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Inhibitors of gelatinase B/matrix metalloproteinase-9 activity Comparison of a peptidomimetic and polyhistidine with single-chain derivatives of a neutralizing monoclonal antibody

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Abstract

Matrix metalloproteinases form a proteinase family with at least 20 members, which are involved in several pathological conditions and which fulfill a large number of physiological functions. Gelatinase A/MMP-2 is a constitutively produced homeostatic enzyme, whereas gelatinase B/MMP-9 is upregulated in acute and chronic inflammations and forms a target for the development of therapeutic inhibitors. We have used a recently developed assay with fluorescent gelatin to analyze gelatinase inhibitors. A peptidomimetic, based on the consensus sequence of the cleavage sites in type II collagen, and various derivatives of a neutralizing antibody were compared as gelatinase inhibitors. A single-chain variable fragment (scFv) derived from the gelatinase B-selective monoclonal antibody REGA-3G12 was tagged with oligohistidine and was also compared with the untagged scFv. Both scFv derivatives inhibited gelatinase B but the peptidomimetic was inefficient. As an extra control and serendipitously it was found that polyhistidine is an inhibitor of gelatinases, presumably by altering the active site by chelation of the catalytic Zn^{2+}.

Keywords: Matrix metalloproteinase; Monoclonal antibody; Histidine tag; Peptidomimetic; Polyhistidine; Inhibitor

1. Introduction

Gelatinase B/MMP-9 is a prototypic member of the MMPs. It is upregulated in many inflammatory conditions, including infections and autoimmune diseases [1]. The regulation of gelatinase B contrasts with that of gelatinase A, the latter of which is constitutively produced by many cell types and functions rather as a homeostatic enzyme. On the basis of biological, biochemical and *in vivo* experiments, gelatinase B has been suggested to be a possible target enzyme in autoimmune diseases such as rheumatoid arthritis [2], diabetes [3] and multiple sclerosis [4] and in acute inflammatory diseases such as bacteremia and endotoxemia [5]. Therefore, the development of specific inhibitors of gelatinase B is in demand for the treatment of a vast array of medical conditions. One approach for the discovery of gelatinase B inhibitors is by generating peptidomimetics. These can be based on the identification of substrate cleavage sites [6–10]. Recently, we described all the cleavages by gelatinase B in collagen type II [2]. The structural requirements for cleavage were deduced from data of substrate proteolysis. This enabled us to define a consensus sequence for the synthesis of analogs, which were used in this study to generate an uncleavable peptidomimetic and to study its properties as an inhibitor.

Other small molecular weight inhibitors of gelatinase B have already been discovered and evaluated for clinical utility, but in general these seem to be rather unselective and to cause side-effects [11]. For instance, hydroxamate

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Abbreviations: DMSO, dimethylsulphoxide; FITC, fluorescein isothiocyanate; MMP, matrix metalloproteinase; scFv, single-chain variable fragment; ZBG, zinc-binding group.

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containing inhibitors [12], tetracyclines [13] and D-penicillamine [14] inhibit several MMPs and have been suggested as potential therapeutics for cancer, autoimmune and other inflammatory diseases. Because the lack of selectivity of these drug candidates is likely due to their small molecular size, we have addressed the issue through the development of antibody inhibitors. One of these, REGA-3G12 [15], possesses a high selectivity, because it does not inhibit the activity of the most closely related MMP, gelatinase A/MMP-2. Furthermore, a scFv was derived by genetic engineering and expressed in *Escherichia coli* [16]. Here, recombinant variants of this reagent, expressed in the yeast *Pichia pastoris*, were compared. By serendipity, we found that poly-histidine is also a potent, albeit nonselective, MMP-inhibitor. This finding has consequences not only for further development of MMP inhibitors, but maybe also for the use of polyhistidine-tagged recombinant proteins.

2. Materials and methods

2.1. Reagents

Polyhistidine (polymer of about one hundred residues), DMSO, FITC, gelatin and bovine serum albumin were purchased from Sigma. Polystyrene microbeads were from Polysciences. The single-chain variable fragment DNA construct of the monoclonal antibody REGA-3G12 against human neutrophil gelatinase B [15] was developed by Zhou *et al.* [16]. Natural gelatinase B was purified and activated as described previously [17,18].

2.2. Standardization of the gelatinase activity assay

Labeling of gelatin with FITC was as described by St-Pierre *et al.* [19]. Subsequently, the fluorescent gelatin was separated from DMSO and free FITC by gel filtration chromatography on a PD-10 column (Pharmacia). The labeled gelatin was coupled to polystyrene microspheres as described [19]. Flow cytometry analysis of the coated beads was performed on a FACS SCAN Plus (Beckton Dickinson). In order to use this as a routine method to test enzyme activity or enzyme inhibition, different factors that influence enzyme activity were investigated.

2.3. Peptide synthesis

Peptides were synthesized by Fmoc chemistry on a peptide synthesizer (Applied Biosystems Inc.), one with amino-acetylation (MIM-3) and the other with free amino-terminus (MIM-3b). Both were carboxy-amidated (Table 1). A peptidomimetic, in which the P'_1 alanine in MIM-3b was replaced by a *N*-methylalanine [Ala(CH_3)], was synthesized (MIM-1) in an analogous way. MIM-3, MIM-3b and MIM-1 were tested as gelatinase B substrates by analysis of the cleavage products with the use of mass spectrometry.

Table 1
Peptide synthesis

Peptide	Sequence	Peak mass
MIM-3	Ac-Pro-Hyp-Gly-Pro-Gln-Gly-Ala-Thr-Gly-Glu-Hyp-Gly-$CONH_2$	1137.4
MIM-3b	H-Pro-Hyp-Gly-Pro-Gln-Gly-Ala-Thr-Gly-Glu-Hyp-Gly-$CONH_2$	1095.6
MIM-1	H-Pro-Hyp-Gly-Pro-Gln-Gly-Ala(CH_3)-Thr-Gly-Glu-Hyp-Gly-$CONH_2$	1109.6

Hyp represents hydroxyproline.

2.4. Digestion of peptides with gelatinase B and mass spectrometry analysis

Amounts of 2500 ng MIM-3 and 2500 ng MIM-3b were incubated together with 460 ng activated gelatinase B in 25 μL assay buffer (100 mM Tris–HCl pH 7.4, 100 mM NaCl, 10 mM $CaCl_2$, 0.01% Tween-20) at 37°. Consecutive aliquots of 5 μL reaction mixture were taken in which the reaction was stopped by the addition of 5 μL 1% TFA. Aliquoting was for 2 hr with 30-min intervals. The reaction products were desalted with the use of C18 ZIPTIP (Millipore) and subjected to mass spectrometry analysis on an Esquire LC ion trap apparatus (Bruker Daltonik). To confirm competition between these peptides, different amounts of MIM-3 (none, 500, 1000 and 2000 ng) were incubated with 500 ng MIM-3b and 92 ng gelatinase B in 5 μL assay buffer. The reactions were terminated with 1% TFA and desalted with C18 ZIPTIP.

