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Analytical Biochemistry 322 (2003) 257–263
ANALYTICAL BIOCHEMISTRY
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A fluorimetric Morgan–Elson assay method for hyaluronidase activity

Tomoko Takahashi,* Mayumi Ikegami-Kawai, Ryozou Okuda, and Kaori Suzuki

Institute of Medicinal Chemistry, Hoshi University, 2-4-41, Ebara, Shinagawaku, Tokyo 142-8501, Japan

Received 8 July 2003

Abstract

Despite their physiological importance, hyaluronidases (HAases) have long been "neglected enzymes," due, presumably, in part to the lack of rapid, sensitive assays. Currently, the colorimetric Morgan–Elson assay method, which is based upon the generation of a new reducing *N*-acetyl-D-glucosamine terminus with each cleavage reaction, is most widely employed but is yet insensitive. We, therefore, reinvestigated the colorimetric method and established the fluorimetric Morgan–Elson assay for HAase activity, with the optimized tetraborate reagent. The fluorimetric assay, requiring neither specialized reagents nor a long time to perform, provided high sensitivity, nearly comparable to that of enzyme-linked immunosorbent assay (ELISA)-like assays, with a detection limit of 5×10^{-3} NFU/ml of bovine testicular HAase after 1-h incubation. The increased sensitivity permitted rapid measurement of low HAase activity in biological samples such as human and rabbit serum HAases, the latter of which has not been detected either by an ELISA-like assay or by zymography. Human serum HAase was easily characterized it along with its optimum pH and kinetic parameters.
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Keywords: Hyaluronidase; Fluorimetric assay; Morgan–Elson reaction; Rabbit serum; Human serum

Hyaluronan (HA)[1] is a high-molecular-mass glycosaminoglycan composed of repeating disaccharide units of D-glucuronic acid (GlcUA) and *N*-acetyl-D-glucosamine (GlcNAc). HA is now recognized as a major participant in such important biological processes as cell motility, proliferation, differentiation, and migration. HA-degrading enzymes, hyaluronidases (HAases), are commonly classified into three groups according to their HA degradation mechanism: hyaluronate 4-glycanohydrolase (hyaluronoglucosaminidase: EC 3.2.1.35), hyaluronate 3-glycanohydrolase (hyaluronoglucuronidase: EC 3.2.1.36), and hyaluronate lyase (EC 4.2.2.1) [1]. The HAases present in various mammalian tissues, belonging to the first group, are of particular biological interest since they have been demonstrated to be involved in the pathophysiology of many human disorders such as cancer and rheumatoid arthritis [2]. Due to their physiological importance, a rapid, sensitive method to measure HAase activity has become increasingly required. A variety of assay methods have been used to measure HAase activity, i.e., classical turbidimetric [3,4], viscometric [5], and colorimetric [6] methods and newer methods such as spectrophotometric [7–9], fluorogenic [10], radiometric [11], agarose plate-based [12,13], ELISA-like [14–16], HPLC- [17], zymography- [18], PAGE- [19], CE- [20], and FACE-based [21], and ECL-assisted [22] assays. The colorimetric method [6], which is based on the Morgan–Elson reaction [23] modified by Reissig et al. [24], is the most practical of these assays and has been widely employed. It is also the most reliable stoichiometrically since it is based upon the generation of a new reducing GlcNAc terminus with each cleavage reaction. However, the colorimetric Morgan–Elson method is insensitive for detecting a small amount

* Corresponding author. Fax: +81-3-5498-5794.
E-mail address: t-tomoko@hoshi.ac.jp (T. Takahashi).

[1] *Abbreviations used:* HA, hyaluronic acid; GlcUA, D-glucuronic acid; GlcNAc, *N*-acetyl-D-glucosamine; HAase, hyaluronidase; NFU, National Formulary Unit; ELISA, enzyme-linked immunosorbent assay; HPLC, high-performance liquid chromatography; FACE, fluorophore-assisted carbohydrate electrophoresis; CE, capillary electrophoresis; PAGE, polyacrylamide gel electrophoresis; ECL, enhanced chemiluminescence; DMAB, *p*-dimethylaminobenzaldehyde; BSA, bovine serum albumin.

