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# Impact of Matrix Metalloproteinases on Inhibition of Mineralization by Fetuin

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Impact of Matrix Metalloproteinases on Inhibition of Mineralization by Fetuin

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Matrilysin; stromelysin; calcium phosphate; mineralization; periodontal diseases

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**Background and Objective:** Human subjects affected by inflammatory diseases such as periodontitis may be at increased risk for the development of cardiovascular diseases and calcification of atheromas but the potential mechanisms are not defined. Alpha-2-Heremans Schmid glycoprotein (fetuin A) is an abundant ~49 kDa serum glycoprotein that inhibits ectopic arterial calcification. We examined whether matrix metalloproteinases (MMPs), which are increased in inflammatory diseases including periodontitis, bind and degrade fetuin and alter its ability to inhibit calcification in vitro.

**Materials and Methods:** Binding and cleavage of fetuin by MMPs were assessed by SDS-PAGE, in silico analyses and mass spectrometry. The effects of intact and MMP-degraded human fetuin on mineralization were measured in a cell-free assay.

**Results:** From in silico analyses and literature review, we found that only matrix metalloproteinases 3 (MMP-3; stromelysin) and 7 (MMP-7; matrilysin) were predicted to cleave human fetuin at levels that were physiologically relevant. In vitro assays showed that MMP-7 and to a lesser extent MMP-3, degraded human fetuin in a time- and dose-dependent fashion. Fetuin peptides generated by MMP-7 cleavage were identified and sequenced by mass spectrometry; novel cleavage sites were found. Hydroxyapatite mineralization in vitro was strongly inhibited by fetuin (>1 μM) as was MMP-3-cleaved fetuin, while MMP-7-cleaved fetuin was 3-fold less effective in blocking mineralization.

**Conclusion:** MMP-7 and less so MMP-3, affect the ability of fetuin to inhibit the formation of hydroxyapatite in vitro. These data suggest that the MMPs increased in inflammatory diseases such as periodontitis could affect regulation of mineralization and potentially enhance the risk of calcified atheroma formation.
Calcification in the intima of blood vessels is associated with several cardiovascular diseases including end-stage renal disease (1, 2). Vascular calcification within atheromas requires the deposition of hydroxyapatite; the amount of calcification is a marker of the atherosclerotic burden and may mediate loss of arterial elasticity (2). Several cohort and cross-sectional studies have found positive associations between periodontitis and increased risk of cardiovascular diseases and vascular calcification (3-8) but the underlying mechanisms that explain if and how periodontitis and other inflammatory diseases enhance the formation of calcifying atheromas are not defined. While there is no definitive evidence for a causal association between cardiovascular diseases and periodontitis (9), it is nevertheless important to identify potential pathophysiological mechanisms that could contribute to this link.

Vascular calcification may be initiated by several mechanisms (2, 10) that include loss of inhibition of calcification, induction of bone formation and cell death, which can nucleate initial calcification processes. Notably, several proteins may be involved in suppressing calcification in atheromas including osteopontin (1, 2, 10, 11), matrix Gla protein (12), osteocalcin and the alpha-2-Heremans Schmid glycoprotein (human homologue of fetuin A) (13, 14). Fetuin is an abundant serum sialoprotein (Mr ~49,000) (15, 16) that is a member of the cystatin superfamily (17, 18) and is synthesized in the liver (19). Fetuin, which is incorporated into mineralized tissues and plays an important role in bone formation and resorption (18, 20, 21), inhibits calcification and prevents precipitation of calcium phosphate (22)(23). Fetuin may act as a crystal poison (23-25) but may also inhibit mineralization by interfering with the differentiation of cells with a mineralizing phenotype (26-29). Low levels of fetuin in the serum are associated with increased levels of vascular calcification (30). In view of the relationship between low levels of intact fetuin in the serum with increased risk for vascular calcification, we considered that MMPs upregulated in periodontitis could reduce levels of fetuin in serum as a consequence
of their ability to degrade this protein (31). Several enzymes that are increased in gingival
crevicular fluid of periodontitis patients, such as alkaline phosphatase (32, 33), ß-glucuronidase
(32, 34-36), aspartate aminotransferase (37-39), lactate dehydrogenase (40, 41), neutrophil
elastase (42, 43) and cathepsins B (40) and D (36), could potentially inhibit the function of
fetuin, but none of these enzymes has been reported to degrade fetuin.

