TSpace

The University of Toronto's research repository

Accepted Manuscript of **Impact of matrix metalloproteinases on inhibition of mineralization by fetuin**

How to cite TSpace items

Always cite the published version, so the author(s) will receive recognition through services that track citation counts, e.g. Scopus. If you need to cite the page number of the TSpace version because you cannot access the published version, then cite the Tspace version¹ **in addition to** the published version².

Published version citation:

Schure R, Costa KD, Rezaei R, Lee W, Laschinger C, Tenenbaum HC, McCulloch CA.
Impact of matrix metalloproteinases on inhibition of mineralization by fetuin. J
Periodontal Res. 2013 Jun;48(3):357-66.

TSpace version citation:

Schure R, Costa KD, Rezaei R, Lee W, Laschinger C, Tenenbaum HC, McCulloch CA.
Impact of matrix metalloproteinases on inhibition of mineralization by fetuin. TSpace.
2013 Jun. Available at <http://hdl.handle.net/XXXX/XXXXX>. Replace the 'XXXX/
XXXXX' with the item ID from the URL.

tspace.library.utoronto.ca

¹ TSpace version: includes the pre-print/original manuscript (version before peer review) and post-print/accepted manuscript (version after peer-review and editing).

² Published version: the publisher's final PDF.

Impact of Matrix Metalloproteinases on Inhibition of Mineralization by Fetuin

Journal:	<i>Journal of Periodontal Research</i>
Manuscript ID:	JRE-05-12-1859.R1
Manuscript Type:	Original Article
Date Submitted by the Author:	13-Jul-2012
Complete List of Authors:	Schure, Ryan; University of Toronto, Dentistry D'Costa, Kerry; University of Toronto, Dentistry Rezaei, Reyhaneh; University of Toronto, Dentistry Lee, Wilson; University of Toronto, Dentistry Laschinger, Carol; University of Toronto, Dentistry Tenenbaum, Howard; University of Toronto, Dentistry MCCULLOCH, CHRISTOPHER; UNIVERSITY OF TORONTO, ROOM 430, Fitzgerald Building
Keywords:	Biochemistry, Coronary heart disease, Matrix metalloproteinase, Periodontal disease

SCHOLARONE™
Manuscripts

Impact of Matrix Metalloproteinases on Inhibition of Mineralization by Fetuin

*R. Schure¹, K. D’Costa², R. Rezaei¹, W. Lee², C. Laschinger², H.C. Tenenbaum¹,
C.A. McCulloch²*

¹Discipline of Periodontology, Faculty of Dentistry, University of Toronto and

²Matrix Dynamics Group, University of Toronto

Correspondence: Christopher McCulloch
Room 244, Fitzgerald Building
150 College Street, Toronto, ON M5S 3E2 Canada
Telephone: 416-978-1258
FAX: 416-978-5956
e-mail: christopher.mcculloch@utoronto.ca

KEY WORDS

Matrilysin; stromelysin; calcium phosphate; mineralization; periodontal diseases

Word Count:

Number of Figures: 5

Number of Tables: 1

Running head: Fetuin inhibition of mineralization

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 ($>1 \mu\text{M}$) 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.

1
2
3 Calcification in the intima of blood vessels is associated with several cardiovascular diseases
4 including end-stage renal disease (1, 2). Vascular calcification within atheromas requires the
5 deposition of hydroxyapatite; the amount of calcification is a marker of the atherosclerotic
6 burden and may mediate loss of arterial elasticity (2). Several cohort and cross-sectional studies
7 have found positive associations between periodontitis and increased risk of cardiovascular
8 diseases and vascular calcification (3-8) but the underlying mechanisms that explain if and how
9 periodontitis and other inflammatory diseases enhance the formation of calcifying atheromas are
10 not defined. While there is no definitive evidence for a causal association between cardiovascular
11 diseases and periodontitis (9), it is nevertheless important to identify potential pathophysiological
12 mechanisms that could contribute to this link.
13
14
15
16
17
18
19
20
21
22
23
24
25
26