0003-2697/$ - see front matter © 2003 Elsevier Inc. All rights reserved.
doi:10.1016/j.ab.2003.08.005

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of HAases in biological samples. Recent methods such as ELISA-like assays [14–16], PAGE-based assay [19], and zymography [18], the latter being semiquantitative, are highly sensitive but either require specialized reagents or take a long time and are troublesome to perform as a routine assay. We, therefore, reinvestigated the Reissig-modified Morgan–Elson method [24] to increase the sensitivity and found that the Morgan–Elson colored product is weakly fluorescent. Here, we describe a fluorimetric Morgan–Elson assay method for HAases, i.e., hyaluronoglucosaminidase and hyaluronate lyase. The established fluorimetric method provided high sensitivity, permitting rapid measurement of very low HAase activity such as rabbit serum HAase, which has not been detected by any assays except for a PAGE-based assay that we developed recently [19].

Materials and methods

Materials

Hyaluronic acid sodium salt from *Streptococcus zooepidemicus*, GlcNAc, *p*-dimethylaminobenzaldehyde (DMAB), and potassium tetraborate ($K_2B_4O_7 \cdot 4H_2O$) were obtained from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). Bovine testicular HAase (EC 3.2.1.35; 295 NFU/mg) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Normal human sera were obtained from laboratory volunteers (19–20 years of age) and normal rabbit sera were from Japanese white rabbits weighing 250–300 g. All aqueous solutions were prepared using water filtered through a Milli-Q water system (Millipore, Bedford, MA, USA). All other chemicals were of reagent grade.

Assay for HAase activity

Colorimetric Morgan–Elson assay method

The Morgan–Elson color reaction [23] was carried out as described by Reissig et al. [24] with slight modifications. DMAB reagent was prepared as described, but tetraborate reagent was prepared by dissolving $K_2B_4O_7 \cdot 4H_2O$ at 0.8 M, without any adjustment of pH to 9.1, and thus the pH was nearly 10.5.

The enzymatic reaction mixture consisted of 125 μl of a 1.5 mg/ml HA solution in 0.1 M buffer containing 0.1 M NaCl and 1.5 mM saccharic acid 1,4-lactone (a β-glucuronidase inhibitor) and 5 μl of enzyme. The buffers used were phosphate buffer (pH 6.0) for bovine testicular HAase and formate buffer (pH 3.9) for human and rabbit serum HAases. Such a purified enzyme as bovine testicular HAase was diluted with digestion buffer containing 1.3 mg/ml bovine serum albumin (BSA) as a stabilizer, and thus the assay mixture included BSA at a final concentration of 0.05 mg/ml. After incubation at 37 °C for an appropriate time, the reaction mixture was heated in a boiling water bath for 5 min to stop the enzyme reaction. After cooling to room temperature, the Morgan–Elson color reaction was started by the addition of 25 μl of tetraborate reagent and subsequent heating for 3 min in a boiling water bath. After cooling to room temperature, 0.75 ml of DMAB reagent was added and incubated at 37 °C for 20 min. After centrifugation at 18,000*g* at 4 °C for 10 min to remove turbidity, the absorbance at 585 nm of the clear supernatant was measured against that of a blank test, which was carried out in the same way except that the enzyme reaction mixture was incubated for 0 time.

Fluorimetric Morgan–Elson assay method

The enzymatic reaction followed by the Morgan–Elson color reaction was carried out in exactly the same way as mentioned above, but the newly released reducing terminal GlcNAc in the supernatant was detected by fluorescence (excitation, 545 nm; emission, 604 nm), with a Hitachi F-4010 fluorescence spectrophotometer, instead of absorbance at 585 nm.

