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Hyaluronidase: a new assay method and its inhibitors

Hiroyuki Nagata, Sayuki Haniu, Ryoko Kojima, Katsukiyo Sakurai,
Hiroyuki Nishimura, Yuji Inada*, Ayako Matsushima and Yoh Kodera
Department of Biomedical Engineering, Toin University of Yokohama,
* Toin Human Science and Technology Center,
1614 Kurogane-cho, Aoba-ku, Yokohama 225-8502, Japan
Fax: 81-45-974-5062, e-mail: kodera@cc.toin.ac.jp

Hyaluronidases are enzymes that degrade hyaluronic acid and are distributed widely in animals and in some bacteria; they play important roles such as in fertilization, wound healing, and infection with bacteria. Inhibition of the hyaluronidase may be as important as their activity in regulation of biological processes. We started developing a new convenient method using a combination of fluorescent hyaluronic acid and gel filtration on a high-performance liquid chromatography. The lower detection limits of hyaluronidase from *Streptococcus dysgalactiae*, *Streptomyces hyalurolyticus* and sheep testis were 70, 3 and 10 μU, respectively. This method was found to be both sensitive and easy, and useful even if inhibitor absorbs UV light or is highly viscous. We then applied the new method to investigate the effects of inhibitors using polysulfonated polysaccharides and catechins. The concentration required for 50% inhibition (IC_{50}) of epigallocatechin gallate (EGCg) towards hyaluronidase was lowest among the catechins tested and was decreased markedly with ageing the EGCg solution. This compound seems to differ from theaflavin nor polymerized EGCg. A structure of the compound is now under investigation. The effect of polysulfonated polysaccharides on hyaluronidase activity was also examined. The degree of inhibition seems to depend on both the number of sulfate introduced and the structure of polysaccharides.
Key words: hyaluronidase, hyaluronidase inhibitors, catechins, polysulfonated polysaccharides

1.INTRODUCTION

Hyaluronidases are enzymes that degrade hyaluronic acid (HA), a polysaccharide of high molecular mass found in extracellular matrix, by hydrolytic or eliminative action. These enzymes are distributed widely in mammalian tissues, organs and in some bacteria; they play important roles in fertilization [1], wound healing [2], tumor invasion [3], and infection with bacteria such as *Staphylococcus aureus* [4]. To study further functions of hyaluronidase, inhibitors may become a useful tool. Moreover hyaluronidase inhibitors seems to have a potential, *e.g.* for the treatment of arthroses or antibacterial therapy of hyaluronidase-producing bacteria [5]. Therefore, we are interested in studying hyaluronidase inhibitors. Because the substrate specificity and mechanism of degradation vary according to origins of hyaluronidases, a variety of methods have been employed to assay enzyme activities [6,7].

We started by developing a new convenient method because existing methods did not fit our purpose. The method, using a combination of fluorescent hyaluronic acid (F-HA) and gel filtration on high-performance liquid chromatography (HPLC), is sensitive and easy. We then applied the new method to study the effects of inhibitors using polysulfonated polysaccharides and catechins. Catechins are known to be good for health [8] and contained in a large amount in green tea [9]. Therefore, we choose catechins to focus on as inhibitors in this study.

2.MATERIALS AND METHODS

Materials

Hyaluronidase from *Streptococcus dysgalactiae* (SD), from *Streptomyces hyalurolyticus* (SH) and from sheep testis were purchased from Seikagaku Corp. (Tokyo, Japan). We extracted and purified HA from rooster comb (MW: 1.3×10^6 Da by viscosity measurement [10]) by the method of Miyazaki and Okuyama [11]. F-HA was prepared by the method of Ogamo et al. [12] using 5-aminofluorescein and 1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide (EDC). We purchased 5-aminofluorescein and EDC from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). Dextran, dextran polysulfate, cellulose polysulfate and chondroitin polysulfate were gifted from Seikagaku Corp. (-)-Epicatechin, (-)-epicatechin gallate, (-)-epigallocatechin and (-)-epigallocatechin gallate (EGCg) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Purified EGCg (≥98%) and theaflavin 3,3'-di-*O*-gallate (TF) (≥90%) were purchased from Nagara Science Co., Ltd. (Gifu, Japan). Other reagents were of analytical grade.

