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Use of a Multiple-Enzyme/Multiple-Reagent Assay System To Quantify Activity Levels in Samples Containing Mixtures of Matrix Metalloproteinases

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ABSTRACT: Matrix metalloproteinases (MMPs) are a family of enzymes that are up-regulated in many diseases, including osteoarthritis (OA) and rheumatoid arthritis (RA). Here we report on a novel technique that can be used to simultaneously measure activity levels for a panel of enzymes, such as the MMPs. The technique, termed the multiple-enzyme/multiple-reagent assay system (MEMRAS), relies on the use of reagents such as substrates with varying selectivity profiles against a group of enzymes. When reaction rates are measured by following a change in fluorescence with time, for mixtures of enzymes, an equation with unknown concentrations for each activity is generated for each reagent used. Simultaneously solving the set of equations leads to a solution for the unknown concentrations. We have applied this mathematical technique to measure activity levels for mixtures of MMPs such as collagenase 3 and gelatinase A. In addition, because we were most interested in determining collagenase 3 levels as a potential biological marker for OA, we developed highly selective substrates for this enzyme by using results found in previous bacteriophage substrate-mapping experiments. Some of the best substrates tested have specific activities for collagenase 3 that are 37 000-, 17 000-, 90-, and 200-fold selective over stromelysin 1, collagenase 1, and gelatinases A and B, respectively.

Osteoarthritis (OA)[1] is a disease of the joints that affects nearly 65 million people. Current techniques that help diagnose the disease and determine disease progression rely on standard X-ray imaging techniques (*1*) as well as clinical symptoms that include swelling and severe joint pain (*2*). Standard treatments for osteoarthritis are typically non-steroidal anti-inflammatory drugs that treat the symptoms but do not alter disease progression (*3*). Hence, there is a

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[1] Abbreviations: Coll 3, collagenase 3; Coll 1, collagenase 1; CSV, comma-separated variable; Dabcyl, 4-dimethylaminophenylazobenzoyl; DNP, dinitrophenylalanyl; ELISA, enzyme-linked immunosorbent assay; FlSub, fluorescent substrate; Flu, fluoresceine; Gel A, gelatinase A; Gel B, gelatinase B; HFBA, heptafluorobutyric acid; HPLC, high-performance liquid chromatography; MEMRAS, multiple-enzyme/multiple-reagent assay system; MMP, matrix metalloproteinase; OA, osteoarthritis; RA, rheumatoid arthritis; SARs, structure−activity relationships; Strom 1, stromelysin 1; TIMP, tissue inhibitor of metalloproteinases; uTIINE, the urinary type II collagen neo-epitope assay.

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great unmet medical need for both diagnostic procedures and novel drugs that prevent cartilage degradation and bone loss associated with OA.

Matrix metalloproteinases (MMPs) are a class of enzymes whose members have been associated with extracellular matrix degradation (*4*). Research efforts have focused on making inhibitors of the MMPs that prevent degradation of type II collagen, as this structural protein is the principal component of cartilage found in joints (*5*). Degradation of type II collagen occurs in what is believed to be a two-step process (*6*). First, enzymes such as collagenases (principally 1 and 3) cleave type II collagen at a specific site (GPQG/LAGQ) (*7*, *8*). Once this cleavage event occurs, the triple-helical collagen begins to unwind and is then subject to degradation by other MMPs such as gelatinase B (*9*).

This type of collagen processing is not limited to OA but also occurs in rheumatoid arthritis (RA). One of the major differences between the two diseases is the varied levels of MMPs in biological fluids (*10*). Hence, for OA, while several MMP levels may be higher in synovial fluid or serum relative to normal controls (*11*), the major degradative enzyme of collagen is thought to be collagenase 3 (*12*, *13*). In RA, biological fluid levels of most MMPs are much higher relative to those from OA patients, indicating that other MMPs may be principal agents for this autoimmune disease (*14*, *15*). Therefore, for diagnostic purposes, measuring a panel of enzyme activities may be more informative than determining amounts of just one component.

Techniques that have attempted to measure MMP activity include type II collagen neo-epitope antibodies, the "uTIINE" assay, and fluorescent substrate assays. When an antibody is made that specifically detects a cleavage site that includes either the free amine or the carboxy terminus in a peptide sequence, it is referred to as a neo-epitope antibody. Using neo-epitope Ab's (*16*) to the sequence GPQG-COOH, a principal cleavage site in type II collagen, Poole and co-workers detected processed type II collagen fragments in synovial fluid from rheumatoid and osteoarthritic patients (*17*, *18*). Another neo-epitope antibody to a new terminus generated by collagenase 1, 2, and/or 3 cleavage of type II collagen has been created by Pfizer (*19*). This antibody, 9A4, recognizes the sequence GPP(OH)GPQG-COOH (*20*). Together with an upstream anti-collagen II-specific antibody, collagen fragments are detected in urine (the urinary type II collagen neo-epitope assay, or uTIINE) (*21*). The urinary TIINE activity has been correlated with OA disease activity (*22*). This demonstrates the importance of collagenase activity in the osteoarthritis disease process. However, these assays are cumbersome and do not discern a particular collagenase or the relative contribution of the various collagenases in different arthritides.

Fluorescent and colorimetric substrates have been used in an attempt to directly measure MMP activities in synovial fluid or plasma. Beekman and co-workers provided preliminary data, using peptide substrates to measure MMP activities in both fluids (*23*−*25*). The fluorescent substrates used were sensitive, but they were not selective enough to determine the contribution of one particular enzyme to the overall MMP activity. For example, since stromelysin is present in abundant quantities in synovial fluid and blood plasma in rheumatoid arthritis (*26*), it will contribute to the measurable activity when less specific substrates are employed.

