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Coupling of size-exclusion chromatography to a continuous assay for Subtilisin using a fluorescence resonance energy transfer peptide substrate: Testing of two standard inhibitors

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Abstract

Liquid chromatography (LC) was coupled on-line to a homogeneous continuous-flow protease assay using fluorescence resonance energy transfer (FRET) as a readout for the screening of inhibitors of an enzyme (e.g., Subtilisin Carlsberg). The inhibitors aprotinin (a protein of ~6500 g/mol) and 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF, 240 g/mol) were mixed with other, non-active compounds and separated on a size-exclusion chromatography column. After the separation, the analytes were eluted to the postcolumn reactor unit where the enzyme solution and subsequently the FRET peptide substrate were added; by measuring the fluorescence intensity the degree of inhibition was monitored on-line. As expected, only the two inhibitors caused a change in the FRET response. Detection limits for aprotinin were 5.8 μM in the flow injection analysis (FIA) mode and 12 μM in the on-line LC mode. System validation was performed by determining IC_{50} values for aprotinin for the FIA mode (19 μM) and the on-line mode (22 μM). These IC_{50} values were in line with the value determined in batch experiments (25 μM). With this system, chemical information (i.e., chromatographic retention time) and biological information (i.e., enzyme inhibition) can be combined to characterize mixtures.

Keywords: Flow injection analysis; FIA; FRET; HIV protease substrate 1; Continuous flow; Bioassay; HTS

1. Introduction

The screening of synthetic and natural chemical sources is the starting point in drug discovery, the main goal being the identification of promising pharmacologically active compounds. High-throughput screening (HTS) technologies have been recently developed and implemented that are able to test tens of thousands of compounds or more per day for their activity in various assay types, ranging from receptor binding and enzyme inhibition to whole-cell assays.

In recent years microtitreplate (MTP) screening technologies are used to perform HTS [1,2]. A large variety of assay formats has been described, with fluorescence in various modes currently being the most important detection technique [3–6]. The most obvious advantage of using MTP screening technology with fluorescence detection is that it allows performing homogeneous assays, i.e., assays where no separation between free and bound reporter molecule is required [7,8].

These days, however, one frequently deals with complex samples composed of natural products or a wide variety of chemicals. To screen such complex samples, fractionation procedures need to be involved, in most cases performed prior to screening or after primary screening [9,10]. This is a major bottleneck as regards efficiency.

In a previous paper we have described a methodology where HTS assays are carried out in a flow injection analysis (FIA) setup, using fluorescence resonance energy transfer (FRET) as a read-out method [11]. The feasibility of a homogeneous, continuous-flow system coupled to FRET was demonstrated using human immunodeficiency virus (HIV)

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protease substrate 1, a protease (Subtilisin Carlsberg) and two inhibitors as a model biological system.

In the present project, the above protease assay is coupled to liquid chromatography (LC) with the goal to establish an analytical screening method for protein inhibitors in complex mixtures. Unlike the small organic molecules which are normally screened in HTS, the screening of proteins is mainly used for biological purpose, e.g., the discovery of biomarkers or proteins involved directly in disease processes. From an assay development point of view, it is particularly interesting to compare the performance of continuous-flow protein assays with MTP type batch assays. Several protein–ligand binding assays were described [12,13], demonstrating the feasibility of measuring protein–receptor or protein–antibody interactions in a continuous-flow system. In the present paper, we investigate the interaction of an inhibitor protein, aprotinin, with HIV protease in a FRET-based assay coupled on-line to size-exclusion chromatography. Particular emphasis is given to the comparison of IC_{50} values between flow and batch assays in order to assess kinetic effects, e.g., slower diffusion, on assay performance.

