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An integrated high-performance liquid chromatography–mass spectrometry system for the activity-dependent analysis of matrix metalloproteases

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Abstract

Matrix metalloproteases (MMPs) comprise a family of enzymes that play important roles in mediating angiogenesis, the remodelling of tissues and in cancer metastasis. Consequently, they are attractive targets for therapeutic intervention in chronic inflammation, cancer and neurological disorders. In order to study MMPs in body fluids in an activity-dependent manner, we have developed an automated, integrated system comprising an immobilized inhibitor cartridge for activity-dependent enrichment, an immobilized trypsin reactor for rapid on-line proteolysis and a capillary or nanoLC–MS system for separation and identification of the obtained peptide fragments. This targeted proteomics system was optimized with respect to recovery and evaluated through the analysis of urine samples that were spiked with recombinant MMP-12. MMP-12 specific peptide fragments were easily detected in a nanoLC–MS analysis of 500 μL crude urine spiked at a level of 8 nM. These results show the feasibility of selective, activity-dependent enrichment of MMPs from a non-treated biofluid at low nM concentrations.

Keywords: Metalloprotease; Proteomics; HPLC; Mass spectrometry; Affinity chromatography; MMPs; Activity-based analysis; Targeted proteomics; Automated online analysis system

1. Introduction

A critical issue in proteomics of body fluids is the high sample complexity and the wide dynamic concentration range, which outweighs the resolving power of all present separation methods such as two-dimensional gel electrophoresis [1]. The labour intensive nature of this method has furthermore driven the development of alternative separation methodologies based on multi-dimensional chromatography [2], which are amenable to automation and direct coupling to mass spectrometry. By increasing the peak and loading capacity, multidimensional approaches increase the number of identified proteins and the dynamic range of the analysis but at the expense of rather long analysis times per sample [3].

An alternative strategy for detection of low abundance proteins in complex biological samples makes use of affinity sorbents for selective enrichment prior to mass spectrometric analysis [4]. Besides selective enrichment, resulting in increased sensitivity for the targeted proteins, additional information is obtained through the nature of the interaction, on which enrichment is based. In the case of enzymes it is possible to enrich only the active form, which is critical when relating enzyme activity to a given disease process [5]. Activity-based profiling has therefore developed into an important branch of chemical biology [6–9].

Affinity sorbents are particularly suited to enrich proteins that are present at low concentrations in large volumes of body fluids. They can furthermore be implemented in automated protein analysis platforms in combination with proteolytic enzyme reactors, thus reducing the need for manual sample preparation steps. However, to date this potential has hardly been realized. In most described integrated systems, where affinity enrichment by immobilized antibodies is followed by on-line digestion and LC–MS analysis, single proteins were monitored without gaining information about the corresponding functionality of the enriched proteins [10–12]. For the activity-based analysis of MMPs in complex body fluids, no integrated, automated approach has been described. A recently developed chemical

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proteomics approach, which allows family-wide MMP analysis based on activity, relies on labelling with reactive, activity-based probes (ABPs) [13,14]. While well-suited to the analysis of cells and tissue, it is less adapted to the analysis of active MMPs in body fluids, since reaction kinetics are concentration-dependent and the amount of ABP needed increases with the volume that needs to be analyzed. As an alternative we describe the development of an automated method to detect active MMPs by using immobilized MMP inhibitors. The method is based on the enrichment of active MMPs by inhibitor affinity chromatography, digestion on an acetylated-immobilized trypsin reactor [15], capturing of peptides on a reversed-phase trap column followed nanoLC–MS/(MS) analysis. This integrated system allows detecting and identifying MMP-12 spiked into crude urine at a concentration of 8 nM from an initial volume of 500 μL. Physiological concentrations of MMPs in biofluids have been described to be as high as 50 nM for MMP-9 in induced sputum after cigarette smoke exposure [16], and as high as 10 nM for MMP-12 in induced sputum from patients suffering from chronic obstructive pulmonary disease (COPD) [17]. Urinary MMP concentrations can be as high as 22 ng/mg total protein (median value in patients diagnosed with malignant bladder cancer) [18], indicating that the system described in this work may be used for determination of endogenous MMPs in biological and clinical samples.