Recently, triple-helical collagen internally quenched fluorescent substrates that were specific to the gelatinases have been used to accurately determine concentrations of these MMPs in cellular-based assays (*27*). By placement of sequences in a triple-helical context, the substrates were more stable to nonspecific degradation and could be used in a cellular system.

In this work, we describe peptide sequences and provide structure−activity relationships for highly selective and novel collagenase 3 substrates. Some of the peptide sequences were converted into fluorescent substrates that are extremely sensitive and can be used in a technique called MEMRAS (multiple-enzyme/multiple-reagent assay system). We describe the MEMRAS technique and use it to measure activity levels for a mixture of two MMPs, collagenase 3 and gelatinase A. These MMPs were chosen because they are typically up-regulated in synovial fluid from OA and RA patients. Therefore, they can serve as potential biological markers to diagnose or follow disease progression in arthritis.

MATERIALS AND METHODS

Materials. All peptides were purchased from SynPep Corp. All buffer components and reagents were obtained from Sigma. Black-coated clear-bottom 96-well plates were purchased from Costar. The MMP inhibitor, GM 6001, was purchased from Chemicon. Filtration plates for removing precipitated protein from samples for HPLC analysis were purchased from Millipore. The C18 column was purchased from Vyadac.

Determination of Enzyme Concentrations. The enzyme concentrations were determined by active-site titration with the tight-binding inhibitor GM 6001 or TIMP 1 using the fluorescent substrate Dabcyl-GPLGMRGC(fluorescein)-NH_2 in buffer containing 50 mM Tris, pH 7.5; 200 mM NaCl; 5 mM $CaCl_2$; 10 μM $ZnCl_2$; and 0.01% Brij 35. Data were fit to the Morrison equation (*28*).

Once the enzyme concentrations were known, the specificity constants were calculated for collagenase 3, collagenase 1, gelatinases A and B, and stromelysin 1 using the consensus sequence substrate 1, dinitrophenyl-GPLGMRG-NH_2. This substrate was always used in subsequent HPLC experiments to serve as an internal control for variability in enzyme concentrations due to preparation conditions and/or for stock solution concentration changes due to storage.

Determination of k_{cat}/K_m for Dinitrophenyl (Dnp) Peptide Substrates. The Dnp substrates listed in Tables 1 and 2 were prepared and assayed as described below. Briefly, a dimethyl sulfoxide stock of 5 mM peptide was diluted in assay buffer so that the substrate concentration was 25 μM in a 200-μL volume. In the case of substrate 35, solubility proved to be a problem. Therefore, the substrate concentration was reduced to 5−10 μM. The assay buffer contained 50 mM Tris, pH 7.5; 200 mM NaCl; 5 mM $CaCl_2$; 10 μM $ZnCl_2$; and 0.01% Brij 35. After addition of enzyme (0.05, 0.17, 0.17, 10, and 17 nM final concentrations for collagenase 3, gelatinase A, gelatinase B, collagenase 1, and stromelysin 1, respectively), time points were taken at 10 min, 30 min, 1 h, and 2 h when a 50-μL aliquot was removed and quenched with 50 μL of 1% heptafluorobutyric acid (HFBA). For collagenase 1 and stromelysin 1, sometimes 100-μL reactions were used and two time points were taken to

Table 1: Specificity and Selectivity Summary for Substrates 1−28

HPLC (colorimetric) substrate	Coll 3 specificity constant (M^{-1} s^{-1})	selectivity vs Coll3 (unitless)			
		Gel A	Gel B	Coll 1	Strom 1
1, DNP-GPLGMRG-NH_2	3.5×10^6	11	5.3	350	4 900
2, DNP-PINLHG-NH_2	3.8×10^5	9.3	3.9	100	820
3, DNP-GPINLHG-NH_2	7.0×10^5	28	15	180	1 100
4, DNP-GPSELKG-NH_2	8.6×10^5	6.9	4.4	470	830
5, DNP-PHPFRG-NH_2	2.3×10^5	28	4.2	2 200	1 100
6, DNP-GPSGIHV−NH_2	1.4×10^5	10	18	1 400	36 000
7, DNP-VTPYNMRG-NH_2	2.7×10^5	31	3.7	39	1 000
8, DNP-GPLQFRG-NH_2	7.0×10^5	32	6.9	3 700	700
9, DNP-GPKGMRG-NH_2	2.3×10^6	7.3	2.8	1 600	13 000
10, DNP-GPYGMRA-NH_2	5.7×10^6	16	5.9	210	410
11, DNP-GPKGITS-NH_2	7.3×10^5	6.3	2.3	550	37 000
12, DNP-GPRPFRG-NH_2	6.3×10^5	8.8	4.4	4 200	
1, DNP-GPLGMRG-NH_2	2.2×10^6	4.7	1.7	180	4 300
13, DNP-GPLSISG-NH_2	1.9×10^5	7.4	1.5	11	
14, DNP-GPMSYNG-NH_2	8.9×10^5	5.3	3.0	200	
15, DNP-GPLSFQG-NH_2	4.5×10^5	21	4.8	690	190
16, DNP-GPLSIQD-NH_2	8.7×10^5	65	6.3	45	590
17, DNP-GPHPFRG-NH_2	9.0×10^5	17	8.0	3 300	2 300
18, DNP-GPSGIHL-NH_2	2.8×10^5	7.7	6.4	3 400	2 000
19, DNP-GPVNLHG-NH_2	1.1×10^6	25	11	460	1 300
20, DNP-PLGMRG-NH_2	9.8×10^5	2.0	1.0	73	
21, DNP-PSGIHL-NH_2	9.2×10^4	2.3	1.6	1 300	
1, DNP-GPLGMRG-NH_2	2.4×10^6	6.8	1.9	590	2 000
22, DNP-GPFGLKG-NH_2	1.3×10^6	26	3.0	410	1 400
23, DNP-GPHPMRG-NH_2	2.3×10^6	12	4.0	530	290
24, DNP-GPLQMRG-NH_2	8.9×10^5	15	1.1	290	310
25, DNP-DEGPMG-LKC(Me)YLG-NH_2	5.3×10^5	9.4	4.0	450	470
26, DNP-GPVNLHGRC(Me)-NH_2	1.0×10^6	8.7	2.2	2 100	510
27, DNP-GPVNLHGC(Me)-NH_2	8.1×10^5	15	2.6	1 400	770
28, DNP-VC(Me)PKGIT-SC(Me)VFR-NH_2	1.5×10^6	7.5	1.4	220	840

