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Enhancement of the Sensitivity of a Fluorometric Lysozyme Assay System by Adding β-*N*-Acetylhexosaminidase

HIDEO FUKUDA,* TSUYOSHI TANIMOTO, and TSUTOMU YAMAHA

National Institute of Hygienic Sciences, 1–18–1, Kamiyōga, Setagaya-ku, Tokyo 158, Japan

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The lysozyme assay using 4-methylumbelliferyl tetra-*N*-acetyl-β-chitotetraoside as a substrate was found to show increased sensitivity for lysozyme in the presence of jack bean β-*N*-acetylhexosaminidase (NAHase).

The assay system containing NAHase gave a linear dose-response curve in the range of 10—70 μg of lysozyme under conditions of 20—60 min incubation at pH 5.2 and 35 °C, and the sensitivity for lysozyme was more than five times higher than that of the fluorometric assay system without the hexosaminidase.

The new assay gave values compatible with those of the cell turbidimetric assay using *Micrococcus lysodeikticus* as a substrate in the estimation of lysozyme in pharmaceutical preparations.

The present assay system has better reproducibility than the cell turbidimetric assay and may serve as a substitute for the latter.

Keywords——lysozyme; fluorometric assay; 4-methylumbelliferyl tetra-*N*-acetyl-β-chitotetraoside; β-*N*-acetylhexosaminidase

The cell turbidimetric assay[1-3] using *Micrococcus lysodeikticus* as a substrate has been used widely for the estimation of lysozyme in pharmaceutical preparations and biological materials. Although the cell assay has a high sensitivity for lysozyme, the reproducibility of the method is not necessarily good because of lack of uniformity of the bacterial cell powder and the sensitivity of the method to the ionic strength of the medium.[4-6]

On the other hand, several kinds of substrates for lysozyme assay other than the cell powder have been reported,[7-10] such as chitooligosaccharides of various sizes or a spin-labeled peptidoglycan. However, they are not always suitable for the routine estimation of lysozyme in pharmaceutical preparations because of the need for relatively complex procedures or special equipment to determine their hydrolysis products.

In the previous study,[11] 4-methylumbelliferyl tetra-*N*-acetyl-β-chitotetraoside (4-MU-$(GlcNAc)_4$) synthesized by Inaba *et al.*[12] was found to be a suitable substrate for the lysozyme assay; the results were not significantly influenced by the ionic strength and were reproducible. The assay using 4-MU-$(GlcNAc)_4$ as a substrate was based on determination of the fluorescence intensity of 4-methylumbelliferone (4-MU) released from 4-MU-$(GlcNAc)_4$ after a 20—40 min incubation with lysozyme at pH 5.2 and 35 °C, and gave a linear dose-response curve in a range of 0—250 μg of lysozyme. However, when the assay was applied to the determination of lysozyme in pharmaceutical preparations, the amounts found were always lower than those obtained by the cell turbidimetric assay. The difference was suspected to be due to interference by some of the pharmaceutical components.

According to Masaki *et al.*,[9,13] an oligosaccharide, $(GlcNAc)_4$, is hydrolyzed by hen egg-white lysozyme mainly into three products, (GlcNAc), $(GlcNAc)_2$ and $(GlcNAc)_3$, at pH 5.0 and 50 °C, though the time courses are not simple. It could therefore be presumed that the

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substrate 4-MU-$(GlcNAc)_4$ may also be hydrolyzed into the corresponding saccharides in the lysozyme assay previously reported.[11] Thus, if it is possible to determine the amounts of the products split from the substrate by measuring fluorescence intensity, the sensitivity of the assay for lysozyme might be increased.

Inaba[14] has found that addition of β-*N*-acetylhexosaminidase (NAHase) from *Turbo cornutus* or *Charonia lampas* to a reaction mixture of egg-white lysozyme and *p*-nitrophenyl di-*N*-acetyl-β-chitobioside ($(GlcNAc)_2$-Ph) considerably increased the release rate of *p*-nitrophenol from $(GlcNAc)_2$-Ph. Since the NAHase hydrolyzed (GlcNAc)-Ph but not $(GlcNAc)_2$-Ph, they concluded that lysozyme produced (GlcNAc)-Ph from $(GlcNAc)_2$-Ph. If a similar reaction occurs in the reaction mixture of the previous assay, NAHase may be able to increase the sensitivity of the assay.

The object of this work was to investigate the feasibility of making the previous assay more sensitive by adding jack bean NAHase[15] which is conveniently similar to hen egg-white lysozyme in optimum pH for each substrate.