MMPs are an important family of zinc-dependent endopeptidases that are involved in the
destruction of the extracellular matrix in periodontal diseases (44-46). Notably, the
concentrations of MMP-3(44-46), MMP-7 (47, 48) and MMP-8 (49) are markedly increased in
the gingival crevicular fluid of patients with periodontitis. MMP-9 is increased in the serum of
patients with periodontitis (31) and among H. pylori-infected subjects, gastric cancer patients
exhibit higher serum levels of MMP-3 and MMP-7 than those with duodenal ulcer and gastritis
(50). Currently there are limited data on the relationship between concentrations in blood of
other MMPs and periodontal status. As fetuin can interact with MMPs (51), we considered that
the binding of MMPs to fetuin may facilitate fetuin degradation (52, 53).

Previous studies have shown that complete digestion of human fetuin occurs within 12
hours when it is incubated with a 10-fold molar excess of either MMP-2 or MMP-7 (52). In
contrast, at equimolar ratios, neither of these enzymes can degrade fetuin, suggesting that MMP-
2 and MMP-7 do not affect regulation of mineralization by fetuin in vivo. While MMP-3 and
MMP-7 can cleave bovine fetuin at specific sites in the C-terminus of the molecule (54), the
impact of these cleavages on the regulation of mineralization has not been examined.
Accordingly, we assessed MMPs that are upregulated in periodontitis and how they interact with
and degrade human fetuin. Second, we examined whether MMP-degraded fetuin loses its ability
to inhibit mineralization in vitro.
Materials and Methods

Reagents

We obtained heparin agarose beads and 4-amino phenylmercuric acetate (APMA) from Sigma-Aldrich (Oakville, ON). Human fetuin was from MyBiosource (San Diego, CA) and antibody to human fetuin was from Alpha Diagnostics (San Antonio, TX). Human recombinant pro-MMP-3, human recombinant MMP-7, manufacturer-activated human recombinant MMP-7 (active), antibody to human MMP-7 and MMP Inhibitor II (N-Hydroxy-1,3-di-(4-methoxybenzenesulphonyl)-5,5-dimethyl-[1,3]-piperazine-2-carboxamide) (55) were from Calbiochem (San Diego, CA).

In silico analysis

We searched online databases (ExPASy PeptideCutter, http://expasy.org/tools/peptidecutter; MEROPS Peptidase Database, http://merops.sanger.ac.uk; CutDB, http://cutdb.burnham.org) for both predicted and reported cleavages of human fetuin by enzymes that are reported to be increased in periodontitis including alkaline phosphatase (32, 33), β-glucuronidase (32, 34-36), aspartate aminotransferase (37-39), lactate dehydrogenase (40, 41), neutrophil elastase (42, 43), cathepsins B (40) and D (34, 36, 44-46), MMP-3 (44-46), MMP-7 (47, 48) and MMP-8 (49).

There is no predicted cleavage of human fetuin by most of these enzymes, however human fetuin is reported to be degraded after overnight incubation with MMP-7 at a very high enzyme:substrate ratio (52). Therefore we focused subsequent analyses on in vitro experiments.

MMP assay

A fluorimetric MMP assay kit (SensoLyte®) was obtained from Anaspec (Fremont, CA). This assay detects MMP-7 and MMP-3 activities in biological samples using a 7-dimethylaminocoumarin-4-acetate (DMC)/nitro-2-1,3-benzoazdiazol-4-yl (NBD) fluorescence resonance energy transfer peptide. When the FRET peptide is not cleaved, the fluorescence of
DMC is quenched by NBD. After cleavage of the peptide by MMP-7 or MMP-3, the fluorescence of DMC is restored and is measured at excitation/emission wavelengths of 370 nm/460 nm in a fluorescence plate reader.

