Journal of Chromatography A, 1216 (2009) 4407–4415

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Development of a liquid chromatography-based screening methodology for proteolytic enzyme activity

Nils Helge Schebb[a], Torsten Vielhaber[a], Alexandre Jousset[b], Uwe Karst[a,*]

[a] Westfälische Wilhelms-Universität Münster, Institut für Anorganische und Analytische Chemie, Corrensstraße 30, 48149 Münster, Germany
[b] Technische Universität Darmstadt, Institut für Zoologie, Schnittspahnstraße 3, 64287 Darmstadt, Germany

ARTICLE INFO

Article history:
Received 16 January 2009
Received in revised form 11 March 2009
Accepted 13 March 2009
Available online 25 March 2009

Keywords:
Protease
MS multi-substrate assay
HPLC with biochemical detection (BCD)
Size-exclusion chromatography (SEC)
Ion-exchange chromatography (IEX)

ABSTRACT

A new methodology for the detection and isolation of serine proteases in complex mixtures has been developed. It combines the characterization of crude samples by electrospray tandem mass spectrometry (ESI-MS/MS) in a multi-substrate assay and the differentiated sensitive detection of the responsible enzymes by means of liquid chromatography hyphenated online to biochemical detection (BCD). First, active samples are identified in the multi-substrate assay monitoring the conversion of eight substrates in multiple reaction monitoring in parallel within 60 s. Hereby, the product patterns are investigated and the suitable peptide as substrate for BCD analysis is selected. Subsequently, the active proteases are identified online in the continuous-flow reactor serving as BCD after non-denaturing separation by size-exclusion chromatography and ion-exchange chromatography. For BCD, the selected *para*-nitroaniline (*p*NA) labeled peptide is added post-column and is cleaved by eluting proteases under release of the coloured *p*NA in a reaction coil (reaction time 5 min). The method was optimized and the figures of merit were characterized with trypsin and chymotrypsin serving as the model proteases. For trypsin, a limit of detection in LC–BCD of 0.1 U/mL corresponding to an injected amount of 0.4 ng protein (~18 fmol) was observed. The BCD signal remained linear for an injected enzyme concentration of 0.3–10 U/mL (1.3–42 ng enzyme). The method was applied to the characterization of the crude venom of the pit viper *Bothrops moojeni* and the extracellular protease of the pathogenic amoeba *Acanthamoeba castellanii*. In the two samples, fractions with proteolytic activity potentially interfering with the blood coagulation cascade were identified. The described methodology represents a tool for serine protease screening in complex mixtures by a fast ESI-MS/MS identification of active samples followed by the separation and isolation of active sample constituents in LC–BCD.

1. Introduction

Enzymatic reactions are important for the regulation of all processes of life. In particular, the large class of proteases takes part in a multitude of physiological processes [1]. Besides simple food digestion, proteases regulate complex biological systems like blood coagulation [2], cell-cycle progression [3], or apoptosis [4]. Proteases account for approximately 2% of the human genes and more than 500 genes in the human genome have been annotated to proteolytic enzymes [5]. Proteases also contribute, for example to the toxicity of snake venom [6–11] or to the invasive potential of microorganisms such as the eye pathogen *Acanthamoeba castellanii* [12–14]. However, most of the proteases have not been investigated so far or only few data are available [1]. Therefore, analytical methods are needed to screen biological samples for proteolytic activity and to characterize the responsible proteins with the goal to achieve an improved understanding of physiological regulation processes. Moreover, the discovery of new proteases is of direct interest for industrial and medical use. Proteases are widely used biocatalysts [15] and serve as diagnostics and pharmaceuticals (e.g., batroxobin, a protease from snake venom [7,9]). Hence, the measurement of enzyme activity is required for the development of new drugs targeting proteases, for example those involved in health threatening processes.