Experimental

Materials——Lysozyme and lysozyme standard were the Lysozyme Reference Substance (Control 832) established by the National Institute of Hygienic Sciences. Pharmaceutical preparations of lysozyme were all commercially available tablets containing 10 mg [potency]/tablet of hen egg-white lysozyme. 4-MU-$(GlcNAc)_4$ was purchased from the Green Cross Corporation, Osaka, Lot No. EH001TI. 4-Methylumbelliferyl *N*-acetyl-β-glucosaminide (4-MU-(GlcNAc)) was purchased from Sigma Chemical Co., Lot No. 113F-5012. 4-MU was purchased from Tokyo Kasei Kogyo Co., Ltd. Jack bean NAHase was purchased from Seikagaku Kogyo Co., Ltd., Lot No. EJ84301; its K_m value for 4-MU-(GlcNAc) in 0.1 M citrate buffer, pH 5.2, at 35 °C was 2.079×10^{-5} M.

Determination of 4-MU——The amount of 4-MU was estimated by measuring the fluorescence intensity (excitation wavelength, 320 nm; emission wavelength, 445 nm) with a Hitachi 650-10S fluorescence spectrophotometer.

Results

Formation of 4-MU-(GlcNAc) from 4-MU-$(GlcNAc)_4$ by Lysozyme

A reaction mixture (2 ml) containing about 0.5 mg of lysozyme and 250 μM 4-MU-$(GlcNAc)_4$ dissolved in 0.01 M citrate buffer, pH 5.2, was incubated at 35 °C for 16 h and then evaporated to dryness. The residue was dissolved with 0.05 ml of distilled water, spotted on a thin layer plate (Avicel, 20 × 10 cm), and developed according to the method described by Masaki *et al.*,[9] with a solvent system of *n*-butanol–pyridine–water (3 : 2 : 1.5). Authentic 4-MU, 4-MU-(GlcNAc) and 4-MU-$(GlcNAc)_4$ were developed on the same plate. After development, the plate was dried and exposed to ultraviolet (UV) light (365 nm). The visualized spots are shown in Fig. 1, indicating the existence of a substance having the same *Rf* value as 4-MU-(GlcNAc) in the reaction mixture. This result suggests that lysozyme catalyzes the hydrolysis of 4-MU-$(GlcNAc)_4$ to 4-MU-(GlcNAc) and consequently that NAHase may serve to increase the sensitivity of the lysozyme assay.

Effect of NAHase on a Lysozyme–4-MU-$(GlcNAc)_4$ Reaction System

A reaction mixture (3.0 ml) consisting of 200 μg of lysozyme, 25 μM 4-MU-$(GlcNAc)_4$ and 0—0.4 unit of NAHase dissolved in 0.1 M citrate buffer, pH 5.2, was incubated at 35 °C for a given time, and released 4-MU was determined. The results are shown in Fig. 2. Under the experimental conditions, the fluorescence intensity was increased up to about five times with increasing amount of NAHase added, though the effect reached a plateau at more than 0.1 unit of NAHase. Thus, it was considered that 0.2 unit of NAHase may be sufficient to increase the sensitivity of the lysozyme assay consistently.

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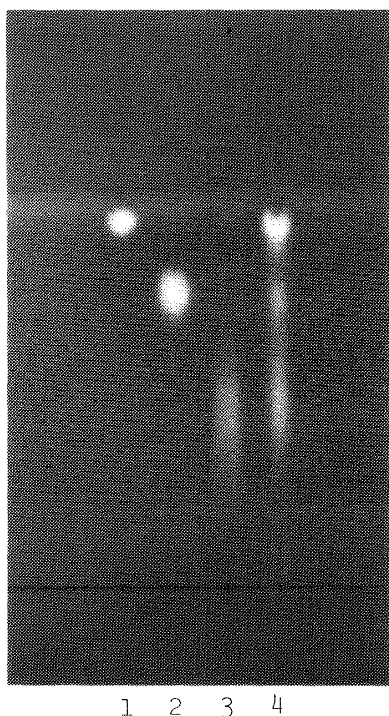

Fig. 1. Detection of 4-MU-(GlcNAc) by Thin-Layer Chromatography (TLC) in a Lysozyme–4-MU-$(GlcNAc)_4$ Reaction Mixture

1, 4-MU; 2, 4-MU-(GlcNAc); 3, 4-MU-$(GlcNAc)_4$; 4, reaction mixture (4-MU-$(GlcNAc)_4$+lysozyme). Development: Avicel SF; *n*-butanol–pyridine–water (3:2:1.5). Detection: UV light (365 nm).

