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Hyaluronidase reaction kinetics evaluated by capillary electrophoresis with UV and high-resolution mass spectrometry (HRMS) detection

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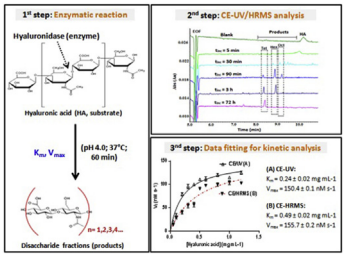
HIGHLIGHTS

- Linearity and stability study of bovine testicular hyaluronidase activity by CE.
- K_m and V_{max} for hyaluronic acid hydrolysis by hyaluronidase obtained by CE-UV/HRMS.
- IC_{50} of epigallocatechin gallate toward hyaluronidase obtained by CE-UV/HRMS.
- Effect of chondroitin sulfate (CS) oligosaccharides on hyaluronidase evaluated by CE.
- Sulfate position and number in CS contribute to anti-hyaluronidase activity.

GRAPHICAL ABSTRACT



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ABSTRACT

The biology of hyaluronidase activity on age related turnover of the hyaluronic acid (HA) in skin dermis and epidermis has not been established. Elucidation of this phenomenon enables discovery of novel compounds for skin health.

As a simple and green technique, capillary electrophoresis (CE) was used for the first time for the determination of the kinetic constants (K_m, V_{max} and IC_{50}) of the enzymatic degradation of HA. Reaction products were identified using CE/high-resolution mass spectrometry (HRMS) after appropriate optimization. Best results in terms of signal sensitivity were obtained using 10 mM ammonium acetate (pH 9.0) BGE, a sheath liquid composed of methanol–water (80:20, v/v) with 0.02% (v/v) formic acid at 10 μL min^{-1} and an ESI voltage at −4 kV. K_m and V_{max} were determined (n = 3) using CE/UV at 200 nm as 0.24 ± 0.02 mg mL^{-1} and 150.4 ± 0.1 nM s^{-1}, respectively. They were also successfully obtained by CE/HRMS (n = 3) with K_m of 0.49 ± 0.02 mg mL^{-1} and V_{max} of 155.7 ± 0.2 nM s^{-1}. IC_{50} of a standard natural inhibitor, epigallocatechin gallate, was also determined by CE-UV/HRMS. Kinetic constant values obtained by CE compared well with literature which validated the developed CE-based assay.

Abbreviation: BGE, background electrolyte; BTH, bovine testicular hyaluronidase; CS, chondroitin sulfate; CPA, corrected-peak area; HA, hyaluronic acid; IB, Incubation buffer; Oligo-HA4 or tetrasaccharide or Tet, oligohyaluronic acid 4.

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In addition, the activity of homemade tetrasaccharides of biotinylated chondroitin sulfate CS-A or CS-C (4- or 6- sulfated in a homogeneous or heterogeneous way) on the hydrolysis reaction of hyaluronidase was evaluated. Hyaluronidase was mostly dose-dependently inhibited by CS-A tetrasaccharides sulfated in a homogeneous way. Two trisaccharides from truncated linkage region of proteoglycans were also tested as inhibitors or activators. CE-based assay showed that even a small modification of one hydroxyl group changes the influence on hyaluronidase activity. CE-based assay can be used for the screening of natural and synthetic inhibitors of hyaluronidase activity for cosmetic and therapeutic applications.

1. Introduction

The key molecule involved in skin moisture, elasticity and structure is hyaluronic acid (HA) since it has a unique capacity in retaining water [1,2]. HA is a non sulfated glycosaminoglycan and a naturally occurring linear polysaccharide with repeating units of D-glucuronic acid and *N*-acetyl-D-glucosamine disaccharide [3]. It is a ubiquitous component of the extracellular matrix. Due to its hydrophobicity, HA can form highly viscous solutions and thereby influence the properties of the skin. It has been implicated in many biological processes including wound healing, inflammation, cell migration and differentiation, and metastasis of tumor cells. On the other hand, hyaluronidase is one of the endotype glycosidases present in the dermis which depolymerizes HA resulting mainly in tetrasaccharide and hexasaccharide fractions [4,5]. HA degradation in epidermis engenders drying and wrinkling of skin. Understanding this mechanism and introducing potent inhibitors or activators [6] of hyaluronidaseactivity is important to maintain structural integrity and elasticity of the skin [7].

The minimum oligosaccharide chain length for hyaluronidase hydrolysis is six saccharide units [8]. Hyaluronidase is also able to degrade chondroitin (C) and chondroitin sulfates (CS), albeit at a slower rate than HA. This hydrolysis occurs at the β-1,4-*N*-acetylgalactosaminide bonds and *N*-acetylgalactosamine (GalNAc) β-(1 → 4)-GlcUA [9]. A fascinating characteristic of this enzyme is to simultaneously display both hydrolytic and transglycosylation activities. More precisely, in the presence of NaCl, at pH 4.0–5.0 and at 37 °C, hyaluronidase will mainly catalyze a hydrolysis reaction with a preference for HA over CS. In the absence of NaCl, at higher pH of 7.0 and at 37 °C or higher, hyaluronidase will mainly catalyze a transglycosylation reaction and prefers CS over HA [4,9,10].