Enzyme assay

Hyaluronidase assay using F-HA was performed as follows. We mixed 100 μl of 10 mM phosphate buffer (optimum pH of an enzyme) containing 10 μg F-HA and 40 μl of enzyme solution in 10 mM phosphate buffer. Subsequently, we incubated it for 10 min at 37°C, followed by heated it for 5 min at 100°C to stop the reaction. The degree of digestion was examined both by HPLC and by measuring the liberated reducing sugars. HPLC was conducted as follows: 10 μl of the reaction

mixture was put on a column for gel chromatography, TSKgel G5000PWXL [Tosoh Corp. (Tokyo, Japan)], equilibrated with 50 mM phosphate buffer pH 9 containing 150 mM NaCl (flow rate 0.5 ml/min). The elution profile was monitored with a fluorescent detector (FS-8010; Tosoh Corp.) (Ex: 492 nm, and Em: 525 nm). Hyaluronidase activity (U) was determined by measuring an amount of liberated reducing sugar according to the Morgan-Elson's method [13] using 100 μl of the same reaction mixture. One unit (U) of the enzyme is defined as the quantity that releases a reducing group corresponding to 1 μmol/min of galactose at 37°C. Hyaluronidase activities (U) in small amounts were calculated using dilution factors because Morgan-Elson's method is not sufficiently sensitive to compare with our method.

In a preliminary experiment, we found that micro-tubes coated with bovine serum albumin (BSA) were free from adsorption of hyaluronidases and some kind of inhibitors to tubes. Therefore, we used micro-tubes coated with BSA.

Inhibitory effects were measured by the same procedure described above in the presence of inhibitors.

3.RESULTS AND DISCUSSIONS

According to viscosity measurements, the molecular weight of F-HA was almost identical to that of unmodified HA. One fluorescein was introduced per 150 hyaluro-disaccharide units, determined with a fluorescent uronic acid as a standard. The resultant F-HA was stable at least a few years at –20°C in the dark as a solid state.

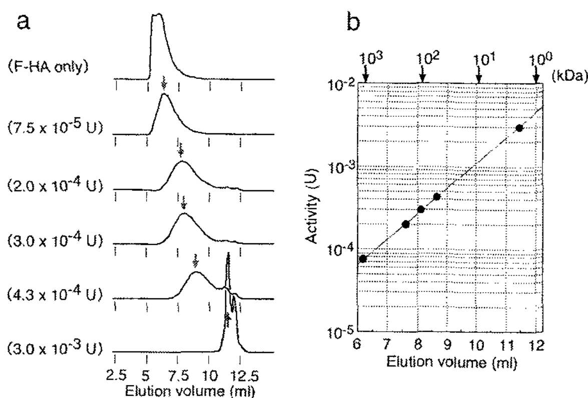

Fig. 1. Chromatograms of the fluorescent hyaluronic acid (F-HA) digests and its relationship. a: Fluorophotometrically monitored chromatogram of F-HA digested for 10 min by hyaluronidase from *Streptococcus dysgalactiae* (SD), b: Relationship between elution volume (or molecular weight estimated using hyaluronic acids with 1.3×10^6, 8.0×10^5, 1.3×10^5 and 6.0×10^4 Da.) and hyaluronidase activity (U) employing the enzyme from SD. One unit (U) of the enzyme catalyzes the release of a reducing group corresponding to 1 μmol/min of galactose at 37°C.

F-HA was incubated with a given amount of hyaluronidase by conditions described in the experimental section. Figure 1a shows chromatograms of F-HA digested for 10 min with hyaluronidase from SD. Data indicate that the main peak positions of the F-HA digests were shifted to larger elution volumes with increasing amounts of enzymes. This phenomenon may indicate that the mean molecular weights of the digested F-HA decrease with increasing amounts of enzyme. As shown in Fig. 1b, the logarithm of the activity (U) used in this experiment versus the elution volume of the peak position on the chromatogram or molecular weight of F-HA digest has a linear relationship (Molecular weights on the upper abscissa were calculated using hyaluronic acids with molecular weights of 1.3×10^6, 8.0×10^5, 1.3×10^5, and 6.0×10^4 Da determined by Laurent's method [10] as standards). A similar correlation between logarithm of activity and elution volume of digests were also observed in the case of hyaluronidases from SH and from sheep testis (data not shown). The lower detection-limits of hyaluronidases from SD, SH, and sheep testis were 70 μU, 3 μU, and 10 μU, respectively. Using the present method, the peak position migrates in a different manner with the enzyme used, as the molecular weights of the degradation products are different between endo-type and exo-type enzymes. The variation of the detection limit may reflect with the substrate specificity of the hyaluronidases. Anyway, these values are much lower than those obtained by the UV, Morgan-Elson's or viscosity methods (300 ~ 5,000 μU). Even though several hyaluronidase assay methods using fluorescently labeled hyaluronic acid (F-HA) have been reported [14–16], the present method seems to be simplest because we can measure digests directly with no operation such as labeling or separation. However, the present method seems not to be useful for enzyme kinetics.

