Comparative Sequence Specificities of Human 72- and 92-kDa Gelatinases (Type IV Collagenases) and PUMP (Matrilysin)†

Sarah Netzel-Arnett,‡ Qing-Xiang Sang,‡ William G. I. Moore,§ Marc Navre,⊥,∥ Henning Birkedal-Hansen,§ and Harold E. Van Wart*,⊥

Department of Chemistry and Institute of Molecular Biophysics, Florida State University, Tallahassee, Florida 32306, Institute of Biochemistry and Cell Biology, Syntex Discovery Research, 3401 Hillview Avenue, S3-1, Palo Alto, California 94304, and Department of Oral Biology, University of Alabama School of Dentistry, Birmingham, Alabama 35294

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ABSTRACT: The sequence specificities of human 72-kDa fibroblast gelatinase (type IV collagenase), human 92-kDa neutrophil gelatinase (type IV collagenase), and putative metalloproteinase (PUMP or matrilysin) have been examined by measuring the rate of hydrolysis of over 50 synthetic oligopeptides covering the P_4 through P_4' subsites of the substrate. The peptides investigated in this paper were those employed in our previous study which systematically examined the sequence specificity of human fibroblast and neutrophil collagenases [Netzel-Arnett et al. (1991) *J. Biol. Chem. 266*, 6747]. The initial rate of hydrolysis of the P_1–P_1' bond of each peptide has been measured under first-order conditions ($[S_0] \ll K_M$), and k_{cat}/K_M values have been calculated from the initial rates. The specificities of these five metalloproteinases are similar, but distinct, with the largest differences occurring at subsites P_1, P_1', and P_3'. The specificities of the two gelatinases are the most similar to each other. They tolerate only small amino acids such as Gly and Ala in subsite P_1. In contrast, larger residues such as Met, Pro, Gln, and Glu are also accommodated well by PUMP. All five enzymes prefer hydrophobic, aliphatic residues in subsite P_1'. PUMP exhibits a stronger preference for Leu in this subsite than is shown by the other enzymes. The P_3' subsite specificities of the gelatinases and collagenases are very similar but different from those of PUMP which particularly prefers Met in this position. The specificity data from this study allow the design of optimized substrates and selective inhibitors for these metalloproteinases.

The matrix metalloproteinases (MMP)[1] are a family of enzymes that are believed to play a leading role in both the normal turnover and pathological destruction of the extracellular matrix (Van Wart & Mookhtiar, 1990; Woessner, 1991; Birkedal-Hansen, 1992). The human MMP family currently consists of eight proteinases that can be grouped into four categories on the basis of similarities in their protein substrate specificities and the domain organizations of their polypeptide chains. The first category includes the fibroblast-type (Stricklin et al., 1977; Goldberg et al., 1986) and neutrophil-type (Mookhtiar & Van Wart, 1990; Mallya et al., 1990; Hasty et al., 1990) interstitial collagenases that hydrolyze type I, II, and III collagens. Fibroblast collagenase also hydrolyzes native type VII (Seltzer et al., 1989a) and X (Schmid et al., 1986; Gadher et al., 1988; Gadher et al., 1989; Welgus et al., 1990) collagens. The interstitial collagenases consist of a propeptide, a catalytic, and a hemopexin-like domain.

The second category is the "gelatinases" or "type IV collagenases" and includes a 72-kDa gelatinase produced by fibroblasts (Salo et al., 1985) and tumor cells (Salo et al., 1983; Collier et al., 1988), and a distinct 92-kDa gelatinase produced by neutrophils (Hibbs & Bainton, 1989; Devarajan et al., 1992), macrophages (Hibbs et al., 1987), and certain transformed cells (Wilhelm et al., 1989; Bernhard et al., 1990). These enzymes hydrolyze native type IV and V collagens and a variety of gelatins (Okada et al., 1990; Wilhelm et al., 1989; Collier et al., 1988). The gelatinases contain the same three domains as the interstitial collagenases, but both contain an additional domain inserted into the catalytic domain that contains three 58 amino acid head-to-tail repeats which are homologous to the type II motif of fibronectin (Collier et al., 1988). The 92-kDa gelatinase has a fifth domain inserted close to the border between the catalytic and hemopexin-like domains that consists of 54 amino acids and is homologous to a segment in the helical region of the $\alpha 2$(V) collagen chain (Wilhelm et al., 1989). Human fibroblasts (Okada et al., 1986; Whitham et al., 1986; Wilhelm et al., 1987; Saus et al., 1988) also produce an enzyme called stromelysin (Chin et al., 1985) which degrades proteoglycans, type IV and IX collagens, laminin, fibronectin, and other substrates (Okada et al., 1986; Okada et al., 1989). Together with the less extensively characterized stromelysin-2 (Muller et al., 1988) and -3 (Basset et al., 1990), these are a third category of MMP that have the same domain structure as the interstitial collagenases, but that do not exhibit collagenase activity. Last, putative metalloproteinase (or matrilysin) consists of only the propeptide and catalytic domains and comprises a category of its own (Muller et al., 1988). It hydrolyzes proteoglycans,

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* To whom correspondence should be addressed: 415-855-5169 (Tel); 415-354-7554 (Fax).