Table 2: Specificity and Selectivity Summary for Substrates 29−44

HPLC (colorimetric) substrate	Coll 3 specificity constant (M^{-1} s^{-1})	selectivity vs Coll3 (unitless)			
		Gel A	Gel B	Coll 1	Strom 1
1, DNP-GPLGMRG-NH_2[a]	4.3×10^6	15	9.3	370	5 800
29, DNP-GPLGLHGC(Me)-NH_2	3.7×10^6	16	4.6	660	10 000
30, DNP-GPLGFRGC(Me)-NH_2	2.4×10^6	10	7.1	11 000	7 300
31, DNP-GPLGFRVC(Me)-NH_2	3.9×10^6	15	20	17 000	4 000
32, DNP-GPLPFHVC(Me)-NH_2	6.0×10^5	20	18	2 900	620
33, DNP-GPSPFHVC(Me)-NH_2	3.4×10^5	18	54	1 600	2 100
34, DNP-GPSPLHGC(Me)-NH_2	1.8×10^6	7.5	6.5	270	3 000
35, DNP-GPVNFRVC(Me)-NH_2	2.6×10^6	88	220	>17 000	400
36, DNP-GPAPFRGC(Me)-NH_2	1.1×10^6	8.7	14	4 400	490
37, DNP-GPAPFRVC(Me)-NH_2	1.8×10^6	37	82	5 800	450
38, DNP-GPAPLHGC(Me)-NH_2	1.7×10^6	12	10	470	620
39, DNP-GPLPFRGC(Me)-NH_2	9.7×10^5	16	8.1	430	810
40, DNP-GPLPFRVC(Me)-NH_2	1.3×10^6	36	21	550	330
41, DNP-GPSPFRGC(Me)-NH_2	1.3×10^6	10	13	2 400	1 400
42, DNP-GPAPLHVC(Me)-NH_2	6.8×10^5	12	11	130	260
43, DNP-GPLGLHVC(Me)-NH_2	4.5×10^6	32	26	180	13 000
44, DNP-GPLPLHGC(Me)-NH_2	1.1×10^6	20	9.8	36	990

[a] Used to correct for variability in enzyme concentrations from preparation to preparation. This value represents the average of three separate experiments.

conserve the amount of enzyme used. Samples were filtered through a Millipore plate coated with a PVDF membrane, using a vacuum manifold, and then run through an HPLC C-18 column from Vyadac using a Hewlett-Packard 1090 HPLC. Turnover of substrates was compared to Dnp-GPLGMRG-NH_2, since the specificity constants for this substrate have already been determined multiple times for collagenase 3, collagenase 1, gelatinases A and B, and stromelysin 1. Using the specificity constant data for Dnp-GPLGMRG-NH_2, the k_{cat}/K_m was calculated for the new substrates. The specificity constant for Dnp peptides is defined as fractional conversion/(reaction time × enzyme concentration), where fractional conversion is determined by analysis of HPLC-separated substrate and product peak areas and enzyme concentration is determined by active-site titration with TIMP 1 or GM 6001.

Determination of k_{cat}/K_m for Dnp Peptide Substrates with Prionex Gelatin. Collagenase 3, collagenase 1, and gelatinases A and B were reacted in a standard assay buffer containing 25 μM Dnp-GPLGMRG-NH_2, with increasing concentrations of Prionex gelatin. Reaction volumes of 100 μL were used, and time points were taken to demonstrate adequate turnover while still operating in the linear range for the turnover versus time curve. Reactions were run at

Table 3: Specificity and Selectivity Summary for Substrate 1 with Prionex Gelatin

HPLC (colorimetric) substrate	Coll 3 specificity constant (M^{-1} s^{-1})	selectivity vs Coll3 (unitless)			
		Gel A	Gel B	Coll 1	Strom 1
DNP-GPLGMRG-NH_2 + H_2O	3.2×10^6	5.9	1.8	250	
DNP-GPLGMRG-NH_2 + 1:1 gelatin	3.4×10^5	41	5.2	91	
DNP-GPLGMRG-NH_2 + 1:4 gelatin	5.6×10^5	46	5.2	96	
DNP-GPLGMRG + 1:16 gelatin	1.9×10^6	85	13	210	
DNP-GPLGMRG-NH_2 + 1:64 gelatin	2.7×10^6	69	13	240	
DNP-GPLGMRG-NH_2 + 1:256 gelatin	4.6×10^6	73	18	380	
DNP-GPLGMRG-NH_2	2.3×10^6	6.8	1.9	590	2 000

room temperature and quenched by addition of an equal volume of 1% HFBA. Samples were analyzed as described above

Determination of k_{cat}/K_m for Fluorescent Peptide Substrates. Human collagenase 3, gelatinase A, gelatinase B, collagenase 1, and stromelysin 1 were thawed from the −80 °C freezer and diluted with assay buffer (50 mM Tris, pH 7.5; 200 mM NaCl; 5 mM $CaCl_2$; 10 μM $ZnSO_4$; 0.01% Brij-35) to achieve the desired concentrations.

