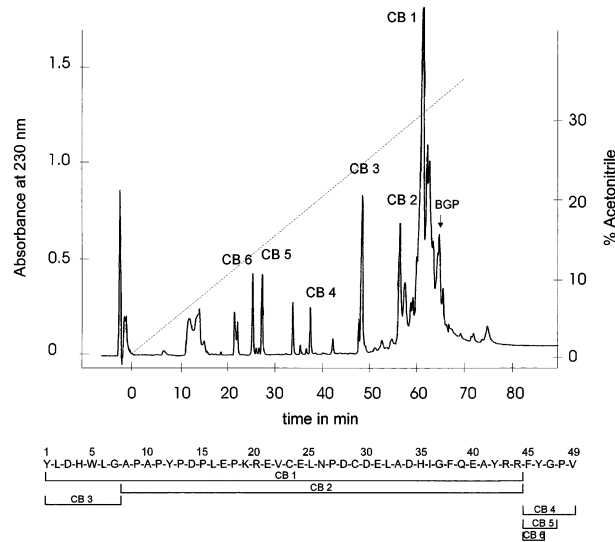
initial cathepsin H cleavage occurred between Gly 7 and Ala 8 to generate 1–7 and 8–49 peptides and that subsequent cleavages within the heptapeptide generated free tryptophan. Because it is known that cathepsin H has aminopeptidase activity, another explanation could be that cathepsin H cleaves as an aminopeptidase until it reaches the resistant Ala8–Pro9 bond. Since we could not detect BGP peptides intermediate in size between intact BGP and the 8–49 peptide, the aminopeptidase hypothesis for cathepsin H would also require that the velocity of the cleavage between the amino acids 1–2 be much slower than the subsequent aminopeptidase cleavages.

The HPLC chromatogram for the cathepsin S digest of bovine BGP, shown in Fig. 5, indicates the presence of three major fragments. Protein sequencing and amino acid analysis showed that these fragments correspond to residues 8–49, 1–7, and 2–7 in the sequence of bovine BGP. Since cathepsin S digests never revealed the presence of a fragment corresponding to 2–49 in BGP, it is likely that the initial cathepsin S cleavage occurs between Gly 7 and Ala 8 and that the 2–7 peptide is derived by a subsequent cathepsin S cleavage within the 1–7 peptide.

The fragmentation pattern produced by cathepsins B and L were more complex than those produced by cathepsins H, D, and S. The HPLC chromatogram for the cathepsin B digest of bovine BGP, shown in Fig. 6, reveals the presence of multiple components, and only the major components were subjected to chemical analysis. Protein sequencing and amino acid analysis showed that fragment CB1 corresponds to residues 1–44 in BGP and that fragment CB2 corresponds to residues 8–44 in BGP. These HPLC purified peptides comigrated with the bands labeled CB1 and CB2 in the SDS-PAGE shown in Fig. 2. Peptide sequencing and amino acid analysis also unambiguously identified the other major peptide fragments shown in Fig. 6 as corresponding to residues 1–7, 45–49, and 45–46 in BGP. These results together with the kinetic evidence indicating the apparent conversion of CB1 to CB2 suggest that the initial cathepsin B cleavage site in bovine BGP lies between Arg 43 and Phe



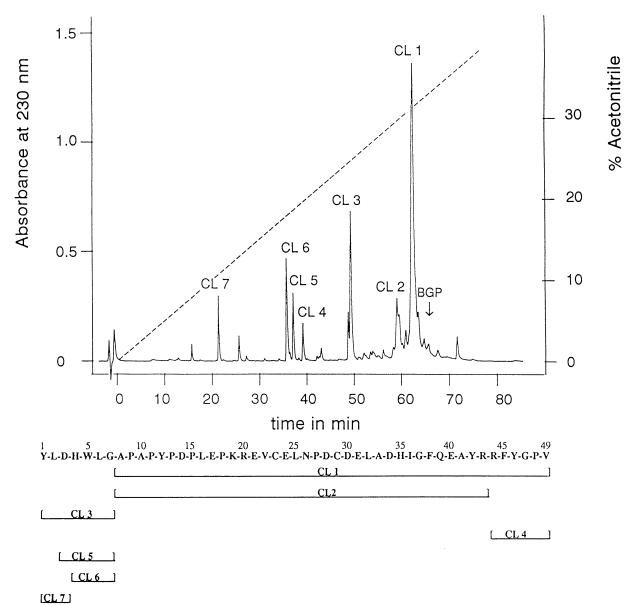
**FIG. 5.** Reverse phase HPLC separation of peptides generated by digestion of bovine BGP with cathepsin S. (For details, refer to Fig. 3).



