Modified proenzymes as artificial substrates for proteolytic enzymes: colorimetric assay of bacterial collagenase and matrix metalloproteinase activity using modified pro-urokinase

Jan H. VERHEIJEN*§, Nancy M. E. NIEUWENBROEK*, Bob BEEKMAN*, Roeland HANEMAAIJER*, Hein W. VERSPAGET†, H. Karel RONDAY*‡ and Arjen H. F. BAKKER*

*Gaubius Laboratory TNO-PG, P. O. Box 2215, 2301 CE Leiden, and Departments of †Gastroenterology and ‡Rheumatology, Academic Hospital Leiden, 2300 CE Leiden, The Netherlands

We describe a new principle for assessment of the activity of proteolytic enzymes of all classes and show the application of this principle for the quantitative assay of bacterial collagenase and human matrix metalloproteinases (MMPs). Central to this new principle is the presence of a proenzyme that can be activated into an active enzyme by a single proteolytic event. The regular activation sequence in the proenzyme is replaced using protein engineering by an artificial sequence recognized by the proteinase to be determined. The latter can act as an activator for the newly engineered proenzyme. In the present paper a simple colorimetric assay for the determination for MMPs is described based on this principle. With the aid of protein engineering, a modified pro-urokinase has been prepared in which the activation sequence normally recognized by plasmin (Pro-Arg-Phe-Lys ↑ Ile-Ile-Gly-

Gly) has been replaced by a sequence expected to be recognized and hydrolysed by many MMPs (Arg-Pro-Leu-Gly ↑ Ile-Ile-Gly-Gly). The active urokinase resulting from activation of the modified pro-urokinase by a MMP could be measured either directly, using a specific chromogenic peptide substrate for urokinase, or indirectly via urokinase-catalysed plasminogen activation. The response of the assay to equal molar quantities of active MMPs decreases in the order MMP-2 > MMP-9 > MMP-1 > MMP-3 > MMP-7. The detection limit for MMP-9 was below 15 pM, corresponding to 3.75×10^{-15} mol per assay. Using the assay, increased MMP activity was detected in synovial tissue extracts from rheumatoid arthritis patients compared with those from osteoarthritis patients, and in stomach tumour extracts as compared with normal stomach tissue extracts.

INTRODUCTION

Proteolytic enzymes occur widely in nature from micro-organisms to humans, and have many different functions (for reviews, see [1,2]). They are involved in digestive processes, both at the level of the organism, e.g. digestive-tract enzymes such as pepsin and (chymo)trypsin, and at the level of individual cells, e.g. lysosomal enzymes such as cathepsin. They play a role in the invasive behaviour of both micro-organisms and cells in multicellular organisms, and in multicellular organisms they are involved in growth and development. Besides these obvious degradation processes, proteolytic enzymes play critical roles in regulatory networks such as blood coagulation and fibrinolysis, blood pressure regulation and activation of growth factors or pro-hormones.

Due to their involvement in many (patho)physiological processes, proteolytic enzymes play a role in diseases, and measurement of the activity of certain enzymes can be important for diagnosis. Measurement of enzymic activity using the natural substrate is not always possible, or leads to complicated assays not suitable for routine application. The development of chromogenic or fluorogenic synthetic peptide substrates has been an important improvement in this area. Such substrates have been developed for many serine proteinases in particular. This kind of substrate is particularly well suited for serine proteinases, since

members of this class of enzymes generally do not recognize the sequence C-terminal to the bond to be hydrolysed. This part can be replaced by a chromogenic or fluorogenic leaving group.

Measurement of aspartic proteinases and particularly metalloproteinases is much more difficult due to particular characteristics of these enzymes. In general such enzymes, unlike serine proteinases, recognize the amino acid residues on both sides of the bond to be cleaved. Thus substrates in which a non-peptide bond is cleaved, such as the above-mentioned chromogenic or fluorogenic substrates, cannot be employed for determination of metalloproteinases or aspartic proteinases. Two different synthetic substrate types currently exist for these enzymes. (a) Peptides containing the essential recognition site for the proteinase; in this case, hydrolysis is followed by a physicochemical separation technique such as HPLC [4–10]. (b) Peptides containing, in addition to the recognition sequence, a potential fluorescent group together with a quenching group. When both groups are in close proximity, fluorescence is quenched; however, after hydrolysis of the peptide, quenching is lost and fluorescence increases [11–15]. In relation to this, a simple and sensitive colorimetric assay for metalloproteinases would be of use for the routine measurement of clinical samples.