In both the colorimetric and the fluorimetric methods, 1 unit of HAase activity was defined as the amount of enzyme required to produce 1 μmol of reducing terminal GlcNAc per minute under the specified conditions, using respective standard curves plotted with known concentrations of GlcNAc.

Determination of optimum pH of HAase activity

HAase activity was determined according to the above fluorimetric Morgan–Elson method, except that 0.1 M buffers of various pH from pH 2.0 to pH 8.0, each containing 0.1 M NaCl and 1.5 mM saccharic acid 1,4-lactone, were used and that the HA concentration was 0.5 mg/ml, instead of the usual 1.5 mg/ml, because such a relatively high HA concentration as 1.5 mg/ml, partly contributing to the sensitivity increase of the assay, showed a tendency to slightly disturb the pH of the buffers with only a weak buffering action. The buffers used were formate buffer for pH 2.0 to pH 5.0 and phosphate buffer for pH 5.0 to pH 8.0. 0.1 M formate and phosphate buffers containing 0.1 M NaCl were prepared by dissolving HCOOH and NaH_2PO_4 at a final concentration of 0.1 M, respectively, and by adjusting the pH to desired values with NaOH after the addition of NaCl at a final concentration of 0.1 M.

Determination of kinetic parameters of HAase

The kinetic parameters, K_m and V_{max}, of HAase were determined from the Lineweaver–Burk plot. Enzymatic assay was carried out according to the fluorimetric method using various HA concentrations from 0.1 to

1.5 mg/ml of 0.1 M formate buffer containing 0.1 M NaCl and 1.5 mM saccharic acid 1,4-lactone (pH 3.9).

Protein determination

Protein concentrations were determined by the bicinchoninic acid assay (Pierce Chemical Co., Rockford, IL, USA) according to the manufacturer's protocol using BSA as a standard.

Results and discussion

In the Morgan–Elson reaction [23], the GlcNAc reducing end is successively transformed into chromogens I and II under alkaline conditions at 100 °C and then into chromogen III by the action of acids, and finally chromogen III reacts with DMAB to give a reddish-purple-colored product which can be detected at 585 nm [26,27]. Fig. 1 shows the proposed structures of the colored product and chromogens I, II, and III in the Morgan–Elson reaction [26]. Since Reissig et al. [24] improved this reaction by using borate (0.8 M, pH 9.1) as an alkali in the first step to increase the production of chromogens, Reissig et al.'s modification has been employed for the Morgan–Elson reaction [23]. However, there has been some confusion with regard to the exact concentration of borate, as pointed out [27,28], owing to their ambiguous description "0.8 M in borate" with respect to the concentration of tetraborate reagent, which could be prepared with either $K_2B_4O_7$ or H_3BO_3 plus KOH. At least some workers [9,29,30] have employed 0.8 M in BO_3^{3-} (or 0.2 M in $B_4O_7^{2-}$), instead of 0.8 M in $B_4O_7^{2-}$ (or 3.2 M in BO_3^{3-}) as possibly directed by Reissig et al. [24]. Of those who have used so called tetraborate reagent, by citing Reissig et al. [24] without any concrete description, some might have employed the lower concentration. Thus, the borate concentrations used differed fourfold among different groups of workers. This difference in the borate concentration may not have such a critical effect, as long as a certain fixed condition (no buffer added, etc.) is consistently used within a series of studies. However, enzyme assays generally use a specific buffer and sometimes require different buffers of various pH from acid to alkaline, for instance, to examine the pH-activity profile of an enzyme. Therefore, we first attempted to reexamine an optimal concentration and pH of tetraborate reagent to be used in the present Morgan–Elson assay method for HAase. Using GlcNAc dissolved in 0.1 M formate and phosphate buffers of pH 2.0 to 8.0, each containing 0.1 M NaCl, different concentrations and pH of tetraborate reagent were tested for their effectiveness in the Morgan–Elson reaction; these buffers are often used in the assay of serum and testicular HAases, respectively. As shown in Fig. 2, when 0.8 M $B_4O_7^{2-}$ (pH 10.5) was employed, the color production was almost the highest over the wide pH range from 2.0 to 8.0. The molar extinction coefficient at 585 nm was calculated to be approximately 20,000 over the pH range, equivalent to the value (18,000–21,000) reported by Reissig et al. [24] for GlcNAc dissolved in water but not in various buffers. Using 0.8 M BO_3^{3-} (pH 9.1), which has been employed by some workers [9,29,30], the color production was approximately 80%