Five different fluorescent substrates were used in these experiments, which were labeled as FlSub1, FlSub3, FlSub4, FlSub15, and FlSub16. FlSub1 is Dabcyl-GPLMRGC(Flu)-NH_2, FlSub3 is Dabcyl-GPVNLHGC(Flu)-NH_2, FlSub4 is Dabcyl-GPHPFRGC(Flu)-NH_2, FlSub 15 is Dabcyl-GPLGFRVC(Flu)-NH_2, and FlSub 16 is Dabcyl-GPSGIHVC(Flu)-NH_2. The substrates were thawed from the −80 °C freezer and diluted with assay buffer (50 mM Tris, pH 7.5; 200 mM NaCl; 5 mM $CaCl_2$; 2 μM $ZnSO_4$; 0.01% Brij-35) to achieve concentrations of 10−20 μM in the final assay solution. Prior to being placed in the freezer, the substrates were diluted from a dry powder to 5 mM concentration in DMSO.

Reactions were performed in a CoStar 96-well plate, black plastic with a transparent flat bottom. All reactions were performed in duplicate. All wells contained 20 μL of the diluted enzyme and 80 μL of the 20 μM substrate mixture. As a control to determine background fluorescence levels, two columns (12 wells total) contained 20 μL of the assay buffer and 80 μL of the substrate mixture. The enzyme dilutions and assay buffer were added first, and then the substrate mixtures were added using a multipipettor. Immediately after addition of the substrates, the plate was inserted into a CytoFluor fluorescence plate reader. Excitation and emission filters were set to 485 and 530 nm, respectively. The CytoFluor's sensitivities were set to 1, 2, 3, and 4. Readings were automatically recorded every 10 or 15 min for 10 or 15 h, respectively.

Data from the CytoFluor plate reader was imported into a Microsoft Excel workbook to calculate the substrate specificity (k_{cat}/K_m) and the substrate selectivity for each enzyme/substrate pair.

Any technique used to determine substrate specificity requires a few basic pieces of information, such as the net fluorescence at complete turnover, the net rate of increase in fluorescence in the linear range, and the enzyme concentration in the reaction well. The sequence of events in the analysis is importation of the fluorescence versus time data, combination of the duplicated well fluorescence counts into a single averaged value, and subtraction of the averaged background fluorescence from the averaged total fluorescence in the well to give the net averaged fluorescence. The slopes of the net averaged fluorescence versus time curves are then calculated, along with manual determination of the maximum net averaged fluorescence for each enzyme and substrate pair. Finally, the substrate specificity and selectivity are calculated from the accumulated data. In these experiments, the selectivities were calculated as the ratio of the specificity of collagenase 3 to the specificity of each of the other enzymes for a given substrate.

The formula used to calculate substrate specificity (k_{cat}/K_m) for fluorescent substrates was

$$k_{cat}/K_m = M/(3600F_eC_e)$$

where M is the slope of the net averaged fluorescence versus time curve in the early linear range (counts/hour), 3600 is the conversion constant (seconds/hour) F_e is the net increase in fluorescence reading at the end point (maximum net averaged fluorescence (counts)), and C_e is the concentration of enzyme in the reaction (molar).

Two-by-Two MEMRAS Experiment with Collagenase 3 and Gelatinase A. Collagenase 3 and gelatinase A were diluted from enzyme stocks stored at −80 °C. The substrate stocks at concentrations of 5 mM for FlSub1 (14 μL) were diluted into assay buffer (3486 μL) as described above, with and without Prionex gelatin added (14 μL). FlSub3 was also diluted to give a final concentration of 20 μM. The Prionex gelatin was used as a specific competitive substrate (inhibitor) of gelatinase A. Inspection of Table 3 shows that a ratio of 1:256 Prionex gelatin to buffer provided about a 10-fold increase in collagenase 3 selectivity over the gelatinases. We chose this concentration of gelatin for the MEMRAS experiment. In addition, three reagents were used (FlSub1, FlSub3, and FlSub1 plus gelatin), so that it could be empirically determined during data analysis which combinations worked the best.

Reactions were run in a 96-well black Costar plate with a transparent bottom. Columns 1−3 contained varying standard concentrations of collagenase 3 (0.0013−0.02 nM) in the presence of FlSub1, FlSub3, and FlSub1 plus gelatin. Columns 4−6 contained varying standard concentrations of gelatinase A (0.013−0.1 nM) in the presence of FlSub1, Flsub3, and FlSub1 plus gelatin. The concentrations chosen were based on literature values for levels of MMPs found in synovial fluid via ELISA.

Columns 7−9 contained mixtures of varied composition in the presence of FlSub1, FlSub3, and FlSub1 plus gelatin. Reactions were run in duplicate. Buffer-only samples (columns10−12) with the appropriate substrate were prepared so that a background rate could be subtracted.

Two-by-Two MEMRAS Data Analysis. A Cytofluor fluorescence plate reader was used to monitor the reaction progress. Typically readings were taken every 5 min for a

total run time of 5 h. Excitation and emission wavelengths were 485 and 530 nm, respectively. Sensitivities were set to three different levels to ensure that data were collected under conditions without saturation of signal.

For the data analysis, the comma-separated variable (CSV) files from the CytoFluor machine were imported into Microsoft Excel. Plots of net fluorescence versus time were generated, and initial velocities were determined by fitting lines to the linear portions of the progress curves. The workbook containing the standard curve slope versus concentration data and the mixed sample response data was then linked to a simultaneous equation solver workbook, where the enzyme concentrations were calculated.

