Matrix Metalloproteinase-3 (Stromelysin-1)

IDENTIFICATION AS THE CARTILAGE ACID METALLOPROTEASE AND EFFECT OF pH ON CATALYTIC PROPERTIES AND CALCIUM AFFINITY*

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Human pro-MMP-3 (pro-matrix metalloproteinase-3) was purified from three sources: articular cartilage and conditioned media from synovial fibroblasts and Chinese hamster ovary cells expressing recombinant pro-MMP-3. All three preparations reacted with two monoclonal antibodies specific for human fibroblast pro-MMP-3. Each preparation of active MMP-3 possessed properties identical to those previously reported for the cartilage acid metalloproteinase (MMP-6; Azzo and Woessner, J. F., Jr. (1986) J. Biol. Chem. 261, 5434–5441): an acid pH optimum of 5.5–5.5 for digestion of cartilage aggrecan; digestion of oxidized insulin B-chain at Ala14-Leu15 and Tyr18-Leu17 in a ratio of 3:1; and heat stability at neutral pH. Further characterization of MMP-3 establishes that the acid pH optimum for cartilage aggrecan is not due to substrate denaturation since the same optimum is found by viscosity assay, by SDS-polyacrylamide gel electrophoresis assay of G domain, and by digestion of aggrecan in fresh cartilage fragments in vitro. Fibronectin was also digested optimally at pH 5.5 and NH2-terminal sequence analysis revealed no pH change in a major proteolytic site of cleavage at the Pro298-Leu300 bond. The specificity constant kcat/Km is maximal at pH 5.5 as determined in a quenched fluorescence peptide assay. This is due to an increase in kcat at pH 5.5 without any substantial effect on Km. The affinity of MMP-3 for calcium is decreased about 10-fold at pH 5.3 compared to neutral pH. Finally, the neutral cartilage metalloproteinase is identified as 72-kDa pro-MMP-2 based on Mr, specificity of insulin B-chain cleavage, and reactivity with a specific polyclonal antibody to human MMP-2.

The metabolic breakdown of the extracellular matrix is governed by a family of matrix metalloproteinases (MMPs). There are now 9 enzymes, including macropage elastase, and they share common properties: zinc is in the active center; calcium ions stabilize the enzymes, zymogen forms are activated by organomercurials, the tissue inhibitor of metalloproteases inhibits all enzymes, and all show sequence homology to collagenase (Woessner, 1991). The name "matrixin" has been proposed for this family. The first three members of the family were identified by their ability to digest collagen, gelatin, and proteoglycan. The matrixin with proteoglycanase activity was subsequently named MMP-3 (stromelysin-1).

Although this enzyme was shown to have a broad substrate specificity and pH optimum (Galloway et al., 1983), it is commonly referred to as a neutral matrix metalloprotease. Thus, Flannery et al. (1992) in reporting the cleavage of human aggrecan by MMP-3 at the Asn341-Phe342 bond in the interglobular domain of aggrecan, studied this cleavage at pH 7.5.

Examination of human osteoarthritic cartilage, in which proteoglycan is one of the major constituents, revealed the presence of two extractable enzymes of the metalloprotease family with proteoglycan degrading activities: one with a acid pH optimum and the other with a neutral pH optimum (Sapolsky et al., 1976; Woessner and Selzer, 1984). Subsequently, Azzo and Woessner (1986) purified and partially characterized this acid metalloprotease activity, sometimes referred to as MMP-6. We have shown that this acid metalloprotease is elevated in extracts of osteoarthritic human articular cartilage (Dean et al., 1989). Campbell et al. (1986) also reported a acid metalloprotease activity secreted by cultures of human articular cartilage explant cultures. Gunja-Smith et al. (1989) purified MMP-3 or stromelysin-1 to homogeneity from cartilage by immunoaffinity chromatography. This enzyme displayed an acid pH optimum of 5.5 for the digestion of aggrecan, but it appeared to be distinct from MMP-6 because it was readily inhibited by 0.1 M maleate and appeared to have a different calcium optimum as measured at neutral pH (Okada et al., 1986).