2. Experimental

2.1. Chemicals

HIV protease substrate 1, a small peptide to which two chromophores, 5-[(2-aminoethyl)amino]naphthalene-1-sulfonic acid (EDANS) and 4-(4-dimethylaminophenylazo)benzoic acid (DABCYL) are covalently attached (see Fig. 1), was purchased from Molecular Probes Europe (Leiden, The Netherlands). Subtilisin Carlsberg, type VIII protease from *Bacillus licheniformis* (EC 3.4.21.62), polyoxyethylenesorbitan monolaurate (Tween 20), aprotinin (inhibitor), bovine serum albumin (BSA) and 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF, inhibitor), were purchased from Sigma–Aldrich (Zwijndrecht, The Netherlands). Methionine enkephalin (m-enkephalin) was purchased from Bachem (St. Helens, UK). Dimethyl sulfoxide (DMSO), spectrophotometric grade 99%, was obtained from Acros Organics (Geel, Belgium). Sodium dihydrogenphosphate monohydrate and disodium hydrogenphosphate were obtained from Merck (Darmstadt, Germany), sodium chloride was from Fluka (Zwijndrecht, The Netherlands). All aqueous solutions were prepared with water purified with a Milli-Q system from Millipore (Bedford, MA, USA).

2.2. Preparation of samples

The mobile phase consisted of 50 mM phosphate buffer (pH 7.5) and 0.4 M sodium chloride, while the assay buffer (batch experiments and post-column additions) contained 50 mM phosphate buffer (pH 7.5) and 0.05% Tween 20. The solutions of aprotinin, AEBSF, BSA and m-enkephalin were dissolved in the mobile phase and prepared prior to use. HIV protease substrate 1 was stored in DMSO at a concentration of 500 μM and was diluted prior to use with the assay buffer to the desired concentration. The enzyme solution in the assay buffer was freshly prepared every day. During all experiments, the substrate was used at a final concentration of 1 μM and the enzyme at 1 μg/ml. In the continuous-flow system, the concentrations of the substrate and the enzyme solutions (before mixing) were four times higher.

2.3. Batch experiments

An HIV protease substrate 1 solution was prepared in an assay buffer (50 mM phosphate buffer; pH 7.5). The fluorescence emission spectrum (excitation wavelength: 340 nm) of the substrate solution without enzyme was recorded in a 1 cm quartz cuvette on a Perkin-Elmer Luminescence Spectrometer LS-50B (Perkin-Elmer, Beaconsfield, UK), thermostated

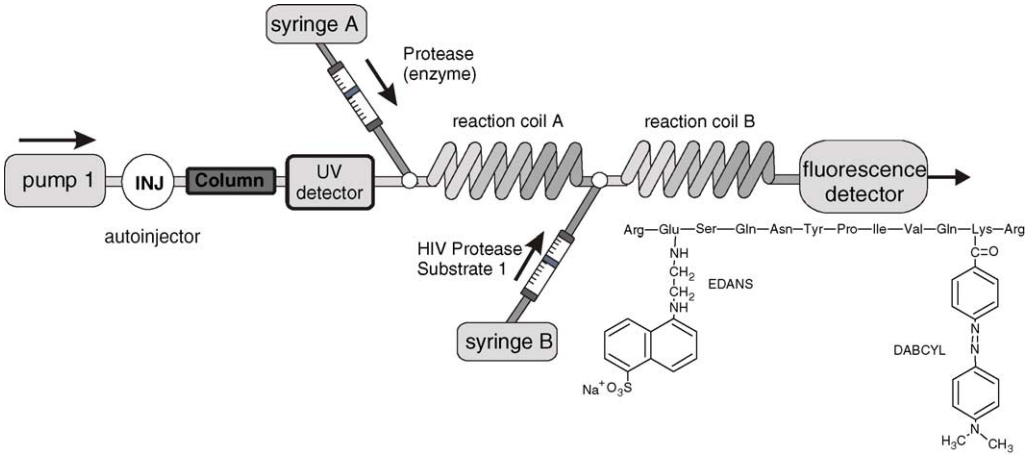

Fig. 1. Schematic to illustrate the continuous-flow bioassay system coupled to LC. The SEC separation of potential inhibitors is carried out with a flow rate of 100 μl/min. For the FRET assay, enzyme and substrate are added with a flow rate of 50 μl/min each. The residence times in reaction coils A and B are 1 and 2 min, respectively. For further details, see text. The chemical structure of doubly labeled HIV protease substrate 1 is also shown.

at 60 °C. After preparing the enzyme solution in the same assay buffer, the substrate solution and the enzyme solution were incubated for 1 min at room temperature and subsequently transferred to the cuvette. Intensity changes in fluorescence emission were recorded at distinct times up to 60 min.