**FIG. 6.** Reverse phase HPLC separation of peptides generated by digestion of bovine BGP with cathepsin B. (For details, refer to Fig. 3).

44 and that subsequent cleavage of the 1–43 fragment CB1 at the Gly 7–Ala 8 site produces the 8–43 fragment CB2.

The HPLC chromatogram of the cathepsin L cleavage of bovine BGP, shown in Fig. 7, also reveals the existence of multiple fragments and multiple corresponding cleavage sites. Peptide sequencing and amino acid analysis demonstrate that fragment CL1 corresponds to residues 8–49 in bovine BGP and fragment CL2 corresponds to residues 8–43. These HPLC purified peptides comigrated with the bands labeled CL1 and CL2 in Fig. 2. Since the kinetic analysis of the cathepsin L digest of bovine BGP by SDS-PAGE indicated CL1 is produced first and that CL2 is produced from CL1, it is likely that the initial cathepsin L cleavage site in bovine BGP lies between Gly 7 and Ala 8 and that a subsequent cleavage of the resulting 8–49 frag-



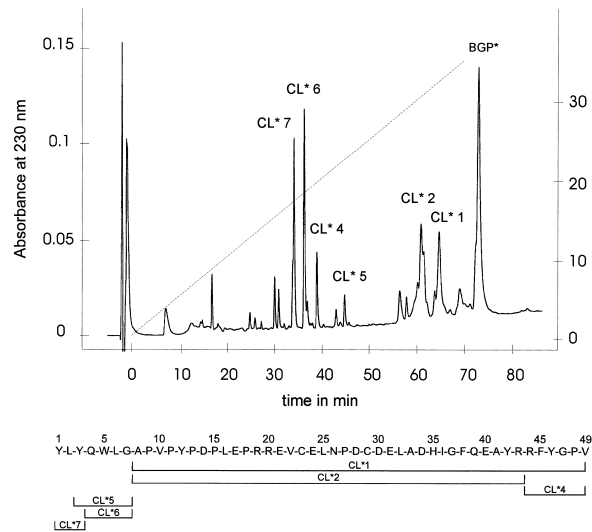
**FIG. 7.** Reverse phase HPLC separation of peptides generated by digestion of bovine BGP with cathepsin L. (For details, refer to Fig. 3).

ment CL1 at the Arg 44–Phe 45 bond produces the 8–43 fragment CL2. Peptide sequencing and amino acid analysis showed that the other peptides seen in Fig. 7 correspond to residues 45–49, 1–7, 2–7, 3–7, and 1–3 in bovine BGP. The failure to detect large BGP fragments in cathepsin L digests which have residue 2 or 3 at the N terminus indicates that peptides 2–7, 3–7, and 1–3 are probably produced by subsequent cathepsin L cleavages within the 1–7 peptide after the release of this peptide from BGP.

#### Digestion of human BGP by cathepsins L, D, H, and S

The fragmentation patterns obtained for human BGP digests with cathepsins L, H, D, and S closely paralleled the cleavage sites identified for the respective enzyme digests of the bovine protein. The HPLC chromatogram for a partial digest of human BGP with cathepsin L is shown in Fig. 8. Peptide sequencing and amino acid analysis revealed that the major cleavage fragments correspond to residues 8–49, 8–43, 44–49, 1–3, 3–7, and 4–7 in human BGP. Although a detailed kinetic analysis of the cathepsin L digest of human BGP was not possible due to limited availability of the protein, the absence of any major component corresponding to residues 1–43 in human BGP suggests that the initial cathepsin L cleavage site is again at Gly 7–Ala 8 and that subsequent cleavage of the resulting 8–49 fragment at the Arg 43–Phe 44 bond produces the 8–43 peptide. Since no large fragments were seen with N-terminal sequences beginning with residues 3 or 4, it is again likely that the 3–7 and 4–7 peptides were generated by subsequent cathepsin L cleavage of the 1–7 peptide following its release from human BGP.