In the present paper we have examined MMP-3 in more detail with respect to acid pH optimum and re-examined its relationship to the acid metalloproteinase (MMP-6) isolated from articular cartilage. In particular we have determined the effects of pH on digestion of matrix components aggrecan and fibronectin, on the specificity constant for the cleavage of a MCA peptide substrate, and on the affinity of MMP-3 for calcium. We have compared the enzyme purified directly from cartilage and the recombinant enzyme expressed by CHO cells to the well-characterized MMP-3 of human synovial fibroblasts in order to be certain there were no differences due to tissue of origin or folding of the recombinant enzyme.
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The results indicate that there is only a single metalloprotease in human cartilage with an acid pH optimum on extracellular matrix macromolecules and that this is MMP-3. Finally, we have re-evaluated the nature of the neutral metalloprotease of cartilage and conclude that it is identical to the 72-kDa progelatinase A (pro-MMP-2).

Materials and Methods

Enzyme Preparation—Human MMP-3 was obtained from three sources: direct extracts of human articular cartilage matrix from synovial fibroblast cultures, and recombinant proenzyme from CHO cells. Human petrellas were obtained at autopsy and the proenzyme was extracted and purified previously described (Gujna-Smith et al., 1989). The fibroblast MMP-3 was purified from conditioned media of cultures of human rheumatoid synovial fibroblasts (Okada et al., 1989) and was generously provided by Dr. Hideaki Nagase, University of Kansas Medical Center.

Recombinant prostromelysin-1 was purified from conditioned media of CHO cells transfected with human fibroblast pro-MMP-3 cDNA as described by Housley et al. (1989). The purified CHO pro-progelatinase A was finally adjusting to pH 7.5, 0.025 M NaCl, 0.005 M CaCl2, and 0.0005% Brij-35 and stored frozen at −80 °C. Human pro-MMP-1 (fibroblast interstitial procollagenase) was purified from conditioned medium of cultured human skin fibroblasts as described by He et al. (1989). Pro-MMP-2 (progelatinase A) purified from rheumatoid synovial fibroblasts (Okada et al., 1986) was a generous gift of Dr. H. Nagase. Protein content of the proenzyme preparations was determined by the bicinchoninic acid method of Hill and Straks (1988).

Latent pro-MMP-1 and pro-MMP-3 were activated by incubation with 0.5–1.5 mM aminophenylmercuric acetate (APMA) for up to 18 h at 37 °C, followed by dialysis against 0.025 M Tris buffer, pH 7.5, containing 0.025 M NaCl, 0.005 M CaCl2, and 0.0005% Brij-35 to remove APMA. The high Mγ, active MMP-3 was prepared by incubation with 1.1-tosylamido-2-phenylethyl chloromethyl ketone-trypsin (1:200 (w/w) ratio of trypsin to pro-MMP-3) as described by Wilhelm et al. (1987). Pro-MMP-2 was activated by the addition of APMA to a final concentration of 0.5 mM in the assays of insulin B-chain digestion described below.

Enzyme Assays—Proteoglycan digestion was assessed by use of bovine nasal proteoglycan monomer (aggrecan) incorporated in polyacrylamide beads (Nagase and Woessner, 1980) dispersed overnight in standard Tris buffer at a concentration of 0.1 mg/ml. Fibroblast MMP-3 (1 μg) was made up to 0.5 ml in standard Tris buffer and mixed with 0.5 ml of aggrecan solution in a 1:1 bovine nasal proteoglycan monomer (aggrecan) incorporated in polyacrylamide beads for 17 h at 37 °C, a microelectrode was touched to the surface of the beads to get a final pH reading. Duplicate samples were assayed, and at each pH a blank containing 1 mM 1,10-phenanthroline was included since the blank is strongly affected by pH. Pepsatin (1 μg) was added to each assay below pH 6.5 to protect against any possible contaminant of cathepsin D. The resulting pH curve is referred to as "low malleate curve." This curve was also repeated using recombinant MMP-3.

A further curve was run using mixtures of 0.1 μl of standard Tris buffer and 0.1 μl of 0.2 M MES buffer (with the same salts as standard Tris buffer). Below pH 7.5, the MES-Tris buffers were formed at pH 7.5 by adding the 0.2 M MES buffer to 0.1 μl of standard Tris buffer and mixing with 0.1 μl of standard Tris buffer the desired final pH was achieved (final MES concentration is 0.1 M). A third curve was prepared in which 0.2 M Tris maleate was added after adjustment to give the correct final pH; in this case the concentration of maleate added was achieved by adding the enzyme in 0.5 ml of 0.2 M MES buffer, pH 5.45; and pH 5.3 was obtained by adding enzyme in 0.5 ml of 0.2 MES buffer, pH 5.0. pH value changes are not linear, rather they follow a second-order kinetic behavior (Woessner, 1973).