As summarized by Schlüter et al., the classical approach for the identification of new proteases is a laborious process comprising several steps [1], starting with the detection of the reaction products in biological samples (I). On the basis of the chemical structure of these products, suitable substrates for the protease are synthesized and identified (II) in order to measure its activity in an assay. This assay is applied to screen biological matrices (III) and their fractions for the presence of the protease. After purification (IV), the sequence of the protease can be elucidated or the enzyme can be characterized biochemically, e.g., by its substrate specificity (V). Recently, we developed an electrospray mass spectrometry (ESI-MS)-based multi-substrate assay to examine protease activity [16].

* Corresponding author. Tel.: +49 251 8333141; fax: +49 251 8336013.
E-mail address: uk@uni-muenster.de (U. Karst).

0021-9673/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2009.03.053

This method meets the demands of the identification of mixtures exhibiting proteolytic activity (step III) as was demonstrated for snake venom fractions [11]. In contrast to classical UV-based assays, ESI-MS-based enzyme assays allow the parallel determination of multiple conversions [17]. By monitoring in parallel, this method is ideal for the identification of suitable substrates (step II) and for the characterization of the protease with respect to their activity pattern (step V) [16]. Besides, many more protease activity screening methods have been described, ranging from MS-based approaches [18,19], to spectrophotometric plate reader assays [20].

However, when it comes to the purification and biochemical characterization (steps IV and V), the existent methods still suffer from major drawbacks. Only the overall activity of a sample is determined, making it impossible to distinguish between active and inactive proteins. Hence, the sample has to be fractionated in a second independent working step, e.g., by size-exclusion chromatography (SEC) [8,11,21]. Hereby, the eluate can only be divided in a large number of arbitrary interval fractions because the eluting substances are monitored by unspecific UV detection. Furthermore, the enzymatic activity has to be measured in additional assays. The whole process is laborious and time-consuming and includes the important risk that enzymes of interest are degraded during fractionation.

In order to overcome these drawbacks, we developed a liquid chromatography (LC) method with biochemical detection (BCD). In this approach, LC is coupled to a continuous-flow reactor serving as BCD system. The biological activity is directly observed on-line after LC separation, allowing immediate identification of active fractions. Until now, LC–BCD has been applied to screen for enzyme inhibitors, including the proteases thrombin, trypsin [22], angiotensin converting enzyme [23], cathepsin B [24] and subtilisin [25] or receptor ligands, e.g., estrogen receptors [26]. However, to our knowledge, the LC–BCD method has only been applied once for the monitoring of enzymatic activities [27]. In this approach, hydrolyzed cytochrome *c* has been screened for microperoxidases by the hyphenation of reversed-phase (RP) HPLC to BCD. This method is well suited for the analysis of microperoxidases, which are very small bioactive peptides (up to 11 amino acids) even in the presence of those organic solvents used in RP-HPLC. On the other hand, RP chromatography is not a suitable technique for the separation of native protein samples, since most other enzymes are denatured and inactivated by organic solvents like methanol (MeOH) or acetonitrile (ACN) or by the contact with the RP-18 surface [22,28].

In the present paper, we applied non-denaturing separation techniques as SEC and ion-exchange chromatography (IEX) in a HPLC–BCD system for the first time to screen for enzymatic activities. In combination with the substrate identification by an improved ESI-MS/MS-based multi-substrate assay (see above), a fundamentally new methodology to screen for proteases in complex mixtures is described. The developed method was thoroughly characterized and optimized with the model proteases trypsin and chymotrypsin. Subsequently, it was applied to analyze the proteases of the venom of the pit viper *Bothrops moojeni* and the extracellular enzymes of the pathogenic amoeba *Acanthamoeba castellanii*.