‡ Florida State University.

§ University of Alabama School of Dentistry.

⊥ Syntex Discovery Research.

∥ Present address: Affymax Research Institute, 4001 Miranda Ave, Palo Alto, CA 94304.

[1] Abbreviations used: Fmoc, 9-fluorenylmethoxycarbonyl; Tricine, *N*-tris(hydroxymethyl)methylglycine; MMP, matrix metalloproteinase; HFC, human fibroblast collagenase; HNC, human neutrophil collagenase; HFG, 72-kDa human fibroblast gelatinase; HNG, 92-kDa human neutrophil gelatinase; PUMP, putative metalloproteinase; PCMB, *p*-chloromercuribenzoate; pNA, *p*-nitroanilide; HPLC, high-performance liquid chromatography; DNP, dinitrophenyl; Dansyl, 5-(dimethylamino)-naphthalene-1-sulfonyl; Dpa, *N*-3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl; Mca, (7-methoxycoumarin-4-yl)acetyl.

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fibronectin, laminin, gelatin, and other substrates (Quantin et al., 1989; Miyazaki et al., 1990; Murphy et al., 1991).

One of the interesting questions relating to the MMP is that of how such a homologous family of proteinases can exhibit such diversity in their protein substrate specificities. The domain structure and organizational pattern of the MMP is unique among known proteinases and may have evolved to facilitate the biologic functions of these enzymes. In particular, the hemopexin-like domain is thought to modulate the protein substrate specificity of certain of these enzymes. For example, when the fibroblast collagenase loses this domain autolytically (Windsor et al., 1991) or when a truncated form of the enzyme is expressed that does not contain this domain (Murphy et al., 1992), it loses collagenase activity but retains activity against nonspecific protein substrates such as gelatin and casein and a peptide substrate. The fibronectin-like and α2(V)-like domains found in the gelatinases may serve analogous substrate recognition functions. Thus, the specificities of these enzymes toward protein substrates may be conceptually divided into their active-site "sequence specificities" toward short peptides localized in the catalytic domain, plus interactions between other parts of the substrate with "exosite" portions of the MMP located on other domains. We have previously examined the sequence specificities of human fibroblast collagenase (HFC) and human neutrophil collagenase (HNC) toward a series of octapeptides and showed a lack of correlation with their collagen specificities (Fields et al., 1987; Mallya et al., 1990; Netzel-Arnett et al., 1991a). In this study, we extend our sequence specificity investigation of the MMP to include human putative metalloproteinase (PUMP), the 72-kDa human fibroblast gelatinase (HFG) and 92-kDa human neutrophil gelatinase (HNG). In addition to helping elucidate the contribution of sequence specificity to the protein specificity of these MMP, the data presented here are also important for the design of optimized synthetic substrates and inhibitors.

MATERIALS AND METHODS

Materials. Pro-HNG, pro-HFG, and pro-PUMP were purified to homogeneity and activated prior to the kinetic experiments. Pro-HNG was isolated from human buffy coats as described earlier (Mookhtiar & Van Wart, 1990). Pro-HFG was purified from the harvest media of human gingival fibroblasts (Birkedal-Hansen, 1987). Pro-PUMP was isolated from Chinese hamster ovary cells carrying an amplified pro-PUMP cDNA (Yuan et al., in preparation). Briefly, conditioned medium was collected from cells grown in serum-free medium, concentrated, applied to a blue-Sepharose column and eluted in 20 mM Tris, 5 mM $CaCl_2$, 0.05% Brij-35, pH 7.4 with a 0–1.5 M NaCl gradient. Fractions containing pro-PUMP were pooled, loaded directly onto a zinc-Sepharose column, and eluted with a 0–600 mM glycine gradient. The final purification was achieved by chromatography over Sepharose using a linear 0–1 M NaCl gradient for elution.

Dansyl chloride and *p*-chloromercuribenzoate (PCMB) were purchased from Sigma; high-performance liquid chromatography (HPLC) grade acetonitrile, mercuric chloride, fluorescamine, dicyclohexylcarbodiimide and Brij-35 from Fisher; constant-boiling HCl and Sequanol-grade trifluroacetic acid from Pierce; Tricine from Behring Diagnostics; Sepharose from Pharmacia; and *N*-α-Fmoc-amino acids and alkoxybenzyl alcohol resin were purchased from Bachem (Torrance, CA).