MEMRAS Simultaneous Equation Definitions. The basic system equation can be written as

$$\sum S_{i,j}C_j = S_i$$

where S_i is the slope of the net fluorescence versus time curve for the well containing the unknown sample reacted with reagent *i* (the unknown sample contains multiple enzymes), $S_{i,j}$ is the slope of the slope of the net fluorescence versus time curve versus enzyme concentration for enzyme *j* reacted with reagent *i*, and C_j is the concentration of enzyme *j*.

In this case, the system equation gives rise to two equations in two unknowns:

$$S_{R_A,E_1}C_{E_1} + S_{R_A,E_2}C_{E_2} = S_{R_A}$$

$$S_{R_B,E_1}C_{E_1} + S_{R_B,E_2}C_{E_2} = S_{R_B}$$

where S_{R_A} is the slope of the net fluorescence versus time curve for the well containing collagenase 3 and gelatinase A combined, reacted with reagent A (fluorescent substrate 1), a known quantity; S_{R_B} is the slope of the net fluorescence versus time curve for the well containing collagenase 3 and gelatinase A combined, reacted with reagent B (fluorescent substrate 1 + gelatin), a known quantity; S_{R_A,E_1} is the slope of the slope of the net fluorescence versus time curve versus enzyme concentration for enzyme 1 (collagenase 3), reacted with reagent A, a known quantity; S_{R_A,E_2} is the slope of the slope of the net fluorescence versus time curve versus enzyme concentration for enzyme 2 (gelatinase A), reacted with reagent A, a known quantity; S_{R_B,E_1} is the slope of the slope of the net fluorescence versus time curve versus enzyme concentration for enzyme 1 (collagenase 3), reacted with reagent B, a known quantity; S_{R_B,E_2} is the slope of the slope of the net fluorescence versus time curve versus enzyme concentration for enzyme 2 (gelatinase A), reacted with reagent B, a known quantity; C_{E_1} is the concentration of enzyme 1 (collagenase 3), an unknown quantity; C_{E_2} is the concentration of enzyme 2 (gelatinase A), an unknown quantity; E_1 is enzyme 1 (collagenase 3); E_2 is enzyme 2 (gelatinase A); R_A is reagent A (fluorescent substrate 1); and R_B is reagent B (fluorescent substrate 1 + gelatin).

RESULTS

Development of Selective Dinitrophenyl Substrates for Collagenase 3. To produce useful reagents that can measure collagenase 3 activity in biological fluids, we needed to develop substrates that had improved selectivity profiles against a panel of MMPs compared to the published sequences. We chose to work with Dnp-labeled substrates and carry out analyses by HPLC, as this was less costly than preparing fluorescent substrates. To develop structure−activity relationships (SARs), we prepared a number of substrates based upon the bacteriophage display mapping data in Deng et al. (*30*) and from BLAST searches performed with the existing sequences (http://www.ncbi.nlm.nih.gov).

By inspecting the experimentally determined selectivities of substrates 1−28, four SARs were determined. First, glycine before phage clone consensus substrate sequence (PLGMRG) increases the reactivity toward collagenase 3 and sometimes leads to better selectivity over the other MMPs (see Table 1 for substrate 20 versus 1 and substrate 2 versus 3). Second, valine at position S3′ increases the selectivity of substrate 6 over 18 for stromelysin and for the gelatinases. Third, placement of phenylalanine at S1′ enhances selectivity for collagenase 3 against collagenase 1 (see substrate 1 versus 8). Finally, a hydrogen-bonding amino acid such as arginine, histidine, or serine at S2 also improves selectivity for collagenase 3 against stromelysin (see substrates 6, 9, and 11).

Next, 12mer substrates were prepared on the basis of the exact sequences found in BLAST searches. For example, substrates 25 and 28 correspond to putative physiological cleavage sites in type IV collagen and TGF beta, respectively. For these substrates, no advantage was gained over our existing bacteriophage sequences. However, the BLAST searches also led to some of the most selective substrates, including the sequence GPVNLHG (see substrates 26 and 27).

From the results in Table 1, a second generation of substrates was prepared and is found in Table 2. Notable examples include substrate 35, Dnp-GPVNFRVC(Me)-NH_2, and substrate 31, Dnp-GPLGFRVC(Me)-NH_2. Substrate 35 had the best selectivities for collagenase 3 against the gelatinases A and B and collagenase 1 with ratios of 88-, 220-, and >17 000-fold, respectively. Substrate 31, Dnp-GPLGFRVC(Me)-NH_2, had ratios of 15-, 20-, 17 000-, and 4000-fold for collagenase 3 over gelatinases A and B, collagenase 1, and stromelysin 1, respectively.

Addition of Gelatin To Improve Selectivities against the Gelatinases. While we developed highly selective substrates for stromelysin 1 and collagenase 1 (>10 000-fold), selectivity improvements were more difficult to achieve for the gelatinases. In order for the MEMRAS technique to work with biological fluids, reagents needed to be identified with variable selectivity profiles for all the MMPs of interest. Thus, if gelatinases were to be measured, some reagent needed to be added to compete with and reduce or eliminate their activities. As a test case, Prionex gelatin was chosen as a competitive substrate for the gelatinases. Prionex gelatin was chosen because it is homogeneous, inexpensive, and readily available. Table 3 shows the results of an experiment using the consensus substrate, Dnp-GPLGMRG-NH_2, with increasing concentrations of gelatin. The selectivity for collagenase 3 against gelatinases A and B was improved approximately 10-fold when Prionex gelatin was diluted 256-fold into the assay. At this concentration of gelatin, the specific activity of collagenase 3 was slightly enhanced relative to the control. A caveat with this procedure is that, depending on the substrate used, the improvement of