27 Vascular calcification may be initiated by several mechanisms (2, 10) that include loss of
28 inhibition of calcification, induction of bone formation and cell death, which can nucleate initial
29 calcification processes. Notably, several proteins may be involved in suppressing calcification in
30 atheromas including osteopontin (1, 2, 10, 11), matrix Gla protein (12), osteocalcin and the
31 alpha-2-Heremans Schmid glycoprotein (human homologue of fetuin A) (13, 14). Fetuin is an
32 abundant serum sialoprotein (Mr ~49,000) (15, 16) that is a member of the cystatin superfamily
33 (17, 18) and is synthesized in the liver (19). Fetuin, which is incorporated into mineralized
34 tissues and plays an important role in bone formation and resorption (18, 20, 21), inhibits
35 calcification and prevents precipitation of calcium phosphate (22)(23). Fetuin may act as a
36 crystal poison (23-25) but may also inhibit mineralization by interfering with the differentiation
37 of cells with a mineralizing phenotype (26-29). Low levels of fetuin in the serum are associated
38 with increased levels of vascular calcification (30). In view of the relationship between low
39 levels of intact fetuin in the serum with increased risk for vascular calcification, we considered
40 that MMPs upregulated in periodontitis could reduce levels of fetuin in serum as a consequence
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

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), β -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-benzoxadiazol-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 CaCl₂, 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 IC₅₀ of 18.4 nM for MMP-3 and an IC₅₀ 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 K_D and B_{MAX} 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 μ l per well) the following solutions were added sequentially: 10 μ l phosphate solution (KH_2PO_4 , 10 mM; Na_2HPO_4 , 20 mM; NaCl, 150 mM; pH 7.4), 10 μ l calcium solution ($CaCl_2$, 50 mM; Hepes, 50 mM; NaCl, 150 mM; pH 7.4) and 80 μ l of test agents in buffer solution (Hepes, 50 mM; NaCl, 150 mM; pH 7.4), which contained 38 μ M fetuin and equal volumes of either vehicle (TNCB) or TNCB with MMP-3 or MMP-7. After incubations (up to 4 hours at 23°C), 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 μ l 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 μ l of cetylpyridinium chloride solution (100 mM) was added

to each well and incubated at 23° 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 r^2 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 μ M) 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 K_D ($2.96 \pm 1.92 \times 10^{-9}$ M; $r^2 = 0.87$), 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).

1
2
3
4 In preliminary trials that examined mineralization in time course experiments,
5
6 spectrophotometric absorbance (540 nm) measurements of mineral-bound alizarin red showed
7
8 that there was a time-dependent increase of absorbance (from 0.5 to 2.2 absorbance units) which
9
10 peaked at 3.5 hours (Fig. 5B). During the conduct of the experiments in which we optimized the
11
12 conditions for the mineralization assay we found that freshly prepared alizarin red staining
13
14 solutions produced somewhat (~30%) lower absorbance measurements than did alizarin red
15
16 preparations that were made at least one week in advance of the assay. Further, we found that
17
18 assays conducted with the aged alizarin red preparations were more sensitive in detecting fetuin-
19
20 induced inhibition of mineralized material than were assays conducted with freshly made alizarin
21
22 red. Accordingly, when the mineralization assays were conducted for 3.5 hours with one week-
23
24 aged alizarin red solutions, the absorbance attributable to alizarin red was ~3 units (Fig. 5C). In
25
26 assays that used 2 μ M human fetuin in the mineralization medium, there was a 10-fold reduction
27
28 of alizarin red absorbance compared to controls ($p < 0.001$; Fig. 5C).
29
30
31
32
33

34 We next conducted mineralization assays with MMP-3 and MMP-7-degraded human
35
36 fetuin. In preliminary trials we found that fetuin-mediated inhibition of mineralization was not
37
38 affected when the fetuin was degraded by MMP-7 for less than 4 hours. Accordingly we
39
40 conducted longer fetuin digestions (24 hours at enzyme:substrate=1:60) in which we anticipated
41
42 there would be degradation of fetuin into fragments of <20 kDa (Fig. 3A). For MMP-7 degraded
43
44 fetuin there was 3-fold more mineralization than with intact fetuin (2 μ M fetuin; $p < 0.001$; Fig.
45
46 5C). In assays that examined MMP-3- degraded fetuin under the same molar ratios and
47
48 incubation times, there was a small but not significant change of alizarin red absorbance ($p > 0.2$).
49
50
51
52
53
54
55
56
57
58
59
60

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) ($IC_{50} = 30 \text{ nM}$), 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