In the present work a new principle for the determination of proteolytic enzymes is described, based on the activation of proenzymes to give easily detectable enzymes. In particular, the

Abbreviations used: MMP, matrix metalloproteinase; uPA, urokinase-type plasminogen activator; S-2444, pyro-Glu-Gly-Arg-*p*-nitroanilide; S-2251, *H*-D-Val-Leu-Lys-*p*-nitroanilide; APMA, *p*-aminophenylmercuric acetate; TIMP, tissue inhibitor of metalloproteinases; BB94 (Batimastat), [4-*N*-hydroxyamino-2*R*-isobutyl-3*S*-(thienylthiomethyl)succinyl]-L-phenylalanine-*N*-methylamide.

§ To whom correspondence should be addressed.

assay of bacterial collagenase and other matrix metalloproteinases (MMPs) will be described.

MATERIALS AND METHODS

Isolation of cDNA coding for human pro-urokinase (pro-uPA)

The complete coding region of the pro-uPA cDNA was isolated from poly(A)⁺ mRNA obtained from the uPA-producing fibrosarcoma cell line HT 1080 [16], using reverse transcriptase-PCR. The oligonucleotides (5' to 3') TAGCGCCCCGGGCTCGCC-ACCAT and ACGGGTCTGGGGAGACCGGT were used as primers to amplify a cDNA fragment spanning nucleotides 99–1620 in human uPA cDNA [17]. To construct an expression vector, this fragment was cloned into the plasmid peV2tPA [18] by replacing the tissue-type plasminogen activator cDNA fragment. To accomplish this, peV2tPA was digested with *Bgl*II and the uPA cDNA fragment was cloned into the peV2 vector using the following linker oligonucleotides: AGCTTCCCGGGAGGCCTGTCGACA and GATCTGTGACAGGCCTCCCGGGA. The resulting expression vector was designated peV2uPA1.1.

Introduction of mutations in human pro-uPA cDNA

Mutations in the uPA expression vector peV2uPA1.1 were introduced using the recombinant circle PCR method [19]. The following oligonucleotides were used (from 5' to 3'): 1, CGGCCGCTCGGGATTATTGGGGGAGAATTCACC (corresponding to amino acids 155–165); 2, CCGGAGCGGCCGCTCAGAGTCTTTTGGCCACA (amino acids 158–148); 3, ATTATTGGGGGAGAATTCACC (amino acids 159–165); 4, CCTCAGAGTCTTTTGGCCACA (amino acids 148–154). Oligonucleotides 1 and 2 contain new codons (underlined) coding for new amino acids Arg-Pro-Leu-Gly replacing the wild-type residues Pro-Arg-Phe-Lys at positions 155–158.

Two independent PCR reactions were performed with the oligonucleotide pairs 1/4 and 2/3. Both PCR products were purified by agarose-gel electrophoresis, cut from the gel and isolated by electro-elution followed by ethanol precipitation. The two isolated PCR products 1/4 and 2/3 were mixed in equimolar quantities, denatured for 5 min at 100 °C and re-annealed by a stepwise decrease in temperature to 55 °C. A small quantity of this material was used to transform *Escherichia coli* JM109 [20]. Colonies were isolated and grown in small cultures for plasmid isolation according to standard procedures. Plasmids were analysed by digestion with the restriction enzymes *Ava*I and *Dra*II. The former enzyme does not digest the wild-type plasmid peV2uPA1.1, but does digest the mutant plasmid at a single position. The latter enzyme cuts the wild-type plasmid four times, whereas the mutant plasmid is cut only three times. Three clones were selected by these criteria and large-scale plasmid preparations were performed. The presence of the desired mutations and the absence of secondary mutations was verified by DNA sequencing [21]. The resulting plasmid was designated peV2UKcol.

Expression of modified pro-uPA

Samples of 10–20 µg of the expression plasmid peV2UKcol were transfected into CHO cells together with 1 µg of pSV2neo plasmid containing a selectable marker. Transfection was performed using the calcium phosphate co-precipitation method [22]. To the culture medium was added 1.2 mg of the neomycin analogue G418/ml, and resistant clones were selected. Selected clones were

grown separately and 24 h conditioned medium was collected. Production of the pro-uPA mutant was quantified by ELISA [23]. High-producing clones were expanded and grown in roller bottles in serum-free medium in the presence of 100 KIE (kallikrein inhibitor units)/ml Trasylol. Medium was collected, centrifuged (200 g, 10 min) and stored at –20 °C until purification.