Peptide Synthesis and Characterization. All peptides were synthesized by the solid-phase method with *N*-α-Fmoc-amino acids according to the procedures described by Stewart and Young (Stewart & Young, 1984) and purified and characterized as described earlier (Netzel-Arnett et al., 1991a).

Kinetic Measurements. Pro-HFG and pro-HNG were activated by incubation with 0.1 mM $HgCl_2$ in assay buffer (50 mM Tricine, 10 mM $CaCl_2$, 0.2 M NaCl, pH 7.5) for 2 h at 37 °C. The $HgCl_2$ was then removed by centrifugation using a Centricon-30 followed by repeated washing with assay buffer. Pro-PUMP was activated by treatment with 1 mM PCMB for 2 h at 37 °C and then dialyzed against 50 mM Tricine, 0.2 M NaCl, 10 mM $CaCl_2$, 50 μM $ZnSO_4$, 0.05% Brij-35, pH 7.5, to remove all traces of PCMB. The activation lowered the molecular weight of each zymogen by 9–10 kDa. Enzyme concentrations were determined spectrophotometrically using extinction coefficients calculated according to the method of Gill and von Hippel (Gill & von Hippel, 1989). The extinction coefficients used for the active enzymes were ϵ_{280} = 123, 104, and 33 mM^{-1} cm^{-1} for HFG, HNG, and PUMP respectively.

The initial rates of hydrolysis (v_i) of most peptides were measured at 30 °C by quantitating the rate of formation of amino groups fluorimetrically after reaction with fluorescamine as described earlier (Netzel-Arnett et al., 1991a). For peptide **35**, which contains Tyr in subsite P_1', corrections were made for the low quantum yield of the resulting fluorescamine-tyrosine adduct (Chen et al., 1978). The rates of hydrolysis of peptides **31** and **32** were measured by quantitating the rate of formation of Gly-Pro-Gln-Gly by HPLC analysis of quenched time points, since these peptides contain Trp and Pro in subsite P_1' respectively. The sites of hydrolysis of all peptides were determined by dansylation of the reaction products followed by HPLC analysis as described earlier (Fields et al., 1987) using the tetrapeptides corresponding to the P_1'–P_4' or P_1–P_4 residues of the substrate as hydrolysis standards. All peptides were hydrolyzed exclusively at the P_1–P_1' bond. In calculating kinetic constants, it was assumed that all of the enzyme molecules were catalytically competent and that each contained a single active site. The specific activities of fully activated HNG and HFG toward type I gelatin, and PUMP toward casein, were determined to be 7800, 12 000, 3500 μg/(min mg), respectively, at 37 °C.

RESULTS

As in our previous specificity study of HFC and HNC (Netzel-Arnett et al. 1991a), the reference peptide for this study is Gly-Pro-Gln-Gly-Ile-Ala-Gly-Gln (peptide **1**) which is modeled after the collagenase cleavage site in the calf/chick α1(I) chain of collagen (Highberger et al. 1982; Glanville et al. 1983). The scissile bond is the Gly-Ile bond and this octapeptide covers the P_4 through P_4' subsites (Schechter & Berger, 1967) of the substrate. All of the other peptides (numbering as in Netzel-Arnett et al., 1991a) differ from **1** by single amino acid substitutions at one of these eight subsites, except for peptides **3–5** and **7–9** which have been altered in length relative to **1**. The substitutions in peptides **10**, **12–28**, **30–40**, **42–56**, and **58** include: Ala and Asn for Pro in P_3; Leu, Hyp, Arg, Asp, Val, Met, and Tyr for Gln in P_2; Met, Glu, Tyr, Ala, Pro, Gln, Phe, Leu, Val, and His for Gly in P_1; Trp, Pro, Glu, Leu, Tyr, Phe, Met, Val, Gln, Ser, and Arg for Ile in P_1'; Phe, Trp, Leu, Hyp, Arg, and Glu for Ala in P_2'; Val, Arg, Met, Ala, and Ser for Gly in P_3'; and Thr, His, Ala, and *p*-nitroanilide (pNA) for Gln in P_4'.

