The initial proteolytic cleavage of transferrin causes formation of additional peptides which can also serve as substrates for the desired protease. Thus, its utility for determining kinetic parameters could not be rationalized. Also, the need for an assay which employed a substrate with a single cleavage site giving rise to a single product which would not interfere with subsequent protease activity on the initial substrate became evident.

A second approach to the desired assay employed a peptide substrate with HPLC detection of reaction products (Harrison et al. 1989). In this assay Substance P, an 11 amino acid peptide, was used to assess stromelysin (MMP-3) activity. MMP-3 cleaves Substance P at the Gln-Phe peptide bond between residues 6 and 7. The advantage of this assay was the high degree of specificity seen with MMP-3 and its amenability to providing solid kinetic data upon which inhibitor design could be based. Unfortunately, the high degree of specificity was also a shortcoming of this assay as it could only be used for MMP-3. In addition, the cumbersome HPLC separation protocol did not allow this assay to be used in a high capacity screening format.

Evolution of our MMP assay was greatly aided by the characterization of a synthetic, fluorogenic peptide substrate which enabled a continuous assay protocol to be developed (Knight et al. 1992). This peptide which we refer to as the MCA peptide (7-methoxy-coumarin-4-yl)Acetyl-Pro-Leu-Gly-Leu-(3-[2,4-dinitrophenyl]-L-2,3-diaminopropionyl)-Ala-Arg-NH₂ takes advantage of the highly fluorescent 7-methoxy-coumarin group which is quenched by energy transfer to the 2,4-dinitrophenyl group in the intact peptide. However, upon cleavage by numerous MMPs (MMP-1, 3, 8, 9, PUMP), an approximate 200-fold increase in fluorescence is observed due to liberation of the MCA-containing peptide fragment (λex 328 nm, λem 393 nm). The broad susceptibility of this peptide to cleavage by numerous MMPs, along with its amenability to a high capacity, continuous assay format makes it ideal for evaluating MMPs which may be involved in cartilage degradation. Michaelis-Menten kinetic constants have been determined for MMP-1, 3, 8, and 9. The Vmax values are 0.93, 1.10, 9.70 and 0.22 fluorescence units/min/ng protein. The Km values are 9.8, 8.2, 4.4, and 2.7 mM, respectively. We are currently using this assay to evaluate various inhibitors which have been described to inhibit the MMP family of proteases.

References

Development of a convenient and sensitive assay for matrix metalloproteinase enzyme activity in synovial fluid samples using fluorogenic peptides

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Matrix metalloproteinases (MMPs) are considered to play a major role in the proteolytic degradation of extracellular matrix in joint and skeletal diseases. Inactive forms of these enzymes are secreted by cells of the joint, together with their tissue inhibitors (TIMPs). Extracellular activation results in active MMPs which can degrade most matrix components of articular cartilage such as proteoglycans and collagen proteins. Matrix degradation is inhibited by interaction of TIMPs with active MMPs. Under physiological conditions, active MMPs are efficiently neutralized, predominantly by TIMPs (MMP/TIMP ratio ≤ 1), so that net proteolytic activity is absent or very low. Under pathological conditions like in rheumatoid arthritis and osteoarthritis, levels of active MMPs as well as TIMPs are increased. More importantly, active enzymes are present in excess over the amount of tissue inhibitor (MMP/TIMP ratio>1).

The actual deleterious species in proteolysis are active MMPs, free of inhibitor. Therefore, ideally assays to monitor the potential for tissue degradation should represent net enzyme activities of MMPs rath-
er than the amounts of (pro- or active) enzyme with or without inhibitor (as is the case in immunological assays). Therefore, a study was initiated to devise simple and sensitive assays for MMP enzyme activity with fluorogenic peptides. These peptide substrates contain besides a fluorophore also a quencher so that the uncleaved peptide does not exert fluorescence. After proteolytic cleavage the fluorophore and quencher reside in different fragments so that the fluorophore is no longer quenched (i.e. fluorescence increases). Advantages of fluorogenic substrates are their high sensitivity and the possibility to continuously monitor substrate cleavage in time.