**Fetuin cleavage by MMPs**

Fetuin was incubated in a buffer (50 mM Tris, pH 7.5, 10 mM CaCl$_2$, 150 mM NaCl, 0.05% BRIJ 35; designated TNCB buffer) with MMP-7 or MMP-3 for 0, 1, 2, 4, 8 or 24 hours and at various concentration ratios of fetuin:MMP as indicated in the Results. Dithiothreitol was not included in the buffer as we did not intend to reduce disulphide bridges in fetuin (56) for degradation assays.

We used MMP-7 and MMP-3 that were either unactivated, supplier pre-activated or that we activated *in vitro* with APMA (1 mM in TNCB for 1 hour at room temperature as described earlier (57)). In some experiments, the reversible, binding site-targeting MMP inhibitor II (58) was co-incubated with fetuin and enzymes in digestion experiments. This inhibitor has an IC$_{50}$ of 18.4 nM for MMP-3 and an IC$_{50}$ of 30 nM for MMP-7 (55, 58).

Proteins from the fetuin digestions were separated by SDS-PAGE gels (12%) and stained with Coomassie blue. Protein bands were excised individually and de-stained with ammonium bicarbonate. Gels were shrunk and alkylated, and the peptides were subjected to trypsin digestion before peptide extraction. Samples were evaporated to dryness and analyzed by tandem mass spectrometry (Protein Technology Service, Hospital for Sick Children, Toronto, ON). For prediction of cleavage sites, Scaffold 3 (Proteome Software, Portland, OR) was used with a web-based database for the amino acid sequence of human fetuin (Swissprot database-http://web.expasy.org) and applied to the analysis of the peptides produced from the digestions.

**Fetuin binding to MMPs**
We assessed whether intact fetuin was bound by MMP-7 prior to potential fetuin digestion by MMP-7. Purified MMP-7 (or BSA as a control) was attached to heparin agarose beads and incubated with fetuin for 20 mins. MMP-7 that had been bound to beads previously was incubated with fetuin at specific dilutions. Quantification of the bound whole fetuin versus free fetuin was estimated by dot blotting proteins on to nitrocellulose and immunodetection with antibody to fetuin. Appropriate dilutions of human fetuin standards were dot-blotted in parallel. Quantification of dot densities were used to estimate the kD and BMAX for MMP7-fetuin binding by Scatchard plots. In separate experiments, the efficacy of MMP-7-mediated digestion of fetuin when the MMP-7 was attached to agarose was assessed by separating fetuin degradation products by SDS-PAGE.

**Mineralization assay**

The assay for measurement of hydroxyapatite crystals formed in vitro was based on an original paper by Chen and co-workers (59) and modified by Dr. G. Hunter (University of Western Ontario, London, ON). In 96 well plates (total volume of 100 µl per well) the following solutions were added sequentially: 10 µl phosphate solution (KH$_2$PO$_4$, 10 mM; Na$_2$HPO$_4$, 20 mM; NaCl, 150 mM; pH 7.4), 10 µl calcium solution (CaCl$_2$, 50 mM; Hepes, 50 mM; NaCl, 150 mM; pH 7.4) and 80 µl of test agents in buffer solution (Hepes, 50 mM; NaCl, 150 mM; pH 7.4), which contained 38 µM fetuin and equal volumes of either vehicle (TNCB) or TNCB with MMP-3 or MMP-7. After incubations (up to 4 hours at 23ºC), the supernatant was removed, leaving the nascent hydroxyapatite crystals bound to the bottom of the well. Electron microscopy (see below) was used to confirm that the crystals did indeed attach to the bottom of the wells.