For purification, the medium was added to a Zn-chelate column. After washing with 6 column volumes of 0.02 M Tris/HCl, pH 7.6, 1 M NaCl and 0.01 % (v/v) Tween 80, mutant uPA was eluted with the same buffer containing 0.05 M imidazole [24]. The mutant-uPA-containing fractions were identified by ELISA [23], pooled, dialysed against 0.05 M sodium phosphate buffer, pH 6.0, 0.05 M NaCl and 0.01 % (v/v) Tween 80, and added to an SP-Sephadex C50 ion-exchange column. After washing with dialysis buffer, the column was eluted with a gradient from 0.05 to 0.5 M NaCl in 0.05 M sodium phosphate, pH 7.4, and 0.01 % (v/v) Tween 80. The mutant-uPA-containing fractions were pooled, dialysed against 0.1 M sodium phosphate, pH 7.0, 0.4 M NaCl and 0.01 % (v/v) Tween 80, treated with 1 mM PMSF for 4 h, passed through a benzamidine-Sepharose column and dialysed against PBS/0.01 % (v/v) Tween 80. All steps were performed at 4 °C.

Assay of uPA activity

The amidolytic activity of (modified) uPA was determined in 0.25 ml of 0.1 M Tris/HCl, pH 7.5, 0.1 % (v/v) Tween 80 and 0.4 mM of the chromogenic substrate pyro-Glu-Gly-Arg-p-nitroanilide (S-2444; Chromogenix). Incubations were performed at 37 °C in 96-well flat-bottomed microtitre plates. Absorbance changes were measured at 405 nm after various time intervals using a Titertek Multiskan 8-channel photometer.

The plasminogen activation activity of (modified) uPA was determined using a coupled reaction system involving plasminogen (Biofine, Leiden, The Netherlands), CNBr-digested fibrinogen and the plasmin substrate *H*-D-Val-Leu-Lys-p-nitroanilide (S-2251; Chromogenix) [25]. Reactions were performed in 96-well plates at 25 °C or 37 °C, and a Titertek Multiskan photometer was used to follow the absorbance change.

Fibrin zymography was carried out as described by Granelli-Piperno and Reich [26]. In short, samples were separated by gel electrophoresis in the presence of SDS under non-reducing conditions. Subsequently the gel was washed in a Triton X-100 solution to remove SDS and renature the proteins, and placed on a layer of agarose containing fibrin and plasminogen. Plasminogen activation results in the lysis of fibrin and reveals itself as dark bands on an opaque background.

Parabolic rate assay for bacterial collagenase and human MMPs

Bacterial collagenase or MMP was incubated with modified pro-uPA (250–750 ng) and S-2444 (0.6 mM) in assay buffer [50 mM Tris/HCl, pH 7.6, 150 mM NaCl, 5 mM CaCl₂, 1 µM ZnCl₂, 0.01 % (v/v) Brij-35] at 37 °C in a total volume of 0.25 ml. Reactions were performed in 96-well flat-bottomed microtitre plates, and a Titertek Multiskan photometer was used to follow the absorbance change at 405 nm.

Activation of mutant pro-uPA by bacterial collagenase

Bacterial collagenase (CLSII from *Clostridium histolyticum*; Worthington) was incubated with various amounts of mutant pro-uPA in 0.1 M Tris/HCl, pH 7.5, and 0.1 % (v/v) Tween 80 at 25 or 37 °C for various periods of time.

Human MMPs and inhibitors

Active human recombinant MMP-1, MMP-1 (19 kDa fragment) and MMP-3 were kindly provided by Dr. G. McGeehan (Glaxo Research, Triangle Park, NC, U.S.A.) [27,28]. Active human recombinant MMP-7 was kindly provided by Dr. P. Cannon (Syntex Research) [29]. Human pro-MMP-2 from fibroblasts [30] was kindly provided by Dr. H. Nagase (University of Kansas Medical Center, Kansas City, KS, U.S.A.). Pro-MMP-9 from human neutrophils was kindly provided by Dr. V. Knäuper (Strangeways Laboratories, Cambridge, U.K.) [31,32]. Pro-MMP-2 and pro-MMP-9 were activated by treatment with 1 mM *p*-aminophenylmercuric acetate (APMA) for 2 h at 37 °C and for 16 h at 4 °C respectively. The activation and integrity of the MMP preparations was checked using gelatin gel zymography as described by Hanemaaijer et al. [33]. All enzymes were at least 90 % active, and no degradation products were visible.