Viscometric Determination of Proteoglycan Digestion—Bovine nasal cartilage aggrecan (Nagase and Woessner, 1980) was dispersed overnight in standard Tris buffer at a concentration of 10 mg/ml. Fibroblast MMP-3 (1 μg) was made up to 0.5 ml in standard Tris buffer and mixed with 0.5 ml of aggrecan solution in a 1-ml Cannon-Fenske viscometer equilibrated in a 37 °C bath. This starting concentration gave an initial relative viscosity of about 3.0 compared to buffer alone. The flow time was measured at 30-min intervals over 3 h. The viscometer was kept in the bath overnight to determine the final viscosity; the digestion was terminated by adding 1 ml of 0.05 M Na2EDTA to achieve an enzyme in 0.5 ml of 0.2 M MES buffer, pH 5.45; and pH 5.3 was obtained by adding enzyme in 0.5 ml of 0.2 MES buffer, pH 5.0. pH value changes are not linear, rather they follow a second-order kinetic behavior (Woessner, 1973).

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Dissociation of Soluble Aggrecan—Bovine nasal aggrecan aggregate (ICN) at a concentration of 7.5 mg/ml in 0.1 M Tris-HCl, pH 7.5, or 0.1 M MES, pH 5.5, containing 0.1 M NaCl, 0.01 M CaCl2, and 0.005% Brij-35 was incubated with 1.0 μg/ml low Mγ, form of recombinant CHO MMP-3 at 37 °C for various times up to 16 h. Aliquots of these reaction mixtures were removed and they were quenched by addition of 0.5 ml of sample buffer (Laemmli, 1975) containing 5% β-mercaptoethanol and analyzed on 6% SDS-polyacrylamide gels with silver staining. In some cases, EDTA was added at a final concentration of 10 mM to reaction mixtures at both pH values to inhibit exogenous MMP-3.

Cartilage Chip Assays—Fresh bovine cartilage from the metacarpal-phalangeal joint of 1–3-month-old calves was collected as chips with a 3-mm curette and chilled on ice. Chips of about 5 mg wet weight were washed in phosphate-buffered saline and incubated in duplicate in 200 μl of pH 7.5 Tris-HCl buffer; pH 5.5 and 0.5 MES-HCl buffer, and pH 5.5 Tris maleate buffer. Each buffer mixture was incubated with a mixture of protease inhibitors: 1 μM E-64, 10 μM leupeptin, and 10 μM pepstatin. Samples were incubated with and without active low Mγ, recombinant MMP-3 (1 μg/ml) for 15 h. Aliquots of fluid were removed at 3 and 15 h and assayed for proteoglycan digestion products by modified chondrosin 6-sulfate as standard.

Dissociation of Plasma Fibronectin—Plasma fibronectin (New York Blood Bank) was diluted to 0.5 mg/ml in either 0.05 M Tris buffer, pH 7.5, or 0.5 MES-HCl, pH 5.5, containing 0.15 M NaCl, 0.01 M CaCl2, and 0.005% Brij-35. This high Mγ, active form of MMP-3 (1 μg) was incubated with 0.15 ml of fibronectin for up to 18 h at 37 °C. Control samples containing either trypsin/soybean trypsin inhibitor (mock activation) or MMP-3 and 1,10-phenanthroline (1 mM) did not result in any detectable cleavage of this substrate. Samples (25 μl) were quenched with an equal volume of SDS sample buffer containing 5% β-mercaptoethanol, 0.5 M phenylmethylsulfonyl fluoride, and 10 mM EDTA and then subjected to SDS-gel electrophoresis in 6%
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acrylamide gel. Substrate controls contained either buffer or trypsin/soybean trypsin inhibitor. Gels were stained with Coomassie Blue R-250. The gels were scanned with an LKB laser densitometer to quantify substrate disappearance. Some samples were electroblotted onto ABI-polyvinylidene fluoride membranes for NH2-terminal sequence analysis by the method of Macnairra (1987).

