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# Activity-based enrichment of matrix metalloproteinases using reversible inhibitors as affinity ligands

J.R. Freije, R. Bischoff*
*University Center for Pharmacy, University of Groningen, Antonius Deusinglaan 1, 9713 AV Groningen, The Netherlands*

**Abstract**

Matrix metalloproteinases (MMPs) are zinc dependent metalloproteases characterized by the ability to cleave extracellular matrix and many other extracellular proteins. MMP activity is tightly regulated but disturbances in this regulation can contribute to various disease processes characterized by a progressive destruction of the extracellular matrix. The ability to profile classes of enzymes based on functionally related activities would greatly facilitate research about the involvement of MMPs in physiological and/or pathological states. Here we describe the characterization of an affinity sorbent using an immobilized reversible inhibitor as a stationary phase for the activity-based enrichment of MMPs from biological samples. With a ligand density of 9.8 m*M* and binding constant of 58 μmol/l towards MMP-12, the capturing power of the affinity sorbent was strong enough to extract MMP-12 spiked into serum with high selectivity from relatively large sample volumes. Experiments with endogenous inhibitors revealed that MMP-12 extraction is strictly activity-dependent, offering powerful means to monitor MMP activities in relation to physiological and/or pathological events by using affinity extraction as a first step in an MMP profiling method.


*Keywords:* Proteomics; Affinity adsorbents; Matrix metalloproteinases; Metalloproteinases; Enzymes; Enzyme inhibitors

## 1. Introduction

The members of the multigene matrix metalloproteinase (MMP) family are characterized by their high primary sequence homology and the requirement of a catalytic $Zn^{2+}$ ion in the active site. MMPs are expressed in tissues and by inflammatory cells and can be secreted into extracellular fluids or localized towards the cell surface. They are capable of degrading all extracellular matrix proteins in tissue remodeling, cell migration and wound repair but also in pathological states. Furthermore, MMPs exhibit proteolytic activity towards other substrates such as other proteinases, proteinase inhibitors and many proteins involved in cellular signaling [1,2]. The activities of all MMPs are tightly regulated at the protein level by post-translational modifications and protein–protein interactions. Like other proteolytic enzymes, MMPs are secreted as inactive proenzymes, which can be activated upon proteolytic removal of the propeptide domain causing the C-terminal cysteine sulfhydryl group that coordinates the active site zinc ion to dissociate [3]. After activation, MMP activity is mainly controlled by tissue inhibitors of metalloproteinases (TIMPs) [4] and the generic protease inhibitor $\alpha_2$-macroglobulin. Because of these regulatory events, high mRNA levels of MMPs do not automatically result in

*Corresponding author. Tel.: +31-50-363-3338; fax: +31-50-363-7582.
*E-mail address:* r.bischoff@farm.rug.nl (R. Bischoff).

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elevated proteolytic activity. It is thought that disturbances in the regulation of the enzymatic activity due to genetic or environmental causes, can contribute to various disease processes such as the development of pulmonary emphysema, rheumatoid arthritis, arteriosclerosis, aneurysms and osteoporosis, which are all diseases where destruction of structural components of the extracellular matrix is an important feature.

Zymography and immunological methods are often used to study the relation between MMPs and disease development. Although these methods can reach high sensitivities they have difficulties to give information about the functional state of MMPs (pro-, active, or inhibited). If the role of MMPs in destructive disease mechanisms shall be analyzed, profiling the levels of MMP activities rather than overall MMP abundance will be necessary because in pathological situations, tissue damage is caused by active enzymes. Moreover, immunological methods are too specific to be used on a family wide scale because different MMP members require a different antibody or substrate. Parallel measurement on a family wide scale would be an attractive feature, to avoid excluding currently unknown members, or strongly related enzyme families which may also be involved in a given disease process.

In the rapidly developing field of proteomics, measurements on a proteome wide scale have become possible but most global approaches suffer from the fact that a significant part of the proteome remains undetected [5,6]. Moreover, global approaches are only capable of measuring protein abundance, which, for the highly regulated MMP family is not predictive for protein activity.

Activity-based proteomic methods aim to measure and identify all proteins with a related activity in a proteome. To date, all known activity based proteomic methods rely on the use of activity-based probes (ABPs), which bear a reactive group to covalently bind to the active site, a tag for detection and quantification and a group with affinity and selectivity for homologous active sites of the enzyme family [7]. To date, most ABPs are targeted towards hydrolytic enzyme families that possess active-site nucleophiles essential for activity like the serine hydrolases [8–10], phospatases [11], cysteine proteases (cathepsins and caspases) [12–15] and proteolytic subunits of the proteasome [16,17]. The strength of these ABP methods lies in the feature that they can zoom into an enzyme family or class of proteins within the whole proteome in an activity dependent way, resulting in sensitive detection of active enzymes, thereby not only giving information about abundance but also about activity and function.

The search for ABPs with reactivity towards new enzyme families resulted in the labeling of members of distinct enzyme classes belonging to oxidoreductase and transferase superfamilies [18,19]. However for MMPs, no such irreversible profiling tools have been described in the literature, most probably because of the nature of catalysis, in which activated water serves as the nucleophile that cleaves the peptide bond.

In an effort to profile active MMPs in biological fluids and tissues, we propose an activity-based proteomic method based on the selective extraction of active MMPs with an affinity sorbent carrying immobilized reversible inhibitors as affinity ligands. Because of the important roles of MMPs in many physiological processes and diseases and the lack of activity-based profiling methods for MMPs, we focused on this family. We argued that such an activity based proteomic MMP profiling method should fulfill a number of requirements: (1) the extraction should be selective for MMPs and functionally related proteases, (2) MMPs should be extracted in an activity dependent manner, (3) the capturing power of the affinity sorbent should be strong enough to reach high extraction yields even for large sample volumes and low MMP concentrations, (4) the solid support should display a low degree of non-specific protein binding.

In this paper, we explore the possibilities for activity-based proteomic MMP profiling using the inhibitor Pro–Leu–Gly–NHOH (where NHOH= hydroxamic acid) that is immobilized as affinity ligand on a Sepharose solid support. Using recombinant human MMP-12 (catalytic domain), we demonstrate that MMP extraction with the Sepharose affinity sorbent is activity dependent and that MMPs can be strongly concentrated and enriched on affinity beads thus providing an excellent basis for developing activity-based proteomic MMP profiling methods.

## 2. Experimental procedures

### 2.1. Materials

*N*-Hydroxysuccinimide (NHS)-activated Sepharose was from Amersham Bioscience (Uppsala, Sweden), Pro–Leu–Gly–NHOH and Mca–Pro–Leu–Gly–Leu–Dpa–Ala–Arg–$NH_2$ [where Mca=(7-methoxycoumarin-4-yl)acetyl and Dpa=*N*-3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl] were from Bachem (Bubendorf, Switzerland), recombinant human MMP-12 (catalytic domain produced in *Escherichia coli*) was provided by AstraZeneca R&D (Lund, Sweden) [20]. ProMMP-12 (murine macrophage), TIMP-1 (human neutrophil granulocyte) and $\alpha_2$-macroglobulin were from Calbiochem (La Jolla, CA, USA). Incubations in 1.5-ml Eppendorf tubes with controlled temperature and rotary shaking were performed on an Eppendorf thermomixer (Hamburg, Germany).