The specific MMP inhibitor BB94 {[4-*N*-hydroxyamino-2*R*-isobutyl-3*S*-(thienylthiomethyl)succinyl]-*L*-phenylalanine-*N*-methylamide} and recombinant tissue inhibitor of metalloproteinases 1 (TIMP-1) were kindly donated by Dr. E. A. Bone (British Biotech, Oxford, U.K.) and Dr. G. Murphy (Strangeways Laboratory, Cambridge, U.K.) respectively.

Determination of active MMP by titration with TIMP-1

Activated MMP preparations were titrated with TIMP-1 and the residual activity was determined using the parabolic rate assay described above. The amount of active MMP was determined from extrapolation of the inhibition curves. A stoichiometry of 1 mol of TIMP/mol of MMP was assumed [34].

Measurement of MMP activity in biological samples

Synovial fluids and tissues were obtained from the Department of Rheumatology, Academic Hospital Leiden. Tumour and normal tissues of the stomach were obtained from the Department of Gastroenterology, Academic Hospital Leiden. Tissue samples (approx. 60 mg) were homogenized with a Potter homogenizer in 0.1 ml of 0.1 M Tris/HCl, pH 7.5, and 0.1 % (v/v) Tween 80. After centrifugation for 15 min at 10000 *g*, supernatants were frozen at −70 °C until use. MMPs were activated by incubation with 1 mM APMA overnight at 37 °C. Samples of 5 µl of activated or non-activated enzyme were assayed in parabolic rate assays as described above.

Miscellaneous procedures and materials

SDS/PAGE, silver staining and Western blotting were performed using established procedures [35–37]. Proteins were visualized on blots with rabbit polyclonal anti-uPA antibodies using a chemiluminescence kit (Boehringer Mannheim, Almere, The Netherlands). Molecular biology was performed using the protocols of Sambrook et al. [20].

Protein was determined by the method of Lowry et al. [38]. Human thrombin was from Leo (Ballerup, Denmark); human plasmin was from Biofine; and human neutrophil elastase purified from polymorphonuclear leucocytes was generously donated by Dr. P. S. Hiemstra (Department of Pulmonology, Academic Hospital Leiden).

RESULTS AND DISCUSSION

Choice of proenzyme/enzyme couple to be modified

The proenzyme/enzyme couple must have certain properties in order to be of use in this application. (a) The difference in activity between the proenzyme and active enzyme must be high. (b) The

Table 1 Substrate recognition sites of various MMPs

Substrate recognition sites of the various MMPs were obtained from references indicated.

Enzyme	Substrate recognition sequence	Ref.
MMP-1	Pro-Gln-Gly ↑ Ile-Ala-Gly-Arg	5
	Pro-Gln-Gly ↑ Ile-Ala-Gly-Gln	7
	Pro-Gln-Gly ↑ Ile-Ala-Gly-Thr	9
	Pro-Gln-Gly ↑ Leu-Ala-Gly-Gln	
	Pro-Leu-Gly ↑ Ile-Ala-Gly-Gln	
MMP-2	Pro-Leu-Gly ↑ Ile-Ala-Gly-Pro	15
	Pro-Leu-Gly ↑ Leu-His-Ala-Arg	
	Pro-Gln-Gly ↑ Ile-Ala-Gly-Trp	12
	Pro-Leu-Gly ↑ Met-Trp-Ser-Arg	
	Pro-Leu-Gly ↑ Val-Arg-Gly	44
MMP-3	Pro-Leu-Gly ↑ Leu-Ala-Gly	
	Pro-Leu-Gly ↑ Tyr-Leu-Gly	
	Pro-Leu-Gly ↑ Leu-Trp-Ala-Arg	13
	Pro-Leu-Ala ↑ Phe-Trp-Ala-Arg	14
MMP-7	Pro-Gln-Gln ↑ Phe-Phe-Gly-Leu	8
MMP-9	Pro-Leu-Gly ↑ Leu-Trp-Ala-Arg	13
MMP-9	Pro-Leu-Gly ↑ Leu-Trp-Ala-Arg	15
Consensus	Pro-(Leu/Gln)-Gly ↑ (Ile/Leu)-Ala-Gly-Xaa	