As MIM-1 was not cleaved by gelatinase B (see Section 3), its inhibitory potential was investigated. Different amounts of MIM-1 (none, 22, 44 and 87 μg) were incubated with 20 μg MIM-3 and 92 ng gelatinase B. A similar procedure as above was used except that the reaction mixture was diluted 10 times with 1% TFA before being desalted with ZIPTIP and analyzed by mass spectrometry.

2.5. Recombinant expression of REGA-3G12 scFv in yeast and purification

Two plasmid constructs were prepared to evaluate the use of *P. pastoris* in the production of scFv constructs of the monoclonal antibody REGA-3G12 (Fig. 1). One construct (p3G12h-pp2.2) contained the sequence encoding a histidine (His_6) tag at 3′ side; the other construct (p3G12-pp11) was produced without a tag. The product (3G12-pp11) of the latter construct is the purified 3G12-scFv protein bearing no tag at the C-terminal extremity. This recombinant protein represents the scFv derivative of the monoclonal antibody REGA-3G12 [15,16], which inhibits the enzymatic activity of human gelatinase B (MMP-9).

A plasmid was constructed containing the cDNA coding for REGA-3G12 scFv (p3G12-pp11) and subsequently

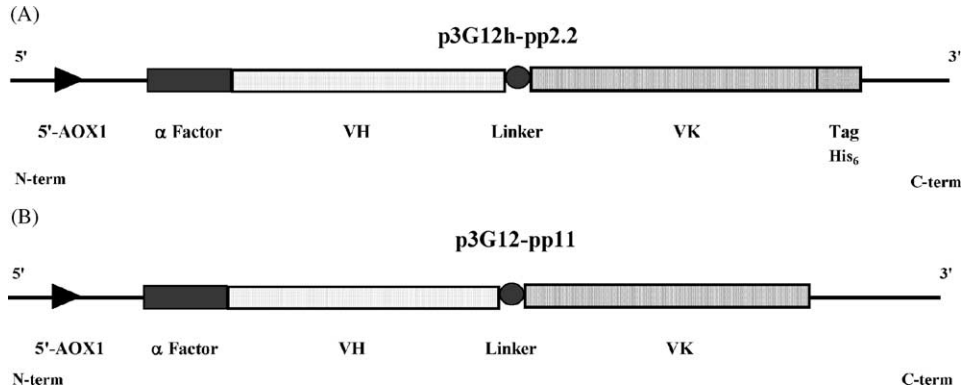

Fig. 1. Construction of plasmids of REGA-3G12 scFv. The REGA-3G12 scFv plasmids [16] formed the basis for construction of a histidine-tagged expression plasmid for *Pichia pastoris*, named p3G12h-pp2.2. An analogous plasmid (p3G12-pp11) was made, generating a protein without histidine tag. VH and VK are the variable immunoglobulin domains of the heavy and the kappa chains, respectively. These are connected with a glycine/serine linker. At the 5′-end of both vectors an alcohol oxidase-1 (AOX1) promoter and an alpha factor secretion signal (α Factor) are included.

used to transform *P. pastoris* GS115 cells. The transformed *Pichia* cells were cloned containing the p3G12-pp11 plasmid. Expressed 3G12-pp11 was produced by the *Pichia* cell culture induced with methanol, and purified by differential ultrafiltration and subsequently by gel filtration chromatography.

The final product (3G12h-pp2.2) is the purified 3G12-scFv protein fused with a histidine (His_6) tag at the carboxy-terminal extremity. The histidine (His_6) tag allows the purification of the fusion protein by affinity chromatography using a Ni-NTA (Qiagen) or an IMAC (Amersham Biosciences) support. A plasmid containing the cDNA of the REGA-3G12 scFv fused to a histidine (His_6) encoding vector (p3G12h-pp2.2) was constructed and used to transform *P. pastoris* GS115 cells. The transformed cells that contain the p3G12h-pp plasmid were cloned. Expressed 3G12h-pp was produced by the *Pichia* cell culture induced with methanol and purified from the medium.

3. Results

3.1. Factors influencing the labeling and stability of the gelatin substrate

Gelatin was fluorescently labeled with FITC, dissolved in DMSO [19]. Since DMSO and free FITC interfere with the binding of fluorescent gelatin to the polystyrene microspheres and the subsequent fluorescence detection, these substances were removed by gel filtration chromatography before fluorescently labeled gelatin was incubated with the microbeads. Non-covalent binding of fluorescent gelatin to polystyrene was independent of the reaction volume (data not shown) and the binding capacity was saturable (Fig. 2).

An important factor for the application of the method is the stability of the non-covalent binding of fluorescent gelatin to polystyrene in the stock solution. At 4°, the fluorescence intensities of the microspheres decreased gradually with time: about 80% fluorescence remained after a storage time interval of 1 month in the dark. Since the substrate is to be incubated at the physiological temperature of 37°, we measured the alterations of fluorescence intensities after prolonged incubation times at 37°. About 50% loss of signal was found after a short incubation of 4 hr and the signal decreased gradually thereafter (Fig. 3A). These data indicate that the binding of fluorescent gelatin to the solid phase generates a substrate with sufficient shelf life for inhibitor screenings. However, appropriate blank controls are necessary to account for changes in the fluorescence signal, when long incubation times are used. During an enzyme reaction at 37°, the difference between the samples incubated with enzyme and their corresponding blank controls is gradually increased. As shown in Fig. 3B, a 16-hr-incubation is adequate as a standard condition, since the percentage of degraded gelatin increases linearly with the incubation time.

The labeled gelatin, bound on the solid phase, sinks to the bottom of the reaction vessels together with the microspheres. The immobilization of the substrate may influence

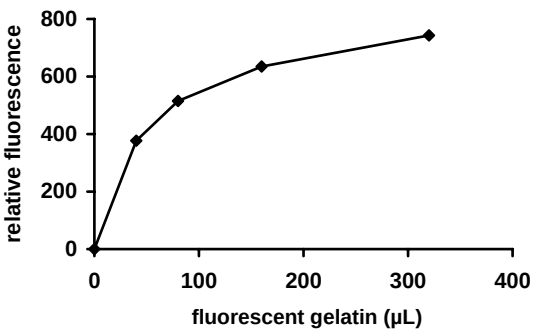

Fig. 2. Binding of fluorescently labeled gelatin on the microspheres. Increasing amounts of a standard substrate preparation (0.124 µg/µL fluorescent gelatin) were incubated with equivalent amounts of polystyrene microbeads. After washing, the fluorescence of coated micobeads was determined by analysis in a flow cytometer.