For the inhibitor assay, the aprotinin solution (final concentration 50 μM unless specified otherwise) was prepared in the same buffer and first incubated with the enzyme solution for 1 min at room temperature. After that, the substrate solution was added to the mixture and again incubated for 1 min. Changes in fluorescence intensity over time were recorded as described above. For the IC_{50} measurements the same conditions were used, with a reaction time of 2 min. Data acquisition was performed by the software package FL Winlab version 3.00 running under Windows 2000.

2.4. On-line LC-FRET setup

In Fig. 1, a schematic of the continuous-flow bioassay system coupled to LC is depicted. This on-line system consisted of three major parts; a sample introduction part, a separation part and a homogeneous bioassay part. In the sample introduction part, the carrier buffer was delivered at a rate of 100 μl/min by pump 1 (Knauer HPLC pump K-500; Knauer, Berlin, Germany). Injection took place (injection volume 20 μl) by a Gilson (Villiers-le-Bel, France) autoinjector 234 equipped with a Rheodyne (Cotati, CA, USA) six-port injection valve. Injections were performed in triplicate, unless stated otherwise.

In the second part, separation was performed by a size-exclusion guard column (Biosep S-2000, dimension 30 mm × 4.6 mm; particle size 5 μm; pore size 145 Å: Phenomenex, Cheshire, UK) with subsequent detection by a 759A absorbance detector (Applied Biosystems, Foster City, USA) at 280 nm.

After UV detection, the analytes entered the homogeneous bioassay part of the system which consisted of a single syringe pump PHD 2000 (Harvard Apparatus Inc., Holliston, MA, USA) with two syringes (syringes A and B). Syringe A was connected to 10 ml Superloop (Pharmacia Biotech, Uppsala, Sweden) to deliver the enzyme solution (4 μg/ml) to the system at a rate of 50 μl/min. Superloops prevent nonspecific binding of compounds to pump surfaces and reduce the consumption of expensive chemicals as the necessary amount of solvent is reduced. In the polytetrafluoroethylene (PTFE)-knitted reaction coil A (volume 150 μl, 250 μm I.D., reaction time 1 min) this flow was mixed with the LC effluent. Then, HIV protease substrate 1 solution was added at 50 μl/min by a second Superloop connected to syringe B. The final mixture was incubated in reaction coil B (volume 400 μl, 250 μm I.D., reaction time 2 min). Both reaction coils A and B were knitted in order to reduce sample dispersion [14] and were immersed in a thermostated waterbath at 60 °C. All other components were kept at room temperature. Finally the fluorescence of the reaction mixtures was measured by a JASCO (Tokyo, Japan) fluorescence detector FP-920 (excitation wavelength, 340 nm; emission wavelength, 490 nm) provided with a 16 μl flow cell.

A separate series of experiments was carried out in FIA mode, in which the LC column was removed but otherwise the same setup and flow rates were used.

3. Results and discussion

As can be seen in Fig. 1, the HIV protease substrate 1 is a small peptide, doubly labeled with a donor fluorophore EDANS and a non-fluorescent acceptor DABCYL. Since the fluorescence emission spectrum of EDANS shows significant overlap with the absorption spectrum of DABCYL, and the average distance between the two chromophores is relatively small, intramolecular FRET is quite efficient and the reduction in EDANS emission intensity due to the presence of DABCYL is substantial. During the incubation of the substrate with the protease enzyme solution, hydrolysis of the peptide chain leads to an increase of the fluorescence intensity: as the EDANS moiety diffuses away from the DABCYL part, FRET quenching is no longer efficient [15,16].