The major peptide fragments produced by the digestion of human BGP by cathepsin H, S, and D were also sepa-



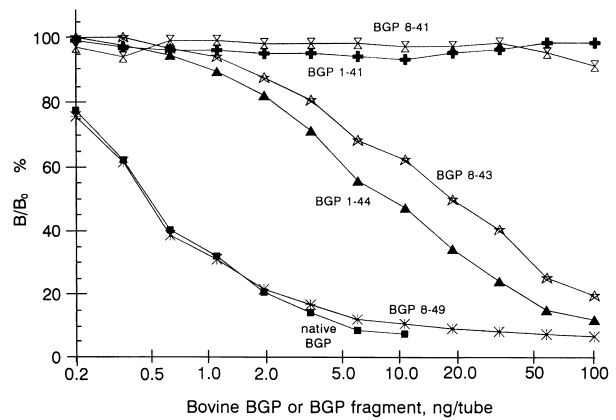
**FIG. 8.** Reverse phase HPLC separation of peptides generated by digestion of human BGP with cathepsin L. (For details, refer to Fig. 3).

rated by HPLC (data not shown) and identified by peptide sequencing and amino acid analysis. The results of these studies are summarized in Table 3 and reveal that cathepsins S and H cleave at the same Gly 7–Ala 8 site in human BGP as seen in bovine BGP, and that cathepsin D cleaves at the same Ala 41–Tyr 42 site in human BGP as seen in bovine BGP.

#### *Antibody recognition of the major BGP fragments produced by cathepsins*

Serum BGP measurement by immunoassay has been used widely for the assessment of bone metabolism in humans and it is therefore of interest to determine the relative immunoreactivity of the major fragments generated by the digestion of BGP with different cathepsins in such assays. As an initial step in these investigations, we evaluated the relative immunoreactivity of the major cathepsin fragments using the original RIA developed for the measurement of serum BGP levels in humans.<sup>(21)</sup> This assay is based on antibodies raised in rabbits against bovine BGP. These antibodies recognize a C-terminal BGP epitope and, since the C-terminal 17 residues of bovine and human BGP are the same,<sup>(22)</sup> this assay cross-reacts identically with human BGP. Studies comparing different assays developed for the detection of serum BGP in humans have shown that this BGP RIA detects the same magnitude of elevation in serum BGP levels with increased bone turnover as do radioimmunoassays subsequently developed by other investigators.<sup>(6)</sup>

As can be seen in Fig. 9, the 8–49 BGP fragment generated by digestion of BGP with cathepsins H, S, and L cross-reacts identically with native BGP, a result that is consistent with the previously reported specificity of this assay for the C-terminal region of BGP. The other major cathepsin L fragment, which corresponds to residues 8–43 in BGP and is generated from the 8–49 fragment by a subsequent cathepsin L cleavage, can also completely dis-



**FIG. 9.** Epitope specificity of the polyclonal antibodies investigated for native bBGP, ■; bBGP fragment 8–49, ×; bBGP fragment 1–44, ▲; bBGP fragment 8–43, ★; BGP fragment 1–41, +; and bBGP fragment 8–41, △. BGP and the fragments were analyzed by competitive immunoassay using radiolabeled bovine BGP as tracer.

place the labeled native BGP tracer from the rabbit anti-BGP antibody but the concentration required for displacement is about 40-fold greater. The major cathepsin B fragment, which corresponds to residues 1–44 in BGP, also fully displaces the labeled native BGP tracer from the rabbit antibody but the concentration required for displacement is about 20-fold greater. The 1–41 fragment generated by digestion of BGP with cathepsin D and the 8–41 fragment generated by subsequent cleavage of the 1–41 peptide with cathepsin H are both unable to significantly displace the labeled native BGP tracer from the rabbit antibody at any concentration tested (Fig. 9). These results reveal a critical contribution to native BGP recognition by this rabbit antibody for residues 42 and 43, and an important but not critical contribution for residues 44 and 45–49. Because of the importance of these C-terminal residues to antibody recognition, it is unlikely that even the most immunoreactive of these C-terminal cleavage fragments, the 1–44 peptide, can contribute significantly to the level of serum BGP unless the concentration of this peptide greatly exceeds the concentration of native BGP in serum.