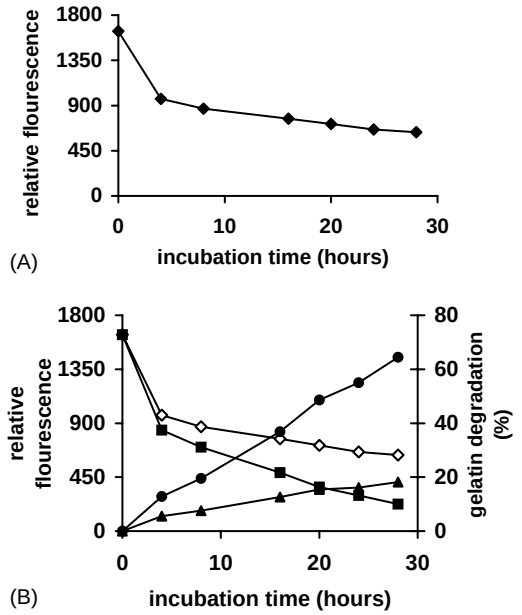

Fig. 3. Stability and degradation of labeled gelatin. (Panel A) A stock solution of fluorescently labeled gelatin-coated microspheres was incubated at 37° for various time intervals and the samples were washed and analyzed for residual fluorescence. (Panel B) Fluorescent substrate was incubated with enzyme for different time intervals and the residual fluorescence was determined (full squares). Their individual blank controls were also tested (open diamonds). The plot (filled triangles) represents the difference between the fluorescence of samples incubated with enzyme and the corresponding controls. As a result, the percentage of cleaved fluorescent gelatin can be deduced (filled circles).

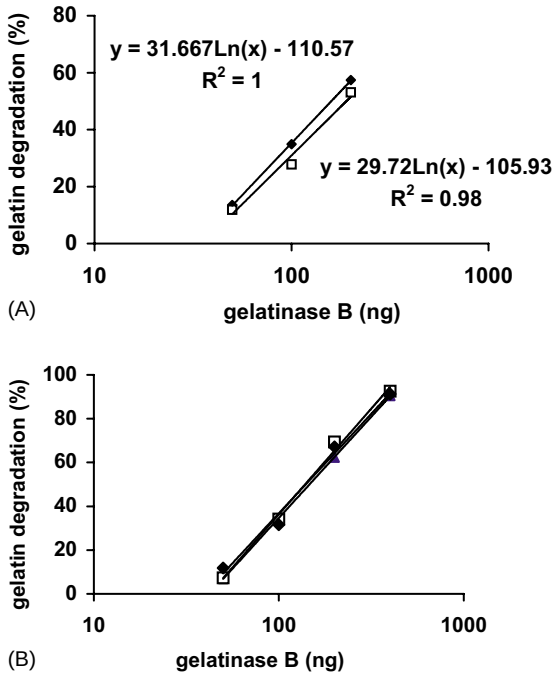

Fig. 4. Effects of rotary incubation and of substrate amount. (Panel A) Stationary vs. rotary incubation. Labeled beads were incubated with 50, 100, 200 ng recombinant mouse gelatinase B at 37° for 16 hr. Substrate conversion was compared between a stationary (open squares) and a rotary incubation (filled diamonds). Parameters of the linear regression analysis are indicated for both curves. (Panel B) Standard lines of substrate conversion by recombinant mouse gelatinase B. For each enzyme concentration, different amounts of labeled substrate from one stock solution were titrated to generate individual standard lines. The plot for 10 μL substrate incubation is indicated with filled triangles ($R^2 = 1$), the plot for 20 μL substrate with open squares ($R^2 = 0.99$) and the plot for 30 μL substrate with filled diamonds ($R^2 = 0.99$).

the enzyme reaction. The substrate conversion in a stationary vs. a stirred vessel was compared. Figure 4A shows that both reaction conditions resulted in equal substrate conversion. This implies that rotary incubation of the reaction is not necessary. In Fig. 4B, the effects of substrate amounts (represented as the amounts of gelatin-coated microspheres) on substrate conversion were evaluated. Similar enzyme activities were obtained with different amounts of fluorescent gelatin labeled microbeads. This implies that the local concentration of gelatin substrate, which is bound to the solid phase and therefore cannot freely move in the solution, cannot be changed by different amounts of coated microspheres. Thus, the method is not suitable for the analysis of enzyme kinetics and K_m and K_i values cannot be obtained with this type of assay. As the substrate conversion only depends on the enzyme concentration of the reaction system, similar standard lines can be obtained even with different batches of solid phase substrate preparations. The assay is useful for the screening of inhibitors.

3.2. Standardization of the assay conditions

The assay was standardized with samples of activated natural human gelatinase B from neutrophils [17,18]. Figure 5A shows the results of two substrate conversion experiments with equivalent enzyme amounts. The resulting semi-logarithmic curves of substrate degradation vs. enzyme amount are sigmoid. With the use of regression analysis the linear segments of the curves can be delineated (Fig. 5B). If the activity of an unknown sample lies in the linear section of such a standard line, the amount of enzyme activity in that sample can be deduced by comparison with a standard enzyme preparation. For adequate titration, we used linear sections that contained at least four data points per sample and linear regression coefficients in excess of $R^2 = 0.99$. Gelatinase B preparations from different sources (e.g. cell lines, species, glycosylation variants) may behave differently in the assay. For instance, the enzyme amounts within the linear part of the standard line ranges from 50 to 400 ng for recombinant mouse gelatinase B [20] and from 60 to 1000 pg for activated natural human neutrophil gelatinase B, as confirmed in at least two different assays.