1
2
3 digestion conditions used here. Further, it would appear that the ~18 kDa fetuin fragment is not
4
5 as effective as the full-length molecule for the inhibition of mineralization. These data indicate
6
7 that MMP-7 may be important for the control of biological mineralization in vivo.
8
9

10 We found that the inhibition of mineralization by fetuin seems to be reliant on intact
11
12 fetuin structure and not by sequestration of fetuin by MMP-7 and MMP-3. Under conditions in
13
14 which fetuin was not substantially degraded by MMP-7 (20 min co-incubations), there was little
15
16 effect on the ability of fetuin to regulate mineralization (data not shown). As fetuin is an
17
18 abundant serum protein and evidently binds MMP-7 with high affinity, it may inhibit the
19
20 catalytic activity of MMPs, analogous to tissue inhibitors of matrix metalloproteinases (62, 63),
21
22 but this possibility has not been examined here.
23
24
25
26

27 There is considerable interest in the potential association between inflammatory
28
29 periodontal diseases and the development of cardiovascular diseases in general (64) and the
30
31 formation of calcified atheromas in particular (8). However, apart from suggestions that
32
33 periodontal pathogens may play a role in this association (65), little is known about other
34
35 putative mechanisms, based on biochemical processes that could explain the statistical evidence
36
37 for this relationship. Conceivably, linkages may exist between different inflammatory diseases
38
39 and cardiovascular diseases (66), which could be mediated by reductions in the levels of intact
40
41 fetuin in serum. In this context, we found a 50% reduction in the level of fetuin in patients with
42
43 severe osteoarthritis (67). Although not investigated in patients with osteoarthritis, MMPs
44
45 released into the bloodstream because of severe inflammatory joint disease may also contribute
46
47 to fetuin degradation.
48
49
50
51
52

53 MMP-7 has been implicated in the pathogenesis of atheroma formation (68). If MMP-7
54
55 that is produced in periodontitis is important for the regulation of calcification of atheromas, then
56
57 there would need to be increased concentrations of MMP-7 in the blood. Notably, the
58
59
60

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.

ACKNOWLEDGEMENTS

We thank Doug Holmyard (Mt. Sinai Hospital, Dept. of Pathology, Toronto, ON) for advice and help with the assessment of mineralization by electron microscopy and by electron diffraction analysis. We are grateful to Graeme Hunter and Yinyin Liao (University of Western Ontario) for providing us with their protocol for assessing hydroxyapatite growth in vitro. The research was supported financially by grants from the Canadian Institutes of Health Research (MOP36332) and the Ontario Heart and Stroke Foundation (T7085) to CAM who is also supported by a Canada Research Chair (Tier 1). The authors report no commercial relationships to the funding sources for this study or no known conflicts of interest related to this study.