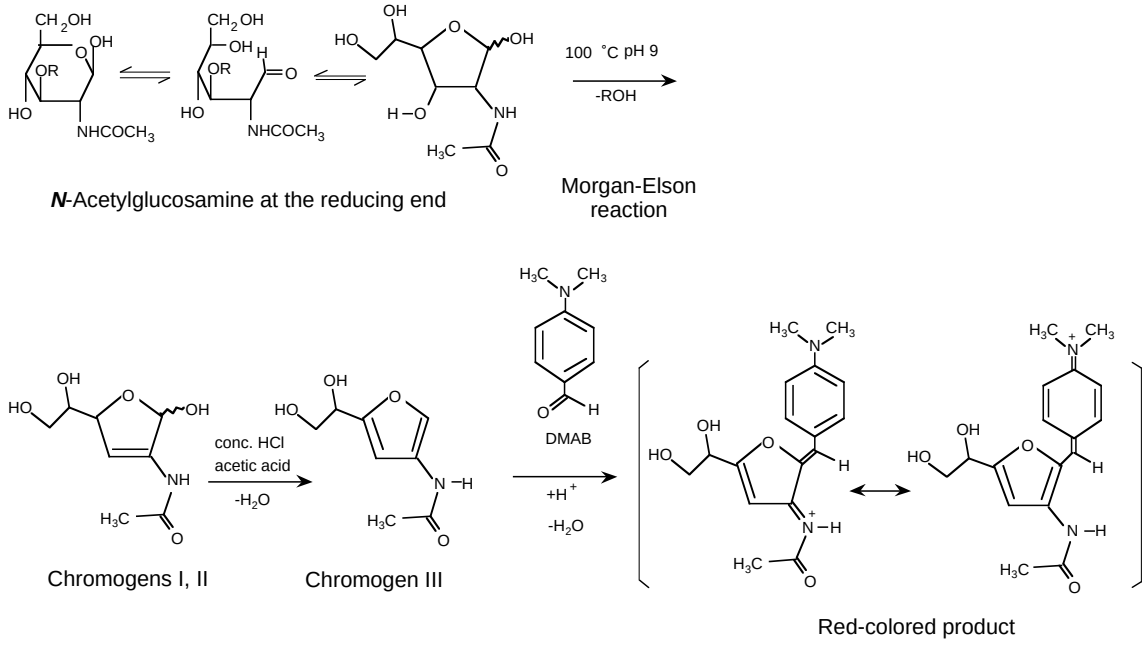


Fig. 1. Proposed structures of the colored product and chromogens I, II, and III in the Morgan–Elson reaction, according to Muckenschnabel et al. [26]. The chromogens I (α configuration) and II (β configuration) have been described by Beau et al. [35].