Standard buffer conditions are also critical: human neutrophil gelatinase B shows 10 times higher activity when incubated in assay buffer (100 mM Tris–HCl pH 7.4, 100 mM NaCl, 10 mM $CaCl_2$, 0.01% Tween-20) than in PBS (100 mM sodium phosphate pH 7.4, 137 mM NaCl) (data not shown). Furthermore, the presence of calcium

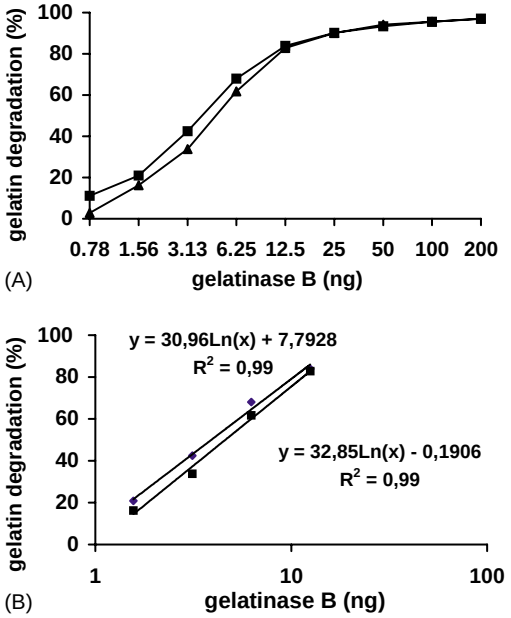

Fig. 5. Effect of gelatinase B concentration on the substrate degradation. (Panel A) Various amounts of activated human neutrophil gelatinase B were incubated with labeled gelatin (coated on microspheres) at 37° for 16 hr and residual fluorescence of the washed microbeads were measured and compared with the fluorescence of the beads incubated without enzyme. Two parallel titrations of an equivalent amount of enzyme are shown. (Panel B) Linear regression analysis of the standard lines from (panel A). Semi-logarithmic plots (inferred from data points obtained between 1.6 and 12.5 ng of human neutrophil gelatinase B) were analyzed.

ions in the digestion buffer stabilizes human neutrophil gelatinase B, especially at low enzyme concentrations. A low concentration of Tween-20 (0.01% in assay buffer) prevents human neutrophil gelatinase B from binding to the surface of the reaction vessels. The latter is particularly important with the use of low enzyme concentrations, for example, 100 pg enzyme in a 100 μL reaction system (data not shown). A last factor to take into account is the incubation time. Equivalent amounts of substrate are converted when lower amounts of enzyme are added for prolonged incubation times. In other words, the detection limit of the assay can be improved by longer incubation times (Fig. 6).

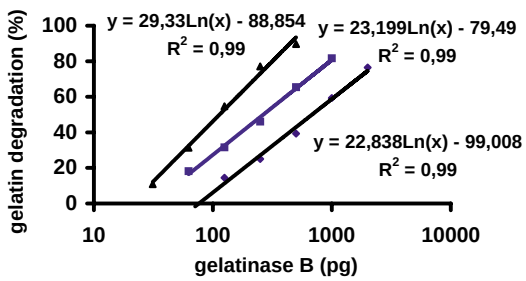

Fig. 6. Effect of incubation time on the fluorescent gelatin conversion. Standard lines of gelatin conversion by activated human neutrophil gelatinase B were generated after different incubation times. Detection limit improves with longer incubation time. Plots represent incubation for 8 hr (filled diamonds), 16 hr (filled squares) and 32 hr (filled triangles), respectively.

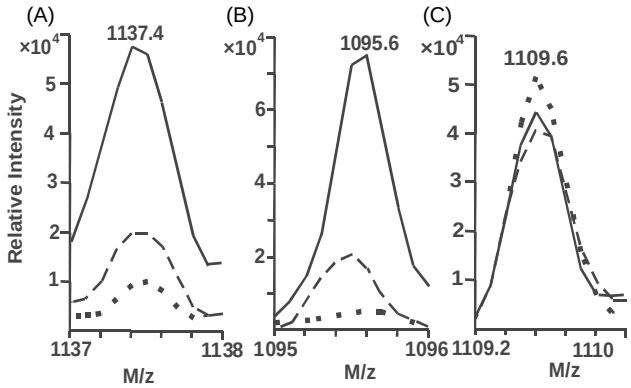

Fig. 7. Degradation of MIM-3, MIM-3b and MIM-1 by gelatinase B. In (panels A, B and C), 500 ng MIM-3, MIM-3b or MIM-1, respectively, were incubated with 92 ng gelatinase B in 5 μL assay buffer at 37°. Reactions were terminated after different incubation times. The reaction products were desalted with the use of C18 ZIPTIP and analyzed by mass spectrometry. The singly charged peak of each peptide is shown. In (A) and (B), full lines represent the amount of peptide incubated with gelatinase B after zero minutes, dashed lines after 30 min incubation, dotted lines after 60 min incubation. In (panel C), full line indicates the amount of MIM-1 incubated with gelatinase B after 0 min, dashed line at 8 hr and dotted line at 24 hr. The theoretical masses of MIM-3, MIM-3b and MIM-1 with a single charge are indicated in Table 1.

3.3. Investigation of a peptidomimetic analog

A consensus peptide with sequence PBGPQGAT-GEBG (single-letter code for amino acids, B is hydroxyproline) was defined based on previously determined gelatinase B cleavage sites in collagen type II [2]. This sequence was used to synthesize peptide substrates for gelatinase B. Both MIM-3 and MIM-3b were cleaved (Fig. 7A and B) by gelatinase B, whereas MIM-1 with *N*-methylalanine on position P_1' was not cleaved (Fig. 7C). No fragment peaks for peptidomimetic (MIM-1) can be detected by mass spectrometry (data not shown). MIM-1 was an inefficient inhibitor of the conversion of MIM-3. An excess of 15.7 mM MIM-1 yielded only 16.7% reduction of the conversion of MIM-3 at 3.5 mM (data not shown).

3.4. Comparison of gelatinase B inhibitors

The inhibitory effects on both gelatinase A and gelatinase B by different inhibitors were tested using the fluorescence assay method. The inhibitors included 1,10-phenanthroline, EDTA, TIMP-1, D-penicillamine, peptidomimetic MIM-1, polyhistidine and scFv REGA-3G12 with and without histidine tag. The linear part of the standard line was from 98 to 782 pg for gelatinase A and from 62 to 500 pg for gelatinase B in a 50 μL reaction system. Different inhibitors were incubated with 109 pM enzyme (391 pg gelatinase A and 500 pg gelatinase B) individually. Positive controls (without inhibitors) were included for both enzymes. The residual activity in a sample in the

Table 2
Inhibition of gelatinase A and gelatinase B

Inhibitor	Concentration	Inhibitory activity on gelatinase B (%)	Inhibitory activity on gelatinase A (%)
1,10-Phenanthroline	10 mM	100	100
EDTA	5 mM	73.8	100
EDTA	10 mM	100	100
TIMP-1	1 nM	88.2	85.2
D-Penicillamine	10 mg/L	43.3	36.5
Peptidomimetic MIM-1	1 mM	No inhibition	No inhibition
Polyhistidine	0.3 μM	43.7	8.2
Polyhistidine	1 μM	67.8	20.5
Polyhistidine	3 μM	85.1	36.8
REGA-3G12 scFv with (His_6) monomer	10 μM	6.5	No inhibition
REGA-3G12 scFv with with (His_6) dimer	5 μM	65.9	75.0
REGA-3G12 scFv no tag	1.25 μM	8.8	No inhibition
REGA-3G12 scFv no tag	2.5 μM	32.4	No inhibition
REGA-3G12 scFv no tag	5 μM	44.4	No inhibition

All percentages inhibitory activity were confirmed in at least two different assays.