Digestion of B-chain by Various Members of the MMP Family—Oxidized bovine insulin B-chain (Sigma) was digested by cartilage and recombinant MMP-3 and by fibroblast MMP-1 and MMP-2. These enzymes were all in their active form except MMP-2, which had 0.5 mM APMA in the reaction mixture. The B-chain (50 µg) was incubated with enzyme in a final volume of 0.1 M Tris-HCl at 37 °C for varying times up to 24 h using the same buffers as described above for plasma fibrocin digestion. The amounts of enzyme used were recombinant MMP-3 (180 ng), cartilage MMP-3 (protein estimate, 90-180 ng of protein), MMP-1 and MMP-2 (240 ng). Aliquots were diluted with 0.1% trifluoroacetic acid, and 0.5 µg of B-chain was injected into a Beckman C-18 ODS reverse-phase column (0.46 X 4.5 cm) and eluted with a linear gradient of 0-40% buffer (70% acetonitrile/water and 0.1% trifluoroacetic acid). Absorbance was monitored at 215 nm. The nomenclature of the insulin B-chain fragments is the following: fragment 1 (residues 1-14), fragment 2 (residues 1-16), fragment 3 (residues 17-30), and fragment 4 (residues 15-30).

The Effect of pH on the Calcium Affinity of MMP-3—The effect of pH on enzyme affinity for calcium was determined by varying the concentration of calcium (0.06-10 mM) at both pH values in the standard [Nle]substance P assay described by Harrison et al., (1989). Briefly, MMP-3 activity was measured by combining 300 µM [Nle1]substance P (Bachem Biosciences) with 2.7 pmol of active MMP-3 in a volume of 300 µl of 0.1 M buffer without azide and adjusting the final Brij-35 concentration to 0.005%. Reaction mixtures were incubated for various times at 37 °C and aliquots of 20 µl were injected into a Beckman C-18 ODS reverse-phase column (0.46 X 4.5 cm) run isocratically in 18% acetonitrile containing 0.1% trifluoroacetic acid at a flow rate of 1 ml/min. The eluant was monitored at 215 nm. Enzyme activity was expressed as nanomoles of the carboxyl-terminal fragment of [Nle]substance P (fragment 7-11) formed per min as described above. Results were expressed percent relative activity, 10 mM Ca2+= 100. The effect of pH on MMP-3 affinity for calcium was also assessed using the proteoglycan bead assay described above. In these assays, standard Tris buffer, pH 7.5; 0.1 M Tris maleate, pH 5.3; and MES-Tris, pH 5.3, were used and calcium was varied from 0.06 to 10 mM. The data were expressed as percent relative activity, 10 mM Ca2+, Tris, pH 7.5 = 100.

Quenched Fluorescent Assay and the Effect of pH on the Specificity Constant (Kj/Km)—The continuous fluorescent assay of Knight et al. (1992) was performed using the newly developed MCA-Pro-Leu-Gly-Leu-Dnp-Ala-Arg-NH2 substrate (Bachem Biosciences) in a Perkin-Elmer Cetus LS-50B spectrofluorometer adapted with a 96-well microtiter plate attachment at 22 °C at various pH values in a final volume of 0.2 ml. Initial velocities using 10 mM MMP-3 were measured continously over a 30-min interval at substrate concentrations ranging from the 1.25 to 40 µM range in a 0.1 M buffer, containing 0.005 M CaCl2, 0.1 M NaCl, 0.005% Brij-35, and 5% dimethyl sulfoxide. Since the dinitrophenol group quenches the fluorescence of the MCA-peptide product at high concentrations of initial substrate, a series of standard curves containing a MCA-reference peptide (MCA-Pro-Leu-Gly-Dnp-Ala-Arg-NH2) dissolved in 0.02 M sodium acetate buffer, pH 4.5, and 5% dimethyl sulfoxide and various concentrations of the MCA-peptide substrate (1.25-40 µM) were prepared in order to determine the concentration of the MCA-peptide formed at various initial substrate concentrations. Nanomolar concentrations of MCA product formed per s were calculated from these standard curves. Michaelis-Menten kinetic constants were calculated by nonlinear regression analysis using the ENZ-FITTER program.