References

- (1) Giachelli CM, Speer MY, Li X, Rajachar RM, Yang H. Regulation of vascular calcification: roles of phosphate and osteopontin. *Circ Res* 2005; **96**: 717-722.
- (2) Giachelli CM. Vascular calcification mechanisms. *J Am Soc Nephrol* 2004; **15**: 2959-2964.
- (3) Beck JD, Slade G, Offenbacher S. Oral disease, cardiovascular disease and systemic inflammation. *Periodontol 2000* 2000; **23**: 110-120.
- (4) Behle JH, Papapanou PN. Periodontal infections and atherosclerotic vascular disease: an update. *Int Dent J* 2006; **56**: 256-262.
- (5) Demmer RT, Desvarieux M. Periodontal infections and cardiovascular disease: the heart of the matter. *J Am Dent Assoc* 2006; **137 Suppl**: 14S-20S; quiz 38S.
- (6) Humphrey LL, Fu R, Buckley DI, Freeman M, Helfand M. Periodontal disease and coronary heart disease incidence: a systematic review and meta-analysis. *J Gen Intern Med* 2008; **23**: 2079-2086.
- (7) Khader YS, Albashaireh ZS, Alomari MA. Periodontal diseases and the risk of coronary heart and cerebrovascular diseases: a meta-analysis. *J Periodontol* 2004; **75**: 1046-1053.
- (8) Guimaraes Henriques JC, Kreich EM, Helena Baldani M, Luciano M, Cezar de Melo Castilho J, Cesar de Moraes L. Panoramic radiography in the diagnosis of carotid artery atheromas and the associated risk factors. *Open Dent J* 2011; **5**: 79-83.
- (9) Lockhart PB, Bolger AF, Papapanou PN, et al. Periodontal Disease and Atherosclerotic Vascular Disease: Does the Evidence Support an Independent Association?: A Scientific Statement From the American Heart Association. *Circulation*.
- (10) Speer MY, Giachelli CM. Regulation of cardiovascular calcification. *Cardiovasc Pathol* 2004; **13**: 63-70.
- (11) Abedin M, Tintut Y, Demer LL. Vascular calcification: mechanisms and clinical ramifications. *Arterioscler Thromb Vasc Biol* 2004; **24**: 1161-1170.
- (12) Luo G, Ducy P, McKee MD, et al. Spontaneous calcification of arteries and cartilage in mice lacking matrix GLA protein. *Nature* 1997; **386**: 78-81.
- (13) Jahnhen-Dechent W, Schafer C, Heiss A, Grotzinger J. Systemic inhibition of spontaneous calcification by the serum protein alpha 2-HS glycoprotein/fetuin. *Z Kardiol* 2001; **90 Suppl 3**: 47-56.
- (14) Schafer C, Heiss A, Schwarz A, et al. The serum protein alpha 2-Heremans-Schmid glycoprotein/fetuin-A is a systemically acting inhibitor of ectopic calcification. *J Clin Invest* 2003; **112**: 357-366.
- (15) Lebreton JP, Joisel F, Raoult JP, Lannuzel B, Rogez JP, Humbert G. Serum concentration of human alpha 2 HS glycoprotein during the inflammatory process: evidence that alpha 2 HS glycoprotein is a negative acute-phase reactant. *J Clin Invest* 1979; **64**: 1118-1129.
- (16) Matsushima K, Cheng M, Migita S. Purification and physicochemical characterization of human alpha 2-HS-glycoprotein. *Biochim Biophys Acta* 1982; **701**: 200-205.
- (17) Kellermann J, Haupt H, Auerswald EA, Muller-Ester W. The arrangement of disulfide loops in human alpha 2-HS glycoprotein. Similarity to the disulfide bridge structures of cystatins and kininogens. *J Biol Chem* 1989; **264**: 14121-14128.
- (18) Triffitt JT, Gebauer U, Ashton BA, Owen ME, Reynolds JJ. Origin of plasma alpha2HS-glycoprotein and its accumulation in bone. *Nature* 1976; **262**: 226-227.

(19) Daveau M, Christian D, Julien N, Hiron M, Arnaud P, Lebreton JP. The synthesis of human alpha-2-HS glycoprotein is down-regulated by cytokines in hepatoma HepG2 cells. *FEBS Lett* 1988; **241**: 191-194.

(20) Triffitt JT, Owen ME, Ashton BA, Wilson JM. Plasma disappearance of rabbit alpha2HS-glycoprotein and its uptake by bone tissue. *Calcif Tissue Res* 1978; **26**: 155-161.

(21) Colclasure GC, Lloyd WS, Lamkin M, et al. Human serum alpha 2HS-glycoprotein modulates in vitro bone resorption. *J Clin Endocrinol Metab* 1988; **66**: 187-192.

(22) Schinke T, Amendt C, Trindl A, Poschke O, Muller-Esterl W, Jahn-Dechent W. The serum protein alpha2-HS glycoprotein/fetuin inhibits apatite formation in vitro and in mineralizing calvaria cells. A possible role in mineralization and calcium homeostasis. *J Biol Chem* 1996; **271**: 20789-20796.

(23) Heiss A, DuChesne A, Denecke B, et al. Structural basis of calcification inhibition by alpha 2-HS glycoprotein/fetuin-A. Formation of colloidal calciprotein particles. *J Biol Chem* 2003; **278**: 13333-13341.

(24) Heiss A, Eckert T, Aretz A, et al. Hierarchical role of fetuin-A and acidic serum proteins in the formation and stabilization of calcium phosphate particles. *J Biol Chem* 2008; **283**: 14815-14825.

(25) Heiss A, Jahn-Dechent W, Endo H, Schwahn D. Structural dynamics of a colloidal protein-mineral complex bestowing on calcium phosphate a high solubility in biological fluids. *Biointerphases* 2007; **2**: 16-20.

(26) Binkert C, Demetriou M, Sukhu B, Szweras M, Tenenbaum HC, Dennis JW. Regulation of osteogenesis by fetuin. *J Biol Chem* 1999; **274**: 28514-28520.