Table 4: Specificity and Selectivity Summary for Fluorescent Substrates

fluorescent substrate	Coll 3 specificity constant (M^{-1} s^{-1})	selectivity vs Coll3 (unitless)			
		Gel A	Gel B	Coll 1	Strom 1
FlSub1					
Dab-GPLGMRGC(Flu)-NH_2	1.6×10^6	3.9	1.2	73	710
(+ gelatin)	1.7×10^6	26	6.3	78	770
normalized	4.4×10^6	11	3.3	210	1 900
normalized (+ gelatin)	4.7×10^6	70	17	210	2 100
FlSub3					
Dab-GPVNLHGC(Flu)-NH_2	4.2×10^5	2.3	0.7	130	71
(+ gelatin)	4.7×10^5	26	6.0	92	73
normalized	1.1×10^6	6.4	1.9	350	190
normalized (+ gelatin)	1.3×10^6	72	16	250	200
FlSub4					
Dab-GPHPFRGC(Flu)-NH_2	3.3×10^4	15	2.6	88	44
(+ gelatin)	2.7×10^4	nd[b]	12	170	54
normalized	8.9×10^4	40	7.0	240	120
normalized (+ gelatin)	7.3×10^4	nd[b]	31	460	150
FlSub15					
Dab-GPLGFRVC(Flu)-NH_2	2.7×10^6	44	7.5	4 600	540
normalized	6.9×10^6	114	19	12 000	1 400
FlSub16					
Dab-GPSGIHVC(Flu)-NH_2	2.5×10^6	20	7.4	1 100	2 800
normalized	6.5×10^6	51	19	2 700	7 300

[a] Values were normalized to specificity constants calculated multiple times for FlSub1. [b] Activity nondetectable under the conditions used in the experiment.

selectivity against the gelatinases may vary. Thus, selectivities for each substrate with and without gelatin should be characterized before proceeding to a MEMRAS experiment.

Specificity Constant Determination for Fluorescent Substrates. Internally quenched fluorescent substrates were synthesized based upon the sequences in Tables 1 and 2. The Dabcyl/fluorescein pair was chosen since substrates with these fluorophores have previously shown good sensitivity (Beekman et al.). The most promising five substrates, FlSub1, FlSub3, FlSub4, FlSub15, and FlSub16, were chosen and are shown in Table 4. These substrates were based on Dnp peptides 1, 19, 17, 31, and 6, found in Tables 1 and 2, respectively. The reaction progress was monitored by following the change in fluorescence with excitation of 485 nm and emission of 530 nm. Specificity constants were determined for all five substrates against the five MMPs (collagenase 3, collagenase 1, gelatinases A and B, and stromelysin 1) and are found in Table 4. Substrate FlSub1, -3, and -4 were tested with and without Prionex gelatin. As shown in Table 4, the gelatin improved the selectivities against both gelatinase A and gelatinase B by a factor of 5−10-fold. The poorest substrate in terms of k_{cat}/K_m was FlSub4, which proved to be unusable. The least selective substrate was FluSub3, with selectivities of only 6.4, 1.9, 350, and 190 against gelatinase A, gelatinase B, collagenase 1, and stromelysin, respectively. The best substrate was FlSub16, with $k_{cat}/K_m = 6.5 \times 10^6$ M^{-1} s^{-1}, and it had improvements in its selectivity against all the MMPs tested. For example, the collagenase 3 specificity constant was 2700- and 7300-fold greater than those of collagenase 1 and stromelysin 1, respectively. FlSub 15 was also an efficient substrate with better selectivity ratios than FlSub1, -3, and -4.

Two-Enzyme/Two-Reagent MEMRAS. We first tested the MEMRAS approach using two enzymes and two reagents: collagenase 3 and gelatinase A, and FluSub1 (reagent A) and FluSub1 + gelatin (reagent B). The five steps in the experiment and analysis are described below. First, in step 1, the enzyme standard dilutions, mixed-enzyme samples, and reagents were added to a 96-well plate. More specifically, four combinations of concentrations of collagenase 3 and gelatinase A were placed in twice-duplicated wells. To sets of duplicated wells were added reagent A and reagent B. Other wells contained standard curves of collagenase 3 and gelatinase A versus reagents A and B. In step 2, the plate reader recorded the fluorescence values every 5 min for 5 h. In step 3, the values of the slope of the net fluorescence versus time curves were plotted versus enzyme concentration for the standard enzyme dilutions, for each enzyme/reagent pair. In step 4, the net fluorescence versus time was plotted for the mixed-enzyme samples when reacted with each reagent. Finally, in steps 5 and 6, the slopes from steps 3 and 4 were combined into a set of two equations in two unknowns and solved for the "unknown" enzyme concentrations in the mixed-enzyme samples.

As a comparison, we contrasted the MEMRAS two-enzyme/two-reagent approach against a single-reagent approach. In a standard single-reagent approach, we hope the substrate is completely selective for the desired enzyme. If the substrate is perfectly selective for the desired enzyme, say enzyme 1, it is converted efficiently by enzyme 1 and not converted at all by enzyme 2. In this case, all activity seen in the mixed-enzyme sample would be due to the desired enzyme. Here, we take the total net activity of the mixed-enzyme sample (counts/h) and divide it by the net activity per unit of desired enzyme for the "perfect" substrate (counts/h/M) to arrive at the concentration of "desired" enzyme.