To investigate the values of the individual kinetic parameters for the hydrolysis of these peptides by PUMP, HFG, and HNG, initial rates (v_i) have been measured as a function of peptide concentration for several peptides and the values of k_{cat} and K_M determined from double reciprocal plots. All of

Table I: Kinetic Parameters for the Hydrolysis of Octapeptides

	peptide	K_m (mM)	k_{cat} (h^{-1})	k_{cat}/K_m (mM^{-1} h^{-1})
	P_4 P_3 P_2 P_1 P'_1 P'_2 P'_3 P'_4			
PUMP				
1	Gly-Pro-Gln-Gly-Ile-Ala-Gly-Gln	2.0	800	400
18	Gly-Pro-**Met**-Gly-Ile-Ala-Gly-Gln	4.2	6900	1600
23	Gly-Pro-Gln-**Ala**-Ile-Ala-Gly-Gln	0.81	1700	2100
34	Gly-Pro-Gln-Gly-**Leu**-Ala-Gly-Gln	7.3	8900	1200
51	Gly-Pro-Gln-Gly-Ile-Ala-**Met**-Gln	2.3	4200	1800
HNG				
1	Gly-Pro-Gln-Gly-Ile-Ala-Gly-Gln	9.8	3300	340
13	Gly-Pro-**Leu**-Gly-Ile-Ala-Gly-Gln	1.1	1100	1000
37	Gly-Pro-Gln-Gly-**Met**-Ala-Gly-Gln	1.9	1100	580
44	Gly-Pro-Gln-Gly-Ile-**Trp**-Gly-Gln	0.42	340	810
HFG				
1	Gly-Pro-Gln-Gly-Ile-Ala-Gly-Gln	15	9700	650
43	Gly-Pro-Gln-Gly-Ile-**Phe**-Gly-Gln	4.8	12000	2500
44	Gly-Pro-Gln-Gly-Ile-**Trp**-Gly-Gln	1.1	2200	2000
53	Gly-Pro-Gln-Gly-Ile-Ala-**Ser**-Gln	3.4	7200	2100

these plots (not shown) are linear over the entire range of substrate concentrations studied, indicating that Michaelis–Menten kinetics are obeyed. The values of k_{cat} and K_M for the hydrolysis of **1** are 800 h^{-1} and 2 mM for PUMP, 3300 h^{-1} and 9.8 mM for HNG, and 9700 h^{-1} and 15 mM for HFG, respectively (Table I). As found in our earlier study (Netzel Arnett et al., 1991a), the K_M values for these reactions are high. Accordingly, most reactions carried out at a substrate concentration of 0.3 mM satisfy the criterion $[S_0] \ll K_M$. Under these conditions, $k_{cat}/K_M = v/[S_0][E_0]$ and the values of k_{cat}/K_M for most of these reactions have been evaluated as the average of at least three determinations made at a single substrate concentration of 0.3 mM. For 10 peptides that were hydrolyzed at unusually high rates by one or more of these enzymes, suggesting that the K_M values could be lower than those for **1**, the values of k_{cat} and K_M were determined individually from double reciprocal plots to ensure that the k_{cat}/K_M value was accurate (Table I). The relative values of k_{cat}/K_M for the hydrolysis of the octapeptides examined here are listed in Tables II–VII, where the value for peptide **1** arbitrarily has been assigned 100%. Also included in these tables for comparative purposes are the hydrolysis rates for HFC and HNC (Netzel-Arnett et al., 1991a).

To investigate the effect of sequential N- and C-terminal truncation on the k_{cat}/K_M values, the rates of hydrolysis of peptides **3–5** and **7–9** have been examined (Table II). The deletion of Gln from subsite P_4' (**7**) decreases the rate for HFG to 60%, while the rates for PUMP and HNG are essentially unaffected. Removal of the Gly residue from subsite P_3' (**8**) reduces the rate for PUMP by 10-fold and the rates for both gelatinases to below measurable values, while further truncation (**9**) abolishes all activity. The similarities between the two gelatinases become more apparent with N-terminal truncation. When the Gly residue in subsite P_4 is removed (**3**), the rates for both HFG and HNG are reduced to approximately 60%, while further deletions (**4**, **5**) result in undetectably low rates. A similar trend is observed with PUMP. These results show that for peptides with unblocked N- and C-termini, occupancy of subsites P_3–P_3' is necessary for rapid hydrolysis by all of these human MMP.

The P_3 and P_2 subsite specificities of PUMP, HFG and HNG are summarized in Table III (peptides **10** and **12–19**). Substitution of either Ala (**10**) or Asn (**12**) for Pro in subsite P_3 leads to a worse substrate for all of these MMP. Asn is a particularly unfavorable substitution for HNG. Replacement of Gln in subsite P_2 by the hydrophobic residues Leu (**13**), Met (**18**), and Tyr (**19**) increases the rates of hydrolysis for all five MMP. The largest effect is the 4.2-fold rate increase for PUMP on replacement of Gln by Leu. Replacement of Gln by Val (**17**) improves hydrolysis marginally for the two gelatinases. HFG is the only MMP that prefers Arg (**15**) over Gln. The rates for both gelatinases and PUMP are markedly lowered when Gln in subsite P_2 is replaced by either Hyp (**14**) or Asp (**16**).