Before developing the on-line assay, batch experiments had to be carried out in order to investigate the kinetics of the substrate–enzyme reaction in absence and presence of an inhibitor. In a continuous-flow assay the reaction time is limited in order to avoid extra band broadening. On the other hand, if the residence time is too short, the signal from the partially hydrolyzed substrate would not be sufficiently strong. In Fig. 2, the change of the fluorescence intensity at 490 nm (the maximum of the EDANS emission) during hydrolysis is shown in absence and presence of the inhibitor aprotinin. It is obvious that in the absence of aprotinin the fluorescence intensity increases dramatically over time especially during the first minutes. However, in the presence of

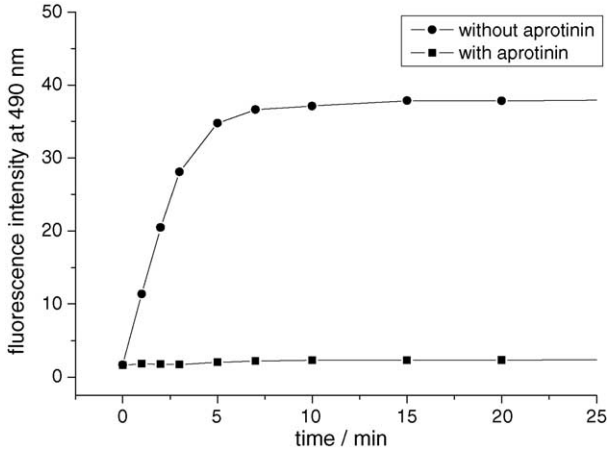

Fig. 2. The fluorescence intensity changes of HIV protease substrate 1 (batch experiment) in the absence and presence of aprotinin (50 μM) as an inhibitor. The solution contained 1 μM substrate, 1 μg/ml enzyme in an assay buffer. Temperature 60 °C.

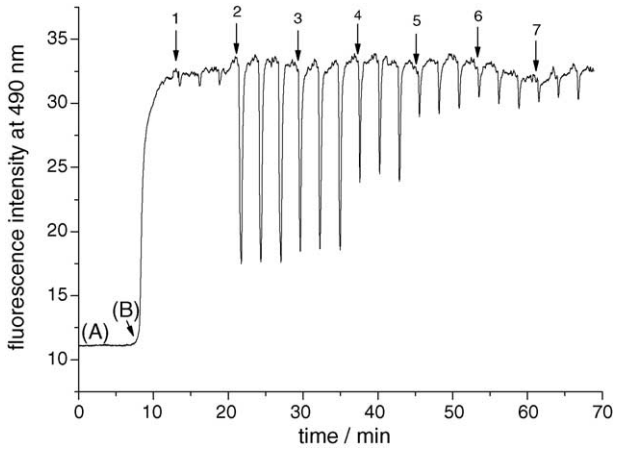

Fig. 3. The flow injection profile. Elution of (A) solely carrier buffer solution ([phosphate] = 50 mM, [Tween 20] = 0.05%, pH 7.5); (B) HIV protease substrate 1 (1 μM) and the enzyme (1 μg/ml); triplicate injections of (1) blank solution (carrier buffer) or aprotinin, (2) 200 μM, (3) 100 μM, (4) 50 μM, (5) 25 μM, (6) 12.5 μM and (7) 6.25 μM (injected concentrations).

inhibitor at the 50 μM level, only a slow increase is observed, indicating that hydrolysis hardly takes place. In the absence of inhibitors a sufficiently strong signal is obtained after some 2 min incubation time; such a time scale is compatible with a continuous-flow bioassay system. Furthermore, in this time window the signal intensity is sensitive to the presence of an inhibitor since in 2 min the reaction has not yet reached its plateau value. Therefore, we decided to use 2 min incubation time for performing the continuous-flow bioassay.