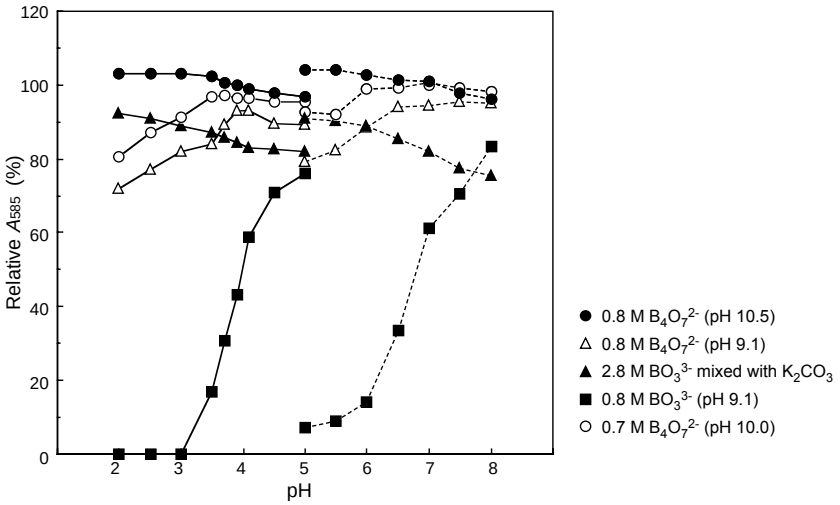


Fig. 2. Comparison of tetraborate reagents of different concentrations and pH in the Morgan–Elson reaction. One hundred thirty microliters of GlcNAc (115 μg/ml) dissolved in 0.1 M buffer containing 0.1 M NaCl was mixed with 25 μl of tetraborate reagent and the Morgan–Elson reaction was started by immediate heating for 3 min, as described under Materials and methods. The buffers used were formate buffer (——) for pH 2.0–5.0 and phosphate buffer (.....) for pH 5.0–8.0. The absorbance at 585 nm of each reaction mixture was measured against a corresponding blank test mixture not containing GlcNAc and expressed as percentage of the absorbance obtained with GlcNAc (0.1 M formate buffer containing 0.1 M NaCl, pH 3.9) using 0.8 M $B_4O_7^{2-}$ (pH 10.5) as tetraborate reagent.

that of 0.8 M $B_4O_7^{2-}$ (pH 10.5) even at the maximum and, surprisingly, little color was produced at the lower pH of both formate (pH 2.0 to 3.0) and phosphate (pH 5.0 to 6.0) buffers. The latter phenomenon seems to indicate that phosphate has a much stronger buffering action than formate and suggests that 0.8 M BO_3^{3-} (pH 9.1) is insufficient in both borate concentration and pH to provide optimal conditions for the Morgan–Elson reaction (e.g., the recommended pH is 8.9 after mixing of reagents [24]). Even 0.8 M $B_4O_7^{2-}$ (pH 9.1) was not sufficient to cover the wide pH range tested, although Natowicz and Wang [31] employed it to obtain the pH-activity profile of human serum HAase. In addition, a 0.8 M $B_4O_7^{2-}$ (or 3.2 M BO_3^{3-}) solution of pH 9.1, but not pH 10.5, was prone to soon deposit crystallines during stocking due to the lower solubility at pH 9.1 than at pH 10.5. Therefore, a slightly lower concentration of 0.7 M $B_4O_7^{2-}$ (pH 10.0) and a mixture of 10 vol of 2.8 M BO_3^{3-} (corresponding to 0.7 M $B_4O_7^{2-}$, pH 9.1) and 1 vol of 6 M K_2CO_3, the latter of which has been used by other workers [26,32], were also tested. Based on the productivity and the pH independence of the Morgan–Elson color production, we finally selected 0.8 M $B_4O_7^{2-}$ (pH 10.5) as the tetraborate reagent in the present Morgan–Elson method.