### 2.2. High-performance liquid chromatography (HPLC) analysis, MMP-12 assays and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis

HPLC analysis was performed with Merck–Hitachi equipment on a 10 cm Discovery $C_{18}$ column (150 mm×2.1 mm I.D., 5 μm; Supelco, Bellefonte, PA, USA) using a diode array detector under the following conditions: flow: 0.25 ml/min, injection volume 10 μl, mobile phase: water–acetonitrile (99:1)+0.1% trifluoroacetic acid (TFA) and detection at 214 nm.

MMP-12 activity assays were performed in 96-well plates (Costar-white) on a Perkin-Elmer LS-50B spectrofluorimeter, with the substrate Mca–Pro–Leu–Gly–Leu–Dpa–Ala–Arg–$NH_2$ in 100 μl assay buffer [0.1 mol/l Tris, pH 7.5, 0.1 mol/l NaCl, 20 m*M* $CaCl_2$, 20 μmol/l $ZnSO_4$, 0.05% (w/v) Brij-35] ($\lambda_{ex}$, $\lambda_{em}$=327, 420 nm, slit widths: 10 nm) [21].

Discontinuous, reducing SDS–PAGE was performed according to Laemmli [22] with 0.75 mm thick slab gels, on the Mini protean III cell assembly from Bio-Rad (Hercules, CA, USA). Acrylamide/bis (*N′N′*-bisacrylamide), ammonium persulfate (APS), *N*,*N*,*N′*,*N′*-tetramethylethylenediamine (TEMED), Laemmli sample buffer and broad range protein markers were from Bio-Rad. SDS, Tris (base) and glycine were from Duchefa (Haarlem, The Netherlands). Dithiothreitol (DTT) was from Sigma (Zwijndrecht, The Netherlands). Experimental conditions were as follows: stacking gel (1 cm), 4% T, 2.67% C, 0.1% (w/v) SDS, 0.05% (w/v) APS, 0.1% (v/v) TEMED, 125 m*M* Tris–HCl, pH 6.8; separating gel (6 cm), 15% T, 2.67% C, 0.1% (w/v) SDS, 0.05% (w/v) APS, 0.05% (v/v) TEMED, 375 m*M* Tris–HCl, pH 8.8; running buffer, 25 m*M* Tris base, 192 m*M* glycine, pH 8.3, 1.0% (w/v) SDS [T=(g acrylamide+g Bis)/100 ml solution; C=g Bis/% T]. Two volumes of sample buffer (+0.35 mol/l DTT) were added to aqueous samples and pure sample buffer was used for dried samples. Samples were heated for 5 min at 95 °C and loaded on the gel directly after heating. Running conditions: 30 min at 50 V (stacking) and 2 h at 100 V. After electrophoresis gels were stained for 3 h in 0.25% (w/v) Coomassie Brilliant Blue (CBB) R250 (Sigma), 40% (v/v) methanol and 7% (v/v) acetic acid, and destained for several hours in 10% (v/v) methanol, 10% (v/v) acetic acid.

### 2.3. Production and characterization of affinity beads

Immobilization of the inhibitor Pro–Leu–Gly–NHOH on 0.5 ml NHS-activated Sepharose was performed in a column, after washing the beads at 4 °C with several volumes of 1 m*M* HCl and coupling buffer [0.2 mol/l $KH_2PO_4$, pH 7.5, 0.5 mol/l NaCl, 5% (v/v) dimethylsulfoxide (DMSO)]. A 0.69-ml volume of 7.3 m*M* Pro–Leu–Gly–NHOH in coupling buffer was added at room temperature while keeping the beads in suspension by vortexing the closed column. The reaction was stopped after 2 h by removing the supernatant and adding several volumes of blocking buffer (0.5 mol/l ethanolamine, pH 8.5, 0.5 mol/l NaCl) to inactivate non-reacted NHS groups. After blocking (1 h, room temperature), the beads were washed alternating with several bead volumes of low pH buffer (0.1 mol/l sodium acetate, pH 4.0, 0.5 mol/l NaCl) and blocking buffer. Sepharose affinity beads were stored at 4 °C in 0.1

mol/l Tris–HCl, pH 7.5, 0.5 mol/l NaCl, 20% ethanol. Samples of 2 μl, obtained prior to and after the reaction, were taken and diluted 25 times with the mobile phase used for HPLC analysis, to determine the amount of immobilized inhibitor indirectly by measuring the decrease of the free inhibitor concentration in the reaction mixture. Control beads, having no immobilized inhibitor, were made similarly to affinity beads by directly coupling ethanolamine in blocking buffer.

The Michaelis–Menten constant ($K_m$) of the fluorogenic substrate Mca–Pro–Leu–Gly–Leu–Dpa–Ala–Arg–$NH_2$ was determined with the MMP-12 activity assay at 40 ng/ml MMP-12 and 4, 8 and 12 μmol/l substrate concentrations (each determination was done twice). The data were fitted to the Michaelis–Menten equation using the GraFit 4.0 software (Erithacus Software).

The binding constants of the inhibitor before ($K_i$) and after ($K_{i(im)}$) immobilization were determined in a competitive MMP-12 assay with 20 ng/ml MMP-12, 4 μmol/l substrate and a varying inhibitor concentration of 0–30 μmol/l for the free inhibitor and 0–80 μmol/l for the immobilized inhibitor (determinations were done four times for each inhibitor concentration). The immobilized inhibitor concentration was varied by diluting drained affinity beads with assay buffer into a homogeneous suspension and transferring different volumes of the suspension into the assay buffer. Both the immobilized and the free inhibitor were preincubated for 10 min at room temperature with MMP-12 to reach equilibrium (see Section 2.4). Assays with 0 μmol/l immobilized inhibitor were performed using the control beads. The assays were started by the addition of substrate to all wells and by mixing the well plate briefly. The beads were allowed to settle for 2 min (to prevent light scattering) prior to measuring the fluorescence ($t=0$). Subsequently, beads were brought into suspension again by rotary shaking at 1000 rpm of the well plate. At $t=13$ min the beads were allowed to settle again before the fluorescence was measured at $t=15$ min. The fluorescent increase was a measure for the rate of substrate conversion $V$ caused by MMP-12 activity. MMP-12 activity in the absence of inhibitor ($V_0$) is given by:

$$V_0 = V_m \cdot [S]/(K_m + [S]) \quad (1)$$

where [S] is the substrate concentration; $V_m$ is the maximal substrate conversion at saturating [S] and $K_m$ is the Michaelis–Menten constant.