resulting active enzyme must be measurable in a simple, reliable and sensitive way. (c) The active enzyme must have a high specificity. (d) The activation step must involve specific limited proteolysis. (e) It must be possible to manipulate the activation recognition sequence of the proenzyme to make it susceptible to activation by the proteinase to be measured.

A number of possible proenzyme/enzyme couples have been investigated, including members of the coagulation and fibrinolysis cascades. In this case the active members are also themselves proteinases, but this is only necessary when a cascade of three or more reactions is built up. We have chosen the pro-uPA/uPA pair as a suitable candidate for the first activation reaction because it meets the criteria described above and a natural amplification step exists.

Modified pro-uPA as a substrate for collagenases and MMPs

Pro-uPA has very low intrinsic activity and can be converted rapidly into active uPA by plasmin (probably its natural activator) and also by kallikrein, cathepsins B and L, thermolysin and trypsin [39]. Activation requires specific hydrolysis of the Lys¹⁵⁸–Ile¹⁵⁹ bond. Hydrolysis of the Arg¹⁵⁶–Phe¹⁵⁷ or Ile¹⁵⁹–Ile¹⁶⁰ bond by thrombin or elastase respectively leads to an inactive product [40]. Comparison of the primary sequences of the activation sites of a number of proenzymes in the serine proteinase family reveals the consensus sequence Xaa ↑ (Ile/Val)-(Ile/Val)-Gly-Gly (sequences obtained from SWISS-PROT, GenBank or PIR databases).

Upon comparison of the substrate preferences of a number of collagenases and MMPs (Table 1), a preference for sequences containing Pro-(Leu/Gln)-Gly ↑ (Ile/Leu)-Ala-Gly-Xaa was revealed. These two consensus sequences were combined to obtain a sequence that, when inserted in uPA, could be hydrolysed by collagenase and MMPs, leading to an active uPA form upon hydrolysis. Therefore it was decided to alter the sequence around the activation site present in normal pro-uPA (Pro-Arg-Phe-Lys ↑ Ile-Ile-Gly-Gly) to Arg-Pro-Leu-Gly ↑ Ile-Ile-Gly-Gly in mutant pro-uPA. To prevent the occurrence of two consecutive Pro residues, the original Pro in uPA was replaced by Arg.

Figure 1 Electrophoretic analysis of modified pro-uPA

Modified pro-uPA partially purified from the culture medium of transfected CHO cells by Zn-chelate chromatography (for details, see the text) was analysed by SDS/PAGE and visualized by silver staining: lanes 1–3, non-reduced; lanes 4–6, reduced. Lanes 1 and 4, 450 ng of modified pro-uPA; lanes 2 and 5, 450 ng of modified pro-uPA after 2 h incubation at 37 °C with 225 ng of bacterial collagenase in 0.1 M Tris/HCl, pH 7.5, 0.1% (v/v) Tween 80; lanes 3 and 6, 450 ng of modified pro-uPA after collagenase treatment in the presence of 1 mM EDTA. Positions and molecular masses (kDa) of marker proteins are indicated.

Figure 2 Zymographic analysis of modified pro-uPA

Modified pro-uPA, purified by Zn-chelate, ion-exchange and affinity chromatography steps, was analysed by SDS/PAGE followed by zymographic detection (for details, see the Materials and methods section). Samples of 100 ng of modified pro-uPA were incubated with 40 ng of bacterial collagenase for 6 h at 37 °C in 0.1 M Tris/HCl, pH 7.5, 0.1% (v/v) Tween 80 in the absence (lane 1) or in the presence (lane 2) of 1 mM EDTA. Lane 3 contains an equal amount of modified pro-uPA not incubated with collagenase. Numbers correspond to positions and molecular masses of marker proteins: tissue-type plasminogen activator (70 kDa), high-molecular-mass uPA (55 kDa) and low-molecular-mass uPA (33 kDa).