Fig. 3 illustrates the operation of the bioassay in FIA mode with aprotinin as the inhibitor. The final concentrations of substrate and enzyme are the same as in the batch experiment. At point (A), solely carrier buffer solution is provided by pump 1, resulting in a stable, low baseline. At point (B), both the enzyme solution and the substrate solution are added to the flow system, leading to an increase of the fluorescence intensity due to the cleavage of the substrate by the enzyme. After stabilization of the system, the blank (same assay buffer) was injected three times (point 1) to check for possible memory effects from previous experiments. Then, six different concentrations of aprotinin were injected, three times each, by the autosampler (points 2 through 7). There is an obvious relationship between the extent of fluorescence decrease and the aprotinin concentrations. A LOD of 5.8 μM was obtained (signal = three times standard deviation of the blank). The other analytical parameters will be discussed below.

A main requirement for the successful on-line coupling of the continuous-flow bioassay to LC is the solvent compatibility. For example, the pH cannot be freely chosen to optimize the separation conditions since it must also be compatible with the enzyme reaction. Therefore, a compromise should be found. This also holds for the use of organic modifiers, which are often used in reversed-phase LC, whereas in general bioassays cannot tolerate the presence of high amounts

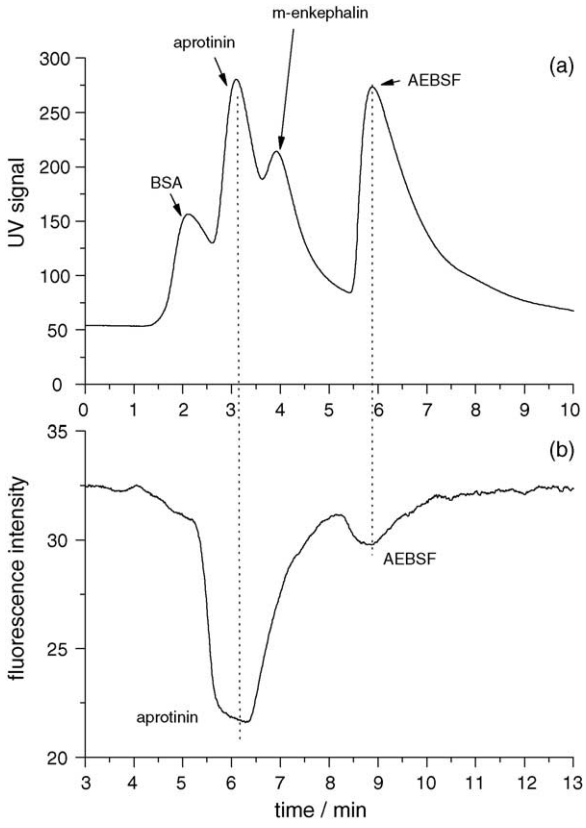

Fig. 4. On-line LC-FRET experiment of a mixture of two inhibitors and two non-active compounds. (a) UV–vis absorption chromatogram (λ = 280 nm) of the mixture (4 mM of AEBSF, 250 μM of aprotinin, 15 μM of BSA and 400 μM m-enkephalin). (b) FRET profile of the same separation, showing the strong inhibition by aprotinin and the weaker inhibition by AEBSF. For ease of comparison, the time axis of the FRET detector was shifted by 3 min to account for the residence time in reaction coils A and B.

of such organic modifiers. That is why for the present paper prior to performing the on-line coupling to the continuous-flow bioassay, the separation as such was studied (data not shown) to find such bio-compatible conditions, using a standard test mixture of four analytes. The mixture contained two Subtilisin Carlsberg inhibitors, namely aprotinin and AEBSF [17–19], and two non-active compounds, i.e., BSA and m-enkephalin. Since there are large differences in their molecular weight (BSA ∼67 000; aprotinin ∼6500; m-enkephalin 631; AEBSF 240 g/mol), a size-exclusion column was selected; the eluent was aqueous phosphate buffer and sodium chloride at pH 7.5, appropriate conditions for the enzyme concerned. As expected, BSA, the largest molecule in the test sample eluted first with a retention time of 2.1 min, followed by aprotinin, m-enkephalin and AEBSF with retention times of 3.1, 3.9 and 5.9 min, respectively.