In a competitive system, MMP-12 activity in the presence of inhibitor ($V_i$) is given by:

$$V_i = V_m \cdot [S]/\{K_m(1 + [I]/K_i) + [S]\} \quad (2)$$

where [I] is the inhibitor concentration.

The ratio $V_0/V_i$ is given by:

$$(V_0/V_i) - 1 = [I]/K_i(1 + [S]/K_m) \quad (3)$$

$V_0$ was determined by averaging the measured enzyme activities in the absence of inhibitor. $(V_0/V_i)-1$ was then determined at the different inhibitor concentrations and plotted against [I] and the slope of the fitted linear regression curve, which is equal to $1/K_i(1+[S]/K_m)$, was used to calculate the $K_i$ values.

### 2.4. MMP-12 extraction yield and binding kinetics

The relation between the extraction yield (EY) and the concentration of immobilized inhibitor was investigated with three binding experiments of 100 μl, containing 1 μg MMP-12 and 1, 5 and 10 μl (corresponding to 98, 490 and 980 μmol/l immobilized inhibitor) of affinity beads at 10 °C and rotary shaking at 1100 rpm for 15 min. A binding experiment with 10 μl of control Sepharose beads was performed to measure non-specific adsorption to the solid support. After the extraction, beads were allowed to settle for 2 min and four samples of 1 μl from each incubation were taken for the analysis of the MMP-12 concentration in the supernatant with the activity assay as described earlier. All bound proteins were analyzed by SDS–PAGE. For this the supernatant was discarded and the beads were washed with 1.5 ml of binding buffer at 0 °C. Bound MMP-12 was desorbed with 2×12.5 μl Laemmli sample buffer, by incubating the beads at 35 °C and rotary shaking at 1000 rpm for 10 min.

The binding kinetics of MMP-12 to the affinity beads were determined in 100 μl batch binding experiments with 10 μl affinity beads and 1.0 μg MMP-12 in binding buffer [10 m*M* Tris, pH 7.5, 10 m*M* $CaCl_2$, 1 mol/l NaCl, 0.05% (w/v) Brij-35] at 1100 rpm rotary shaking and 10 °C. At the given

times, 1 μl supernatant samples were obtained to determine the MMP-12 concentration in the supernatant. Prior to sampling, shaking was stopped for 45 s to let the beads settle. The samples were analyzed with the MMP-12 activity assay (4 μmol/l substrate) as described earlier and the measured increase in fluorescent was converted to MMP-12 concentration by measuring the uninhibited MMP-12 activity of reference solutions with different MMP-12 concentrations (10, 2.5 and 0.25 μg/ml). Binding experiments with control Sepharose beads were performed to measure the degree of non-specific binding to the stationary phase independent of the immobilized inhibitor.

### 2.5. Activity dependence of MMP-12 binding

The inhibition of 0.9 nmol/l MMP-12 by 1 nmol/l TIMP-1 was measured with the activity assay, both without preincubation of TIMP-1 and MMP-12 and with a preincubation in the assay buffer for 1.75 h at 15 °C and 300 rpm rotary shaking. MMP-12 activity was measured after the addition of substrate (4 μmol/l), and compared to uninhibited MMP-12 activity.

TIMP-1 dependent MMP-12 binding to the affinity beads was investigated as follows: 9.1 pmol MMP-12 were preincubated with 0 to 9.1 pmol TIMP-1 in binding buffer for 3 h at 600 rpm rotary shaking and 15 °C. After preincubation, binding experiments were performed in binding buffer with 10 μl affinity beads in a total volume of 50 μl, for 30 min at 0 °C while suspending the beads every 2 min. Both supernatant and beads were analyzed by SDS–PAGE. After binding, proteins in the supernatant were precipitated with trichloroacetic acid (TCA) [6% (w/v) final concentration] on ice for 30 min and centrifuged for 10 min (4 °C, 11300 *g*). The pellet was washed twice with 75 μl 3% (v/v) water in acetone kept at −20 °C, dried under vacuum for 10 min and dissolved in 10 μl of sample buffer. The beads were washed with 100 μl of cold binding buffer and bound proteins were desorbed with 2× 12.5 μl Laemmli sample buffer by incubating the washed beads for 10 min at 35 °C at 1000 rpm rotary shaking. Proteins were visualized by silver staining as described by Yan et al. [23].

$\alpha_2$-Macroglobulin dependent MMP-12 binding was investigated in the same way as TIMP-1 dependent binding but with different quantities and a preincubation at 25 °C. A 45-pmol MMP-12 sample was preincubated with 0 to 45 pmol of $\alpha_2$-macroglobulin in binding buffer for 3 h at 25 °C and 600 rpm rotary shaking. After preincubation, binding experiments were performed in 50 μl with 10 μl affinity beads in binding buffer, for 30 min at 0 °C while suspending the beads every 2 min. After incubation, the supernatant was removed and the beads were washed twice with 2 ml of cold binding buffer. Bound proteins were desorbed with 2×12.5 μl Laemmli sample buffer, by incubating for 10 min at 35 °C and 1000 rpm rotary shaking. SDS–PAGE was performed as described earlier.

Batch binding experiments with 0.5 μg murine proMMP-12 were performed in 100 μl with 10 μl affinity beads in binding buffer, for 30 min at 0 °C and suspending the beads every 2 min. The samples were prepared for SDS–PAGE analysis as described for the TIMP-1 dependent experiments. SDS–PAGE analysis was performed as described earlier.

### 2.6. Extraction of MMP-12 spiked into human serum

Different amounts of MMP-12 (varying from 0 to 1.5 μg) were spiked into 15 μl human serum, obtained from a healthy volunteer and preincubated prior to the extraction with affinity beads for 1 h at 37 °C and 600 rpm rotary shaking. A sample, containing approximately 8 μg serum proteins from the incubation with spiked MMP-12, was taken for SDS–PAGE analysis. The extraction was started by addition of 10 μl affinity beads and adjustment to 50 μl with binding buffer followed by incubation for 20 min at 10 °C and 1100 rpm rotary shaking. An identical serum extraction (without MMP-12) was performed with control Sepharose beads. After the extraction, supernatant was discarded and the beads were washed twice with 1.5 ml of binding buffer at 0 °C. Positive controls were performed with MMP-12 in binding buffer. Bound proteins were desorbed with 2×12.5 μl Laemmli sample buffer, by incubating the washed beads for 10 min at 35 °C and 1000 rpm rotary shaking. SDS–PAGE analysis was performed on a 12.5% acrylamide gel and staining was performed as described earlier.