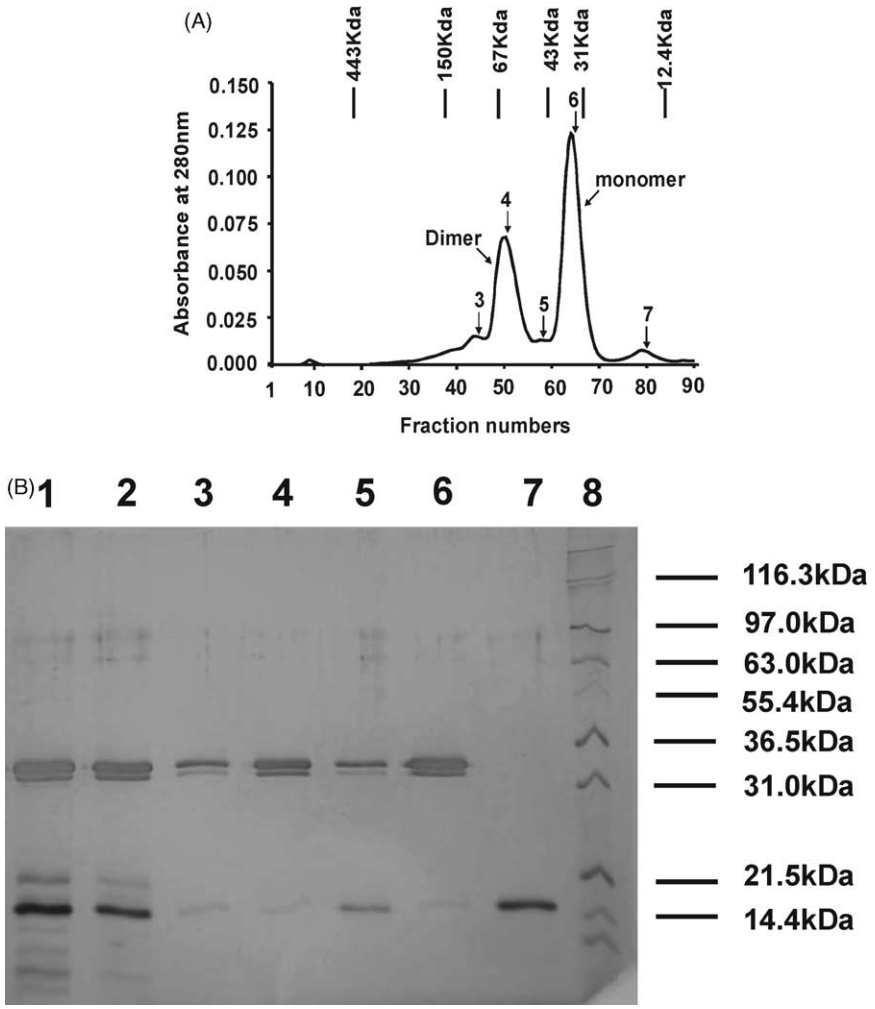

Fig. 8. Analysis of the REGA-3G12 scFv with a histidine tag. (Panel A) Gel filtration chromatography of REGA-3G12 scFv with a histidine Tag. Six hundred microliters crude sample (3.43 mg/mL) was loaded on a Superdex S-200 column. The flow rate was 0.5 mL/min with 2 min/fraction. The arrows indicate the monomeric and dimeric forms. The molecular standardization is indicated in kilodaltons on top and the numbers in the graph refer to the SDS–PAGE lanes in (panel B). (Panel B) SDS–PAGE of protein fractions after gel filtration chromatography of REGA-3G12 scFv with a histidine tag. All samples were chemically reduced before loading on the gel. Lanes 1 and 2, crude sample 0.5 and 0.25 μL; lane 3, fraction 45; lane 4, fraction 51; lane 5, fraction 58; lane 6, fraction 65; lane 7, fraction 80; lane 8, molecular marker proteins, indicated in kilodaltons (kDa).

presence of inhibitor was calculated. The inhibitory activities are summarized in Table 2. Polyhistidine inhibited gelatinase B efficiently at 3 μM. The only selective inhibitor of gelatinase B was the REGA-3G12 scFv without histidine tag.

3.5. Comparison of REGA-3G12 scFv variants

REGA-3G12 is a mouse monoclonal antibody that specifically inhibits human gelatinase B [15]. It has been derivatized into a single chain variable fragment (scFv) [16]. Here, we expressed variants of this scFv in the yeast *P. pastoris*, separated monomeric and dimeric variants by gel filtration chromatography (Fig. 8, panels A and B) and demonstrated the differences between the recombinant proteins (Fig. 9A and B). For example, the 3G12h-pp2.2 protein reacted with an oligohistidine-specific antibody and the selectivity of 3G12-pp11 for gelatinase B is evident from the Western blot analysis in Fig. 9B. In Table 2, the various forms are compared. The monomeric REGA-3G12 scFv with an oligohistidine tag has low inhibitory activity and is selective. As a contrast the dimeric form had higher inhibitory activity but also inhibited gelatinase A. Finally, the most selective inhibitor was the untagged REGA-3G12 scFv.