The P_1 subsite specificities have been investigated by comparing the initial rates of hydrolysis of peptides **1**, **20–28**, and **30** (Table IV). PUMP and the two gelatinases behave differently toward substitutions at this subsite. HFG and HNG appear to tolerate only small amino acids in subsite P_1, since all substitutions except Ala (**23**) give a significant decrease in hydrolysis rate. The trend for PUMP is more complex and more closely resembles that for the collagenases. The substitution of Gly by large, bulky hydrophobic residues leads to a decrease in hydrolysis rate with Phe (**26**) > Leu (**27**) > Tyr (**22**) > Val (**28**). In contrast, substitution by five residues (**20**, **21**, **23–25**) of markedly different size and polarity leads to an increase in hydrolysis rate. The 5.3-fold increase for Ala (**23**) is most profound, while that observed for Pro (**24**), Met (**20**), Glu (**21**), and Gln (**25**) is more moderate. Apparently, the amino acid preference observed at this site reflects a variety of types of interactions.

The P_1' subsite specificities of HFG, HNG and PUMP are reflected by the rates of hydrolysis of peptides **31–40** and **42** (Table V) in which the Ile residue has been replaced by a variety of positively charged, negatively charged, hydrophobic, and aromatic amino acids. In spite of this variety of substitutions, very few produce better substrates for these enzymes. Only the conservative replacement of Ile by Leu (**34**) increases the rate for PUMP, while only the substitution by Met (**37**) produces a better substrate for HFG and HNG. In general, the two gelatinases respond in a parallel fashion to substitutions at this subsite. The rate of hydrolysis for all three enzymes drops to low or undetectable levels when Trp (**31**), Pro (**32**), Glu (**33**), Ser (**40**), and Arg (**42**) are present in subsite P_1'.

The effect of replacing the Ala in subsite P_2' by selected residues is illustrated in Table VI. In contrast to subsite P_1', most of the substitutions investigated result in faster rates of hydrolysis. Phe (**43**), Trp (**44**), Leu (**45**), and Arg (**47**) all increase the rate of hydrolysis substantially for both PUMP and the gelatinases. On the other hand, Glu (**48**) causes a small decrease in rate for HFG and PUMP, but a slight increase for HNG. Hyp (**46**) is detrimental for all five MMP. In general, it appears that all of these MMP prefer a large hydrophobic residue in subsite P_2', but will also accept Arg.

Finally, the P_3' and P_4' subsite specificities are summarized in Table VII. When the Gly residue of **1** in subsite P_3' is replaced by Ala (**52**) or Ser (**53**), the rate of hydrolysis increases for all five MMP. PUMP is distinguished from the two gelatinases and collagenases in that substitution of Val (**49**), Arg (**50**), and Met (**51**) for Gly leads to better substrates for PUMP only. The Met substitution produces a 4.5-fold higher rate, one of the largest increases observed for any substitution. Changes in the identity of the residue in subsite P_4' do not produce many large effects. It is interesting to note, however, that substitution of Gln by Thr (**54**) in this position increases the rate for HNG, but decreases it for HFG. When pNA (**58**) is placed in subsite P_4', the rate is halved for the gelatinases, and there is a moderate decrease for PUMP. In general, substitutions in subsite P_4' have little effect on the rate of hydrolysis by PUMP.

DISCUSSION

The results of this study on HFG, HNG, and PUMP, together with our earlier one on HFC and HNC (Netzel-

Table II: Effect of N- and C-Terminal Truncation on the Hydrolysis of Peptides by Five MMP

	peptide P_4 P_3 P_2 P_1 P'_1 P'_2 P'_3 P'_4	activity (%) PUMP	HFG	HNG	HFC[a]	HNC[a]
1	Gly-Pro-Gln-Gly-Ile-Ala-Gly-Gln	100	100	100	100	100
3	Pro-Gln-Gly-Ile-Ala-Gly-Gln	43	62	65	150	100
4	Gln-Gly-Ile-Ala-Gly-Gln	8.3	<5.0	<5.0	7.3	<5.0
5	Gly-Ile-Ala-Gly-Gln	<5.0	<5.0	<5.0	<5.0	<5.0
7	Gly-Pro-Gln-Gly-Ile-Ala-Gly	99	60	93	68	54
8	Gly-Pro-Gln-Gly-Ile-Ala	10	<5.0	<5.0	13	<5.0
9	Gly-Pro-Gln-Gly-Ile	<5.0	<5.0	<5.0	<5.0	<5.0

[a] Data taken from Netzel-Arnett et al., 1991a.