In order to establish whether the interference of serum with MMP-12 binding was due to $\alpha_2$-macroglobulin, 6.25 μl serum was preincubated with 0.5 μg trypsin [*N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK)-treated, Sigma] for 1 h, 37 °C and 600 rpm rotary shaking. A 0.5-μg amount of MMP-12 was spiked into both pretreated and untreated serum and samples equivalent to approximately 8 μg serum proteins were taken. The extractions were performed in 50 μl with 10 μl affinity beads in binding buffer (15 min, 10 °C, 1100 rpm rotary shaking). Sample preparation for SDS–PAGE analysis and staining was performed as described earlier.

### *2.7. Binding of serum proteins*

Serum proteins were extracted in binding buffer with and without 2 m*M* Pro–Leu–Gly–NHOH or in metal depletion buffer (50 m*M* Tris–HCl, pH 7.5, 1.0 mol/l NaCl, 6 m*M* EDTA, 0.05% (w/v) Brij-35). Extractions (20 min, 10 °C, 1100 rpm rotary shaking) were performed in 100 μl containing 40% (v/v) human serum, one of the buffers and 10 μl affinity or control beads (negative controls). Extractions with affinity beads were also performed as described above but without serum and with 1.0 μg MMP-12 (positive controls). Extracted proteins were prepared for SDS–PAGE analysis and stained as described earlier.

## 3. Results

### *3.1. Production and characterization of affinity beads*

Following immobilization of the inhibitor, the ligand concentration and the $K_{i(im)}$ were determined by HPLC analysis and enzyme activity assays, respectively. HPLC analysis showed that 95% of the added inhibitor were immobilized which resulted in a ligand concentration of 9.8 m*M*. Comparison with the estimated ligand concentration of 16–23 m*M* NHS groups (manufacturers' data) of the NHS-activated Sepharose beads, shows that approximately 50% of the theoretically available NHS groups were derivatized.

The $K_m$ value between MMP-12 and the substrate Mca – Pro – Leu – Gly – Leu – Dpa – Ala – Arg – $NH_2$, which is needed for the subsequent $K_i$ determinations, was measured to be 17 μmol/l. While ideally this value should be determined using substrate concentrations around the $K_m$ value, this was not possible due to a strong decrease in emitted fluorescence at substrate concentrations above 12 μmol/l probably caused by quenching. Although determination of the $K_m$ with low substrate concentrations results in a less accurate value, the influence of this inaccuracy on the determined $K_i$ values was decreased by performing the $K_i$ measurements at a low substrate concentration of 4 μmol/l (see Eq. (3)). The $K_i$ determinations were done by measuring the fluorescent increase ($V$) caused by MMP-12 activity, at a constant substrate concentration [S] and varying concentrations of immobilized or free inhibitor [I]. The $K_i$ values were obtained by plotting the $(V_0/V_i)-1$ values against the inhibitor concentration [I] and using the slope (which is equal to $1/\{K_i(1+[S]/K_m)\}$) of the fitted linear regression curve (see Eq. (3)). The linearity of the $(V_0/V_i)-1$ against [I] plots (Fig. 1) confirms the competitive nature of inhibition by both free and immobilized inhibitor, which means that binding occurs at the active site. The $K_{i(im)}$ determination (Fig. 1A) also shows that the affinity of the immobilized inhibitor for its target protein decreases by roughly a factor of five when compared to the free inhibitor indicating that immobilization affects MMP-12 binding. However, it also demonstrates that the binding properties have not changed dramatically thus indicating that a functional affinity sorbent was obtained.

### *3.2. MMP-12 extraction yield and binding kinetics*

With both the concentration of the immobilized inhibitor and the $K_{i(im)}$ known, it is possible to calculate the theoretical EY as a function of inhibitor concentration and $K_{i(im)}$. The reversible binding of an enzyme (E) to an immobilized inhibitor (I) at equilibrium can be described by the equation:

$$E + I \leftrightarrow EI \quad (4)$$

where the equilibrium constant, $K_{i(im)}$, is an inversely

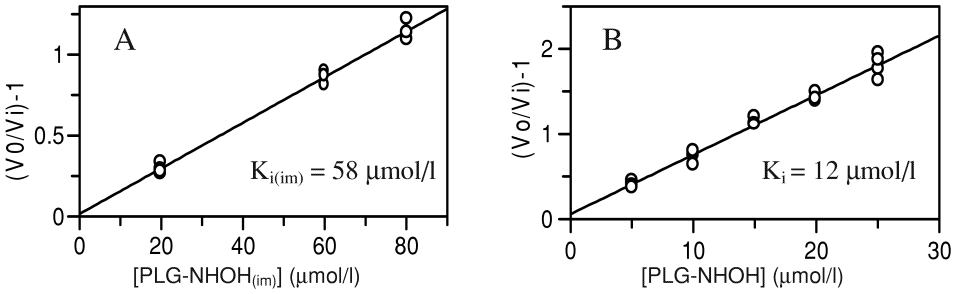


Fig. 1. $K_i$ determination of (A) the immobilized inhibitor and (B) the free inhibitor. MMP-12 activity ($V_i$) in the presence of different inhibitor concentrations was measured at 4 µmol/l substrate concentration and related to uninhibited MMP-12 activity ($V_0$). Eq. (3) and the slope of the plot are used to calculate the $K_i$ values.

proportional measure for the strength of this interaction, defined by:

$$K_{i(im)} = [[E][I])/[EI] \quad (5)$$

This equation can be rearranged to:

$$[EI]/[E] = [I]/K_{i(im)} \quad (6)$$

which shows that when [I]>>[E] (which holds true for m*M* concentrations of immobilized inhibitor), [I] is effectively constant, and the degree of binding is only determined by the ratio $[I]/K_{i(im)}$ and not by the enzyme concentration [E]. When we define the EY as the fraction of bound enzyme concentration [EI], relative to the total enzyme concentration [E]+[EI] in percentages, Eq. (6) can be transformed into the following form:

$$EY\ (\%) = 100/\left[1 + (K_{i(im)}/[I])\right] \quad (7)$$

which allows the EY to be calculated independent of the enzyme concentration. In batch extractions, [I] decreases depending on beads and sample volume ($V_{beads}$, $V_{sample}$), resulting in lower EY values. Taking the beads and sample volume into account, EY is given by:

$$EY\ (\%) = 100/\left\{1 + \left(K_{i(im)}V_{sample}/[I]V_{beads}\right)\right\} \quad (8)$$

The relation between EY and immobilized inhibitor concentration was investigated in batch binding experiments using different amounts of affinity beads (Fig. 2). Extracted MMP-12 was measured directly by SDS–PAGE, and indirectly by quantifying the remaining MMP-12 concentration in the supernatant. At 0.45 µmol/l enzyme and 98–980 µmol/l immobilized inhibitor concentrations there was no significant difference in the intensities of the extracted MMP-12 bands. This indicates that the measured EY is higher than the theoretical EY values calculated with Eq. (8). Table 1 shows the theoretical EY values for the different immobilized inhibitor concentrations based on the measured $K_{i(im)}$ and ligand density. Indeed, the measured EY values are significantly higher than predicted, indicating that the