2. Experimental

2.1. Chemicals

The protease substrates pefachrome TH, FIXa (IX), TRY (TR), FXa (X), FVIIa (VI), PCa (PC), C1E (C1) as well as freeze-dried venom of *B. moojeni* were a kind gift of Pentapharm (Basel, Switzerland). Pefaflour TH (TF) was purchased from Loxo (Dossenheim, Germany). The sequence of the peptides and their chemical structures are displayed in Fig. 1. Trypsin from hog (11,909 U/mg), bovine α-chymotrypsin (59 U/mg), ribonuclease A [isoelectric point (p*I*) 9.5], formic acid (FA), potassium hydrogenphosphate, potassium dihydrogenphosphate and ammonium acetate were purchased from Fluka Chemie (Buchs, Switzerland). Acetonitrile (ACN) and methanol (MeOH) in gradient grade quality were obtained from Merck (Darmstadt, Germany). Bovine thrombin (43 U/mg), myoglobin (*pI* 7.0), α-chymotrypsinogen (*pI* 9.5), cytochrome *c* (*pI* 10.45), lysozyme (*pI* 11), bovine serum albumin (BSA, 66 kDa), ovalbumin (43 kDa), 4-amino benzoic acid (ABA), trypsin inhibitor from soybean type I-S (20 kDa), ammonium formate and all other chemicals were obtained from Sigma Aldrich Chemie (Steinheim, Germany). Purified water for HPLC analysis and sample dilution was generated by a Milli-Q Gradient A 10 system and filtered through a 0.22 μm Millipak 40 filter unit (Millipore, Billerica, MA, USA). All chemicals were of the highest quality available unless noted otherwise.

2.2. Preparation of amoeba supernatant

A. castellanii was cultivated axenically at 17 °C in PGY medium (peptone 20 g/L, yeast extract 5 g/L, glucose 10 g/L) as described by Jousset et al. [29]. Stationary phase cultures (2 weeks old) were collected by centrifugation (400 × *g*, 1 min) and resuspended in 50 mM Tris buffer. After 1 week, the cultures were pelleted (17,000 × *g*, 1 min), the supernatant filtered through a 0.2 μm membrane (Millipore, Schwalbach, Germany) and fractionated by ultrafiltration through a Vivaspin 20, 3000 MWCO (Sartorius, Göttingen, Germany). The concentrated fraction (molecular weight >3 kDa) was diluted in 1 mL of 50 mM Tris buffer and directly used for flow-injection analysis (FIA)–ESI-MS/MS and HPLC–BCD analysis.

2.3. Multi-substrate assay

In order to characterize the substrate specificity, 50 μL of enzyme containing solution was mixed with 950 μL substrate solution containing eight substrates (Fig. 1, Table 1) with a concentration of 50 μM each. Both solutions were prepared in 100 mM ammonium acetate buffer pH 7.4. The mixture was incubated at room temperature up to 200 min and aliquots of 5 μL were analyzed by FIA–ESI-MS/MS at least every 3.5 min. For the proteases trypsin, α-chymotrypsin and thrombin, an enzyme concentration of 100 U/mL was used and *B. moojeni* venom was analyzed at a concentration of 0.2 mg/mL. As the amoeba supernatant exhibited a lower conversion activity, 50 μL was incubated with 450 μL multi-substrate solution.

2.4. FIA–ESI-MS/MS analyses

FIA–ESI-MS/MS analyses were carried out on an API 2000 tandem mass spectrometer (Applied Biosystems, Darmstadt, Germany) equipped with a turbo ionspray source (pneumatically assisted ESI) operating in positive ion mode. The $[M+H]^+$ ions of the analytes were formed in the interface with an ionspray voltage of 5500 V, 50 psi nebulizer gas and 80 psi drying gas with a temperature of 400 °C. For each substrate and product (formed by complete conversion of substrate solution by overnight incubation with trypsin) the parameters like declustering potential (DP), entrance potential (EP) and focussing potential were optimized. The collision energy (CE) and the collision cell exit potential (CXP) were optimized for the five most abundant fragment ions, whereas the most abundant one was used for quantification. The *m*/*z* of the analyzed $[M+H]^+$ ions, MS/MS transitions and all optimized MS parameters are provided in Table 1.

Aliquots of the incubation mixture were injected by an SIL-HT-A autosampler (Shimadzu, Duisburg, Germany) into a 300 μL/min solvent stream of ACN/water (80/20 (v/v)) containing 0.1% FA deliv-