Zymography and Immunoprecipitation of the 72-kDa Gelatinase (MMP-2) —Gelatin zymography was a modification of the procedure described by Herron et al. (1986). SDS-PAGE was performed in 10% acrylamide gels containing 0.5% gelatin. The gels were rinsed with Triton X-100 and then incubated at 37 °C for 18 h in 0.05 M Tris-HCl, pH 7.5, 0.2 M NaCl, 0.01 M CaCl2, 1.0 µM ZnCl2, 1 mM phenylmethylsulfonyl fluoride, 0.02% NaN3, and 0.005% Brij-35. Gels were stained with Coomassie Blue R-250. Both latent and active forms of MMP-2 protein could be clearly visualized in the gel. Cartilage extracts were prepared as previously described by Azzo and Woesner (1986). The extract was passed through DEAE-Sepharose to remove aggrecan and then chromatographed on a Aca-54 column. Fractions (26-28) containing MMP-2 activity by gelatin zymography were concentrated 5-fold by ultrafiltration and 50 µl were reacted with protein A-agarose (50-95 µg) containing 5.0 µg of either rabbit anti-human MMP-2 IgG (Collier et al., 1988) or non-immune rabbit IgG in 10 mM Tris-HCl, pH 7.5, 0.05 M NaCl, 0.5% Nonidet P-40, and 0.1% bovine serum albumin. Samples were incubated at 4 °C and washed three times with buffer. Immune complexes were dissolved in SDS sample buffer without reducing agent and analyzed by zymography.

RESULTS

Comparison of Cartilage, Synovial Fibroblast, and Recombinant CHO MMP-3—Recombinant CHO MMP-3 and synovial fibroblast MMP-3 were found to have the same specific activities on proteoglycan (1050 µg digested per min/mg enzyme) and on transferrin (710 µg/min/mg enzyme), based on the protein measurements of latent proenzyme of M, 56,000. The cartilage enzyme could not be compared because its low protein content precluded accurate determination of values of protein concentration. No differences in pH curves, substrate specificity, or rates of hydrolysis were found. All three preparations were equally stable to heating at 60 °C and pH 7.5 for 4 h, losing less than 20% of their activity over this period (data not shown).

As shown in Fig. 1, Western blotting demonstrated that all three preparations reacted similarly with two different MMP-3 monoclonal antibodies (mAb). MAB 188.7 reacts with an epitope in the NH2-terminal propeptide domain of the glycosylated (66-kDa) and nonglycosylated (58-kDa) forms of pro-MMP-3. MAB 188.2 recognizes an epitope in the COOH-terminal hemopexin domain and, therefore, both the latent proenzyme and high M, activated enzyme forms are recognized by this monoclonal antibody. As shown in Fig. 1, the recombinant CHO pro-MMP-3 is partially active.

pH Curves of Proteoglycan Digestion—Three different buffer systems were used to construct the pH curves for synovial fibroblast MMP-3 shown in Fig. 2: MES buffer, 0.1 M Tris maleate as used earlier by Gunja-Smith et al. (1989) and low Tris maleate. It is seen that the pH optimum depends on the level of maleate in these assays. In the low Tris maleate assays the actual concentration of maleate at pH 5.5 is about 20 mM which is not inhibitory and so an optimum is observed
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Fig. 2. pH dependence of proteoglycan bead digestion by synovial fibroblast MMP-3. Enzyme assay and preparation of pH curves are described under "Materials and Methods." The curve labeled MES contains MES buffer at 0.1 M at all pH values below 7; 0.1 M maleate contains that concentration of Tris maleate at all pH values below 7; and low maleate is obtained by adjusting pH 7.5 Tris buffer with varying concentrations of Tris maleate. All buffers contain 10 mM CaCl₂.

Fig. 3. Viscosimetric study of soluble proteoglycan digestion by synovial fibroblast MMP-3. The effect of 3 pH conditions is shown as a second-order plot (described under "Materials and Methods").

Fig. 4. Time course of digestion of cartilage aggrecan by recombinant MMP-3. Digestion and electrophoresis are described under "Materials and Methods." Each lane represents 5.0 µg of total protein detected by silver staining. The 58-kDa G₁ domain is shown running at the bottom of the gels. Addition of EDTA (10 mM) totally blocked appearance of this band at both pH values.