Characterization of modified pro-uPA

Modified pro-uPA was partially purified from serum-free culture medium using Zn-chelate chromatography. The resulting preparation was analysed by PAGE and visualized by silver staining. One major band at the expected molecular mass of 55 kDa was observed, and only small quantities of contaminants were present (Figure 1, lanes 1 and 4). Fibrin zymography revealed the presence of traces of tissue-type plasminogen activator activity (results not shown). Therefore the material was further purified using ion-exchange chromatography to remove contaminating tissue-type plasminogen activator. Finally, traces of active uPA were removed using benzamidine–Sephadex. Analysis using fibrin zymography revealed that the purified material displayed hardly any proteolytic activity (Figure 2, lane 3), as expected for a pro-uPA preparation. The absence of activity was confirmed by incubation with the chromogenic uPA substrate S-2444 (results not shown).

Modified pro-uPA was incubated with bacterial collagenase and subsequently analysed by SDS/PAGE and fibrin zymography. On collagenase treatment, single-chain modified

Figure 3 Effect of bacterial collagenase on modified pro-uPA

Modified pro-uPA (32 ng) was incubated with various amounts of bacterial collagenase as indicated for various time periods. Part of the material was analysed by SDS/PAGE under reducing conditions followed by Western blotting (upper panel). Another portion was analysed for uPA activity (lower panel) (for details, see the Materials and methods section). Lane 1, 15 ng of bacterial collagenase; lanes 2–5, 32 ng of modified pro-uPA after 0, 1, 2 and 6 h incubation respectively with 15 ng of bacterial collagenase; lane 6, 32 ng of modified pro-uPA after 6 h incubation with 15 ng of collagenase in the presence of 1 mM EDTA; lanes 7 and 8, 32 ng of modified pro-uPA after 6 h incubation with 7.5 ng and 30 ng of collagenase respectively; lane 9, standard 33 kDa uPA.

pro-uPA was converted into the two-chain form, as expected (Figure 1, lanes 4 and 5), with a concomitant increase in activity as shown on the fibrin zymogram (Figure 2, lanes 1 and 3). When EDTA, a non-specific inhibitor of collagenase, was added during the incubation, the conversion to the two-chain form (Figure 1, lane 6) and the increase in activity (Figure 2, lane 2) were much smaller.

In another experiment, modified pro-uPA was incubated with collagenase for various time periods and at various collagenase concentrations. Samples were analysed by gel electrophoresis under reducing conditions followed by Western blotting. Aliquots of the same samples were used for amidolytic activity measurements. The results of this experiment revealed a time- and concentration-dependent decrease of the 55 kDa single-chain mutant pro-uPA and a concomitant increase in the 33 kDa uPA light chain upon incubation with collagenase, as would be expected upon cleavage of the newly introduced Gly¹⁵⁸–Ile¹⁵⁹ bond (Figure 3, upper panel). Conversion of the single-chain form of the mutant pro-uPA into the double-chain form was accompanied by an increase in activity, as measured with chromogenic substrate (Figure 3, lower panel). In the presence of EDTA both the conversion and the activation were inhibited.

Stability and specificity of modified pro-uPA

Modified pro-uPA could possibly be cleaved by proteinases other than MMPs. Cleavage at the newly engineered activation site would lead to an active enzyme and thus to non-specificity of the assay. Cleavage at other sites in modified pro-uPA would not result in activation, but could lead to substrate depletion and therefore might influence the assay result. Therefore stability and specificity were studied with a number of purified proteinases at high concentration and with biological samples likely to be used

Figure 4 Effects of various proteolytic enzymes on modified pro-uPA

Modified pro-uPA at 2 $\mu\text{g/ml}$ (36 nM) was incubated at 37 °C for 0 h (lane 1) or 6 h with the following amounts of purified proteinases: none (lane 2), 1 $\mu\text{g/ml}$ bacterial collagenase (lane 3), bacterial collagenase + 1 mM EDTA (lane 4), 20 nM plasmin (lane 5), 20 nM elastase (lane 6) or 50 nM thrombin (lane 7). After incubation, samples were analysed by SDS/PAGE after reduction, followed by Western blotting with a uPA-specific antibody (for details, see the Materials and methods section). The numbers on the right correspond to the positions of molecular mass marker proteins.