2. Materials and methods

2.1. Materials

N-Hydroxysuccinimide (NHS)-activated Sepharose was from Amersham Bioscience (Uppsala, Sweden), Pro-Leu-Gly-NHOH was from Bachem (Bubendorf, Switzerland), TAPI-2 (*N*-(*R*)-(2-(hydroxyaminocarbonyl)methyl)-4-methylpentanoyl -L-*t*-butyl-glycine-L-alanine 2-aminoethylamide) was from Calbiochem (La Jolla, CA, USA). Calcium chloride (>99% pure), ethanolamine (>98% pure) and tris(hydroxymethyl)aminomethane (Tris, >99.5% pure) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Sodium chloride (>99.5% pure) and EDTA (ethylenediaminetetraacetic acid, >99% pure) were from Fluka (Seelze, Germany). Brij-35 was from ICN Biomedicals (Zoetermeer, The Netherlands), Poly(ethylene glycol) 400 monooctyl ether and poly(ethylene glycol) 600 monooctyl ether were from Acros Organics (Geel, Belgium), dodecyl-β-D-maltoside was from MP Biomedicals (Solon, OH, USA) and octylglucopyranoside was from Sigma–Aldrich.

Trypsin (TCPK-treated (tosyl phenylalanyl chloromethylketone), bovine pancreas, 10 000–15 000 BAEE units/mg protein) was from Sigma–Aldrich. Acetic acid *N*-hydroxysuccinimide (NHS) ester (AANHS) was from ICN Biomedicals, Poroszyme immobilized-trypsin beads were from Applied Biosystems (Foster City, CA, USA).

Acetonitrile (Supragradient grade) was from Biosolve (Valkenswaard, The Netherlands), formic acid (Suprapure grade) was obtained from Merck (Darmstadt, Germany). Ultrapure water, produced in-house by an Elga purification system, was used for all buffer and mobile phase preparations, which were filtered (0.22 μm pore size) before use.

Recombinant human MMP-12 (catalytic domain produced in *E. coli* as described elsewhere [19]) was provided by AstraZeneca R&D (Lund and Moelndal, Sweden).

2.2. Preparation of immobilized inhibitor cartridges

Seph-PLG-NHOH and Seph-TAPI-2 affinity cartridges (10 mm length × 2 mm I.D.) with a ligand density of 5 mmol/L were prepared as described earlier [20]. Briefly, NHS-activated Sepharose beads were washed with several bead volumes of coupling buffer (0.2 mol/L K_2HPO_4, pH 7.5) at 4 °C. The washed beads were then incubated with an equal volume of a 5 mmol/L solution of either PLG-NHOH or TAPI-2 in coupling buffer for 2 h in a shaking incubator (Eppendorf thermomixer) maintained at 25 °C. The remaining NHS-groups were quenched with blocking buffer (0.5 mol/L ethanolamine in coupling buffer) for 1 h at 25 °C. Control beads were prepared by immediate quenching with ethanolamine. Beads were stored in 20% (v/v) ethanol at 4 °C until use.

Extraction cartridges were prepared by slurry packing the inhibitor beads in 10 cm × 2 mm I.D. solid-phase extraction cartridges (Spark Holland, Emmen, the Netherlands) fitted with a 0.2 μm stainless steel inlet frit on one side.

2.3. Preparation of immobilized trypsin reactor

AANHS-modified immobilized trypsin reactors were prepared as described earlier [15]. Immobilized trypsin was either prepared in-house by incubating NHS-activated Sepharose beads with an equal volume of 20 mg/ml trypsin in coupling buffer (0.1 M K_2HPO_4, pH 7.8) for 25 min with rotary shaking at 1100 rpm at 25 °C, or purchased ready-made (Applied Biosystems; Porozyme). The immobilized trypsin was modified by acetylation with an equal volume of modification buffer (22 mmol/L AANHS in coupling buffer) for 20 min. Trypsin beads were stored at 4 °C in 50 mM Tris, pH 8.2, 1 mM $CaCl_2$, 0.02% NaN_3 until use.

For the experiments the immobilized trypsin beads were slurry-packed into 10 cm × 2 mm I.D. solid-phase extraction cartridges (Spark-Holland) fitted with a 0.2 μm stainless steel inlet frit on one side. The packed cartridges were housed in a clamp (Spark-Holland).

2.4. On-line coupling of the extraction–digestion steps to capillary LC–MS

The integrated system (Fig. 1) consisted of a solid-phase extraction (SPE) workstation (Prospekt 2, Spark-Holland), comprising a Triathlon autosampler (with sample cooling to 4 °C), an automated cartridge exchanger (ACE) unit, and a micro high pressure dispenser (μ-HPD, flow rate range: 12–1000 μL/min). Experiments with the integrated system were performed with 2-mm I.D. Seph-PLG-NHOH cartridges, 2-mm I.D. AANHS modified Poroszyme trypsin cartridges, a Vydac C_8 trapping cartridge (10 mm, 1 mm I.D., 5 μm, 300 Å pore size) and