Table III: P_3 and P_2 Subsite Specificities of Five MMP

	peptide P_4 P_3 P_2 P_1 P'_1 P'_2 P'_3 P'_4	activity (%) PUMP	HFG	HNG	HFC[a]	HNC[a]
1	Gly-Pro-Gln-Gly-Ile-Ala-Gly-Gln	100	100	100	100	100
10	Gly-**Ala**-Gln-Gly-Ile-Ala-Gly-Gln		22	9.4	50	23
12	Gly-**Asn**-Gln-Gly-Ile-Ala-Gly-Gln	25	60	<5.0	17	46
13	Gly-Pro-**Leu**-Gly-Ile-Ala-Gly-Gln	420	330	290	150	270
14	Gly-Pro-**Hyp**-Gly-Ile-Ala-Gly-Gln	17	32	15	11	15
15	Gly-Pro-**Arg**-Gly-Ile-Ala-Gly-Gln	13	160	83	17	31
16	Gly-Pro-**Asp**-Gly-Ile-Ala-Gly-Gln	7.0	11	10	30	44
17	Gly-Pro-**Val**-Gly-Ile-Ala-Gly-Gln	57	130	110	32	29
18	Gly-Pro-**Met**-Gly-Ile-Ala-Gly-Gln	400	120	180	160	160
19	Gly-Pro-**Tyr**-Gly-Ile-Ala-Gly-Gln	240	200	150	110	110

[a] Data taken from Netzel-Arnett et al., 1991a.

Table IV: P_1 Subsite Specificities of Five MMP

	peptide P_4 P_3 P_2 P_1 P'_1 P'_2 P'_3 P'_4	activity (%) PUMP	HFG	HNG	HFC[a]	HNC[a]
1	Gly-Pro-Gln-Gly-Ile-Ala-Gly-Gln	100	100	100	100	100
20	Gly-Pro-Gln-**Met**-Ile-Ala-Gly-Gln	150	22	12	200	140
21	Gly-Pro-Gln-**Glu**-Ile-Ala-Gly-Gln	170	15	29	28	330
22	Gly-Pro-Gln-**Tyr**-Ile-Ala-Gly-Gln	34	58	30	130	180
23	Gly-Pro-Gln-**Ala**-Ile-Ala-Gly-Gln	530	96	110	640	330
24	Gly-Pro-Gln-**Pro**-Ile-Ala-Gly-Gln	140	32	46	260	190
25	Gly-Pro-Gln-**Gln**-Ile-Ala-Gly-Gln	180	25	13	37	150
26	Gly-Pro-Gln-**Phe**-Ile-Ala-Gly-Gln	63	15	26	95	170
27	Gly-Pro-Gln-**Leu**-Ile-Ala-Gly-Gln	49	21	8.8	27	54
28	Gly-Pro-Gln-**Val**-Ile-Ala-Gly-Gln	<5.0	<5.0	<5.0	5.5	7.9
30	Gly-Pro-Gln-**His**-Ile-Ala-Gly-Gln		65	44	160	50

[a] Data taken from Netzel-Arnett et al., 1991a.

Table V: P_1' Subsite Specificities of Five MMP

	peptide P_4 P_3 P_2 P_1 P'_1 P'_2 P'_3 P'_4	activity (%) PUMP	HFG	HNG	HFC[a]	HNC[a]
1	Gly-Pro-Gln-Gly-Ile-Ala-Gly-Gln	100	100	100	100	100
31	Gly-Pro-Gln-Gly-**Trp**-Ala-Gly-Gln	<5.0	<5.0	<5.0	<5.0	48
32	Gly-Pro-Gln-Gly-**Pro**-Ala-Gly-Gln	<5.0	<5.0	<5.0	<5.0	<5.0
33	Gly-Pro-Gln-Gly-**Glu**-Ala-Gly-Gln	8.0	<5.0	<5.0	<5.0	<5.0
34	Gly-Pro-Gln-Gly-**Leu**-Ala-Gly-Gln	300	88	80	130	180
35	Gly-Pro-Gln-Gly-**Tyr**-Ala-Gly-Gln	21	50	96	45	480
36	Gly-Pro-Gln-Gly-**Phe**-Ala-Gly-Gln	24	55	24	<5.0	46
37	Gly-Pro-Gln-Gly-**Met**-Ala-Gly-Gln	89	230	170	110	100
38	Gly-Pro-Gln-Gly-**Val**-Ala-Gly-Gln	17	30	25	9.1	9.0
39	Gly-Pro-Gln-Gly-**Gln**-Ala-Gly-Gln	<5.0	34	20	28	10
40	Gly-Pro-Gln-Gly-**Ser**-Ala-Gly-Gln	5.5	15	<5.0	5.9	<5.0
42	Gly-Pro-Gln-Gly-**Arg**-Ala-Gly-Gln	<5.0	<5.0	<5.0	<5.0	<5.0

[a] Data taken from Netzel-Arnett et al., 1991a.