(27) Szweras M, Liu D, Partridge EA, et al. alpha 2-HS glycoprotein/fetuin, a transforming growth factor-beta/bone morphogenetic protein antagonist, regulates postnatal bone growth and remodeling. *J Biol Chem* 2002; **277**: 19991-19997.

(28) Rittenberg B, Partridge E, Baker G, et al. Regulation of BMP-induced ectopic bone formation by Ahsg. *J Orthop Res* 2005; **23**: 653-662.

(29) Demetriou M, Binkert C, Sukhu B, Tenenbaum HC, Dennis JW. Fetuin/alpha2-HS glycoprotein is a transforming growth factor-beta type II receptor mimic and cytokine antagonist. *J Biol Chem* 1996; **271**: 12755-12761.

(30) Reynolds JL, Skepper JN, McNair R, et al. Multifunctional roles for serum protein fetuin-a in inhibition of human vascular smooth muscle cell calcification. *J Am Soc Nephrol* 2005; **16**: 2920-2930.

(31) Soder PO, Meurman JH, Jogestrand T, Nowak J, Soder B. Matrix metalloproteinase-9 and tissue inhibitor of matrix metalloproteinase-1 in blood as markers for early atherosclerosis in subjects with chronic periodontitis. *J Periodontol* 2009; **44**: 452-458.

(32) Nakashima K, Giannopoulou C, Andersen E, et al. A longitudinal study of various crevicular fluid components as markers of periodontal disease activity. *J Clin Periodontol* 1996; **23**: 832-838.

(33) Nakashima K, Roehrich N, Cimasoni G. Osteocalcin, prostaglandin E2 and alkaline phosphatase in gingival crevicular fluid: their relations to periodontal status. *J Clin Periodontol* 1994; **21**: 327-333.

(34) Harper DS, Lamster IB, Celenti R. Relationship of subgingival plaque flora to lysosomal and cytoplasmic enzyme activity in gingival crevicular fluid. *J Clin Periodontol* 1989; **16**: 164-169.

- (35) Loos BG, Tjoa S. Host-derived diagnostic markers for periodontitis: do they exist in gingival crevice fluid? *Periodontol 2000* 2005; **39**: 53-72.
- (36) McCulloch CA. Host enzymes in gingival crevicular fluid as diagnostic indicators of periodontitis. *J Clin Periodontol* 1994; **21**: 497-506.
- (37) Imrey PB, Crawford JM, Cohen RL, Alves ME, McSwiggin TA, Chambers DA. A cross-sectional analysis of aspartate aminotransferase in human gingival crevicular fluid. *J Periodontol Res* 1991; **26**: 75-84.
- (38) Persson GR, DeRouen TA, Page RC. Relationship between levels of aspartate aminotransferase in gingival crevicular fluid and gingival inflammation. *J Periodontol Res* 1990; **25**: 17-24.
- (39) Persson GR, Page RC. Diagnostic characteristics of crevicular fluid aspartate aminotransferase (AST) levels associated with periodontal disease activity. *J Clin Periodontol* 1992; **19**: 43-48.
- (40) Lamster IB, Ahlo JK. Analysis of gingival crevicular fluid as applied to the diagnosis of oral and systemic diseases. *Ann NY Acad Sci* 2007; **1098**: 216-229.
- (41) Lamster IB, Oshrain RL, Harper DS, Celenti RS, Hovliaras CA, Gordon JM. Enzyme activity in crevicular fluid for detection and prediction of clinical attachment loss in patients with chronic adult periodontitis. Six month results. *J Periodontol* 1988; **59**: 516-523.
- (42) Gustafsson A, Asman B, Bergstrom K, Soder PO. Granulocyte elastase in gingival crevicular fluid. A possible discriminator between gingivitis and periodontitis. *J Clin Periodontol* 1992; **19**: 535-540.
- (43) Palcanis KG, Larjava IK, Wells BR, et al. Elastase as an indicator of periodontal disease progression. *J Periodontol* 1992; **63**: 237-242.
- (44) Alpagot T, Bell C, Lundergan W, Chambers DW, Rudin R. Longitudinal evaluation of GCF MMP-3 and TIMP-1 levels as prognostic factors for progression of periodontitis. *J Clin Periodontol* 2001; **28**: 353-359.
- (45) Haerian A, Adonogianaki E, Mooney J, Docherty JP, Kinane DF. Gingival crevicular stromelysin, collagenase and tissue inhibitor of metalloproteinases levels in healthy and diseased sites. *J Clin Periodontol* 1995; **22**: 505-509.
- (46) Unemori EN, Bair MJ, Bauer EA, Amento EP. Stromelysin expression regulates collagenase activation in human fibroblasts. Dissociable control of two metalloproteinases by interferon-gamma. *J Biol Chem* 1991; **266**: 23477-23482.
- (47) Emingil G, Tervahartiala T, Mantyla P, Maatta M, Sorsa T, Atilla G. Gingival crevicular fluid matrix metalloproteinase (MMP)-7, extracellular MMP inducer, and tissue inhibitor of MMP-1 levels in periodontal disease. *J Periodontol* 2006; **77**: 2040-2050.
- (48) Tervahartiala T, Pirila E, Ceponis A, et al. The in vivo expression of the collagenolytic matrix metalloproteinases (MMP-2, -8, -13, and -14) and matrilysin (MMP-7) in adult and localized juvenile periodontitis. *J Dent Res* 2000; **79**: 1969-1977.
- (49) Lee W, Aitken S, Kulkarni G, et al. Collagenase activity in recurrent periodontitis: relationship to disease progression and doxycycline therapy. *J Periodontol Res* 1991; **26**: 479-485.
- (50) Yeh YC, Sheu BS, Cheng HC, Wang YL, Yang HB, Wu JJ. Elevated serum matrix metalloproteinase-3 and -7 in H. pylori-related gastric cancer can be biomarkers correlating with a poor survival. *Dig Dis Sci*; **55**: 1649-1657.
- (51) Ochieng J, Warfield P, Green B. Interactions of gelatinases with soluble and immobilized fetuin and asialofetuin. *Arch Biochem Biophys* 1995; **322**: 250-255.