in the assay. Plasmin (the natural activator of normal uPA), elastase and thrombin at concentrations much higher than physiological and after long incubation periods did not cleave modified pro-uPA. Cleavage at the activation recognition sequence was only observed with bacterial collagenase (Figure 4). Enzymic activity of modified pro-uPA was only observed after

Figure 5 Stability of modified pro-uPA in tissue extracts

Upper panel: modified pro-uPA (1.5 μg) was incubated at 37 °C for 0 or 24 h with an extract of synovial tissue from a rheumatoid arthritis patient (18 μg of protein) in a total volume of 0.3 ml of assay buffer. After incubation, 15 μl samples were electrophoresed and blotted. uPA-related material was visualized using immunostaining. Lane 1, modified pro-uPA + 0.5 nM MMP-9; lanes 2 and 3, modified pro-uPA + tissue extract + 30 mM EDTA after 24 h and 0 h respectively; lanes 4 and 5, modified pro-uPA + tissue extract after 24 h and 0 h respectively; lanes 6 and 7, tissue extract only after 24 h and 0 h respectively; lanes 8 and 9, modified pro-uPA + 30 mM EDTA after 24 h and 0 h respectively. Lower panel: modified pro-uPA (5 $\mu\text{g/ml}$) ($\square$), modified pro-uPA mixed with synovial tissue extract ($\circ$) and modified pro-uPA preincubated overnight at 37 °C with synovial tissue extract ($\bullet$) were used in an activity assay with 0.4 mM S-2444 with (solid lines) or without (broken lines) added MMP-9 (1 nM). Absorbance measured at 405 nm is shown

Table 2 Specificity of the MMP assay

Control buffer, synovial fluid or synovial fluid treated with APMA (1 mM, 16 h, 37 °C) was incubated with S-2444 (0.4 mM) and modified pro-uPA (5 $\mu\text{g/ml}$) in the presence of the specific MMP inhibitor BB94 (5 $\mu\text{g/ml}$), its solvent DMSO or EDTA (33 mM) as indicated. As a control, modified pro-uPA was omitted. The activity was determined and expressed in units of $1000 \times (\Delta A \cdot h^{-2})$.

	Activity (units)				
	No additions	BB94	DMSO	EDTA	No modified pro-uPA
Buffer	0.3	0.2	0.2	0.2	0
Synovial fluid	0.6	0.5	0.5	0.3	0.2
APMA-treated synovial fluid	2.2	0.4	1.9	0.2	0.2

incubation with bacterial collagenase, whereas plasmin, elastase and thrombin were not able to activate modified pro-uPA (results not shown).

In order to test the stability of modified pro-uPA against cleavage by non-metalloproteinases present in biological samples, a synovial tissue extract (not APMA-activated) was preincubated with modified pro-uPA. No degradation of modified pro-uPA was observed (Figure 5, upper panel). In another experiment, synovial tissue extract was preincubated overnight with modified pro-uPA; after the preincubation, purified MMP-9 was added and the activity was measured and compared with that obtained with modified pro-uPA not preincubated with tissue extract. In both cases similar responses were observed (Figure 5, lower panel), indicating that no degradation of modified pro-uPA had occurred.

The specificity of the assay for MMPs was investigated further using synovial fluid from rheumatoid arthritis patients. Synovial fluid showed low activity which increased after activation with APMA, and this activity could be inhibited by the specific MMP inhibitor BB94 or the more general inhibitor EDTA. This indicates that the observed activity is due to MMPs and not to other proteinases present in the sample. When no modified pro-uPA was present, hardly any response was measured, indicating that direct hydrolysis of the substrate S-2444 by proteinases in the sample does not occur (Table 2). Based on these results, it can be concluded that modified pro-uPA is stable in biological fluids and can be used as a specific substrate for MMPs in such fluids.

Kinetics and concentration-dependence of activation of modified pro-uPA by various MMPs

Human MMP-1, MMP-2, MMP-7 and MMP-9 were incubated at various concentrations (determined by active-site titration with TIMP-1) with modified pro-uPA and S-2444. The absorbance change at 405 nm was measured after various incubation periods. For all MMPs tested, the absorbance change was found to be linear with respect to the square of the duration of incubation (Figure 6, lower panels), indicating that the conditions required for a parabolic rate assay [40a] were met. In the presence of EDTA hardly any response could be detected. The response in the assay was found to be linear with respect to concentration for all MMPs, although the sensitivities for the various MMPs were different (Figure 6, upper panels).

