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Human neutrophil elastase inhibition studied by capillary electrophoresis with laser induced fluorescence detection and microscale thermophoresis☆

Fayad Syntia, Reine Nehmé*, Bérengère Claude, Philippe Morin

Institut de Chimie Organique et Analytique, Université d'Orléans, CNRS FR 2708, UMR 7311, Orléans, France

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ABSTRACT

Capillary electrophoresis-laser induced fluorescence (CZE-LIF) and microscale thermophoresis (MST) were used for the first time to study the inhibition of human neutrophil elastase (HNE). We recently studied HNE kinetics (K_m and V_{max}) by developing an in-capillary CZE-LIF assay based on transverse diffusion of laminar flow profiles (TDLFP) for reactant mixing. In this work, the former assay was adapted to monitor HNE inhibition. Two natural well known HNE inhibitors from the triterpene family, ursolic acid and oleanolic acid, were tested to validate the developed assay. Since the solubility of pentacyclic triterpenes in aqueous media where the enzymatic reaction will take place is limited, the effect of DMSO and ethanol on HNE was studied using microscale thermophoresis (MST). An agglomeration of the enzyme was revealed when preparing the inhibitor in 5% (v/v) DMSO. This phenomenon did not occur in the presence of ethanol. Therefore, ethanol was used as inhibitor solvent, at a limited percentage of 20% (v/v). In these conditions and after optimization of the TDLFP approach, the repeatability (RSD on migration times and peak-areas inferior to 2.2%) of the CZE-LIF assay and the sensitivity (LOQ of few nM) were found to be satisfactory for conducting inhibition assays. IC_{50} values for ursolic and oleanolic acid were successfully determined. They were respectively equal to 5.62 ± 0.10 μM ($r^2 = 0.9807$; $n = 3$) and to 8.21 ± 0.23 μM ($r^2 = 0.9887$; $n = 3$). Excellent agreement was found between the results obtained by CE and those reported in literature which validates the developed method. Particularly, the CE-based assay is able to rank HNE inhibitors relative to each other.

Furthermore, MST technique was used for evaluating HNE interaction with the ursolic acid. Up to 16 capillaries were automatically processed to obtain in one titration experiment the dissociation constant for the HNE-ursolic acid complex. K_i was found to be 2.72 ± 0.66 μM ($n = 3$) which is in excellent agreement with the value determined by CE enzyme inhibition studies ($K_i = 2.81$ μM) confirming the reliability of the developed CE assay and the competitive inhibition mode of ursolic acid.

1. Introduction

Neutrophils are the body's first line of antibacterial defense. They degrade microorganisms using an array of weapons that include antimicrobial peptides and proteases such as neutrophil elastase [1,2]. The rapid interaction of these enzymes with their substrates or inhibitors makes difficult the measurement of their activity in blood or body fluids [3]. Particularly, human neutrophil elastase (HNE) has been the subject of intensive studies because of its ability to destroy tissues. This potent proteolytic enzyme is capable of degrading or activating various proteins, including extracellular matrix proteins, receptors and cytokines [1,4,5]. HNE is involved in skin aging and lung inflammatory diseases [6–10]. Therefore, its inhibition can be a promising approach to prevent premature skin aging and many other diseases. Natural inhibitors like pentacyclic triterpenes attract more attention compared to chemically synthesized compounds due to reduced side effects. Feng et al. [11] determined the IC_{50} of several triterpene acids by following the enzymatic hydrolysis of the substrate MeO-Suc-Ala-Ala-Pro-Val-pNA at 405 nm because of the release of 4-nitroaniline.

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* Corresponding author at: Institut de Chimie Organique et Analytique (ICOA), Université d'Orléans, BP 6759, Rue de Chartres, 45067 Orléans Cedex 2, France. Fax: +33 2 38 41 72 81.
E-mail address: reine.nehme@univ-orleans.fr (R. Nehmé).

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Ursolic acid and oleanolic acid were shown to inhibit HNE with the highest potency. These triterpenoid compounds exist widely in medicinal herbs and other plants, and have been extensively used in cosmetics and health products. They have many important pharmacological activities including anticancer, antioxidant and anti-inflammatory effects; their similar activities are due to their similar chemical structures [12,13]. The inhibition of HNE by pentacyclic triterpenes was found to be purely competitive with tripeptide or tetrapeptide substrates and non competitive with a shorter dipeptide substrate [12]. Alongside with spectrophotometry [11,14], surface plasmon resonance (SPR) [15] was also used to detect the effect of inhibitors on elastase activity or on its release from neutrophils. Nowadays, miniaturizing enzymatic assays is essential due to the high cost of reactants and/or to their limited quantities. Therefore, capillary electrophoresis (CE) is being widely used for assaying enzymatic activity and for the determination of different kinetic constants (K_m, V_{max} and IC_{50}) [16,17]. In this case, laser induced fluorescence (LIF) detection is particularly advantageous due to its high sensitivity [18]. CE-based assays can be categorized into offline or pre-capillary assays and online or in-capillary assays [19–21]. Considering offline assays, the enzymatic reaction takes place in a vial and few nanoliters of the reaction mixture are injected into the capillary. The product formed is then separated, detected and quantified. In the second case, the enzymatic reaction takes place directly in the CE capillary which acts as an enzymatic nanoreactor. In this case, the incubation and the analysis can be performed by transverse diffusion of laminar flow profiles (TDLFP) or *via* electrophoretic phenomenon, referred thus as electrophoretically mediated microanalysis (EMMA). A complementary technique named microscale thermophoresis or MST was developed in early 2008 to quantify biomolecular interactions in free solution (see review [22] and references therein). MST is based on the unique physical principle of thermophoresis which is the directed motion of molecules in temperature gradients [23–27]. Under constant buffer conditions, thermophoresis probes the size, charge and hydration shell or conformation (solvation entropy) of the molecules. Upon binding of a ligand to a target *e.g.*, an inhibitor to an enzyme, at least one of these parameter changes and thus the binding constant can be quantified by measuring the change in the MST signal. An infrared laser is used for local heating. The infrared radiation is absorbed by the sample solvent inside a glass capillary to create a temperature gradient. Molecule mobility in the induced temperature gradient is analyzed via fluorescence detection. In standard MST, one binding partner is fluorescently labeled and the visible light is used for fluorescence excitation.