Finally, the LC separation was coupled to the bioassay system in the configuration depicted in Fig. 1 with the UV detector and FRET detection system in series. Fig. 4(a) shows the LC chromatogram obtained by UV detection and Fig. 4(b) the FRET profile of the on-line protease assay. In order to compare the chromatogram and FRET signals, the time axis of the

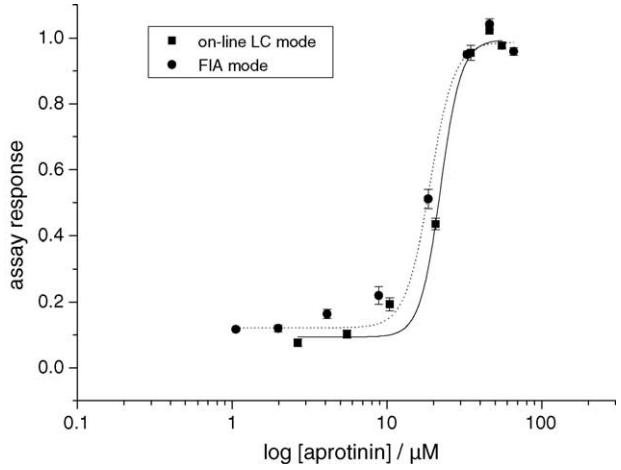

Fig. 5. Effect of varying the concentration of aprotinin inhibitor on the substrate–enzyme reaction in FIA mode and in on-line LC mode. The assay response is given as the peak height relative to the maximum peak height observed, as a function of the final inhibitor concentration in the reaction mixture. Conditions are given in Section 2. The data points were fitted with a sigmoidal curve. The error bars represent the standard deviation ($n = 3$).

latter was shifted by 3 min. Since BSA and m-enkephalin are not inhibitors for the enzyme, they did not give any significant response in the FRET profile. Two compounds, aprotinin and AEBSF, inhibited the activity of the enzyme, resulting in a negative peak in the FRET trace. The peak heights depend on the concentration of the inhibitor and its binding affinity. Even though the analytes were not baseline separated, the FRET assay still indicated which compounds are active as inhibitors.

In order to validate the on-line LC/bioassay system, quantitation parameters were determined using aprotinin as an inhibitor. First of all, IC_{50} values were determined in both the FIA and the on-line mode. The negative peak heights in the FRET profile (relative to the maximum peak height observed) were plotted against the final concentration of the inhibitor aprotinin, as shown in Fig. 5. The inhibitor concentration in the final mixture was calculated from the injected concentrations and the observed peak widths in the FIA and LC chromatograms; the latter were a factor of 1.8 broader due to the SEC column. The IC_{50} value found was $22.3 \pm 0.4\ \mu M$ when measured in on-line LC mode and $18.9 \pm 0.5\ \mu M$ when measured in FIA mode. These values agree very well with that obtained in batch experiments, $25 \pm 2\ \mu M$. A LOD of $12\ \mu M$ (injected concentration) was obtained (signal = three times standard deviation of the blank). This is also consistent with the LOD of the FIA system ($5.8\ \mu M$ injected). Due to the bandbroadening in the SEC column the analyte is diluted and therefore approximately two times higher concentrations are required in order to obtain the same extent of inhibition in the on-line LC/bioassay. Of course one should realize that in case the enzyme concentration would be too high in the on-line system (without adjusting the reaction coil residence time) the reaction would reach a plateau value at the detection window. In that case inhibition effects would be smaller than in Fig. 5.

4. Conclusions

We have developed an analytical on-line LC/bioassay system with which both chemical and biological information can be obtained simultaneously. Proteins are separated using a size-exclusion column and subsequently the homogeneous assay enables the on-line distinction between active inhibitors and non-active compounds, based on FRET detection. A comparison of IC_{50} values between continuous-flow and batch assays reveals that protein inhibitors can be screened with a similar assay sensitivity compared to small molecule inhibitors.

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