The fluorescent EDANS group in combination with the DABCYL moiety as a quencher were selected because of their optimal spectral overlap (i.e. optimal quenching properties). In comparison to other known fluorophore/quencher combinations the EDANS-DABCYL peptides exhibit favorable optical characteristics (i.e. high excitation and emission wavelengths; 340 and 485 nm, resp.), and good solubility (due to the charged EDANS and DABCYL groups). Peptides were designed to be selective for MMPs (in comparison to other proteolytic enzymes) by selecting gelatin/collagen-like amino acid sequences.

Incubations of EDANS-DABCYL peptides with MMPs resulted in an easily detectable change of fluorescence in time. Compared to other fluorogenic substrates basal fluorescence was very low. Typically, incubation with MMP-9 (4 nM) resulted in a 18-fold increase of fluorescence in 10 minutes. The rate of increase in fluorescence (AFLU/TIME) was proportional to the amount of peptide cleaved (as determined by HPLC), and increased linearly with time. Enzyme kinetic studies with purified MMPs (1, 2, 3, 7 and 9) showed that all these enzymes efficiently hydrolyzed the EDANS-DABCYL substrates with Michaelis Menten-type kinetics.

Inherent to the use of synthetic peptides as MMP substrates is their high turnover by gelatinases (MMP-2 and MMP-9) and poor cleavage by collagenases (MMP-1 and MMP-8) which require a triple helix structure. For instance this is the case with the first described fluorogenic substrate for MMPs (Knight et al., FEBS Lett 1992; 296: 263-266); kcatal/Km of MMP-9 is 80-fold higher than that of MMP-3. The EDANS-DABCYL peptides have more favorable characteristics in that they are relatively efficiently cleaved by other MMPs than the gelatinases. For instance, kcatal/Km of MMP-9 is only 5 times that of MMP-3, and 10 times that of MMP-1. Thus, the EDANS-DABCYL substrates present the best general MMP substrates described as yet.

The new fluorogenic substrates could conveniently be used to determine MMP enzyme activity in small, 20-μl samples of synovial fluid (SF; untreated, i.e. not activated). In SF from osteoarthritis (OA) and rheumatoid arthritis (RA) patients, fluorescence increase linear with time (for at least 4 hours). Extremely low enzyme activities (in control SF, obtained at autopsy) could still be monitored by simply increasing the incubation time (tested up to 24 hours). The assay was concluded to be selective for MMPs without cleavage by other proteinases since ΔFLU/TIME was completely inhibited by the addition of EDTA or phenanthroline to the SF incubations. Furthermore, addition of the serine proteinase inhibitor trasylol did not effect ΔFLU/TIME.

Preliminary studies showed that ΔFLU/TIME in OA (n=7) and RA (n=5) samples were 7- and 12-fold higher than in control SF (n=5). In all groups interindividual differences were large; standard deviations of 97, 50 and 26% were observed in SF samples of control, OA and RA subjects, respectively. In OA and RA samples ΔFLU/TIME values were significantly higher than in control SF (P 0.004 and 0.0001, respectively). Furthermore, ΔFLU/TIME values were significantly higher in RA samples than in OA samples (P 0.02).

Conclusion

The fluorogenic EDANS-DABCYL peptides were efficiently and selectively hydrolyzed by MMPs and can be conveniently used to monitor MMP enzyme activity in small aliquots of SF (only 20 μl required). In comparison to other substrates the EDANS-DABCYL peptides have favorable properties such as good solubility and applicability for overall assessment of MMP activity (i.e. other MMPs than gelatinases cleave the substrates relatively well). MMP activity in untreated SF samples increased in the order: C < OA < RA. Inasmuch as MMP activity was significantly higher in OA subjects than in control individuals, the frequent use of OA samples as controls in studies on RA can be debated.