Next, to enhance the sensitivity, we tried to excite the Morgan–Elson colored product at 545 nm, where it has the absorption maximum, in addition to that at 585 nm. Upon excitation, the colored product emitted characteristic fluorescence with an emission maximum at 602–604 nm (Fig. 3). The fluorescence intensity at 604 nm, although not so strong, could be determined reliably and increased linearly with the amount of GlcNAc up to at least 1.9 μg/ml (Fig. 4A). The detectable limit of GlcNAc by the fluorimetric method was lowered to 0.016 μg/ml, compared with 0.44 μg/ml by Reissig et al.'s colorimetric method [24], which we performed under assay conditions the same as those of the present fluorimetric method except that 0.8 M $B_4O_7^{2-}$ (pH 9.1) was used as the tetraborate reagent. The increase in sensitivity of the fluorimetric method ranged approximately 30- to 40-fold, owing to the pH of the GlcNAc-containing samples used. The Morgan–Elson red-colored product is not stable and it slowly converts to other uncharacterized compound(s). Since the fading rate of the colored product measured by the absorbance at 585 nm was essentially the same as that measured by the fluorescence (excitation, 545 nm; emission, 604 nm), both measurements seemed to detect the identical product. It was also noted that the excitation of the colored product at another absorption maximum, 585 nm, resulted in lower quantum yield than that at 545 mn.

When bovine testicular HAase was assayed at pH 6.0 by the present fluorimetric method, 4.2 NFU corresponded to 1.0 mU, with a detection limit of 5×10^{-3} NFU/ml after 1-h incubation (Fig. 4B). This sensitivity appears to be nearly comparable to those of sensitive ELISA-like assays (1×10^{-2} [15], 1×10^{-3} [16], 5×10^{-4} [14] NFU/ml), if compared on the basis of the same 1-h incubation. The increased sensitivity permitted rapid measurement of human serum HAase with an incubation time of as short as 10–30 min (Fig. 4B), compared with 18–24 h [6,33] or 4 h [31] by the colorimetric Morgan–Elson methods, and the activity was 5.1 ± 0.91 mU/ml ($n = 8$). With rabbit serum HAase, which has not previously been detected by ELISA-like

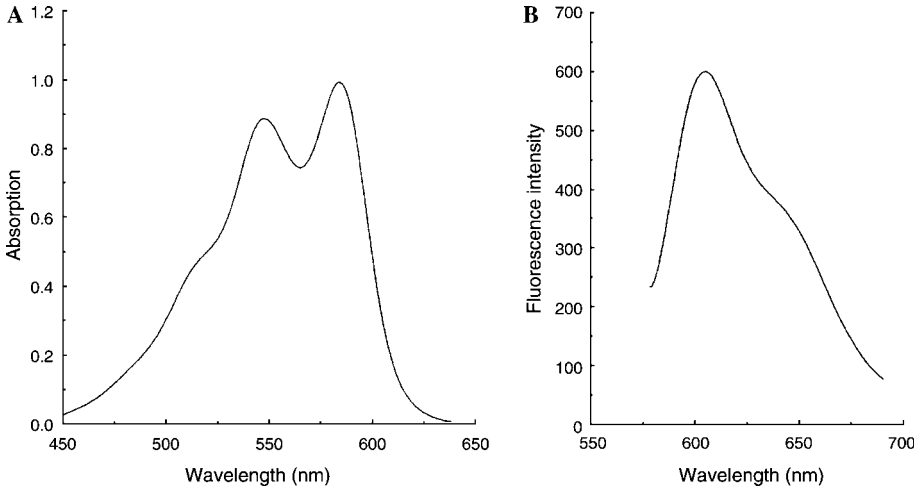


Fig. 3. Absorption and fluorescence spectra of the Morgan–Elson colored product. (A) Absorption spectrum obtained for GlcNAc (final concentration of 12 μg/ml of 0.1 M formate buffer containing 0.1 M NaCl, pH 3.9). (B) Fluorescence spectrum obtained for GlcNAc (final concentration of 0.98 μg/ml of 0.1 M formate buffer containing 0.1 M NaCl, pH 3.9). Excitation wavelength was 545 nm.