Arnett et al., 1991a), demonstrate that these five MMP have similar, yet distinct, sequence specificities. As with the collagenases, PUMP and the two gelatinases are endopeptidases that exhibit no amino- or carboxypeptidase activity toward any of the penta- through octapeptides studied. For peptides with unblocked N- and C-termini, subsites P_3–P_3' must be occupied for effective hydrolysis by these enzymes.

With respect to the amino acid preferences in these six sites, there are many similarities among the five MMP. Of the amino acids studied, Pro is preferred by all five MMP in subsite P_3. In addition, they all prefer hydrophobic residues in subsites P_2 and P_2'. The biggest differences in specificity are found at subsites P_1, P_1', and P_3'. The two gelatinases have very similar specificities at all of these sites. Unlike PUMP and the collagenases, virtually all substitutions for Gly in subsite P_1 are detrimental for the gelatinases and only the substitution of Met for Ile in subsite P_1' gives a better substrate. With respect to substitutions in subsite P_3', the

Table VI: P_2' Subsite Specificities of Five MMP

	peptide P_4 P_3 P_2 P_1 P_1' P_2' P_3' P_4'	activity (%) PUMP	HFG	HNG	HFC[a]	HNC[a]
1	Gly-Pro-Gln-Gly-Ile-Ala-Gly-Gln	100	100	100	100	100
43	Gly-Pro-Gln-Gly-Ile-**Phe**-Gly-Gln	140	380	390	430	310
44	Gly-Pro-Gln-Gly-Ile-**Trp**-Gly-Gln	330	310	240	730	830
45	Gly-Pro-Gln-Gly-Ile-**Leu**-Gly-Gln	250	400	240	210	400
46	Gly-Pro-Gln-Gly-Ile-**Hyp**-Gly-Gln	8.0	32	11	<5.0	<5.0
47	Gly-Pro-Gln-Gly-Ile-**Arg**-Gly-Gln	270	180	200	180	170
48	Gly-Pro-Gln-Gly-Ile-**Glu**-Gly-Gln	86	85	130	35	58

[a] Data taken from Netzel-Arnett et al., 1991a.

Table VII: P_3' and P_4' Subsite Specificities of Five MMP

	peptide P_4 P_3 P_2 P_1 P_1' P_2' P_3' P_4'	activity (%) PUMP	HFG	HNG	HFC[a]	HNC[a]
1	Gly-Pro-Gln-Gly-Ile-Ala-Gly-Gln	100	100	100	100	100
49	Gly-Pro-Gln-Gly-Ile-Ala-**Val**-Gln	170	26	49	77	56
50	Gly-Pro-Gln-Gly-Ile-Ala-**Arg**-Gln	220	35	45	55	33
51	Gly-Pro-Gln-Gly-Ile-Ala-**Met**-Gln	450	40	35	41	33
52	Gly-Pro-Gln-Gly-Ile-Ala-**Ala**-Gln	300	180	140	220	120
53	Gly-Pro-Gln-Gly-Ile-Ala-**Ser**-Gln	150	320	130	130	130
54	Gly-Pro-Gln-Gly-Ile-Ala-Gly-**Thr**	100	59	160	160	140
55	Gly-Pro-Gln-Gly-Ile-Ala-Gly-**His**	87	150	120	91	140
56	Gly-Pro-Gln-Gly-Ile-Ala-Gly-**Ala**	91	85	110	86	110
58	Gly-Pro-Gln-Gly-Ile-Ala-Gly-**pNA**	70	54	51	130	110

[a] Data taken from Netzel-Arnett et al., 1991a.

two gelatinases behave like the two collagenases. PUMP behaves most differently from the other MMP. Its preference for Met, Ala, and Pro in subsite P_1 resembles that of the collagenases, but its response to Tyr in this subsite resembles that of the gelatinases. PUMP exhibits a strong preference for Leu in subsite P_1' that is not shown by the other four MMP. Most notably, the replacement of the Gly residue in subsite P_3' by Val, Arg, and particularly Met enhances catalysis by PUMP but gives lower rates for the other four MMP.