Comparison of response of the assay with various MMPs

From the experiments described above (Figure 6), the relative activities of MMP-1, MMP-2, MMP-7 and MMP-9 were

Figure 6 Time- and concentration-dependence of the MMP assay

Lower panels: activated MMP-1, MMP-2, MMP-7 and MMP-9 were each incubated at a single concentration for various time periods with modified pro-uPA and S-2444 in the absence or the presence of 1 mM EDTA. The absorbance at 405 nm was followed over time and plotted against the square of the incubation time. Upper panels: activated MMP-1, MMP-2, MMP-7 and MMP-9 at various concentrations, as determined by active-site titration with TIMP-1, were incubated for various time periods as indicated above. Activities, expressed as absorbance change/time² (corresponding with the slope of the graphs in lower panels), were plotted against the enzyme concentration.

Table 3 Comparison of response of various MMPs

From the experiments shown in Figure 6, the relative activities of MMP-1, MMP-2, MMP-7 and MMP-9 were calculated. The activities of 19 kDa MMP-1 and MMP-3 were based on determinations at a single concentration and their quantities were based on protein determination. The molar activity of MMP-2 was arbitrarily assigned a value of 100. The relative molar concentrations of MMP-1, -2, -7 and -9 were determined by active-site titration with TIMP-1.

Enzyme	Relative molar activity
MMP-1	6.8
19 kDa MMP-1	7.0
MMP-2	100
MMP-3	0.46
MMP-7	0.27
MMP-9	60.0

calculated. As shown in Table 3, the sensitivity was highest for MMP-2 and MMP-9, intermediate for MMP-1 and its 19 kDa fragment, and much lower for MMP-3 and MMP-7.

Measurement of MMP activity in tissue extracts

To investigate the ability of the assay to detect MMP activity in clinical samples, synovial tissue extracts obtained from rheumatoid arthritis patients and osteoarthritis patients were tested. Furthermore, tissue extracts from normal stomach and stomach carcinoma were investigated. Activities were expressed in all cases as $1000 \times (\Delta A \cdot h^{-2} \cdot mg \text{ of protein}^{-1})$.

In synovial tissue extracts, total MMP activity as determined after activation with APMA was found to be significantly higher ($P < 0.03$; Mann-Whitney unpaired test) in tissue from rheumatoid arthritis patients ($n = 13$; one patient with no detectable activity; mean $\pm$ S.D. 13.28 ± 11.21 ; median 7.38; range 0–28.56) compared with tissue from osteoarthritis patients ($n = 11$; six patients with no detectable activity; mean $\pm$ S.D. 6.63 ± 13.05 ; median 0; range 0–41.23). Without APMA activation, activity was absent or low in most cases.

In stomach tumour extracts, total MMP activity after APMA activation (52.4 ± 16.6 , mean $\pm$ S.E.M.; $n = 4$) was significantly higher ($P < 0.01$; Student's *t* test) than in extracts of normal tissue (1.4 ± 0.7 ; $n = 4$). Without APMA activation no activity could be detected.

Conclusions

In the present paper, a simple and sensitive colorimetric assay for MMPs is described, based on a novel principle. In the present form of the assay the activities of many members of the MMP family can be measured. A cascade system of detecting enzyme activities is complicated and prone to interference, e.g. trypsin-like enzymes might directly hydrolyse S-2444, or inhibitors of uPA might inhibit the active modified uPA formed. No such interference was observed, however, in a variety of biological samples, indicating that in many cases these effects are small, as shown for parabolic rate assays for plasminogen activators that have been in use for many years [25,41].

Several improvements to the current assay are possible. Since only a single recognition sequence has been made and tested, it

is conceivable that the reactivity and specificity for a particular MMP can be increased considerably by modification of this consensus-like sequence. Another way to obtain specificity for a particular MMP is the use of specific antibodies to 'catch' or inhibit a particular enzyme. In particular, the use of catching antibodies is interesting, since by this means not only would specificity for a particular MMP be introduced, but also interference by other enzymes or inhibitors would be prevented [43].

The new assay is sufficiently specific and sensitive to be used with biological samples, as illustrated by the detection of increased MMP levels in synovial tissue extracts from rheumatoid arthritis patients compared with those from osteoarthritis patients, and in extracts of stomach tumour tissue compared with normal stomach tissue.

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