Hydroxyapatite crystals were stained with 75 µl Alizarin Red S (0.5% Alizarin Red S; pH4.2; 5 mins of staining). Then, the alizarin red solution was removed by pipetting. To dissolve the crystal-bound alizarin red, 100 µl of cetylpyridinium chloride solution (100 mM) was added.
to each well and incubated at 23º C for 30 mins. The relative abundance of hydroxyapatite crystals formed in each well was estimated from the amount of Alizarin Red S stain remaining in the cetylpyridinium chloride solution, which was measured by absorbance at 540 nm with a spectrophotometer.

The authenticity of the hydroxyapatite crystals that were formed was assessed by embedding crystals in electron microscopy embedding resin, thin sectioning and examination of unstained sections by transmission electron microscopy. Briefly, the dried mineral was scraped from the bottom of the culture well, dropped into molds and embedded in Quetol-Spurr resin. Sections (100 nm thick) were cut on an RMC MT6000 ultramicrotome, placed on formvar coated-grids and viewed on an FEI Tecnai 20 transmission electron microscope. The samples were not fixed or stained. Electron diffraction analysis of crystals (60) was conducted (Mt. Sinai Hospital, Dept. of Pathology, Toronto, ON) using comparisons with hydroxyapatite standards.

Statistical analyses

For all data shown, experiments were repeated at least 3 times and were conducted on different days. For each individual experiment, at least 3 replicates were analyzed. For continuous variables, means and standard deviations were computed. For the binding experiments, means, standard errors and r² were computed. Comparisons between groups were assessed with analysis of variance and individual group differences were analyzed post hoc with Tukey’s test. The type I error threshold for estimation of statistical significance was set at p<0.05.
Results

We quantified the enzyme activity of three different MMP-7 formulations (unactivated, manufacturer-activated and APMA-activated (1 mM APMA in TNCB for 1 hour at room temperature as described earlier (56)). Enzyme activity was estimated with a fluorimetric MMP assay that provides increased fluorescence after MMP-3 or MMP-7-induced cleavage of the MMP substrate. In time-course experiments that evaluated substrate degradation by the MMP-7 formulations, there was a plateau of enzyme activity approaching one hour (Fig. 1), which was considered to be the optimal time used for APMA activation. While the APMA-activated MMP-7 exhibited the highest activity of the three formulations throughout the time course, the activity levels were not markedly different (<5% difference between groups at one hour). For all subsequent experiments, we used APMA-activated MMP-7 and APMA-activated MMP-3.

Time-course studies were conducted (at 37°C) using MMP-3 and MMP-7 incubated with fetuin at an enzyme to substrate ratio of 1:60 and analysis of fetuin degradation was assessed by SDS-PAGE. MMP-3 and MMP-7 were not detected in the PAGE gels because they were present at much more lower molar concentrations than fetuin. MMP-3 produced limited cleavage of human fetuin that was detectable only after 24 hours of incubation while marked degradation of fetuin was readily observed after 1 hour incubation with MMP-7 (Fig. 2A). By 24 hours there was no intact fetuin remaining after incubation with MMP-7. The prominent fetuin degradation fragments after MMP-7 treatment for 2 hours were ~40 and ~18 kDa; after longer incubations the 18 kDa fragment predominated. Notably, based on densitometry of intact fetuin, MMP-7-mediated degradation of fetuin was inhibited by >90% with the MMP II inhibitor (1 µM; Fig. 2B; IC50 = 30 nM; (55)), suggesting that the cleavage was attributable to the catalytic activity of MMP-7 and not due to non-specific degradation by undefined mechanisms in the assay.
To complement the time-course experiments, digestions of human fetuin by MMP-3 and MMP-7 were conducted using various enzyme:substrate ratios for 4 hours and the relative amounts of full-length fetuin were evaluated by densitometry of stained gels. For MMP-7, 24% of fetuin was degraded at enzyme:substrate=1:60, 71% at E:S=1:30 and 100% at E:S=1:15. For MMP-3, even at E:S=1:10, only 6% of fetuin was degraded. As the relative amount of MMP-7 was increased relative to the amount of substrate (i.e. elevated enzyme:substrate), MMP-7 caused more extensive fetuin degradation and the relative amount of intact fetuin was decreased. Therefore, MMP-7 but not MMP-3 appeared to be relevant for study of pathophysiological fetuin degradation.