TABLE I

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Tris pH 7.5</th>
<th>MES pH 6.5</th>
<th>MES pH 5.5</th>
<th>Maleate pH 5.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer alone</td>
<td>15*</td>
<td>15</td>
<td>25</td>
<td>50</td>
</tr>
<tr>
<td>Enzyme added</td>
<td>56</td>
<td>48</td>
<td>191</td>
<td>172</td>
</tr>
<tr>
<td>Net enzyme effect</td>
<td>41</td>
<td>33</td>
<td>166</td>
<td>122</td>
</tr>
</tbody>
</table>

*µg of chondroitin 6-sulfate equivalents/ml/3 h/chip. Data were obtained from a single experiment.
FIG. 5. Time course of digestion of plasma fibronectin by recombinant MMP-3. Digestion and electrophoresis are described under "Materials and Methods." Panel A, disappearance of intact fibronectin subunit as quantified by LKB laser densitometry of panel E. Results are expressed as % of fibronectin remaining. Panel B, SDS-PAGE gel (6% acrylamide) of time course of fibronectin digestion stained with Coo massie Blue. Control samples incubated with trypsin/soybean trypsin inhibitor (M) used to activate pro-MMP-3 revealed no digestion at either pH.

The Affinity of MMP-3 for Calcium is pH-dependent—The affinity of MMP-3 for calcium is pH-dependent as seen in Fig. 6. The calcium ion concentration required for optimum activity at pH 7.5 in Tris-HCl buffer is 0.5 mM. This same result was reported by Okada et al. (1986) and Housley et al. (1993). However, at pH 5.3 in MES-Tris (Fig. 6A) or MES-HCl (Fig. 6B) the requirement is increased about 10-fold to 5-6 mM. This effect of pH on affinity of MMP-3 for calcium results in a decrease in enzyme stability at acid pH since preincubation of MMP-3 at acid pH at 0.5 mM calcium was shown to significantly increase the rate of enzyme autolysis under these conditions.

Effect of pH on the Kinetic Constants—We have utilized the newly designed fluorescent MCA-peptide assay reported by Knight et al. (1992) to measure the effect of pH on catalytic efficiency and kinetic constants of MMP-3. As shown in Table II, the 46-kDa active form of MMP-3 exhibited approximately a 2-fold increase in the $k_{cat}/K_m$ at pH 5.5 compared to neutral pH. This increase in catalytic efficiency observed for MMP-3 at acid pH is due to an increase in $k_{cat}$ without any significant effect on $K_m$.

Digestion of Insulin B-Chain—Digestion with both cartilage and recombinant MMP-3 resulted in the cleavage of two bonds at Ala$^{14}$-Leu$^{15}$ and Tyr$^{16}$-Leu$^{17}$ as determined by high performance liquid chromatography (Fig. 7) and the amino acid quantitation of each peptide. There is no decrease in total Leu or Tyr in the products, indicating that the enzyme makes only a single cleavage in one chain, and does not excise the intervening Leu-Tyr dipeptide.

FVNQHLCGSHLVEA*LY*LVCQGQRGFYYTPKA

These are the same two cleavage points and in the same proportion as reported for the acid metalloprotease of cartilage (Azzo and Woessner, 1986). Furthermore, we find that at pH 5.3 the specificity is not altered. However, the rate of cleavage of the B-chain is the same at pH 5.3 and 7.5. This is
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**FIG. 6. Calcium affinity of MMP-3 as a function of pH.** Panel A, pH dependence of proteoglycan bead digestion by recombinant MMP-3. The pH was adjusted using standard buffer, pH 7.5, or equal volumes of standard buffer and 0.2 M Tris maleate, pH 5.02, to give final pH 5.3 (details under "Materials and Methods"). Calcium could not be lowered to 0 because the enzyme preparation carried some calcium with it (about 0.06 mM final concentration).

**TABLE II**
**Effect of pH on the catalytic efficiency (kcat/Km) for the hydrolysis of MCA-peptide substrate by recombinant MMP-3**

<table>
<thead>
<tr>
<th>pH</th>
<th>Buffer</th>
<th>kcat</th>
<th>Km</th>
<th>kcat/Km</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.5</td>
<td>Tris</td>
<td>0.19</td>
<td>47</td>
<td>4040</td>
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<tr>
<td>6.5</td>
<td>MES</td>
<td>0.32</td>
<td>65</td>
<td>4920</td>
</tr>
<tr>
<td>5.5</td>
<td>MES</td>
<td>0.34</td>
<td>42</td>
<td>8090</td>
</tr>
<tr>
<td>5.5</td>
<td>Acetate</td>
<td>0.36</td>
<td>43</td>
<td>8370</td>
</tr>
</tbody>
</table>

