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Assaying human neutrophil elastase activity by capillary zone electrophoresis combined with laser-induced fluorescence

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ABSTRACT

Skin aging is a progressive process determining the ultimate skin appearance. Human neutrophil elastase (HNE) has been shown to play an important role in the degradation of the extracellular matrix. In order to assay HNE kinetics, a novel online capillary zone electrophoresis (CZE) assay has been developed in this study for the determination of the maximum velocity (V_{max}) and of the Michaelis–Menten constant (K_m) of HNE regarding several potential substrates. These assays are based on short-end injection to shorten analysis time, on transverse diffusion of laminar flow profiles (TDLFP) for in-capillary reactant mixing, and on UV or laser-induced fluorescence (LIF) detection. Kinetic constants for a referenced peptidic substrate were determined using not only online assays but also offline (pre-capillary) mode. The results obtained were cross compared and compared to the literature in order to validate the developed assays. The hydrolysis of three new potential fluorogenic substrates by HNE was also monitored. Two new peptidic substrates for HNE were identified through this study. K_m values of these novel substrates were successfully determined using online CZE assay (K_m ~0.07 mM). This value was in the same order of magnitude of that of the referenced substrate despite the presence of the labeling group 5-carboxyfluorescein (5-FAM). HNE activity has never been assessed using online CZE-based assay, neither with UV nor with LIF detection. The developed assay conducted with the new labeled substrates is particularly sensitive (LOQ of few nM), does not require the presence of micelles in the BGE (which is the case for the reference substrate) and only necessitates few nanoliters of reactants making it particularly adapted for screening studies.

1. Introduction

Skin aging is influenced by several factors [1–3], particularly by solar UV radiation, a process known as photoaging [4]. The deleterious effects of UV are both direct and indirect. The indirect effects are due to the production of reactive oxygen species that may cause; cellular, extracellular and membrane structure damages; increasing release of pro-inflammatory mediators from a variety of skin cells (keratinocytes and fibroblasts); and induction of an inflammatory infiltrate. This infiltrate contains, in order of entrance in dermis, neutrophilic granulocytes, macrophages and T-lymfocytes (see [5,6] and references therein). Skin-infiltrating neutrophils are the major source of photoaging-associated production of neutrophil elastase, a serine protease, and matrix metallo proteinases (MMPs) [6–9]. Human neutrophil elastase (HNE) has broad substrate specificity and can cleave the peptide or amide linkage not only in elastin but also in other extracellular matrix (ECM) proteins, such as collagen, fibronectin and proteoglycan [10,11]. Apart from skin aging, HNE is also involved in the development of chronic obstructive pulmonary diseases and in non small-cell lung cancer progression [12,13]. Therefore, it is necessary to develop simple, efficient and robust methods to study its activity in cosmetic as well as in therapeutic fields. Recently, capillary electrophoresis (CE) has been shown to be a useful and interesting approach for studying enzyme catalyzed reactions due to the low consumption of samples and reagents, fast analysis time and ability to use several detectors. The CE assays can be divided into two general categories: pre-capillary (or offline) assays and in-capillary (or online) assays [14]. In pre-capillary assays, the incubation is performed offline and the reaction mixture is subsequently analyzed by CE for quantification. With in-capillary assays, enzyme reaction and separation of the analytes are performed inside the same capillary. The latter is thus used as an enzyme-nanoreactor and separation tool. This has the advantage of reducing the amounts of reagents to few tens of nanoliters. Two main methods have been used so far for mixing solutions inside a capillary. The first method relies on differences in electrophoretic mobilities of the

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solutes so it is referred to as electrophoretically mediated microanalysis (EMMA). The analyte injected first is the one with the lower electrophoretic mobility. After the application of an electric field, the plugs of enzyme and substrate interpenetrate due to the differences in their apparent mobilities [15,16]. Enzyme reaction is thus triggered. The resultant reaction products as well as the unreacted substrate are electrophoretically transported toward the detector, where they are individually detected. Van Schepdael et al. [17] developed the partial filling mode of EMMA by injecting a plug of incubation buffer to separate the reactant plugs from the background electrolyte (BGE). The second method for in-capillary mixing has been developed in 2005 by Krylov et al. [18]. It is based on the transverse diffusion of laminar flow profiles (TDLFP). The solutions of reactants are injected inside the capillary by pressure as series of consecutive plugs. Due to the laminar nature of flow inside a capillary, the plugs have parabolic profiles with predominantly longitudinal interfaces between the plugs. After injection, the reactants are mixed by transverse diffusion [19,20]. As a consequence, dilution occurs during reactant mixing; however there are no defined dilution coefficients as the solute profiles are not uniform [20]. To characterize the quality of micromixing in a capillary, a general quantitative measure was successfully developed by Krylov et al. [21]. They introduced a predictive measure of micromixing termed "quantitative overlap" that depends on the distribution of reactants throughout the capillary nanoreactor and that can be calculated by solving equations of diffusion [22]. Based on these equations, Krylov et al. [23] developed a software able to accurately simulate the spatial overlapping of plugs taking into account injection pressure, plug length, reactant coefficient of diffusion and concentration, and capillary inner radius and length. UV, mass spectrometry or laser-induced fluorescence (LIF) may used for detection in CE.