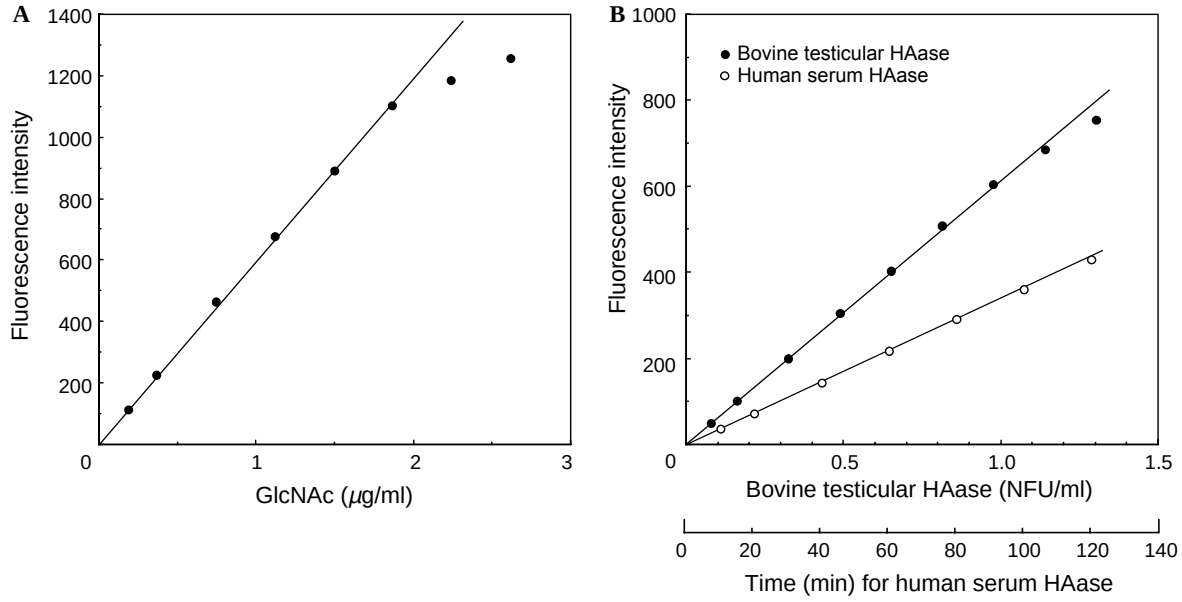


Fig. 4. Determination of *N*-acetyl-D-glucosamine (A) and hyaluronidase activity (B) by the fluorimetric Morgan–Elson method. (A) Various concentrations of GlcNAc in 0.1 M formate buffer containing 0.1 M NaCl (pH 3.9) were determined. (B) Various concentrations of bovine testicular HAase were assayed in 0.1 M phosphate buffer containing 0.1 M NaCl (pH 6.0) with a 20-min incubation. Human serum HAase (5 μl) was assayed in 0.1 M formate buffer containing 0.1 M NaCl (pH 3.9) with 10- to 120-min incubations. The other assay conditions were as described in the text. The abscissas for bovine testicular HAase and human serum HAase express concentration (NFU/ml) and time (min), respectively.

assay [15], by zymography [25], or viscosimetry [34], an incubation time of 10 h (with a standard volume of 5 μl of serum) or 5 h (with a double volume of 10 μl, because of its very low activity) was sufficient. The HAase activity of rabbit serum was 0.052 ± 0.0057 mU/ml ($n = 8$), indicating that it is as low as approximately 1/100 that of human serum, in support of our previous result by a PAGE-based assay [19].