Following MMP-7 degradation (24 hours), fragments were cut out of PAGE gels at sites in the gels that were expected to contain fetuin and fetuin fragments (Fig. 3A), and were analyzed by tandem mass spectrometry using Scaffold 3 analysis of the Swiss-Prot protein sequence data base. From a series of analyses, continuous amino acid sequences (i.e. without interruption) of fetuin digestions by MMP-7 were compared to the reported cleavage sites of bovine fetuin by MMP-7 (54). Based on these comparisons and our examination of predicted sequences obtained from all of the peptides identified in the various analyses, we identified a high probability (>95%) cleavage site in human fetuin at R317-H318. We also identified novel predicted cleavage sites (A167-L168; P192-L193), which were based on examination of the fetuin fragments of <20 kDa (Fig. 3B) that included assembly and contiguity analyses of the sequences of the various tryptic peptides that were detected in all samples. In contrast to our analyses, based on the online data bases ExPASy PeptideCutter, MEROPS Peptidase Database, CutDB and Swissprot, MMP-7 was predicted to cleave fetuin at the sequence YDLR-HTFM (amino acid positions 312-313 at the cleavage site).
As MMP-7 evidently cleaved human fetuin (Fig. 2), we examined the initial interaction of MMP-7 with fetuin by in vitro binding studies with MMP-7 bound to heparin agarose beads. We first measured the amount of MMP-7 that bound to beads by dot blot analysis and compared this with known amounts of MMP-7 as standards (Fig. 4A). For assessment of the binding of fetuin to MMP-7, fetuin at various concentrations (1.1-111 µM) was incubated with MMP-7 bound to heparin agarose beads and the relative amounts of bound and free fetuin after 20 min binding were quantified by immunodetection and densitometry of dot blots (Fig. 4B). The time for binding (20 min) was chosen as we anticipated that there would be very limited fetuin degradation by MMP-7 at this time. Measurements of the free versus bound amounts of fetuin allowed computation of BMAX (0.87±0.17) and kD (2.96±1.92 x 10^-9 M; r²=0.87), indicating high affinity binding of fetuin to MMP-7 (Fig. 4B).

As MMP-7 binds tightly to heparin (59), we attached MMP-7 to heparin agarose in binding buffer and the catalytic efficacy of the bound enzyme was assessed. The binding of the MMP-7 to heparin agarose did not affect catalytic activity and bead-bound MMP-7 efficiently degraded fetuin (24 hours digestion; Fig. 4C). Therefore the heparin that was used to attach MMP-7 for the binding assays (Fig. 4B) did not evidently occlude functionally the catalytic site of MMP-7.

For assessing the effect of hydroxyapatite mineralization in vitro by fetuin, we first examined the authenticity of the hydroxyapatite crystals that were formed in the in vitro mineralization assays. Transmission electron microscopy showed small, crystalline structures that formed at the bottom of the dishes (Fig. 5A). Electron diffraction analysis of the mineralized material showed that the d-spacings and intensities of the pattern of the hydroxyapatite formed in the in vitro assay matched the published standards for hydroxyapatite (Table 1).
In preliminary trials that examined mineralization in time course experiments, spectrophotometric absorbance (540 nm) measurements of mineral-bound alizarin red showed that there was a time-dependent increase of absorbance (from 0.5 to 2.2 absorbance units) which peaked at 3.5 hours (Fig. 5B). During the conduct of the experiments in which we optimized the conditions for the mineralization assay we found that freshly prepared alizarin red staining solutions produced somewhat (~30%) lower absorbance measurements than did alizarin red preparations that were made at least one week in advance of the assay. Further, we found that assays conducted with the aged alizarin red preparations were more sensitive in detecting fetuin-induced inhibition of mineralized material than were assays conducted with freshly made alizarin red. Accordingly, when the mineralization assays were conducted for 3.5 hours with one week-aged alizarin red solutions, the absorbance attributable to alizarin red was ~3 units (Fig. 5C). In assays that used 2 µM human fetuin in the mineralization medium, there was a 10-fold reduction of alizarin red absorbance compared to controls (p<0.001; Fig. 5C).