Six hyaluronidases having similar catalytic mechanisms have so far been identified in the human body: Hyaluronidase-1, -2, -3, -4, PH-20 and Hyaluronidase-P1 [7]. Hyaluronidase-1, the major form in somatic tissues, hydrolyzes specifically HA of high molecular weight. Human Hyaluronidase-1 is not suitable for isolation and purification [11]. Many studies were done with bovine testicular hyaluronidase (BTH) that presents similar activity [12,13]. To determine kinetics of BTH reaction, Vercruysse et al. [14] used a spectrophotometric assay that consists of adding in the reaction mixture a chelating agent, neocuproine, which absorbs at 450 nm after reacting with reducing Cu^{+}. The kinetic constants K_m and V_{max} for BTH were respectively 0.46 mg mL^{-1} and 126 nM s^{-1}. This assay is limited by interference with any reducing substance and by the use of a chelating ligand that may reduce enzyme activity. The same group used a viscosimetric assay to obtain K_m value (0.51 mg mL^{-1}) [15]. This approach provides an empirical estimation of enzymatic activity based on the change of HA polymeric properties.

Capillary electrophoresis (CE) has recently emerged as a promising analytical tool for assaying enzymatic activity due to the high separation efficiency and versatility as well as its very low consumption of chemicals (few nanoliters to few microliters) and the possibility of using several complementary detectors (UV, LIF, MS, C^4D) [16–23]. CE-based enzyme assays cover all aspects of kinetic analysis including the evaluation of substrate affinity and any activity modulator (inhibitor or stimulator) as well as their identity by using high-resolution mass spectrometry (HRMS) [16,18,23–29]. BTH activity was monitored by CE [30–32] and HA depolymerization products were investigated by CE/ESI-MS for the first time in 2003 by Kuhn et al. [33]. The identification of several HA oligomers up to 16-mer which was not possible by CE/UV was done using an ion trap mass spectrometer. Grundmann et al. [31] used CE-electrospray ionization-time-of-flight mass spectrometry (CE-ESI-TOF-MS) for the analysis of HA oligomer mixtures. Matysiak et al. [32] recently developed a new method for the determination of BTH activity using CZE/UV. The activity of bovine testes and honeybee venom hyaluronidase was evaluated using multiple regression analysis in which sizes of the peaks of the main HA degradation products were used. However, none of these studies evaluated the kinetic constants of BTH reaction by CE.

As already mentioned, a large number of studies are focusing on identifying new inhibitors of hyaluronidase to prevent skin aging and many other diseases [1,6,7,34,35]. Particularly, Ratnasooriya et al. [34] assessed BTH inhibitory activity of black tea and of epigallocatechin gallate (EGCG) as reference inhibitor. The spectrophotometric method described by Reissig J.L et al. [36] was adapted to measure the amount of *N*-acetylglucosamine formed from HA degradation. BTH was activated by adding calcium chloride at 37 °C. Dimethylbenzaldehyde was added to the reaction mixture to be able to use spectrophotometric determination at 585 nm. In these conditions, EGCG exhibited a dose-dependent anti-hyaluronidase activity with an IC_{50} value of 0.09 ± 0.00 mg mL^{-1}. Suzuki et al. [37] were the first to test the effect of fully O-sulfated hyaluro-oligosaccharides on BTH by the flow injection assay after 2 h incubation at 37 °C [38]. The products of enzymatic action on HA were detected by using fluorimetric detection with the fluorogenic reagent 2-cyanoacetamide. The major products were confirmed by H-1 NMR spectroscopy and CE/UV. The inhibitory action of the oligosaccharides of 16- to 20- mer range corresponded to 79% of that shown by fully O-sulfated hyaluronic acid (MW 100 kDa). The 20-mer O-sulfated HAoligos showed the highest inhibition for the enzyme with an IC_{50} value of 3.54 μg mL^{-1} [37]. Chemically O-sulfonated glycosaminoglycans showed IC_{50} values for hyaluronidase inhibition that correlated with the degree of O-sulfonation [38]. Recently, Kakizaki et al. [39] studied the effect of oligosaccharides of HA and CS on the hydrolysis and transglycosylation reactions of BTH. CS oligosaccharides inhibited BTH in a dose-dependent mode but not the HA or the chondroitin oligosaccharides. Results also showed that the tetra- or larger oligosaccharides of chondroitin 4-sulfate (C-4S) and chondroitin 6-sulfate (C-6S) inhibited both the hydrolysis and the transglycosylation activities of BTH. The effect of homogeneous and heterogeneous sulfation was not addressed.

In this study, pre-capillary CE-UV/HRMS assay was used to