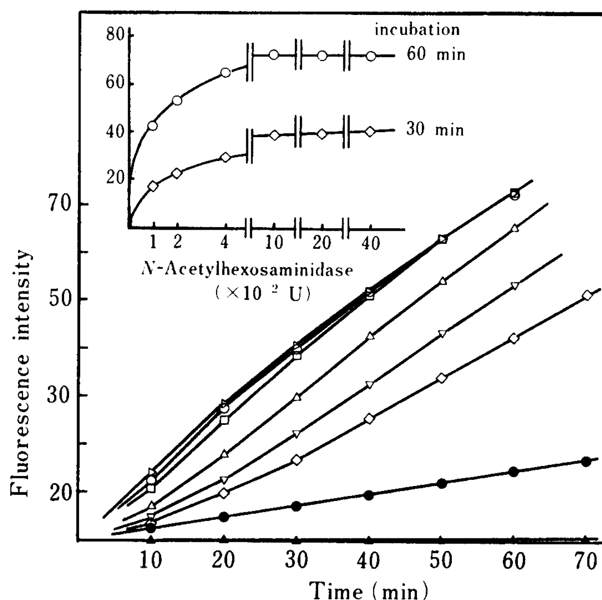

Fig. 2. Effect of β-*N*-Acetylhexosaminidase (NAHase) on a Lysozyme–4-MU-$(GlcNAc)_4$ Reaction System

Each was plotted after subtracting of a fluorescence intensity obtained from a 1-min incubating reaction mixture consisting of 25 μM 4-MU-$(GlcNAc)_4$, 0.4 unit NAHase and no lysozyme from each observed value.

Lysozyme: 200 μg, 4-MU-$(GlcNAc)_4$: 25 μM. NAHase (unit) ●---●: 0, ◇—◇: 0.01, ▽—▽: 0.02, △—△: 0.04, □—□: 0.1, ○—○: 0.2, ▷—▷: 0.4, ▲—▲: 0.4 (−lysozyme).

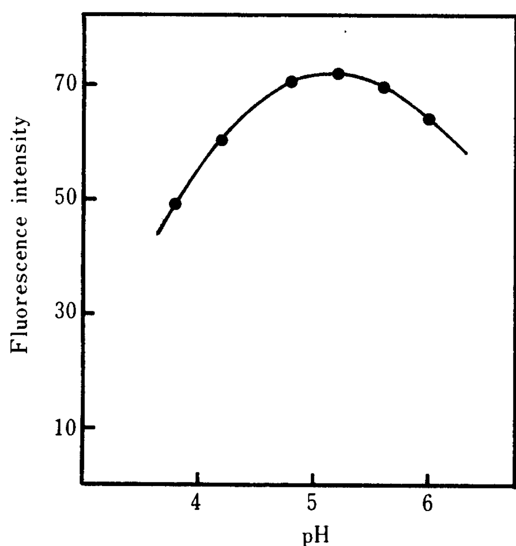

Fig. 3. Optimum pH of a NAHase-Containing Lysozyme–4-MU-$(GlcNAc)_4$ Reaction System

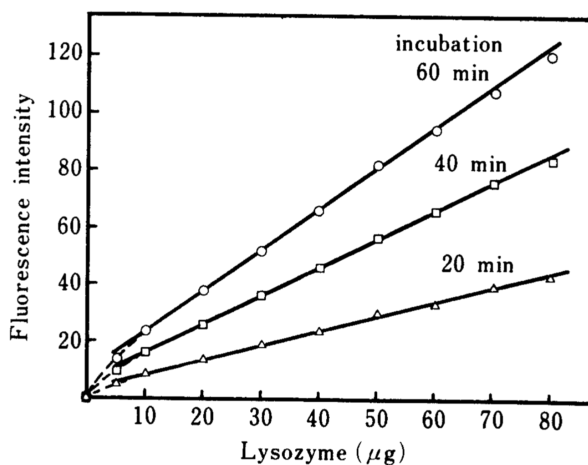

Fig. 4. Dose–Response Relationships for Lysozyme Obtained in a NAHase-Containing Lysozyme–4-MU-$(GlcNAc)_4$ Reaction System

4-MU-$(GlcNAc)_4$: 25 μM, NAHase: 0.2 unit.

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Optimum pH of a Lysozyme–4-MU-(GlcNAc)$_4$ Reaction System Containing NAHase

A reaction mixture (3.0 ml) consisting of 200 μg of lysozyme, 25 μM 4-MU-(GlcNAc)$_4$ and 0.2 unit of NAHase dissolved in 0.1 M citrate buffer of a given pH was incubated at 35 °C for 60 min, and released 4-MU was determined. The optimum pH of the system was the same as that of the assay system without NAHase, *i.e.*, around 5.2 (Fig. 3).

Dose–Response Relationship of the Assay System Containing NAHase

A reaction mixture (3.0 ml) consisting of 25 μM 4-MU-(GlcNAc)$_4$, 0.2 unit of NAHase and a given amount of lysozyme dissolved in 0.1 M citrate buffer, pH 5.2, was incubated at 35 °C for 20, 40 and 60 min, and released 4-MU was determined. Good linear dose–response relationships were found in all cases in the range of 10 to 70 μg of lysozyme (Fig. 4). These straight lines, however, seemed not to pass through the origin.