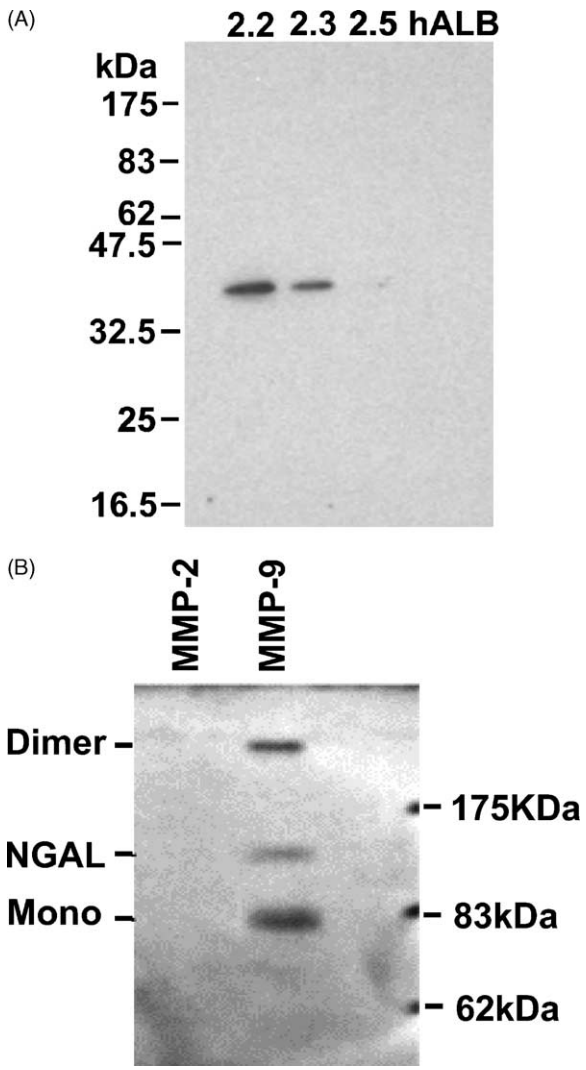

Fig. 9. Analysis of recombinant scFvs. Single-chain variable fragments derived from REGA-3G12 were expressed in *Pichia pastoris* with the use of the plasmids described in Fig. 1. (Panel A) Western blot analysis of the product of p3G12h-pp2.2 and two other clones (2.3 and 2.5). The blot was probed with an anti-histidine antibody. Human albumin (hALB) was used as negative control. (Panel B) The scFv p3G12h-pp11, expressed in yeast, reacted selectively with neutrophil gelatinase B monomers (Mono), dimers and neutrophil gelatinase B-associated lipocalin (NGAL) complexes, but not with gelatinase A (MMP-2). To visualize the immunoreaction, we used a horse radish peroxidase-conjugated anti-mouse IgG.

4. Discussion

In the present study, the conversion of fluorescently labeled gelatin by gelatinases was used to characterize gelatinase B inhibitors. As an example, a small inhibitory peptidomimetic compound was synthesized. This peptidomimetic showed a limited inhibition of cleavable gelatin-derived peptides, but was ineffective as inhibitor of intact gelatin degradation.

A common problem in the development of active site inhibitors of MMPs is the absence of selectivity. In addition, as the synthetic substrates are typically small, the assay method is also a key element in the definition of inhibitors. Early approaches for the identification of MMP inhibitors were based on the information of the sequence at the cleavage site [21–25]. Apparently, all inhibitors needed a ZBG and showed only moderate inhibitory activity. At a later stage, research groups successfully developed hydroxamate-containing peptidomimetics with inhibitory activity against gelatinase B [26–34]. Small peptidomimetics using thiol ZBG instead of hydroxamate also appeared to possess high inhibitory activity against gelatinase B [35–37]. Recently, some natural peptidomimetic inhibitors were found [38,39]. However, all these small molecules showed a broad-spectrum inhibitory effect and were not selective between different MMPs, especially not between gelatinase A and gelatinase B.

Small peptidomimetics interact with the catalytic site, whereas intact gelatin makes additional interactions with the three fibronectin repeats. Therefore, intact gelatin has a much higher affinity with gelatinase B than peptidomimetics. This, in addition to the absence of a ZBG, may explain why the peptidomimetic MIM-1 was a poor inhibitor.

Elongation of the peptide sequence may be a way to increase inhibitor selectivity. From the present data, it seems to be difficult to obtain a good inhibition from a peptide with only a natural sequence devoid of any ZBG(s). Therefore, the use of combinatorial synthesis [40] to deconvolute natural sequences incorporated with different zinc-binding groups in different positions, together with structural data [41–43] and structure–function studies [7],

are a promising but unexplored solution to obtain effective and selective gelatinase B inhibitors.

Gelatinase A and gelatinase B are unique in the MMP family as they have three fibronectin type II repeats inserted in the catalytic domain. These are important to endow the gelatinases with gelatinolytic [44] and elastinolytic activity [45]. The X-ray crystallographic model of a progelatinase A variant [46] implied that the prodomain peptide protrudes into the fibronectin domain. This suggests that a useful peptidomimetic inhibitor would need to be extended into that domain. From the present data, it seems that a natural peptide with a sequence from P'_6 to P_6 is not long enough to confer selective inhibition. As a result, we are now approaching the issue of selectivity by the synthesis of peptidomimetic inhibitors interacting with the fibronectin domain.

To address the issue of assay sensitivity, we used the FASC method with intact gelatin as substrate and with gelatinase A or gelatinase B as enzymes to compare a series of known and new inhibitors. Accordingly, we found that polyhistidine at micromolar concentrations is an inhibitor of both gelatinase A and B with tenfold higher inhibitory activity against gelatinase B than against gelatinase A. This may represent concentration dependence as a recent study evaluating a histidine-tagged scFv in a renal ischemia model did not uncover a histidine-specific inhibition of gelatinase B/MMP-9 when used at nanomolar levels.[1] Previously, we developed a monoclonal antibody with selectivity against gelatinase B [15]. Various novel forms of the single-chain Fv of this monoclonal REGA-3G12 were produced and compared in the present study. It was found that all monomeric forms with a histidine tag are less efficient as inhibitors, whereas the dimeric forms inhibited better, but also lost selectivity. This may be explained by the presence of two histidine tags which may be enough to confer inhibitory activity. As expected, when the scFv was expressed without histidine tag, it inhibited gelatinase B but not gelatinase A, emphasizing the fact that large size inhibitors may indeed possess better selectivity against gelatinase B than small peptidomimetics.

In conclusion, so far the best way to target specifically gelatinase B activity, and to leave gelatinase A unaffected is through the use of large size inhibitors such as monoclonal antibody derivatives. By serendipity we also found that polyhistidine at certain concentrations acts as an unselective gelatinase B inhibitor. As it is common practice in developmental pharmaceutics to use histidine tails as protein tags to enable a fast preparation of recombinant protein on nickel columns, it is clear from our data that the *in vivo* use of histidine-tagged proteins may cause unsuspected side-effects by inhibition of MMPs, especially when applied in micromolar concentrations.

[1] Ilenchuk TT, Caron A, Desrosiers RR, Houde M, Graziano M, Opdenakker G, Beliveau R, unpublished results.