## DISCUSSION

The present investigations show that there are relatively few bonds in BGP that are cleaved rapidly by the five cathepsins studied and that these sensitive sites consist of the Gly<sub>7</sub>–Ala<sub>8</sub> bond and three sites between residues 41 and 45. The most likely explanation for the highly restricted sequence distribution of cleavage sites in BGP is that only these regions of the polypeptide chain are accessible to the active site of proteases. The peptide bonds in the central core of BGP would be protease resistant because the folded conformation of the protein is such that these bonds are inaccessible to a protease active site.<sup>(23–25)</sup> There are examples in the literature of conformation-dependent differences in protease susceptibility dependent on pH and ion strength which lend support to this hypothesis.<sup>(26)</sup> If the

TABLE 2. MAJOR BOVINE BGP FRAGMENTS GENERATED BY CATHEPSIN L, H, S, D AND B

<i>Enzyme</i>	<i>Major cleavage sites</i>	<i>Fragments isolated by HPLC</i>	<i>Evidence for fragment structure</i>	<i>Correspondence to fragments in SDS-gel</i>	<i>Correspondence to fragments in HPLC</i>
Cathepsin L	↓	8-49	Sequencing, composition	Fragment CL 1	Fragment CL 1
	Gly <sub>7</sub> -Ala <sub>8</sub>	1-7	Sequencing, composition		Fragment CL 3
	Minor:	8-43	Sequencing, composition		Fragment CL 2
Cathepsin H	↓	44-49	Composition	Fragment CH 1	Fragment CL 4
	Arg <sub>43</sub> -Arg <sub>44</sub>	8-49	Sequencing, composition		Fragment CH 1
	Gly <sub>7</sub> -Ala <sub>8</sub>	8-49	Sequencing, composition		Fragment CS 1
Cathepsin S	↓	8-49	Sequencing, composition	Fragment CS 1	Fragment CS 1
	Gly <sub>7</sub> -Ala <sub>8</sub>	1-7	Sequencing, composition		Fragment CS 2
Cathepsin D	↓	1-41	Composition	Fragment CD 1	Fragment CD 1
	Ala <sub>41</sub> -Tyr <sub>42</sub>	42-49	Sequencing, composition		Fragment CD 2
Cathepsin B	↓	1-44	Sequencing, composition	Fragment CB 1	Fragment CB 1
	Arg <sub>44</sub> -Phe <sub>45</sub>	45-49	Composition		Fragment CB 4
	Minor:	8-44	Sequencing, composition		Fragment CB 2
	↓	1-7	Sequencing, composition		Fragment CB 3
	Gly <sub>7</sub> -Ala <sub>8</sub>				Fragment CB 3

TABLE 3. MAJOR HUMAN BGP FRAGMENTS GENERATED BY CATHEPSIN L, H, S, AND D

<i>Enzyme</i>	<i>Major cleavage sites</i>	<i>Fragments isolated by HPLC</i>	<i>Evidence for fragment structure</i>	<i>Correspondence to fragments in HPLC</i>
Cathepsin L	↓	8-49	Sequencing, composition	Fragment CL* 1
	Gly <sub>7</sub> -Ala <sub>8</sub>	4-7	Sequencing	Fragment CL* 6
	Minor:	3-7	Sequencing	Fragment CL* 5
	↓	8-43	Sequencing, composition	Fragment CL* 2
	↓	44-49	Sequencing, composition	Fragment CL* 4
Cathepsin H	Arg <sub>43</sub> -Arg <sub>44</sub>	8-49	Sequencing, composition	
	Gly <sub>7</sub> -Ala <sub>8</sub>			
Cathepsin S	↓	8-49	Sequencing, composition	
	Gly <sub>7</sub> -Ala <sub>8</sub>			
Cathepsin D	↓	1-41	Composition	
	Ala <sub>41</sub> -Tyr <sub>42</sub>	42-49	Sequencing, composition	

protease sensitivity of different regions of BGP is in fact attributable to the folded conformation of the protein, it is likely that other proteases found in bone or in blood will likewise prove to cleave BGP preferentially in these same N- and C-terminal regions of the polypeptide chain.