The only substrate that did not show an acid pH optimum; however, only a single relatively high concentration of substrate was tested. In contrast, human synovial fibroblast gelatinase A (MMP-2, Fig. 7) cleaves the B-chain at the same two bonds as MMP-3, but in reverse proportions of 0.3:1 at neutral pH. This is directly comparable to the ratio of 0.5:1 reported by Azzo and Woessner (1986) for the neutral metalloproteinase of cartilage. The B-chain is a poor substrate for MMP-2 since the results shown in Fig. 7 are for 14-h digestion with MMP-2 versus 1.5 h with MMP-3 at similar molarities of enzyme. Human fibroblast MMP-1 (Fig. 7), which cleaved insulin B-chain at a single Tyr16-Leu17 site, is included to show that its action is distinct from that of MMP-2 and MMP-3.

**DISCUSSION**

**MMP-3 as an Acid Metalloprotease**—This study had three main goals: 1) to establish that MMP-3 extracted from human cartilage and the recombinant enzyme expressed by CHO cells did not differ from MMP-3 secreted by human synovial fibroblasts; 2) to explore in more detail the novel acid pH optimum observed for MMP-3; and 3) to ascertain whether the previously reported acid metalloproteinase of cartilage (MMP-6, Azzo and Woessner (1986)) might not, in fact, correspond to MMP-3 while the neutral cartilage metalloproteinase might be identified as gelatinase A (MMP-2). All three enzymes reacted similarly with two monoclonal antibodies specific for fibroblast MMP-3; exhibited the same limited specificity on the B-chain of insulin, and were heat stable. Therefore, we have not been able to find any
A-Sepharose-fraction 26 blank. The MMP-2 with a specific I& fraction to human pro-MMP-2.

proteoglycan digestion (Azzo and Woessner, 1986; Gunja-beads might alter or constrain the conformation of aggrecan, beads as a substrate, it might be argued that the acetone used immunoprecipitates of fractions 26 and 28, respectively; Sepharose-anti MMP-2 filtration chromatography, and subjected to immunoprecipitation and

Therefore, several additional assays have been employed. First, soluble aggrecan which had been lyophilized but not exposed to organic solvents was analyzed by viscosimetry and by SDS-polyacrylamide gel electrophoresis assay. Second, exogenous MMP-3 was added to fresh cartilage chips in which aggrecan remained in its native state in situ. Both methods confirmed the acid pH optimum of 5.3-5.5 for digestion of aggrecan by MMP-3. The viscosimetric study further showed that there was no sudden change in viscosity (conformation) as the pH was lowered from 7.5 to 5.3. There might still be subtle alterations in the G1 and G2 globular domains which would not significantly affect viscosity but which render scissile bonds more accessible to hydrolysis. However, other metalloproteinases such as MMP-7 of rat uterus (Woessner and Taplin, 1988) and Ht-d metalloprotease of Crotalus atrox venom3 show pH optima of about 7 in the same proteoglycan bead assay.

We present evidence that aggrecan and fibronectin are both digested optimally at pH 5.3 by MMP-3. Fosang et al. (1991) have shown that MMP-3 cleavage of the aggrecan fragment G1-G2 between the interglobular domain is also optimal at pH 5.5. Murphy et al. (1991) also report an acid pH optimum on this substrate but did not observe a 3:1 ratio in activity between acid and neutral pH. We also showed a pH optimum of about 6.2 for the digestion of Azocoll by MMP-3 (Gunja-Smith et al., 1989). On the other hand, the uterine metalloproteinase (MMP-7) digesting beads or Azocoll (Woessner and Taplin, 1988) and MMP-2 digestion of gelatin (Seltzer et al., 1981) do not show acid pH optima, indicating that MMP-3 appears to be unique in this respect.

Is MMP-3 to be Considered as an Acid Metalloproteinase?—In the case of the thermostable bacterial zinc metalloproteinase, thermolysin, the digestion of small peptide substrates shows two pK values: one near pH 5.0 and the other at 8.5. There is a broad, relatively flat, pH optimum between these two limits when the second-order rate constant (k_m/K_m) is plotted against pH (Kunugi et al., 1982). The case of MMP-3 is more complex, with at least one more pK value for macromolecular (Fig. 1) or peptide substrates (Harrison et al., 1992). Our data on the effect of pH on the catalytic efficiency of recombinant MMP-3 hydrolysis of the synthetic MCA-peptide substrate shown in Table II is in agreement with the results of Harrison et al. (1992) who reported pKs of 5.4, 6.1, and 9.5 for recombinant MMP-3. Harrison et al. (1992) concluded that the enhanced catalytic efficiency of MMP-3 at acidic pH was primarily due to ionization of a critical residue in the active site. When considering macromolecular substrates for which the kinetic constants are unknown, it is probable that protein concentrations (below 10^-4 M) are below their respective K_m values, and therefore, one sees rates of that are as much as 3-4 times higher at acidic pH compared to neutral pH. Additional studies will be needed in order to determine whether the increased catalytic efficiency of MMP-3 in the hydrolysis of macromolecules at acid pH is the result of either improved binding of these substrates and/or an increased turnover number.