The increased sensitivity of the fluorimetric assay also permitted one to quickly determine the optimum pH and kinetic parameters of human serum HAase. When the activity of human serum HAase was determined as a function of pH, it showed maximum activity at pH 3.9, similar to the reported values of the optimum pH [6,26,31,33], and no activity at pHs above 5.0 (Fig. 5A). The shoulder of activity at pH 3.4 shown by Natowicz and Wang [31] for human serum HAase was not observed at present, suggesting that the shoulder might have come from their tetraborate reagent (0.8 M $B_4O_7^{2-}$, pH 9.1), which has irregular color production around

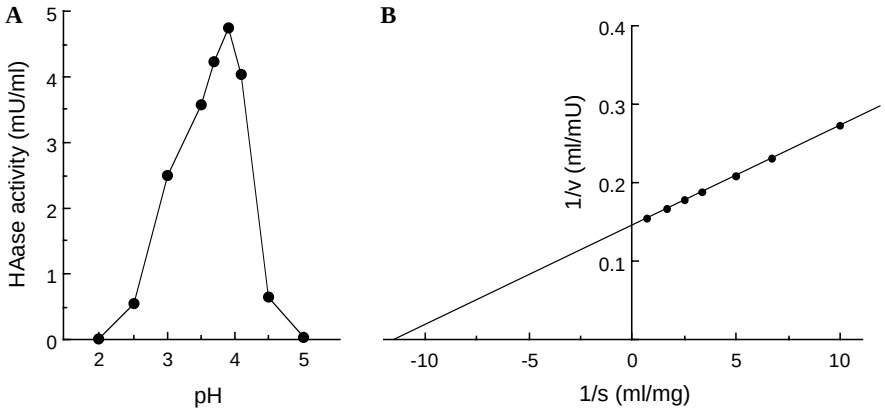


Fig. 5. Determination of pH optimum (A) and kinetic parameters (B) of human serum HAase. The activity of human serum HAase was determined with 5 μl of serum and an incubation time of 60 min, as described in the text except as indicated. (A) The buffers used were 0.1 M formate (pH 2.0–5.0) and phosphate (pH 5.0–8.0) buffers containing 0.1 M NaCl. (B) The HA concentration was changed from 0.1 to 1.5 mg/ml. Data show means of two different experiments and are expressed in a Lineweaver–Burk plot.

that pH (Fig. 2). The apparent K_m and apparent V_{max} values of human serum HAase at pH 3.9 and 37 °C were precisely ($R^2 = 0.998$) estimated from the Lineweaver–Burk plot to be 0.087 mg/ml and 6.9 mU/ml, respectively (Fig. 5B). These values are in good agreement with the sole reported values ($K_m = 0.114$ mg/ml, $V_{max} = 5.1$ mU/ml) [31] for human serum HAase, which have been determined by the colorimetric Morgan–Elson method.

It should be noted that the centrifugation at as high as 18,000*g*, prior to fluorescence (and absorbance) measurement, was important to completely remove the turbidity produced during the enzyme reaction. Asteriou et al. [29] proposed that the contribution of such turbidity in the absorbance at 585 nm could be estimated only by using a curvilinear interpolation, without centrifugation. However, their proposal was not always suitable, especially for assays of serum HAases performed at acidic pH, since the resulting turbid colored reaction mixtures were often unstably suspended.

Fluorescence-based assays for HAase activity as fluorogenic [10] and FACE-based [21] methods have also been reported. However, both methods are not suitable for the routine assay, because of their tediousness requiring a lengthy fluorescence labeling process of substrate [10] or digested oligosaccharides [21]. Furthermore, the sensitivity (1×10^{-2} NFU/ml or less) of the former method is not higher than that of the present fluorimetric method, and the sensitivity of the latter method, which seems to be suited to the analysis of digested oligosaccharides rather than the assay of enzyme activity, appears to be lower than that of our method.

This is the first study to determine the Morgan–Elson colored product fluorimetrically. The fluorimetric Morgan–Elson assay method, with the optimized tetraborate reagent, provided high sensitivity, permitting rapid, simple measurement of low HAase activity in various biological samples. The most remarkable advantage of the present method, compared with other techniques including the colorimetric Morgan–Elson method, is the good and long linearity of the dose (or time)-activity curves.

Acknowledgments

This work was supported by a grant from the Ministry of Education, Science, Sports, and Culture of Japan.

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