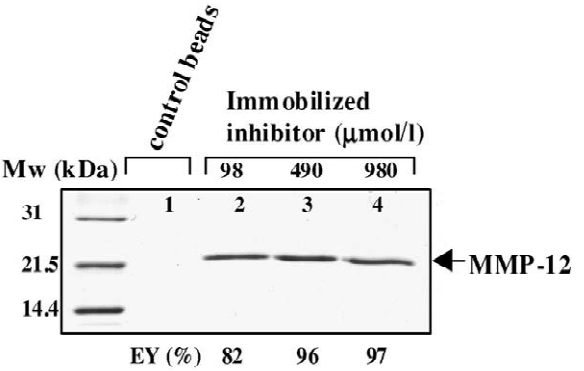


Fig. 2. The dependence of the MMP-12 extraction yield on the immobilized inhibitor concentration measured by SDS–PAGE. Binding experiments were performed in 100 µl binding buffer, with 1 µg MMP-12 (0.45 µmol/l), 10 µl of control beads or different volumes of affinity beads (1, 5 and 10 µl). MMP-12 was not extracted with the control beads (lane 1). The intensities of the MMP-12 bands extracted with different immobilized inhibitor concentrations (lanes 2, 3 and 4) show that the amount of extracted proteins is almost constant. The measured EY, based on the remaining MMP-12 activity measured in the supernatant after the binding experiment is given below the lanes with the different immobilized inhibitor concentrations. Mw = Molecular mass; kDa = kilodaltons.

Table 1
Comparison of the theoretical EY values (based on determined $K_{i(im)}$ and ligand density) and the measured EY values

| [I] (μmol/l) | EY (%) | | $K_{i(im)}$ (μmol/l) |
|---|---|---|---|
| | Theoretical | Measured | |
| 980 | 94.5 | 97.3±0.2 | 27.4±1.7 |
| 490 | 89.5 | 95.8±0.2 | 21.3±1.1 |
| 98 | 63.0 | 82.2±0.5 | 21.3±0.7 |

corresponding $K_{i(im)}$ values are approx. 2.5 times lower under these conditions than under the conditions of the competitive enzyme assay. An explanation for this significant increase in affinity could be the fact that the extraction experiments were performed with a 10 times higher NaCl concentration and a 10 times lower Tris concentration than the activity experiments used to measure the $K_{i(im)}$. The higher salt concentration may lead to a salting out effect, which increases the affinity between the hydrophobic amino acid side chains of the inhibitor and the hydrophobic S3, S2 and S1 substrate binding pockets in the active site of MMP-12 [24]. The metal chelating properties of the higher Tris concentration used in the enzyme assay may also have contributed to an increase in the measured $K_{i(im)}$.

The binding kinetics of MMP-12 were investigated in batch binding experiments. Binding was measured indirectly by following the decrease in MMP-12 concentration in the supernatant based on activity measurements. Because of the sensitivity of the enzyme assay, only small sample volumes are required and sampling does not affect the overall binding experiment conditions. Fig. 3 shows that little if any MMP-12 activity is bound to the control Sepharose beads confirming that binding to the affinity beads was specific. The binding of MMP-12 to the affinity beads reaches equilibrium after approximately 10 min of incubation, resulting in a constant level of 4.2% (0.42 μg/ml) unbound MMP-12 relative to the starting level (10 μg/ml). This is equivalent to an extraction yield of 95.8%, which is in good agreement with the EY of 97.3% measured in the binding experiment under the same conditions given in Table 1. As predicted by Eq. (8), the capturing power of the affinity sorbent depends on two properties namely, the ligand density and the $K_{i(im)}$ (which, as described above, also depends on the binding conditions such as buffer composition). An important consequence of this is that the MMP-12 concentration as such does not influence the EY. In batch experiments, EY is only influenced by the sample volume relative to the volume of added affinity beads. Table 1 shows that even for relatively large sample volumes (100 μl) and rather small bead volumes (1 μl) it is feasible to extract the larger part of MMP-12 despite the fact that $K_{i(im)}$ is in the moderate μmol/l range while the MMP-12 concentration is only 0.45 μmol/l. These characteristics are important for future experiments with biological samples containing unknown concentrations of active MMPs.

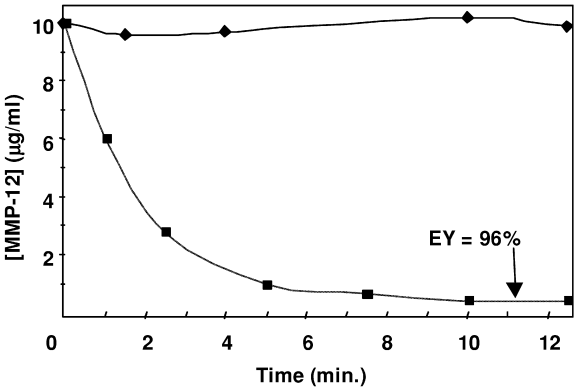


Fig. 3. Kinetics of MMP-12 binding to the affinity beads (■) and control beads (♦), performed in 100 μl binding buffer with 1.0 μg MMP-12 at 10 °C and 1100 rpm. The MMP-12 concentration in the supernatant was followed by analyzing 1 μl samples for MMP-12 activity. After approximately 10 min, equilibrium was reached and the MMP-12 concentration in the supernatant leveled off at 0.42 μg/ml, which corresponds to an extraction yield (EY) of 95.8%.

### 3.3. Activity dependence of MMP-12 extraction

Considering that many MMPs exist in vivo as inactive complexes with endogenous inhibitors or as inactive proMMPs we tested the ability of the affinity beads to extract inhibited MMP-12 and proMMP-12. As relevant endogenous inhibitors, TIMP-1 and $\alpha_2$-macroglobulin were tested for the degree to which they influence the extraction yield. The TIMP-1–MMP-12 interaction was first studied with the activity assay. Addition of 1 nmol/l TIMP-1 to 0.9 nmol/l MMP-12 showed a slow decrease of