Calcium Requirement of MMP-3—The present studies indicate that the affinity of MMP-3 for calcium is highly dependent on pH. This could be due to changes in ionization of one or more side chains in the enzyme that are involved in calcium binding. In thermolysin there are stretches of 5 acidic residues in three of four calcium binding sites (Matthews et al., 1972). Calcium has recently been shown to be responsible for the thermostability properties of MMP-3 (Housley et al., 1993). Therefore, the effect of acid pH in reducing calcium affinity of MMP-3 is consistent with a role of calcium binding sites speculated to be conserved within the low M_2 catalytic domain of MMP-3. A similar observation on the effect of calcium on mouse bone collagenase (MMP-1) activity at pH 5.5 has been reported by Eeckhout (1990). Although MMP-3 has evolved to function efficiently at acidic pH in the extracellular matrix, the concentrations of free extracellular calcium in the range of 1–2 mM would have an important role in regulating MMP-3 activity in acidic pH microenvironments. That is, the level might be too low to preserve activity for long, except in acidic environments such as resorptive zones of the bone matrix where the free calcium might rise to 10–20 mM (Eeckhout, 1990).

Relationship of MMP-3 to the Cartilage Acid Metalloprotei-

nase.—The calcium affinity, thermal stability, and digestion of the oxidized B-chain of insulin demonstrated here for MMP-3 match exactly the properties previously reported for the cartilage acid metalloproteinase (Azzo and Woessner, 1986). The only difference was in the pH curves. In the earlier study, the acid enzyme digested aggrecan optimally at pH 5.3 even in the presence of 0.1 M maleate; whereas in a subsequent study of MMP-3, Gunja-Smith et al. (1989) found that 0.1 M maleate shifted this pH optimum to the neutral range. Further support to the idea that the acid activity was distinct from MMP-3 came from two additional observations. First, the shift in the calcium optimum for the cartilage acid metalloprotease and second, the activity present in cartilage extracts assayed at pH 5.5 in 0.1 M maleate was 5-fold higher than the

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3 J. F. Woessner, Jr. and C. J. Taplin, unpublished data.
activity measured at neutral pH. These results led to the conclusion that acid cartilage metalloproteinase was distinct from MMP-3 which was initially identified as the neutral metalloproteinase from cartilage. A re-examination of the earlier study of Azzo and Woessner revealed that the actual concentration of maleate in these assays was only 20 mM, a concentration which is not inhibitory. In addition, we see from the data in Table I, that 0.1 M maleate is not nearly as inhibitory in this chip assay. We speculate that this is due to interaction of maleate with other components present in the cartilage matrix and crude extracts. We now conclude that the acid cartilage enzyme (initially designated as MMP-6) is not a novel cartilage-specific proteinase, but is identical to the previously cloned and characterized metalloproteinase MMP-3 (stromelysin-1, Okada et al. (1986), Whitham et al. (1987), and Wilhelm et al. (1987)).

Identity of the Neutral Metalloproteinase of Cartilage—Several lines of evidence indicate that the neutral metalloproteinase of cartilage (Azzo and Woessner, 1986) is actually the 72-kDa gelatinase (MMP-2, gelatinase A). First, the digestion of insulin B-chain by MMP-2 (Fig. 7) matches that reported by Azzo and Woessner (1986). Second, we have now shown that MMP-2 is present in extracts of articular cartilage by use of a specific antibody against this enzyme (Fig. 8). Finally, the pH optimum for this enzyme is slightly alkaline (Seltzer et al. 1992) that recombinant MMP-2 cleaves the Asn \(^{341}\).Phe\(^{182}\) bond in the interglobular domain of the core protein of cartilage aggrecan.

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