There are BGP fragments in serum, but the structure and significance of these fragments are still unclear.<sup>(27-30)</sup> Our present results suggest that the most likely BGP fragments that would be found in serum are those missing the N-terminal heptapeptide, a C-terminal peptide of variable length, or both. This is in good agreement with results of other investigators. There is a report in the literature that discusses serum BGP peptides with small C- and/or N-terminal changes of BGP assayed by two different two-site assays with monoclonal antibodies against the C and N terminus. The BGP concentration obtained differed by more than 2-fold. The authors concluded that the different antibodies recognize different epitopes within the peptide

antigen (1-12 and 38-49) used for their generation.<sup>(31)</sup> Additionally, BGP fragments have also been found in bone preparations. These fragments also had a removal of amino acids in the C- or N-terminal region, presumably caused by an endogenous proteolytic action in bone: peptide 7-49 from human BGP,<sup>(32)</sup> peptide 1-41, and 3-50 from chicken BGP.<sup>(33,34)</sup> Our results suggest that cysteine proteinases may be responsible for these degradations.

Furthermore, recent research provides evidence to support the hypothesis that cysteine proteinases play a key role in osteoclastic bone resorption and therefore in the enzymatic hydrolysis of collagen and noncollagenous proteins, including BGP. (1) It has been shown by immunohistochemical experiments that cathepsins L, B, C, H, G, and D are localized in osteoclasts. In particular, the cathepsins L, B, and C have been found in the ruffled border region and cathepsins L, B, and G outside the osteoclasts, in the extracellular space.<sup>(13-15,35)</sup> Recently, a novel cysteine pro-

teinase named cathepsin K or O and predominantly expressed in osteoclasts was cloned.<sup>(36-38)</sup> (2) Cathepsin L, cathepsin L-like enzyme, and cathepsin B were isolated from bone tissues.<sup>(39,40)</sup> (3) In vitro resorption experiments determined that there is cathepsin L and B activity in the culture medium of bone cells.<sup>(41,42)</sup> PTH increases and calcitonin suppresses the cathepsin L and/or cathepsin L-like proteinase activity in the culture medium.<sup>(41,42)</sup> (4) E-64 and other specific cysteine proteinase inhibitors suppress bone resorption in vivo and in vitro. Collagenase inhibitors do not suppress this process.<sup>(43-47)</sup> There is no correlation between the resorption of cultured mouse bone cells and their secretion of collagenase.<sup>(48)</sup> E-64 inhibits the osteoclastic resorption of collagen from dentine slices but does not suppress the process of demineralization, evaluated by electron microscopy.<sup>(49)</sup> (5) The ruffled border region has optimal conditions for the action of cathepsins (acidified bone surface underneath the osteoclasts), but no optimal conditions for collagenase (optimum at neutral or near basic pH).<sup>(50,51)</sup> (6) Cathepsin B can generate collagenase by the activation of procollagenase.<sup>(52)</sup> (7) Cathepsins L, S, and B have a high affinity to collagen, cathepsin L having the highest.<sup>(12,51)</sup> (8) Cathepsins L and B can degrade the following noncollagenous proteins: BGP, osteonectin, and  $\alpha_2$ -HS-glycoprotein.<sup>(53)</sup>

The present study shows that not only cathepsins L and B but also the cysteine proteinases cathepsins H and S and the aspartic proteinase cathepsin D can degrade human and bovine BGP. The activity of the enzymes are different. The activity of cathepsin L against BGP is about 10 times that of cathepsin B. Interestingly, it was reported that the activity of cathepsin L against collagen is also nine times that of cathepsin B.<sup>(54)</sup> Against BGP, cathepsin L possesses the highest activity, nine times more than cathepsin B. Cathepsin S also has a high affinity for BGP. Cathepsins H and D are less active. Experiments with the novel cysteine proteinase cathepsin K could not be included because the active enzyme was not available at the time that these studies were undertaken.

It has been recognized for some time that the presence of BGP fragments in serum would have the potential to introduce discrepancies in serum BGP levels which would depend upon the epitope specificity of the particular immunoassay used.<sup>(6-8)</sup> As a first step in the analysis of the possible effects of such proteolytic cleavages on serum BGP immunoassays, we have here examined the immunoreactivity of each of the major fragments generated in the present studies using the original RIA developed for the detection of human serum BGP. These studies reveal that any of the protease cleavages in the 41-45 residue region reduce the immunoreactivity of BGP to the point that it is unlikely that such fragments would ever contribute significantly to the serum BGP levels measured by this assay. In marked contrast, protease cleavage at Gly<sub>7</sub>-Ala<sub>8</sub> produces a fragment that is indistinguishable from native BGP on this assay. These results indicate that the human and bovine BGP peptides we have prepared may provide useful tools for future studies on the precise epitope specificity of other assays currently used for the measurement of human serum BGP levels in clinical studies.

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