MMP-12 activity from almost fully active, just after addition, to 38% residual activity after 50 min. After a preincubation of 1.75 h at the same concentrations, residual activity decreased to about 9% of the initial value. This slow and almost stoichiometric inhibition suggest a slow-tight-binding mechanism with a subnanomolar $K_i$, which is in agreement with the $K_i$ values found for TIMP-1 and MMP-1, 2 and 3 (0.25, 0.14 and 0.24 nmol/l, respectively [25]). Next, the influence of TIMP-1 on MMP-12 extraction was investigated. Incubations between TIMP-1 and MMP-12 prior to incubation with affinity beads were performed for 3 h to reach complete complex formation. Fig. 4A shows that TIMP-1 stoichiometrically decreases the MMP-12 extraction yield, demonstrating that MMP–TIMP complexes in biofluids would not be recognized. This is an important finding in view of later applications, where such complexes might well be present. Interestingly, despite a molar excess of almost 11000-fold of immobilized inhibitor over TIMP-1, there is no detectable competition between the immobilized inhibitor and TIMP-1 for MMP-12, which would result in liberation of MMP-12 and binding to the affinity beads under the conditions of the experiment (30 min incubation at 0 °C). This can be explained by the slow kinetics of the MMP-12–TIMP-1 complex formation which, together with the very strong interaction (low $K_i$), results in an extremely slow dissociation rate. Kinetic analysis of MMP-2–TIMP-2 complex formation revealed a two step binding mechanism with a relatively low-affinity intermediate and a very slow dissociation rate of the final stable complex of $2 \cdot 10^{-8}$ $s^{-1}$ [26]. Equilibrium in

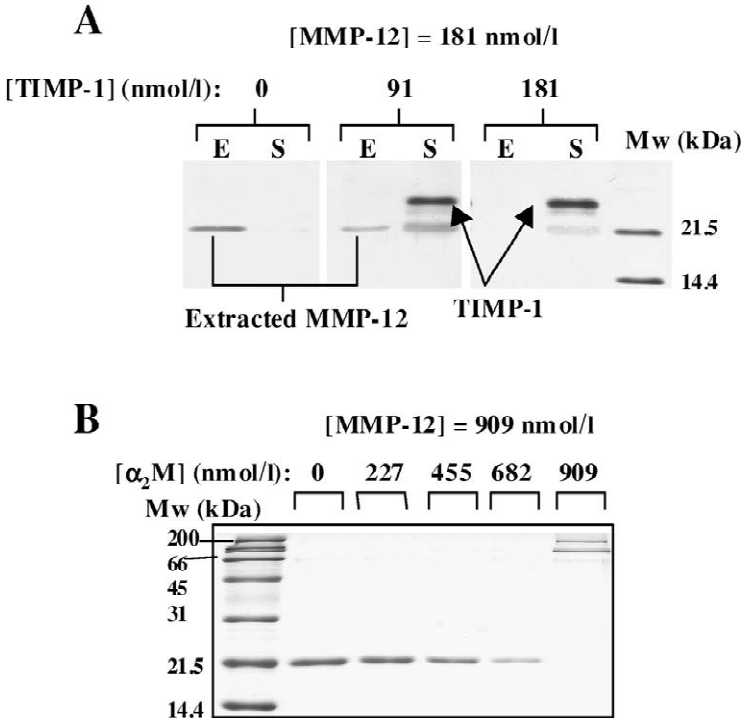


Fig. 4. MMP-12 is extracted with affinity beads in an activity-dependent manner. (A) MMP-12 (181 nmol/l) was preincubated with different TIMP-1 concentrations for 3 h, followed by extraction with affinity beads. Unbound proteins in the supernatant (S) and proteins extracted with the affinity beads (E) were analyzed. Silver staining confirmed that TIMP-1 stoichiometrically inhibits MMP-12 extraction. (B) MMP-12 (909 nmol/l) was preincubated with different $\alpha_2$-macroglobulin concentrations for 3 h, followed by extraction with affinity beads. Extracted proteins were analyzed by SDS–PAGE. Coomassie staining confirmed that $\alpha_2$-macroglobulin stoichiometrically inhibits MMP-12 extraction with affinity beads. The absence of intact or cleaved $\alpha_2$-macroglobulin monomer bands amongst the adsorbed proteins demonstrates that the active site of encapsulated MMP-12 is not accessible to the immobilized inhibitor.

the competition between TIMP-1 and the immobilized inhibitor therefore can not be reached within an incubation time of 30 min, and will not result in MMP-12 extraction, even if the equilibrium would be in favor of the immobilized inhibitor–MMP-12 complex.

The non-specific endopeptidase inhibitor $\alpha_2$-macroglobulin can be considered as a general back-up defense in body fluids against an excess of free proteolytic activity. It is known that proteinases are irreversibly trapped within the $\alpha_2$-macroglobulin cavity after cleavage of one of the solvent exposed bait regions, leaving the active site of the trapped proteinase accessible to small substrates and inhibitors via openings to the cavity on the surface of the $\alpha_2$-macroglobulin–proteinase complex but preventing any macromolecular substrates to be cleaved [27]. Like TIMP-1, $\alpha_2$-macroglobulin inhibits the binding of MMP-12 to the affinity beads (Fig. 4B). The absence of protein bands corresponding to the intact or cleaved monomer of $\alpha_2$-macroglobulin ($M_r$ 180000 and 90000, respectively) shows that the active site of trapped MMP-12 is not accessible to the immobilized inhibitor and as a consequence does not lead to binding of the $\alpha_2$-macroglobulin–MMP-12 complex. Although trapped MMP-12 may still have activity towards low-molecular-mass synthetic substrates, it is justifiable to speak of activity dependent binding, because large endogenous substrates will not be able to reach the active site of the trapped MMP-12. Thus, from a physiological point of view, trapped MMP-12 is ''non-active'' and consequently not recognized by the affinity beads.

The activity dependent binding to the affinity beads was also tested with the inactive zymogen of MMP-12, which may occur in vivo in higher concentrations than the active enzyme itself. Due to unavailability of human proMMP-12, we used the highly homologous mouse proMMP-12 (from macrophages). The extraction experiments showed that proMMP-12 did not bind to the affinity beads (data not shown). This result confirms that MMP-12 binding is dependent on an accessible active site.

### *3.4. Extraction of MMP-12 from biological matrices*

Spiking experiments were performed to investigate if the extraction yield of MMPs from a biological matrix is influenced by components in that matrix. Serum was chosen as a biological matrix because of its very high protein concentration and its relevance to future research in biofluids from patients. We argued that if a very complex matrix like serum would not influence the extraction yield dramatically, than biological matrices with a lower degree of complexity should also not influence MMP binding to the immobilized inhibitors.