We next conducted mineralization assays with MMP-3 and MMP-7-degraded human fetuin. In preliminary trials we found that fetuin-mediated inhibition of mineralization was not affected when the fetuin was degraded by MMP-7 for less than 4 hours. Accordingly we conducted longer fetuin digestions (24 hours at enzyme:substrate=1:60) in which we anticipated there would be degradation of fetuin into fragments of <20 kDa (Fig. 3A). For MMP-7 degraded fetuin there was 3-fold more mineralization than with intact fetuin (2 µM fetuin; p<0.001; Fig. 5C). In assays that examined MMP-3- degraded fetuin under the same molar ratios and incubation times, there was a small but not significant change of alizarin red absorbance (p>0.2).
Discussion

We examined the cleavage of fetuin by two specific MMPs that are known to be increased in the gingival crevicular fluid of patients with periodontitis, MMP-3 (44-46) and MMP-7 (47, 48). The rationale for focusing on MMP-3 and MMP-7 was based on the initial in silico analyses indicating that of those enzymes known to be increased in tissues affected by periodontitis, cleavage of fetuin was predicted for only these two MMPs. Despite the predicted cleavage of fetuin by MMP-3 and MMP-7 (54), we found that only MMP-7 degraded human fetuin efficiently. Notably, the digestion of human fetuin by MMP-7 was strongly reduced by an MMP inhibitor (55) (IC$_{50}$ = 30 nM), suggesting that the digestion was MMP-specific. Even when incubated at high enzyme-substrate ratios, human fetuin was not efficiently degraded by MMP-3. Based on these findings we focused subsequent studies on the interaction between purified MMP-7 and fetuin. These experiments indicated very tight binding of these two proteins, consistent with our data showing that MMP-7-mediated degradation of fetuin increased proportionately when the enzyme-substrate ratio was increased.

Bovine fetuin and human fetuin exhibit ~70% amino acid identity (61) and the similarity of the arrangement of cysteine residues and of disulfide loops has led to the proposal that bovine and human fetuin are equivalent proteins (61). We found that MMP-7 digestion fragments of human fetuin exhibited novel and unexpected cleavage sites that were different than the predicted cleavage sites for bovine fetuin (54). These variations between predicted and actual cleavages by MMP-7 indicate that small, species-dependent differences of fetuin structure could explain the discrepancies of the observed versus predicted cleavage sites.

We found that human fetuin inhibited mineralization in an in vitro model of hydroxyapatite formation. The inhibitory effect of fetuin on this mineralization assay was reduced after digestion by MMP-7 but was not substantially altered by MMP-3 under the
digestion conditions used here. Further, it would appear that the ~18 kDa fetuin fragment is not as effective as the full-length molecule for the inhibition of mineralization. These data indicate that MMP-7 may be important for the control of biological mineralization in vivo.

We found that the inhibition of mineralization by fetuin seems to be reliant on intact fetuin structure and not by sequestration of fetuin by MMP-7 and MMP-3. Under conditions in which fetuin was not substantially degraded by MMP-7 (20 min co-incubations), there was little effect on the ability of fetuin to regulate mineralization (data not shown). As fetuin is an abundant serum protein and evidently binds MMP-7 with high affinity, it may inhibit the catalytic activity of MMPs, analogous to tissue inhibitors of matrix metalloproteinases (62, 63), but this possibility has not been examined here.

There is considerable interest in the potential association between inflammatory periodontal diseases and the development of cardiovascular diseases in general (64) and the formation of calcified atheromas in particular (8). However, apart from suggestions that periodontal pathogens may play a role in this association (65), little is known about other putative mechanisms, based on biochemical processes that could explain the statistical evidence for this relationship. Conceivably, linkages may exist between different inflammatory diseases and cardiovascular diseases (66), which could be mediated by reductions in the levels of intact fetuin in serum. In this context, we found a 50% reduction in the level of fetuin in patients with severe osteoarthritis (67). Although not investigated in patients with osteoarthritis, MMPs released into the bloodstream because of severe inflammatory joint disease may also contribute to fetuin degradation.