(52) Ochieng J, Green B. The interactions of alpha 2HS glycoprotein with metalloproteinases. *Biochem Mol Biol Int* 1996; **40**: 13-20.

(53) Jahnen-Dechent W, Trindl A, Godovac-Zimmermann J, Muller-Esterl W. Posttranslational processing of human alpha 2-HS glycoprotein (human fetuin). Evidence for the production of a phosphorylated single-chain form by hepatoma cells. *Eur J Biochem* 1994; **226**: 59-69.

(54) Kubler D, Gosenca D, Wind M, et al. Proteolytic processing by matrix metalloproteinases and phosphorylation by protein kinase CK2 of fetuin-A, the major globulin of fetal calf serum. *Biochimie* 2007; **89**: 410-418.

(55) Sung JY, Park SM, Lee CH, et al. Proteolytic cleavage of extracellular secreted {alpha}-synuclein via matrix metalloproteinases. *J Biol Chem* 2005; **280**: 25216-25224.

(56) Nawratil P, Lenzen S, Kellermann J, et al. Limited proteolysis of human alpha2-HS glycoprotein/fetuin. Evidence that a chymotryptic activity can release the connecting peptide. *J Biol Chem* 1996; **271**: 31735-31741.

(57) Romanelli R, Mancini S, Laschinger C, Overall CM, Sodek J, McCulloch CA. Activation of neutrophil collagenase in periodontitis. *Infect Immun* 1999; **67**: 2319-2326.

(58) Pikul S, McDow Dunham KL, Almstead NG, et al. Discovery of potent, achiral matrix metalloproteinase inhibitors. *J Med Chem* 1998; **41**: 3568-3571.

(59) Chen CC, Boskey AL, Rosenberg LC. The inhibitory effect of cartilage proteoglycans on hydroxyapatite growth. *Calcif Tissue Int* 1984; **36**: 285-290.

(60) Cuisinier F, Bres EF, Hemmerle J, Voegel JC, Frank RM. Transmission electron microscopy of lattice planes in human alveolar bone apatite crystals. *Calcif Tissue Int* 1987; **40**: 332-338.

(61) Dziegielewska KM, Brown WM, Casey SJ, et al. The complete cDNA and amino acid sequence of bovine fetuin. Its homology with alpha 2HS glycoprotein and relation to other members of the cystatin superfamily. *J Biol Chem* 1990; **265**: 4354-4357.