Mathematically, in the standard single-reagent approach, the system equations reduce to

$$S_{R_A,E_1} C_{E_1} = S_{R_A}$$

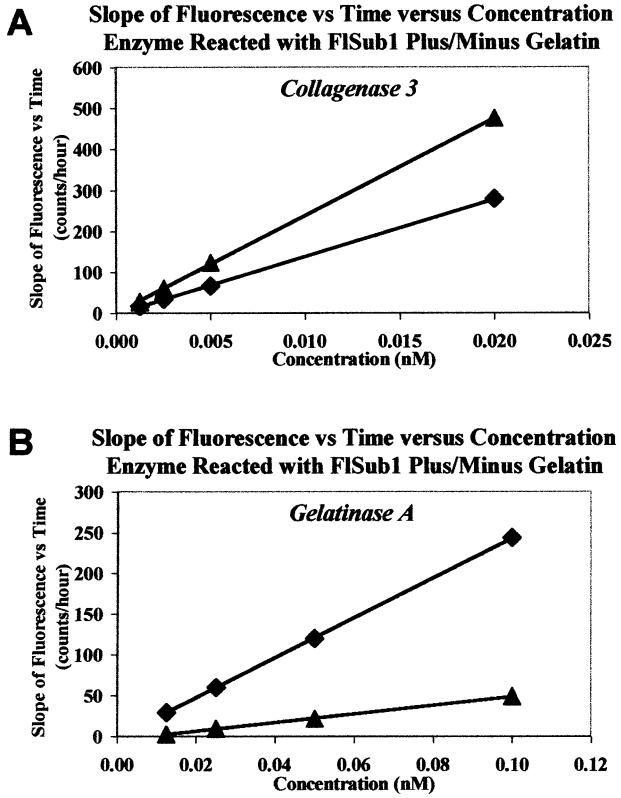

FIGURE 1: Plots of the slope of the net average fluorescence versus time versus concentrations. (A) Collagenase 3 initial velocities, from a fit to the data for net average fluorescence versus time, were plotted against enzyme concentrations. (B) Gelatinase A initial velocities, from a fit to the data for net average fluorescence versus time, were plotted against enzyme concentrations. The two plots represent reactions performed with substrates FlSub 1 (◆) and FlSub1 + gelatin (▲). R^2 values were all greater than 0.99. Slopes from these graphs were used in the determination of concentrations of enzymes in sample mixtures.

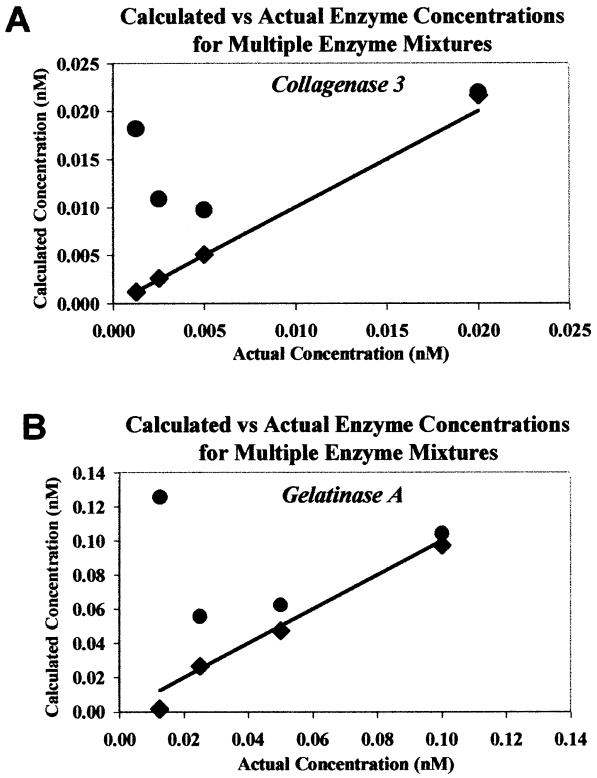

FIGURE 2: Actual versus calculated concentrations of MMPs in sample mixtures. Actual concentrations are represented by the straight line (—). Calculated MEMRAS concentrations are represented by ◆, and calculated single-reagent concentrations are denoted by ●. (A) The plot represents concentrations for collagenase 3 in four sample mixtures. (B) The plot represents concentrations gelatinase A in four sample mixtures.

or, solving for C_{E_1},

$$C_{E_1} = S_{R_A}/S_{R_A,E}$$

A detailed explanation of all symbols used in the equations is included in the Materials and Methods section.

Figure 1 displays the standard curves for collagenase 3 and gelatinase A, all of which fit well to a straight line ($R^2 > 0.99$). The slopes calculated from the plots in Figure 1 were used in determining the concentrations of the two MMPs in the mixed samples. Figure 2 displays the plots of the actual versus calculated concentrations of collagenase 3 and gelatinase A using the MEMRAS two-reagent approach. Table 5 contains the numerical representation of Figure 2. As a comparison, the calculated concentrations for the MMPs, based on a single-reagent approach, are also depicted in Figure 2.

As can be seen in Figure 2 and Table 5, the two-by-two MEMRAS accurately predicted the concentrations of collagenase 3 and gelatinase A in four different multiple-enzyme samples (even with collagenase 3 concentrations in the low picomolar range). The percent errors in the MEMRAS-calculated concentrations ranged from 2 to 8% for collagenase 3. The largest error occurred when calculating the lowest concentration of gelatinase A in the presence of the highest concentration of collagenase 3. However, even in this case, while there was an 86% error, MEMRAS did correctly indicate a relatively low concentration of gelatinase A and a relatively high concentration of collagenase 3 (8% error).