The degree of non-specific serum protein binding to the control beads is very low (Fig. 5, extract CB), which is an important feature for the further development of an integrated, selective MMP profiling method. Comparison of the extraction of MMP-12 spiked into human serum and the equivalent extractions with the optimized binding conditions of MMP-12 in binding buffer (Fig. 5) shows that serum gave rise to a reduced extraction yield. In view of

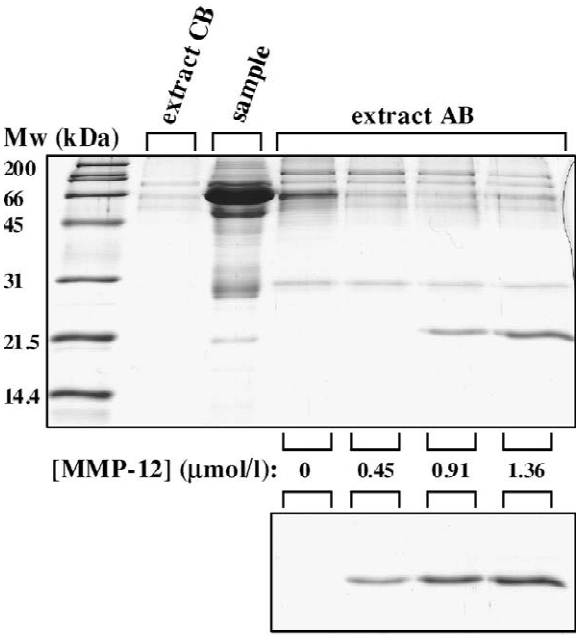


Fig. 5. Different concentrations of MMP-12 were spiked into 30% human serum in binding buffer. After preincubations, the samples were extracted with control beads (CB) and affinity beads (AB). A sample (sample) was taken prior to extraction to follow the enrichment (upper panel). Positive controls were performed with the same MMP-12 concentrations but without serum (lower panel). The affinity beads extractions were performed at an immobilized inhibitor concentration of 2 m*M*.

our results with $\alpha_2$-macroglobulin and its effect on MMP-12 binding, one possible explanation for this serum effect might be the presence of this generic protease inhibitor at levels of 2–4 mg/ml [28]. This results in an $\alpha_2$-macroglobulin concentration of 0.8–1.7 μmol/l in the extraction experiments which might well be able to trap all MMP-12 at a concentration of 0.45 μmol/l making it inaccessible for extraction. This hypothesis was investigated by pretreating serum with trypsin to inactivate $\alpha_2$-macroglobulin prior to the addition of MMP-12. Fig. 6 shows that the serum effect is almost completely abolished after pretreatment with trypsin so that the extraction yield reaches essentially the same level as the positive control performed in binding buffer. This result indicates that the decreased binding of MMP-12 to the immobilized inhibitor in serum is mainly caused by the presence of trypsin-sensitive endogenous inhibitors most likely to $\alpha_2$-macroglobulin.

The extraction results of MMP-12 in untreated and pretreated serum clearly demonstrate that MMP-12 can be selectively enriched and concentrated by just one extraction step from an undetectable low abundance protein (lane 1) to the most abundant protein in the extract (lane 4). Together with the activity dependent extraction results shown above, these results demonstrate the suitability of affinity extraction to be used as the first step in an activity-based proteomic MMP profiling method.

### 3.5. Binding of serum proteins

Figs. 5 and 6 also show that other serum proteins are enriched by the affinity beads. Comparison with the serum extraction profile obtained with control beads indicates that these proteins are bound to the immobilized inhibitor. The nature of the interaction between these serum proteins and the immobilized inhibitor was investigated by performing the extraction in buffers containing a metal chelating agent (EDTA) or 2 m*M* of free inhibitor. Fig. 7 shows that an enriched serum protein of $M_r$ 30000 (lane 1) does not bind in the presence of the metal chelating agent EDTA (lane 2), and exhibits decreased binding in the presence of 2 m*M* of the free inhibitor (lane 3).

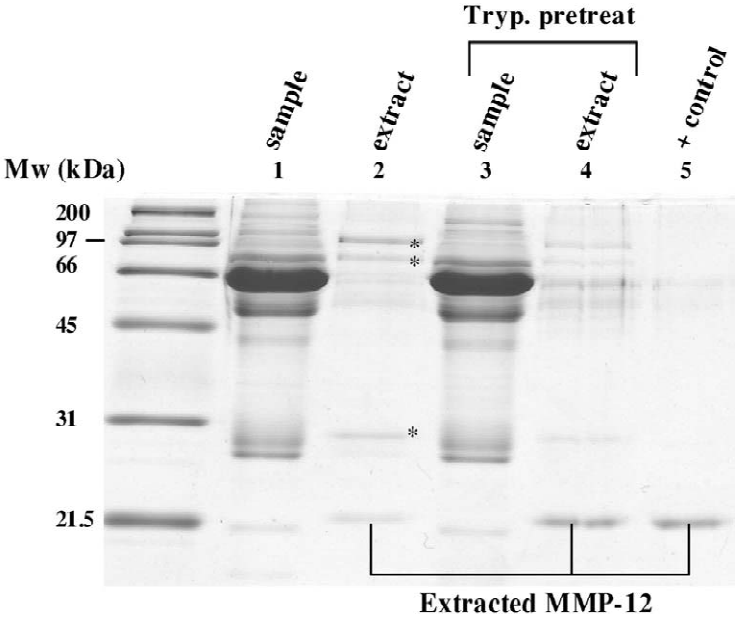


Fig. 6. MMP-12 extraction yield after spiking into serum is dependent on trypsin sensitive endogenous inhibitors like $\alpha_2$-macroglobulin. MMP-12 was spiked into untreated (1) and tryptic pretreated (3) human serum with a total serum protein level of 10 mg/ml and an MMP-12 level of 0.01 mg/ml. Both samples were extracted with affinity beads (2, 4) and compared to extraction of MMP-12 in binding buffer (5) at the same concentrations.

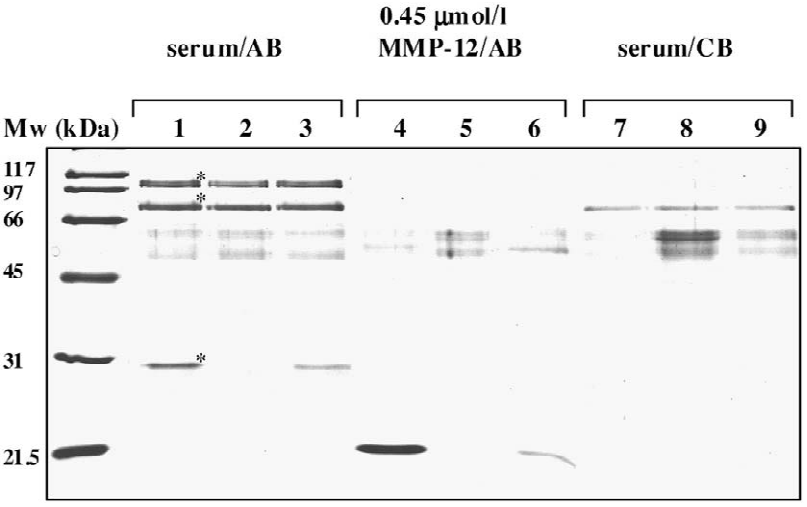


Fig. 7. Extraction of an $M_r$ 30000 serum protein is metal dependent. Serum proteins were extracted in binding buffer (lanes 1, 4 and 7) or in metal depletion buffer with 6 m*M* EDTA (lanes 2, 5 and 8) or in binding buffer with 2 m*M* H-PLG-NHOH (lanes 3, 6 and 9). Beside extractions of serum proteins with affinity beads (serum/AB), extractions were done with MMP-12 without serum (0.45 μmol/l MMP-12/AB) as positive control and with control beads and serum (serum/CB) as negative control. Extracted proteins were analyzed by SDS–PAGE and Coomassie staining.