MMP-7 has been implicated in the pathogenesis of atheroma formation (68). If MMP-7 that is produced in periodontitis is important for the regulation of calcification of atheromas, then there would need to be increased concentrations of MMP-7 in the blood. Notably, the
concentration of MMP-9 is increased in the blood of patients with periodontitis (31) and MMP-3 and MMP-7 are increased in serum of patients with gastric cancer (50). Therefore to evaluate the potential biological impact of MMP-7 degradation of fetuin in periodontitis, it will be important to determine whether concentrations of MMP-7 are increased in the blood of patients with periodontitis. We conclude that based on the data described above, MMP-7 may be able to cleave serum fetuin, potentially increasing the risk of vascular calcification. If this notion is correct, MMP-7 levels in the blood may be instructive for assessing risk of vascular calcification in inflammatory diseases such as periodontitis. The definition of such a mechanism, if it were demonstrated in vivo, could explain why there may be a relationship between inflammatory periodontal diseases and calcification in cardiovascular diseases.

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Table 1  Electron diffraction analysis of hydroxyapatite mineralization

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Data are derived from electron diffraction analysis and compared with published standards. Comparison of crystals formed in vitro with standards show close agreement, indicating that with the assay conditions used here, calcium hydroxyapatite crystals formed were authentic.
Figure Legends

**Fig. 1.** MMP catalytic activities of recombinant MMP-7 preparations measured with a DMC/NBD fluorescence resonance energy transfer peptide. After cleavage by MMP-7, the fluorescence of DMC is restored. In this assay the fluorescence was measured at excitation/emission wavelengths of 370 nm/460 nm in a fluorescence plate reader. The three different MMP-7 preparations evaluated were: APMA-activated, unactivated (control) and manufacturer-activated (active MMP-7). Data are relative fluorescence units (RFU) and were measured after indicated incubation times (secs) with a fluorescence peptide.

**Fig. 2.** A. Digestion of human fetuin by MMP-7 and MMP-3. Fetuin was incubated with purified enzymes at a 1:60 enzyme:substrate ratio for the indicated times. The degradation fragments were separated by SDS-PAGE and stained with Coomassie blue. B. Inhibition of MMP-7-mediated degradation of fetuin with the MMP Inhibitor II ($\text{C}_2\text{H}_7\text{N}_5\text{O}_8\text{S}_2; 1 \mu\text{M}$). MMP-7 and human fetuin were co-incubated for 1 or 24 hours or with addition of the MMP inhibitor for 1 or 24 hours as indicated.

**Fig. 3.** A. Typical SDS-PAGE gel of fetuin after no treatment (0 hr) or degradation by MMP-7 (1:60 enzyme:substrate ratio) for 24 hrs that was used for mass spectrometry analysis of fetuin degradation. In this example, an ~18 kDa fragment (circled area) was cut out of gel, subjected to tryptic digestion and analyzed by mass spectrometry. Data from a single sample are shown here. Four uninterrupted, matched (>95% probability) tryptic peptides from the cut-out fragment are shown in yellow highlighter and are overlaid over the whole known amino acid sequence for human fetuin. Green shading shows amino acids with post-translational modifications detected by mass spectrometry. B. The full amino acid sequence of human fetuin indicating location of a disulphide bond (C16-C358, red letters), predicted cleavage site by MMP-7 based on reported data of bovine fetuin (position changed in human fetuin to R317-H318, green letters), and
predicted novel cleavage sites based on data from mass spectrometry of cleavage fragments formed after MMP-7 digestion of fetuin for 24 hours (A167-L168 and P192-L193, blue letters).