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References

[1] Van den Steen PE, Dubois B, Nelissen I, Rudd PM, Dwek RA, Opdenakker G. Biochemistry and molecular biology of gelatinase B or matrix metalloproteinase-9 (MMP-9). Crit Rev Biochem Mol Biol 2002;37:375–536.

[2] Van den Steen PE, Proost P, Grillet B, Brand DD, Kang AH, Van Damme J, Opdenakker G. Cleavage of denatured natural collagen type II by neutrophil gelatinase B reveals enzyme specificity post-translational modifications in the substrate, and the formation of remnant epitopes in rheumatoid arthritis. FASEB J 2002;16:379–89.

[3] Descamps FJ, Van den Steen PE, Martens E, Ballaux F, Geboes K, Opdenakker G. Gelatinase B is diabetogenic in acute and chronic pancreatitis by cleaving insulin. FASEB J 2003;17:887–9.

[4] Opdenakker G, Van Damme J. Cytokine-regulated proteases in autoimmune diseases. Immunol Today 1994;15:103–7.

[5] Paemen L, Jansen PM, Proost P, Van Damme J, Opdenakker G, Hack E, Taylor FB. Induction of gelatinase B and MCP-2 in baboons during sublethal and lethal bacteraemia. Cytokine 1997;9:412–5.

[6] Netzel-Arnett S, Sang QX, Moore WG, Navre M, Birkedal-Hansen H, Van Wart HE. Comparative sequence specificities of human 72- and 92-kDa gelatinases (type IV collagenases) and PUMP (matrilysin). Biochemistry 1993;32:6427–32.

[7] Bickett DM, Green MD, Berman J, Dezube M, Howe AS, Brown PJ, Roth JT, McGeehan GM. A high throughput fluorogenic substrate for interstitial collagenase (MMP-1) and gelatinase (MMP-9). Anal Biochem 1993;212:58–64.

[8] McGeehan GM, Bickett DM, Green M, Kassel D, Wiseman JS, Berman J. Characterization of the peptide substrate specificities of interstitial collagenase and 92-kDa gelatinase. Implications for substrate optimization. J Biol Chem 1994;269:32814–20.

[9] Xia T, Akers K, Eisen AZ, Seltzer JL. Comparison of cleavage site specificity of gelatinases A and B using collagenous peptides. Biochim Biophys Acta 1996;1293:259–66.

[10] Kridel SJ, Chen E, Kotra LP, Howard EW, Mobashery S, Smith JW. Substrate hydrolysis by matrix metalloproteinase-9. J Biol Chem 2001;276:20572–8.

[11] Coussens LM, Fingleton B, Matrisian LM. Matrix metalloproteinase inhibitors and cancer: trials and tribulations. Science 2002;295:2387–92.

[12] Whittaker M, Floyd CD, Brown P, Gearing AJH. Design and therapeutic application of matrix metalloproteinase inhibitors. Chem Rev 1999;99:2735–76.

[13] Paemen L, Martens E, Norga K, Masure S, Roets E, Hoogmartens J, Opdenakker G. The gelatinase inhibitory activity of tetracyclines and chemically modified tetracycline analogues as measured by a novel microtiter assay for inhibitors. Biochem Pharmacol 1996;52:105–11.

[14] Norga K, Paemen L, Masure S, Dillen C, Heremens H, Billiau A, Carton H, Cuzner L, Olsson T, Van Damme J, Opdenakker G. Prevention of acute autoimmune encephalomyelitis and abrogation