In a previous work [28], we studied HNE activity by developing an online capillary zone electrophoresis (CZE) assay with LIF detection. Two novel HNE substrates labeled with the fluorogenic compound 5-carboxyfluorescein (5-FAM) were introduced. The corresponding Michaelis-Menten constant (K_m) was successfully determined and showed a good affinity of these substrates towards HNE. This assay has been adapted in this work and used for assaying HNE inhibition. The ursolic acid and the oleanolic acid were chosen for the development of the inhibition assay. Pentacyclic triterpenes are soluble in organic solvents such as ethanol and DMSO that may affect the activity and/or the affinity of HNE. This potential influence of the solvent (nature and proportion) on HNE was evaluated using CE and MST. In the optimal conditions, the half maximal inhibitory concentration (IC_{50}) and the inhibition dissociation constant of the enzyme-inhibitor complex (K_i) were evaluated by taking advantage of the complementarily of CE and MST. Results were cross-compared and also compared when available to those reported in literature to validate the developed CE assay.

2. Materials and methods

2.1. Chemicals

All reagents were of analytical grade and used as received without further purification. HEPES (4-(2-Hydroxyrthyl)) piperazine-1-ethanulfonic acid (purity ≥ 99.5%), oleanolic acid (purity ≥ 99.5%), sodium acetate (CH_3COONa, purity ≥ 99%), sodium chloride (NaCl, purity ≥ 99%), sodium hydroxide (NaOH, purity ≥ 98%) and sodium tetraborate decahydrate ($B_4Na_2O_7{\cdot}10H_2O$, purity ≥ 99.5%) were purchased from Sigma–Aldrich (Saint-Quentin Fallavier, France). Human neutrophil elastase (HNE, 29.5 kDa, pI of 8.8–9.6, specific activity ≥22.0 U mg^{-1}) was purchased from Merck Millipore (Molsheim, France). 5-FAM-Arg-Glu-Ala-Val-Val-Tyr-OH and 5-FAM-Arg-Glu-Ala-Val-OH were purchased from Genscript (Piscataway, USA). Ursolic acid (purity ≥ 99.5%) was provided from Extrasynthese (Genay, France). Dimethyl sulfoxide (DMSO) was purchased from Fluka (Saint-Quentin-Fallavier, France). Hydrochloric acid (HCl) and ethanol (EtOH) HPLC grade were purchased from VWR International (Fontenay-sous-Bois, France). Ultra-pure water (18 MΩ cm) was produced from an Elga apparatus (Elga, Villeurbanne, France). Syringes and hydrophilic polyvinylidenedifluoride (PVDF) Millex-HV Syringe Filters, pore size 0.45 μm, were purchased from Millipore (Molsheim, France). Fluorescent label (NT-647) and Tween-20 were purchased from NanoTemper Technologies GmbH (Munich, Germany).

2.2. Solutions

Solutions were filtered through 0.45 μm PVDF Millex-HV Syringe Filter before use and stored at 4 °C. The pH of the buffers was measured with a MeterLab PHM201 Portable pH-Meter (Radiometer Analytical, Villeurbanne, France) and adjusted if necessary. The different buffers were prepared fresh each day and their parameters were given by PHoEBuS software (Analis, Namur, Belgium). The parameters given by PHoEBuS software are the chemical characteristics (pH, ionic composition, buffer capacity), the electrical characteristics (conductivity, estimation of the current) and the electrophoretic characteristics (mean electrophoretic mobilities of the co-ion and the counter ion).

2.2.1. Incubation buffer (IB)

The IB was prepared by dissolving 25 mM HEPES and 20 mM of NaCl in ultra-pure water. The buffer pH was fixed at 7.5 using 10 mM NaOH.

2.2.2. Background electrolyte (BGE)

50 mM tetraborate buffer pH 8.7 adjusted with 1 M HCl.

2.2.3. Microscale thermophoresis buffer (MST buffer)

For thermophoresis measurements, the IB presented above containing Tween20 at 0.05% (v/v) was used for reactant preparation. It was referenced as MST buffer in this study.

2.2.4. Substrate (S), product (P) and inhibitor (I) solutions

Stock solutions of the substrate 5-FAM-Arg-Glu-Ala-Val-Val-Tyr-OH (1 mM) and of the product 5-FAM-Arg-Glu-Ala-Val-OH (1 mM) were prepared weekly in the IB and stored at −20 °C. The tested inhibitors were ursolic acid and oleanolic acid. They differ only in the position of one methyl group in their chemical structure (Table 1). Two organic solvents, DMSO and EtOH, were used to prepare inhibitor stock solutions (1 mM). These solutions were stored at 4 °C until use. These solutions were diluted using IB to concentrations ranging from 1.25 to 70 μM. The reaction rate was determined with inhibitor concentrations ranging from 1.25 to 70 μM. The