In Table 5, the MEMRAS approach accurately measured a collagenase 3 concentration of 0.0013 nM (2% error), even in the presence of 77 times higher levels of gelatinase A (0.1 nM) in the same sample. In contrast, a standard single-reagent approach accurately measures enzyme concentration only if the "desired" enzyme concentration is relatively high and the "other" enzyme concentration is relatively low (Table 6). A standard single-reagent approach reports large errors in enzyme concentration if the "desired" enzyme level is relatively low and the "other" enzyme level is relatively high (Table 6). Thus, when collagenase 3 is 0.0013 nM and gelatinase A is 0.10 nM, the error for the collagenase 3 concentration is approximately 1400%. Likewise, when the gelatinase A and collagenase 3 levels approach one another, the error on the gelatinase A concentration exceeds 900%.

CONCLUSION

On the basis of the preliminary work reported by Deng et al. (*29*), we have developed collagenase 3 substrates with improved selectivities against several MMPs. The substrate sequences were chosen on the basis of the development of SARs by combining BLAST search results with the testing of published phage sequences. The best Dnp-labeled substrates were substrates 6, 17, 19, 31, and 35. These substrates showed improved selectivities over the consensus sequence, substrate 1, and were translated into useful fluorescent substrates. The best fluorescent substrate, FlSub 15, was

Table 5: Actual versus Calculated Concentrations of Collagenase 3 and Gelatinase A Using a Two-by-Two MEMRAS Approach

	enzyme 1−collagenase 3			enzyme 2−gelatinase A		
enzyme mixture (Col3:GelA)	actual concn (nM)	calcd conc (nM)	calcd concn error (%)	actual concn (nM)	calcd concn (nM)	calcd concn error (%)
1:0.63	0.0200	0.0216	8	0.013	0.002	−86
1:5	0.0050	0.0051	2	0.025	0.027	6
1:20	0.0025	0.0026	4	0.050	0.048	−5
1:77	0.0013	0.0012	−2	0.100	0.097	−3

Table 6: Actual versus Calculated Concentrations of Collagenase 3 and Gelatinase A Using a Standard Single-Reagent Approach

	enzyme 1−collagenase 3			enzyme 2−gelatinase A		
enzyme mixture (Col3:GelA)	actual concn (nM)	calcd concn (nM)	calcd concn error (%)	actual concn (nM)	calcd concn (nM)	calcd concn error (%)
1:0.63	0.0200	0.0219	10	0.013	0.126	910
1:5	0.0050	0.0097	94	0.025	0.056	120
1:20	0.0025	0.0109	340	0.050	0.062	25
1:77	0.0013	0.0182	1400	0.100	0.104	4

based on Dnp substrate 31. Unfortunately, stromelysin 1 preferred substrates with the internally quenched fluorophore pair Dabcyl/fluorescein relative to the dinitrophenyl colorimetric labeled substrates. For example, for peptide 6, Dnp-GPSGIHV-NH_2, the collagenase 3 to stromelysin 1 selectivity was 36 000-fold. However, in the corresponding fluorescent substrate, FlSub 16, the selectivity against stromelysin 1 was only 2800-fold. The increase in k_{cat}/K_m for stromelysin 1 relative to that for collagenase 3 was also found by comparing colorimetric substrate 31, Dnp-GPLG-FRV-NH_2, and fluorescent substrate FlSub 15, Dabcyl-GPLGFRVC(fluorescein)-NH_2. Since multiple features were changed on going from a Dnp-labeled to a fluorescent substrate, it is unclear at this time what structural feature the stromelysin prefers in the internally quenched substrates. What is clear is that our substrate 31, GPLGFRV, has improved selectivity over the consensus substrate, GPLG-MRG, for collagenase 3 over all the MMPs tested.

Also noteworthy is that addition of gelatin improves selectivities against the gelatinases. This is a simple alternative to using gelatinase-specific inhibitors that are typically unavailable commercially. Any of the substrates, with or without gelatin, could be useful in the MEMRAS technique to determine gelatinase concentrations in addition to the other MMPs. We have successfully tried, for example, a two-by-two MEMRAS with reagents FlSub 1 with or without gelatin and enzymes collagenase 3 and gelatinase A. The results proved to be very successful, giving an accurate determination of all concentrations in mixtures of the two enzymes. This technique could theoretically be used in the determination of plasma or synovial fluid levels since the two enzymes are elevated in patients with OA and RA (*10*). The gelatinases could also be predictive indicators of cancer (*30*). Thus, there is a great interest in quantitatively measuring gelatinase levels for a variety of disease states.

The substrates developed, even if not very selective, also became useful when the MEMRAS technique was developed. As opposed to a single reagent that is highly selective for a given MMP, MEMRAS relies on using as many reagents as there are enzymes, and the reagents do not necessarily have to be extremely selective. One note of caution is that careful choice of substrates is critical in making the MEMRAS technique work. For example, with the two-by-two MEMRAS, we also tried using substrates FlSub1 and FlSub3 with or without gelatin (data not shown). The errors were larger, in part because the substrate, FlSub3, is not as sensitive as FlSub1.

The two-by-two MEMRAS system can quantify how much of the total activity is due to collagenase 3 and how much is due to gelatinase A simply by using the fluorescence versus time curves of the enzyme mixtures and the enzyme standard curves. The measurement error of the MEMRAS system is significantly less (10-fold) than the error arising from a single-reagent system. The MEMRAS system is a unique solution to the problem that substrates are never quite selective enough to directly assay activities in biological fluids. This technique could also be applied to measurements of nonenzymatic activities, as long as an assay was available for measurement. For example, receptors could be quantified by binding assays, even if the receptors were not specific to a given ligand. Alternatively, an ELISA could be used where the antibodies cross-react with proteins of interest. We have chosen to use this system with members of the matrix metalloproteinase family because these enzymes are typically up-regulated in diseases such as arthritis and cancer. A larger MEMRAS, where stromelysin and collagenase 1 levels are also quantified, could be even more useful than the two-by-two MEMRAS described herein. Experiments are underway to assess the usefulness of this system in biological fluids.

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