(62) Cawston TE. Metalloproteinase inhibitors and the prevention of connective tissue breakdown. *Pharmacol Ther* 1996; **70**: 163-182.

(63) Woessner JF, Jr. Matrix metalloproteinases and their inhibitors in connective tissue remodeling. *FASEB J* 1991; **5**: 2145-2154.

(64) Cotti E, Dessi C, Piras A, Mercurio G. Can a chronic dental infection be considered a cause of cardiovascular disease? A review of the literature. *Int J Cardiol* 2011; **148**: 4-10.

(65) Grau AJ, Urbanek C, Palm F. Common infections and the risk of stroke. *Nat Rev Neurol* 2010; **6**: 681-694.

(66) Zakynthinos E, Pappa N. Inflammatory biomarkers in coronary artery disease. *J Cardiol* 2009; **53**: 317-333.

(67) Albilal JB, Tenenbaum HC, Clokie CM, et al. Serum levels of BMP-2, 4, 7 and AHSX in patients with degenerative joint disease requiring total arthroplasty of the hip and temporomandibular joints. *J Orthop Res* 2012; **In press**.

(68) Fu X, Kassim SY, Parks WC, Heinecke JW. Hypochlorous acid oxygenates the cysteine switch domain of pro-matrilysin (MMP-7). A mechanism for matrix metalloproteinase activation and atherosclerotic plaque rupture by myeloperoxidase. *J Biol Chem* 2001; **276**: 41279-41287.

Table 1 Electron diffraction analysis of hydroxyapatite mineralization

Hydroxyapatite: JCPDS d-spacing (Å)	Hydroxyapatite: JCPDS intensity (%)	Sample: Calculated d-spacing (Å)	Sample: Visual intensity (%)
3.44	40	3.39	40
3.08	19	3.03	20
2.81	100	2.76	100

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 ($C_{21}H_{27}N_3O_8S_2$; 1 μ 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 μ M fetuin as indicated in the inset. Data are mean $\pm$ 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 μ M) or with human fetuin (2 μ M) that had been pre-digested with MMP-3 or MMP-7 for 24 hrs at enzyme:substrate=1:60. Data are mean $\pm$ standard deviation of absorbance measured at 540 nm.

Fig. 1

Fig. 2

A

MKSLVLLCL	AQLWGCHSAP	HGPGLIYRQP	NCDDPETEEA	ALVAIDYINQ	NLPWGYKHTL
NQIDEVKVWP	QQPSGELFEI	EIDTLETTCH	VLDPTPVARC	SVRQLKEHAV	EGDCDFQLLK
LDGKFSVVYA	KCDSSPDSAE	DVRKVCQDCP	LLAPLNDTRV	VHAAKAALAA	FNAQNNGSNF
QLEEISRAQL	VPLPPSTYVE	FTVSGTDCVA	KEATEAAKCN	LLAEKQYGFC	KATLSEKLG
AEVAVTCTVF	QTQPVTSQPQ	PEGANEAVPT	PVDPDAPPS	PPLGAPGLPP	AGSPDPSHVL
LAAPPQHQLH	RAHYDLRHTF	MGVSLGSPS	GEVSHPRKTR	TVVQPSVGAA	AGPVVPPCPG
RIRHFKV					

B

MKSLVLLCL AQLWGCHSAP HGPGLIYRQP NCDDPETEEA ALVAIDYINQ NLPWGYKHTL NQIDEVKVWP⁷⁰
QQPSGELFEI EIDTLETTCH VLDPTPVARC SVRQLKEHAV EGDCDFQLLK LDGKFSVVYA KCDSSPDSAE¹⁴⁰
DVRKVCQDCP LLAPLNDTRV VHAAKAALAA FNAQNNGSNF QLEEISRAQL VP LPPSTYVE FTVSGTDCVA²¹⁰
KEATEAAKCN LLAEKQYGFC KATLSEKLG AEVAVTCTVF QTQPVTSQPQ PEGANEAVPT PVDPDAPPS²⁸⁰
PPLGAPGLPP AGSPDPSHVL LAAPPQHQLH RAHYDLRHTF MGVSLGSPS GEVSHPRKTR TVVQPSVGAA³⁵⁰
AGPVVPPCPG RIRHFKV³⁶⁷

Fig. 3

Fig. 4

Fig. 5