A micellar electrokinetic capillary chromatography (MEKC) method has been successfully developed in 1998 by Viglio et al. [24] to determine HNE activity. This assay is very simple to conduct and efficient. However it presents some limitations due to the use of SDS micelles, low detection sensitivity and relatively high reagent consumption (few tens of microliters).

This work aims to develop a novel sensitive and robust online capillary zone electrophoresis (CZE) assay for studying HNE kinetics. First of all, a pre-capillary CE-UV assay was implemented based on the method previously described by Viglio et al. [24]. It involved the measurement of HNE activity against synthetic referenced peptidic substrate and the determination of the corresponding catalytic constants (V_{max} and K_m). Subsequently, online approaches based on EMMA and TDLFP for in-capillary mixing were used for the first time to study HNE activity. Moreover, LIF detection was employed to increase assay sensitivity and selectivity [25]. For this, three synthetic peptides were tested as novel potential substrates for HNE by evaluating the corresponding catalytic constants. The amino acid sequence of the tested substrates was chosen taking into account their ionization properties (p*K*a) to avoid the use of MEKC mode, and the narrow cleavage site specificity of HNE [26]. The different results obtained through this study were cross-compared in terms of kinetic constants, and sample- and time-consumption. They were also compared when available with those reported in literature.

2. Materials and methods

2.1. Chemicals

All reagents were of analytical grade and used as received without further purification. HEPES (4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (purity ≥99.5%), N-methoxysuccinyl-Ala-Ala-Pro-Val-p-nitroaniline (S_1: N-MeO-Suc-Ala-Ala-Pro-Val-pNA, HNE substrate), 4-nitroaniline (pNA, purity ≥99%), sodium acetate (CH_3COONa, purity ≥99%), sodium chloride (NaCl, purity ≥99%), sodium dodecyl sulfate (SDS, purity ≥98.5%), 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, specific activity ≥22.0 U mg^{-1}) was purchased from Merck Millipore (Molsheim, France). H-Ala-OH, H-Ala-Ala-OH, H-Ala-Ala-Ala-OH, H-Ala-Ala-Ala-Tyr-OH (S_2) and 5-carboxyfluorescein (5-FAM) were purchased from Bachem (Weil am Rhein, Germany). 5-FAM-Ala-Ala-Ala-Phe-Tyr-Asp-OH (S_3), 5-FAM-Ala-Ala-Ala-OH (Pr_3), 5-FAM-Arg-Glu-Ala-Val-Val-Tyr-OH (S_4) and 5-FAM-Arg-Glu-Ala-Val-OH (Pr'_2) were purchased from Genscript (Piscataway, NJ, USA). Hydrochloric acid (HCl) and methanol (MeOH) (of 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).

2.2. Solutions

Ultra-pure water (18 MΩ cm) was used. Solutions were filtered through 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).

Incubation buffer (IB): The IB was optimized by dissolving appropriate amounts of HEPES (25, 50 and 100 mM) and NaCl (0, 10, 20, 50 and 100 mM) in ultra-pure water. The pH was fixed at 7.5 by adding 10 mM NaOH.

Background electrolyte (BGE): A 35 mM tetraborate buffer (pH 9.3) containing 65 mM SDS and 15% MeOH was used as BGE for MEKC-UV assays. For CZE-LIF assays, the BGE was optimized by using sodium tetraborate at 10, 20, 50, 75 and 100 mM concentrations with various pHs (7.5, 8.0, 8.5, 8.75, 9.0 and 10.0) adjusted with 1 M HCl.

Substrate and product solutions: Stock solutions of the referenced substrate S_1 at 4 mM and the product pNA at 2 mM were prepared weekly in the optimized IB containing 25 mM HEPES and 20 mM NaCl (pH 7.5). The solutions were stored at 4 °C. Stock solutions of peptidic substrates (S_2, S_3 and S_4) and products (Pr_1, Pr'_2, H-Ala-Ala-Ala-OH, H-Ala-Ala-OH and H-Ala-OH) were prepared at 2 mM in the appropriate IB and stored in dark at −20 °C.

HNE solution: A 50 μg (85 μM) of HNE solution was prepared in 50 mM sodium acetate and 500 mM NaCl (pH 5.5) and stored at −20 °C when not in use (stable for 1 year) [27]. A solution of 0.9 μM HNE was prepared in the optimized IB (25 mM of HEPES and 20 mM of NaCl, pH 7.5) and stored at 4 °C (stable for 1 month). A daily fresh solution was prepared by dilution of the stock solution and used for all enzymatic assays.

Derivatization mixture: 2 mM stock solution of 5-FAM was prepared in 10 mM sodium tetraborate (pH 9.5) and diluted with the corresponding compound at the appropriate concentration in the IB and stored at −20 °C. For the microwave-assisted (MW) derivatization reaction, experiments were carried out with a MW synthesis labstation (Start Synth, Milestone, Bergamo). The compounds labeled by the optimized MW-assisted derivatization procedure were S_2, H-Ala-Ala-OH (Pr_2) and H-Ala-OH (Pr_1) (Table 1).