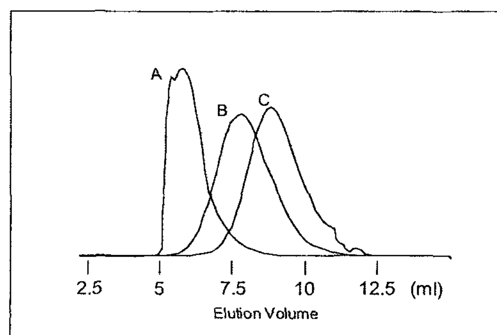

Fig. 2. Chromatograms of fluorescent hyaluronic acid (F-HA) digested by the hyaluronidase (SD) in the presence and absence of catechin as inhibitor. A: only F-HA, B: with EGCg, C: without EGCg.

We then applied the new method to investigate the effects of inhibitors using catechins. Figure 2 shows chromatograms of F-HA digested with hyaluronidase from SD in the presence and the absence of EGCg. Data indicate that the main peak position of the F-HA digests shifted to smaller elution volumes with addition of the

catechin. This phenomenon may indicate that the mean molecular weight of the digested F-HA increases by addition of catechin, namely, hyaluronidase activity was inhibited with catechin.

Effect of other catechins as well as EGCg on the hyaluronidase activity using the enzyme from SD was examined and the concentration required for 50% inhibition (IC_{50}) of catechins for hyaluronidase activity are summarized in Table I. EGCg was shown to be the most effective inhibitor among catechins tested.

Table I. The concentration required for 50% inhibition (IC50): catechins

inhibitors	IC50 (μg/ml)	structure
(-)-epigallocatechin gallate	41	
(-)-epicatechin gallate	80	
(-)-epigallocatechin	129	
(-)-epicatechin	—*	
		R: galloyl group

* HAase activity remained by 60% at 300 μg/ml of inhibitor.

During this experiment, we recognized that the degree of inhibition varied widely even if the same concentration of the catechin was used, and found that the difference in inhibition seems to come from times after dissolving catechins. Table II shows IC_{50} of the purified EGCg, TF (a conjugate of ECg and EGCg) and the EGCg preincubated at pH 6.5 for 16 hr at 37 °C or for 1 hr at 70 °C. This indicates, surprisingly, that the IC_{50} of EGCg decreased markedly from 40 μg/ml to 2 μg/ml by the preincubation. It is known that the catechins in tea were oxidized with enzymes and oxygen to make polymers such as TF and their oligomers [17]. The preincubated, namely aged, EGCg seems to be not TF nor polymerized form judging from spectrophotometory and MALDI-TOF-MS analysis (data not shown). The structure of incubated EGCg was now under investigation.

Table II. Effect of preincubation

inhibitors	preincubation time* (inhibitors only)	IC50 (μg/ml)	structure
(-)-epigallocatechin gallate (EGCg)	fresh	40	
	37°C, 16 hr	2	
	70°C, 1 hr	3	
theaflavin 3, 3'-di-*O*-gallate (TF)	fresh	2	
			R: galloyl group

*EGCg was preincubated in 10 mM phosphate buffer pH 6.5, and was added into the hyaluronidase assay.

As heparin and related sulfonated polysaccharides were reported as inhibitors for hyaluronidases [15,18], effect of other polysulfonated polysaccharides were also tested using the new method. As is shown in Table III, hyaluronidase activity was inhibited both with dextran polysulfate (IC_{50} = 40 μg/ml), chondroitin polysulfate (IC_{50} = 2 μg/ml) and cellulose polysulfate (IC_{50} = 0.3 μg/ml). Toida et al. [15] reported that chemically fully *O*-sulfonated glycosaminoglycans inhibit hyaluronidase. Considering the above results, degree of inhibition seems to depend on both number of sulfate in a molecule and structure of polysaccharides.