**Fig. 4 A.** Dot-blot assay to assess amount of MMP-7 bound to heparin agarose beads. The MMP-7 was eluted from a fixed volume of pelleted beads, dotted on to the membrane and then stained with antibody to MMP-7. MMP-7 standards were included to estimate the amount of MMP-7 bound to the beads. **B.** Estimates of binding affinity based on measurements of free versus bound fetuin with MMP-7 attached to heparin agarose beads. Computations of BMAX and kDa were performed by Scatchard analysis. **C.** MMP-7 or BSA was bound to heparin agarose beads and incubated with human fetuin for 24 hours. Supernates or materials eluted from beads were separated by SDS-PAGE on 12% gels and stained with Coomassie blue. For MMP-7-bound beads, the bead eluent protein migrates at ~20 kDa, which is the approximate size of fetuin digestion products identified in Fig. 2. For beads with bound BSA, fetuin is found only in the supernate fraction and there is no fetuin or digestion products attached to beads.

**Fig. 5 A.** Transmission electron micrograph of a thin section of unstained hydroxyapatite crystals formed at the bottom of a culture dish with scale bar for magnification. **B.** Measurements of alizarin red absorbance in hydroxyapatite mineralization assays conducted over time with 0 or 2 µM fetuin as indicated in the inset. Data are mean±standard deviation of absorbance at 540 nm. Mineralization assays were conducted as described in the Methods. **C.** Mineralization assays were conducted with TNCB buffer (no fetuin) or with human fetuin (2 µM) or with human fetuin (2 µM) that had been pre-digested with MMP-3 or MMP-7 for 24 hrs at enzyme:substrate=1:60. Data are mean±standard deviation of absorbance measured at 540 nm.
Fig. 1
### Table A

<table>
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<tr>
<th>Mr</th>
<th>1 HR</th>
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<th>4 HR</th>
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<tr>
<td>MMP-7</td>
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<td>55 kDa</td>
<td>43 kDa</td>
<td>34 kDa</td>
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<tr>
<td>MMP-3</td>
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### Table B

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<th>Digest 1 hr</th>
<th>Digest 24 hrs</th>
<th>Digest + Inhibitor 1 hr</th>
<th>Digest + Inhibitor 24 hrs</th>
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**Fig. 2**
Fig. 3

A

24 HR
0 HR
0 HR
Mr

92 kDa
72 kDa
55 kDa
43 kDa
34 kDa
17 kDa
14 kDa

Normalized Spectrum Count

Biological Sample

B

MKSLVLLCL AQLWGCSSAP HPGLIYRQP NCDDPETEEA ALVAIDYINQ NLPWGKHTL NQIDEVKVWP
QQPSGELFEI EIDTLETTCH VLDPTPVARC SRVQLKEHAV EGDQDFQLLK LDGKFVVA KCDSSPDAE
DLGKFVVA KCDSSPDAE DVRKVCQDCP LLAALNDTRV VHAAKAA LAA FNAQNGSNF QLEEISRAQL VP LPPSTYVE FTVPSTDCVA KEATEAACKN LLAEEKQYDFGC KATLSEEKLGQ AEAVTCTVF QTPQVTSPQ PEGANEAVPT PVDPDAPPSS
PLPLGAPGLPP AGSPPDHSV LLAAPGHQLH RAHYDLR HTF MGVVLGSPS GEVSHPRKTR TVVQPSVGAA
AGPVVPPCPG RIRHFKV

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**Fig. 4**

### A

MMP-7 Std

MMP-7 bound to resin sample

Dilution from neat

1 ug  0.5 ug  0.25 ug  0.125 ug  62.5 ng

### B

![Graph showing moles of bound fetuin vs. moles of free fetuin.](image)

Moles of bound fetuin x 10^11

Moles of free fetuin x 10^11

### C

<table>
<thead>
<tr>
<th>Mr</th>
<th>Supernate</th>
<th>Eluent</th>
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<tr>
<td></td>
<td>Digest (MMP-7)</td>
<td>Control (BSA)</td>
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<tr>
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Fig. 5

A

B

C

No Fetuin 2μM Fetuin MMP3 Degraded Fetuin MMP7 Degraded Fetuin

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