Very little data have been reported previously concerning the sequence specificities of the MMP. Except for data for HFC discussed earlier (Netzel-Arnett et al., 1991a), only the specificity of HFG has been studied in any detail. Seltzer et al. (1981, 1989b, 1990) have quantitated the rate of hydrolysis of a series of peptide substrates by HFG. Their studies investigated primarily the effect of substitutions in subsites P_1–P_3' and the results are generally in good agreement with the more extensive data reported here. These same authors have also reported the sequence of sites in α1(I) gelatin chains that are hydrolyzed by HFG (Seltzer et al., 1981; Seltzer et al., 1990), as summarized in Table VIII. The hydrolysis of these sites in gelatin is generally consistent with the trends reported in Tables II–VI. The only exception is the Gly–Glu cleavage site which both Seltzer et al. (1990) and ourselves would have predicted from specificity data on peptides to be cleaved slowly due to the presence of Glu in subsite P_1'. Thus, the specificity of HFG toward bonds in the α1(I) gelatin chain is well accounted for by the sequence specificity of the enzyme alone. This contrasts with the collagen cleavage site specificities of the collagenases (Fields et al., 1987; Mallya et al., 1990; Netzel-Arnett et al., 1991a). The sites cleaved by PUMP in porcine aggrecan (Fosang et al., 1992) and prourokinase (Marcotte et al., 1992) have been reported. In addition, its own autolytic activation sites (Crabbe et al., 1992) have been identified. The sequences surrounding these five sites are shown in Table VIII. All of them have a bulky hydrophobic residue in subsite P_1' and four of the five have a Glu or Asp residue in subsite P_1. These features are generally consistent with our finding that Glu is a favorable residue in subsite P_1 (Table IV) and that nonaromatic hydrophobic residues are preferred in subsite P_1' (Table V).

Table VIII: Protein Sequences Hydrolyzed by HFG and PUMP

	P_4 P_3 P_2 P_1 P_1' P_2' P_3' P_4'
	HFG
α1(I) collagen chain[a]	Gly-Ala-Hyp-Gly-Leu-Glx-Gly-His
	Gly-Pro-Gln-Gly-Val-Arg-Gly-Glu
	Gly-Pro-Ser-Gly-Leu-Hyp-Gly-Pro
	Gly-Pro-Ala-Gly-Phe-Ala-Gly-Pro
	Gly-Pro-Ile-Gly-Asn-Val-Gly-Ala
	Gly-Pro-Hyl-Gly-Ser-Arg-Gly-Ala
	Gly-Pro-Ala-Gly-Glu-Arg-Gly-Ser
	Gly-Pro-Ala-Gly-Glx-Asp-Gly-Pro
	Gly-Pro-Ala-Gly-Val-Gln-Gly-Pro
	Gly-Ala-Lys-Gly-Leu-Thr-Gly-Ser
	PUMP
aggrecan[b]	Thr-Ser-Glu-Asp-Leu-Val-Val-Gln
	Ile-Asp-Glu-Asn-Phe-Phe-Gly-Val
PUMP (autolytic)[c]	Asp-Val-Ala-Glu-Tyr-Ser-Leu-Phe
	Arg-Val-Ile-Glu-Ile-Met-Gln-Lys
	Gly-Met-Ser-Glu-Leu-Gln-Trp-Glu
prourokinase[d]	Pro-Pro-Glu-Glu-Leu-Lys-Phe-Gln

[a] Data taken from Seltzer et al., 1981, 1990. [b] Data taken from Fosang et al., 1992. [c] Data taken from Crabbe et al., 1992. [d] Data taken from Marcotte et al., 1992.

These specificity studies permit the identification of substrates and inhibitors with optimized sequences in subsites P_3–P_3'. Stack and Gray (1989) have prepared a synthetic substrate for HFC and HFG that has the fluorescent Trp residue in subsite P_2', the dinitrophenyl (DNP) quenching group on the N-terminus and an Arg residue for enhanced solubility in subsite P_4'. The quenching of the Trp fluorescence by the DNP group in the intact peptide is relieved on hydrolysis of the P_1–P_1' bond and forms the basis for a continuously recording fluorescent assay. On the basis of the data summarized here, a series of similar peptides have already been prepared in which the residues in subsites P_2–P_1' and P_3' were chosen so as to maximize specificity for individual MMP (Netzel-Arnett et al., 1991b). Thus, DNP-Pro-Leu-Gly-Met-Trp-Ser-Arg serves as a good substrate for HFG and HNG, DNP-Pro-Leu-Ala-Leu-Trp-Ala-Arg for HFC, and DNP-

Pro-Leu-Ala-Tyr-Trp-Ala-Arg for HNC. Although no substrate was prepared for PUMP, a good candidate would be DNP-Pro-Met-Ala-Leu-Trp-Met-Arg. Most recently, Knight et al. (1992) have reported that Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 is hydrolyzed by PUMP and HFG at rates that are over 100-fold faster than the best substrates reported here. This implies that either the Mca or Dpa groups in subsites P_4 or P_2', respectively, have particularly favorable interactions with these enzymes.

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