Table III. The concentration required for 50% inhibition (IC50): polysulfonated polysaccharides

inhibitors	incubation time	IC50 (μg/ml)	structure	SO3H : H**
dextran	37°C, 1 hr	—*		0 : 6
dextran polysulfate	37°C, 1 hr	40		1.8 : 4.2
	37°C, 1 hr	40		3.7 : 2.3
chondroitin polysulfate	37°C, 1 hr	2		2.6 : 2.4
cellulose polysulfate	37°C, 1 hr	0.3		4.5 : 1.5
			X: SO_3H or H	

* The activity was not inhibited at all with 40 μg/ml of dextran.
** number per disaccharide unit

As is seen above, the novel method for hyaluronidase assay is easy and sensitive. This method did not affected by varied contaminations. The presence of colored compound interferes the UV absorption method and that of viscous polymers interfere the viscosity method. Using this assay method, we surveyed various compounds as inhibitors of hyaluronidase, and found that catechins act as suitable inhibitors. Especially, preincubated EGCg suppressed the hyaluronidase activity at a low concentration of 2 μg/ml, which value may be small enough for catechins to act in tissues or organs even if the degree of incorporation of orally taken catechins into organs or tissues are small amount [19].

Hyaluronidases from mammalian and bacteria may strongly associate with inflammatory diseases. The findings that catechins and its derivatives markedly suppress the enzyme activity are interest in the relation between health and natural compounds.

REFERENCES

[1] M. Gmachl, S. Sagan, S. Ketter and G. Kreil, *FEBS Lett.*, **336**, 545-548 (1993).
[2] P. H. Weigel, G. M. Fuller and R. D. LeBoeuf, *J. Theor. Biol.*, **119**, 219-234 (1986).
[3] D. Liu, E. Pearlman, E. Diaconu, K. Guo, H. Mori, T. Haqqi, S. Markowitz, J. Willson and M-S. Sy, *Proc. Natl. Acad. Sci. U S A*, **93**, 7832-7837 (1996).
[4] J. J. Leyden, R. R. Marples and A. M. Kligman, *Br. J. Dermatol.*, **90**, 525-530 (1974).
[5] A. Botzki, D. J. Rigden, S. Braun, M. Nukui, S. Salmen, J. Hoechstetter, G. Bernhardt, S. Dove, M. J.

Jedrzejas, A. Buschauer, *J. Biol. Chem.*, **279**, 45990-45997 (2004).
[6] W. L. Hynes and J. J. Ferretti, "Methods Enzymol" **235**, Ed. by V. R. Clark and P. M. Baviol. Academic Press, San Diego (1994) pp.606-616.
[7] G. I. Frost and R. Stern, *Anal. Biochem.*, **251**, 263-269 (1997).
[8] H. Mukhtar and N. Ahmad, *Am. J. Clin. Nutr.*, **71**, (suppl.)1698S-1702S (2000).
[9] A. D. Balentine, "Phenolic Compounds in Food and Their Effects on Health" Ed. by H. Chi-Tang, Y. L. Chang and H. Mou-Tuan, ACS Washington, DC (1992) pp. 103-117.
[10] T. C. Laurent and M. Ryan, *Biochim. Biophys. Acta,* **42**, 476-485 (1960).
[11] T. Miyazaki, T. Okuyama, Purification of acidic polysaccharides (in Japanese), Japan Patent S60-9042 (1985).
[12] A. Ogamo, K. Matsuzaki, H. Uchiyama and K. Nagasawa, *Carbohydr. Res.*, **105**, 69-85 (1982).
[13] J. L. Reissig, J. L. Strominger and L. F. Leloir, *J. Biol. Chem.*, **217**, 959-966 (1955).
[14] T. Takahashi, M. Ikegami-Kawai, R. Okuda and K. Suzuki, *Anal. Biochem.*, **322**, 257-263 (2003).
[15] T. Toida, Y. Ogita, A. Suzuki, H. Toyoda and T. Imanari, *Arch. Biochem. Biophys.*, **370**, 176-182 (1999).
[16] T. Nakamura, M. Majima, K. Kubo, K. Takagaki, S. Tamura and M. Endo, *Anal. Biochem.*, **191**, 21-24 (1990).
[17] T. Tanaka, K. Inoue, Y. Betsumiya, C. Mine and I. Kouno. *J. Agric. Food Chem.*, **49**, 5785-5789 (2001).
[18] R. A. Wolf, D. Glogar, L. Y. Chaung, P. E. Garrett, G. Ertl, J. Tumas, E. Braunwald, R. A. Kloner, M. L. Feldstein and J.E. Muller, *Am. J. Cardiol.*, **53**, 941-944 (1984).
[19] M. Suganuma, S. Okabe, M. Oniyama, Y. Tada, H. Ito and H. Fujiki, *Carcinogenesis,* **19**, 1771-1776 (1998).

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