Furthermore, this protein does not bind to the control beads (lanes 7–9). The effect of EDTA on binding suggests that the interaction with the immobilized inhibitor is metal dependent. However, it is not possible to conclude at present whether metal(s) of this unknown protein have a structural role or whether they are involved in biological activity or an active site. Strikingly, MMP-12 binding follows the same pattern (lanes 4–6) meaning that a metal containing enzyme cannot be excluded. This is presently further investigated. The binding of another enriched serum protein of $M_r$ 85000 (lanes 1–3) is not affected by the presence of EDTA or the free inhibitor, indicating that it is probably not a metal containing protein which binds to the solid support. This protein is, as one of the few proteins, also extracted with the control beads, confirming that it binds to the solid support. A third enriched protein at $M_r$ 105000 (lanes 1–3) does not bind to the control beads, but the enrichment by affinity beads is not affected by the presence of the free inhibitor or EDTA. It is presently unclear how the interaction with the immobilized inhibitor is mediated but this protein may have a higher affinity for the immobilized inhibitor than for the free inhibitor. Such a difference could be explained by the charge carried at the N-terminus of the free inhibitor, which is not present at the immobilized inhibitor.

## 4. Discussion

Proteomics is an approach to gain knowledge about all proteins in a given sample. Mostly, proteomics compares the abundance of proteins in samples thus giving information on the relative abundance of each protein. Comprehensive proteomics, albeit an impressive set of techniques, suffers from the fact that most low-abundance proteins are not displayed and that a number of protein families, such as membrane proteins, are not well represented.

The analysis of low-abundance proteins is generally performed based on high-affinity, specific interactions between the target protein and a chemical or biochemical ligand such as an antibody. Indeed, immunological methods are dominating the field of clinical and biochemical analysis of low-abundance

proteins due to their sensitivity and ease of use. While powerful for a given target protein, these methods suffer at the same time from the lack of providing a comprehensive picture of a proteome.

Somewhere in between are methods such as the one described here that allow the profiling of a subset of proteins in a proteome based on a well-defined molecular property. Most notable are the so-called activity-based profiling methods, all using ABPs to profile active hydrolytic enzymes within one family. These ABPs interact specifically with an enzyme's active site, label it covalently, and introduce a tag for sensitive detection or enrichment. Along these lines but extending the range of such methods, we describe a method for the activity based enrichment of a class of related proteases, based on the use of an affinity sorbent, carrying an immobilized reversible inhibitor as a stationary phase. We show here that this enrichment method fulfills a number of criteria, which are critical for its successful application with real-life samples:

(a) Selectivity for MMPs.

(b) Extraction in an activity-dependent manner.

(c) Efficient extraction that is independent of the target enzyme's concentration in the sample.

(d) A low degree of non-specific protein binding.

Although we did not test the selectivity of our affinity sorbent with other MMPs besides MMP-12, literature evidence suggests that it also has enough affinity for the parallel enrichment of other MMP members or functionally related metalloproteases. This inhibitor was already used for the purification of active MMP-1, MMP-8, MMP-7 (collagenases 1 and 2 and matrysilin) and an astacin (which like the MMPs also belongs to the metzincin superfamily) indicating its broad spectrum [29–32]. The well characterized substrate specificities of other MMP members within their S3, S2 and S1 binding pockets indicate further that other MMPs such as MMP-2 and MMP-9 (gelatinases A and B), MMP-3 and MMP-10 (stromelysins 1 and 2) MMP-18 (collagenase-4) and the MMP-14 to 17 (MT-MMPs) will probably also have considerable affinity for this inhibitor and thus should be enriched from biological samples [33,34].

Although ABP methods have been applied to a range of biological questions, not all of them have addressed the criteria listed above. Only limited attention has been paid to the question whether the probes react only with free, active enzymes. The degree in which activity dependent probing takes place was only investigated qualitatively with ABPs directed towards serine proteases. These ABPs were found to exhibit reduced reactivity towards the soybean trypsin inhibitor–trypsin complex and the inactive zymogens of trypsin and chymotrypsin [8,10]. The degree to which the reactivity towards the inhibitor–trypsin complex and other enzyme–inhibitor complexes was reduced has not been investigated yet. This can, however, be of importance because competition between the ABP and endogenous inhibitors for active sites could lead to increased levels of measured activity.

Many of the reactive covalently modifying ABPs do also show some degree of activity-independent labeling of non-related proteins, probably caused by the intrinsic reactivity of the reactive group. Selectivity is then often established by comparing heat-inactivated samples with native samples. This means that most of the presently available activity-based profiling methods are still in their early stages of development and more work on controls is needed to render results obtained in complex biological samples reliable.

Notwithstanding this fact, these methods have a great potential to complement comprehensive proteomics strategies by adding information about the activity status of a given class of proteins and by enriching previously undetectable low-abundance proteins.

The use of immobilized inhibitors with $\mu M$ affinity towards our model protease MMP-12 resulted in high extraction yields and efficient preconcentration on the affinity sorbent even for diluted large volume samples. The activity dependent binding experiments with both inactive proMMP-12, and MMP-12 inhibited by two relevant in vivo inhibitors of MMPs shows that MMP-12 extraction is strictly activity dependent, an important prerequisite for the development of activity-based proteomic MMP profiling methods. Potentially interfering highly abundant proteins, present in complex biological samples, were efficiently depleted. Because sample complexity is greatly reduced, the use of sophisticated and labor-intensive separation methods such as two-dimensional gels can be avoided and protein analysis

may be simplified. Automation of analytical procedures is a prerequisite to reach a higher throughput, which is necessary for clinical studies, as well as to achieve greater reproducibility and robustness. The use of other types of stationary phases with better mass transfer properties and high pressure stability will facilitate the high throughput analysis of active MMPs in biological samples. An activity-based affinity solid-phase extraction (SPE) step could be an ideal starting point of such an integrated functional proteomic profiling system. Affinity-SPE followed by for, example, on-bead digestion and liquid chromatography–mass spectrometry (LC–MS) would give the opportunity to investigate the role of active target proteases in physiological and/or pathological events related to some of the major diseases. Such an assay could also be used to guide the development of novel pharmaceutical agents directed against MMPs in preclinical and clinical research.

Last not least, our findings that at least one serum protein binds in a specific, metal-dependent manner to the immobilized hydroxamic acid inhibitor indicates that the selectivity of this class of compounds may have been overestimated and that some of the observed side effects in clinical trials may have resulted from such interactions. We are presently identifying these proteins and will study them further in light of their possible role in toxicity of hydroxamate-based MMP inhibitors.

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