of relapses in murine models of multiple sclerosis by the protease inhibitor D-penicillamine. Inflamm Res 1995;44:529–34.
[15] Paemen L, Martens E, Masure S, Opdenakker G. Monoclonal antibodies specific for natural human neutrophil gelatinase B used for affinity purification, quantitation by two site ELISA and inhibition of enzymatic activity. Eur J Biochem 1995;234:759–65.
[16] Zhou N, Paemen L, Opdenakker G, Froyen G. Cloning and expression in *Escherichia coli* of a human gelatinase B-inhibitory single-chain immunoglobulin variable fragment (scFv). FEBS Lett 1997;414: 562–6.
[17] Masure S, Proost P, Van Damme J, Opdenakker G. Purification and identification of 91-kDa neutrophil gelatinase Release by activating peptide interleukin-8. Eur J Biochem 1990;198:391–8.
[18] Van den Steen PE, Proost P, Wuyts A, Van Damme J, Opdenakker G. Neutrophil gelatinase B potentiates interleukin-8 tenfold by amino-terminal processing whereas it degrades CTAP-III, PF-4, and GRO-alpha and leaves RANTES and MCP-2 intact. Blood 2000;96: 2673–81.
[19] St-Pierre Y, Desrosiers M, Tremblay P, Estève P-O, Opdenakker G. Flow cytometric analysis of gelatinase B (MMP-9) activity using immobilized fluorescent substrate on microspheres. Cytometry 1996; 25:374–80.
[20] Masure S, Paemen L, Van Aelst I, Fiten P, Proost P, Billiau A, Van Damme J, Opdenakker G. Production and characterization of recombinant active mouse gelatinase B from eukaryotic cells and *in vivo* effects after intravenous administration. Eur J Biochem 1997;244: 21–30.
[21] Moore WM, Spilburg CA. Purification of human collagenases with a hydroxamic acid affinity column. Biochemistry 1986;25:5189–95.
[22] Odake S, Okayama T, Obata M, Morikawa T, Hattori S, Hori H, Nagai Y. Vertebrate collagenase inhibitor. I. Tripeptidyl hydroxamic acids. Chem Pharm Bull (Tokyo) 1990;38:1007–11.
[23] Odake S, Okayama T, Obata M, Morikawa T, Hattori S, Hori H, Nagai Y. Vertebrate collagenase inhibitor. II. Tetrapeptidyl hydroxamic acids. Chem Pharm Bull (Tokyo) 1991;39:1489–94.
[24] Beckett RP, Davidson AH, Drummond AH, Huxley P, Whittaker M. Recent advances in matrix metalloproteinase inhibitor research. Drug Discovery Today 1996;1:16–26.
[25] Johnson WH, Roberts NA, Borkakoti N. Collagenase inhibitors: their design and potential therapeutic use. J Enz Inhib 1987;2:1–22.
[26] Tamaki K, Tanzawa K, Kurihara S, Oikawa T, Monma S, Shimada K, Sugimura Y. Synthesis and structure–activity relationships of gelatinase inhibitors derived from matlystatins. Chem Pharm Bull (Tokyo) 1995;43:1883–93.
[27] Tomczuk BE, Gowravaram MR, Johnson JS, Delecki D, Cook ER, Ghose AK, Mathiowetz AM, Spurlino JC, Rubin B, Smith DL, Pulvino T, Wahl RC. Hydroxamate inhibitors of the matrix metallo-proteinases (MMPs) containing novel P_1' heteroatom based modifications. Bioorg Med Chem Lett 1995;5:343–8.
[28] Wahl RC, Pulvino TA, Mathiowetz AM, Ghose AK, Johnson JS, Delecki D, Cook ER, Gainor JA, Gowravaram MR, Tomczuk BE. Hydroxamate inhibitors of human gelatinase B (92 kDa). Bioorg Med Chem Lett 1995;5:349–52.
[29] Gowravaram MR, Tomczuk BE, Johnson JS, Delecki D, Cook ER, Ghose AK, Mathiowetz AM, Spurlino JC, Rubin B, Smith DL. Inhibition of matrix metalloproteinases by hydroxamates containing heteroatom-based modifications of the P_1' group. J Med Chem 1995;38:2570–81.
[30] Pratt LM, Beckett RP, Bellamy CL, Corkill DJ, Cossins J, Courtney PF, Davies SJ, Davidson AH, Drummond AH, Helfrich K, Lewis CN, Mangan M, Martin FM, Miller K, Nayee P, Ricketts ML, Thomas W, Todd RS, Whittaker M. The synthesis of novel matrix metalloproteinase inhibitors employing the Ireland–Claisen rearrangement. Bioorg Med Chem Lett 1998;8:1359–64.
[31] Xue CB, He X, Roderick J, DeGrado WF, Cherney RJ, Hardman KD, Nelson DJ, Copeland RA, Jaffee BD, Decicco CP. Design and synthesis of cyclic inhibitors of matrix metalloproteinases and TNF-alpha production. J Med Chem 1998;41:1745–8.
[32] Levy DE, Lapierre F, Liang W, Ye W, Lange CW, Li X, Grobelny D, Casabonne M, Tyrrell D, Holme K, Nadzan A, Galardy RE. Matrix metalloproteinase inhibitors: a structure–activity study. J Med Chem 1998;41:199–223.
[33] Yamamoto M, Tsujishita H, Hori N, Ohishi Y, Inoue S, Ikeda S, Okada Y. Inhibition of membrane-type 1 matrix metalloproteinase by hydroxamate inhibitors: an examination of the subsite pocket. J Med Chem 1998;41:1209–17.
[34] Krumme D, Wenzel H, Tschesche H. Hydroxamate derivatives of substrate-analogous peptides containing aminomalonic acid are potent inhibitors of matrix metalloproteinases. FEBS Lett 1998;436:209–12.
[35] Baxter AD, Bhogal R, Bird JB, Buckley GM, Gregory DS, Hedger PC, Manallack DT, Massil T, Minton KJ, Montana JG, Neidle S, Owen DA, Watson RJ. Mercaptoacyl matrix metalloproteinase inhibitors: the effect of substitution at the mercaptoacyl moiety. Bioorg Med Chem Lett 1997;7:2765–70.
[36] Levin JI, DiJoseph JF, Killar LM, Sharr MA, Skotnicki JS, Patel DV, Xiao XY, Shi L, Navre M, Campbell DA. The asymmetric synthesis and *in vitro* characterization of succinyl mercaptoalcohol and mercaptoketone inhibitors of matrix metalloproteinases. Bioorg Med Chem Lett 1998;8:1163–8.
[37] Campbell DA, Xiao XY, Harris D, Ida S, Mortezaei R, Ngu K, Shi L, Tien D, Wang Y, Navre M, Patel DV, Sharr MA, DiJoseph JF, Killar LM, Leone CL, Levin JI, Skotnicki JS. Malonyl alpha-mercaptoketones and alpha-mercaptoalcohols, a new class of matrix metalloproteinase inhibitors. Bioorg Med Chem Lett 1998;8:1157–62.
[38] Demeule M, Brossard M, Page M, Gingras D, Beliveau R. Matrix metalloproteinase inhibition by green tea catechins. Biochim Biophys Acta 2000;1478:51–60.
[39] Mattei M, Carnieri E, Politi V, D'Alessio S, Sella A, Cassol M, Robeva A, Colizzi V, Sumerska T. Inhibition of contact hypersensitivity reaction to picryl chloride: effect of small molecular weight peptidomimetic compounds possessing inhibitory activity against metalloproteinases. Int Immunopharmacol 2002;2:699–710.
[40] Gilles F, Jean A, Boutin GA, Fauchere J-L, Gordon C. Selection of a histidine-containing inhibitor of gelatinases through deconvolution of combinatorial tetrapeptide libraries. Mol Diversity 1996;2:135–46.
[41] Kiyama R, Tamura Y, Watanabe F, Tsuzuki H, Ohtani M, Yodo M. Homology modeling of gelatinase catalytic domains and docking simulations of novel sulfonamide inhibitors. J Med Chem 1999;42: 1723–38.
[42] Rowsell S, Hawtin P, Minshull CA, Jepson H, Brockbank SM, Barratt DG, Slater AM, McPheat WL, Waterson D, Henney AM, Pauptit RA. Crystal structure of human MMP9 in complex with a reverse hydroxamate inhibitor. J Mol Biol 2002;319:173–81.
[43] Elkins PA, Ho YS, Smith WW, Janson CA, D'Alessio KJ, McQueney MS, Cummings MD, Romanic AM. Structure of the C-terminally truncated human ProMMP9, a gelatin-binding matrix metalloproteinase. Acta Crystallogr D: Biol Crystallogr 2002;58:1182–92.
[44] Kaur K, Zhu K, Whittemore MS, Petersen RL, Lichte A, Tschesche H, Pourmotabbed T. Identification of the active site of gelatinase B as the structural element sufficient for converting a protein to a 22 metalloprotease. Biochemistry 2002;41:4789–97.
[45] Shipley JM, Doyle GA, Fliszar CJ, Ye QZ, Johnson LL, Shapiro SD, Welgus HG, Senior RM. The structural basis for the elastolytic activity of the 92-kDa and 72-kDa gelatinases. Role of the fibronectin type II-like repeats. J Biol Chem 1996;271:4335–41.
[46] Morgunova E, Tuuttila A, Bergmann U, Isupov M, Lindqvist Y, Schneider G, Tryggvason K. Structure of human pro-matrix metalloproteinase-2: activation mechanism revealed. Science 1999;284:1667–70.