Application of the Assay System Containing NAHase

On the basis of the results obtained here, the standard procedure for the NAHase-containing fluorometric lysozyme assay was chosen (Fig. 5).

Samples from six kinds of pharmaceutical preparations were assayed by three different methods, the present method, the assay method without NAHase and the cell turbidimetric method. The results are shown in Table I.

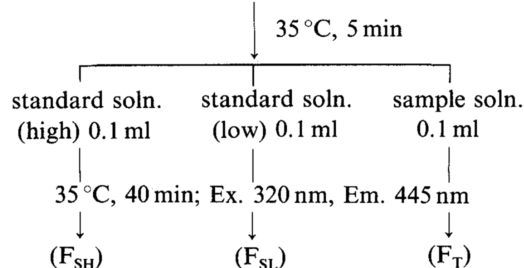
The present method was found to give results comparable with those of the cell method, while the method without NAHase gave values about 20% lower than those of the cell method, as found in the previous study.[11]

Reproducibility of the Present Assay

From the values of fluorescence intensity in the present method and of optical density in the cell method obtained from both sample and standard solutions, the coefficients of

1) substrate soln.: 4-MU-(GlcNAc)$_4$, 3.7 mg→10 ml (water)
2) standard soln. (high): lysozyme, 60 mg [potency]→100 ml (0.01 M phosphate buffer containing 0.4 M NaCl, pH 6.2)
3) standard soln. (low): standard soln. (high), 25 ml→50 ml (0.01 M phosphate buffer containing 0.4 M NaCl, pH 6.2)
4) sample soln.: sample, 100 mg [potency] corresponding amount→ 200 ml (0.01 M phosphate buffer containing 0.4 M NaCl, pH 6.2)
5) hexosaminidase soln.: NAHase (from jack bean), 5 units→2.5 ml (0.01 M phosphate buffer, pH 7.0)
6) procedure:

substrate soln.	0.2 ml
hexosaminidase soln.	0.1 ml
0.1 M citrate buffer (pH 5.2)	2.6 ml



7) calculation: lysozyme mg [potency]/100 ml of sample soln.

$$=\frac{F_T - F_{SL}}{F_{SH} - F_{SL}} \times 30 + 30$$

Fig. 5. Standard Procedure for the Fluorometric Assay of Lysozyme in the Presence of NAHase

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Table I. Comparison of Lysozyme Potencies Found in Some Pharmaceutical Preparations by Three Different Assay Methods

Sample No.	Found mg [potency]/tablet (means)		
	Fluorometry		Turbidimetry
	Not added[a]	Added[a]	
1	8.41, 8.38 (8.40)	10.79, 10.71 (10.75)	10.43, 11.73 (11.08)
2	8.81, 8.69 (8.75)	10.34, 10.50 (10.42)	10.57, 11.20 (10.89)
3	6.55, 6.53 (6.54)	9.43, 9.51 (9.47)	8.64, 10.31 (9.48)
4	7.81, 7.93 (7.87)	9.17, 9.10 (9.14)	8.32, 9.27 (8.80)
5	6.45, 6.43 (6.44)	9.24, 9.28 (9.26)	8.92, 9.66 (9.29)
6	6.59, 6.49 (6.54)	9.15, 8.66 (8.91)	8.49, 8.12 (8.31)

a) NAHase.

variation (C.V.) were calculated. All the assays were carried out by the same worker. The C.V. of the present method and the cell method were 1.4 and 8.2%, respectively.

Discussion

The sensitivity of the lysozyme assay using 4-MU-$(GlcNAc)_4$ as a substrate was found to be increased by up to about five times by addition of NAHase. Under the assay conditions, NAHase does not hydrolyze 4-MU-$(GlcNAc)_4$ directly, and the effect on the 4-MU release rate reaches a plateau with more than 0.1 unit of NAHase. These observations seem to indicate that NAHase does not directly affect the 4-MU-$(GlcNAc)_4$ hydrolysis rate of lysozyme. On the other hand, lysozyme was found not to hydrolyze 4-MU-(GlcNAc) under the assay conditions. Therefore, the sensitivity-increasing effect of NAHase is considered to be due merely to the release of 4-MU from 4-MU-(GlcNAc) which is produced from 4-MU-$(GlcNAc)_4$ only by lysozyme. NAHase is relatively stable and inexpensive, having a lower K_m value for 4-MU-(GlcNAc), and a similar optimum pH to lysozyme for each substrate. Therefore, it seems to be practically useful for the present purpose.

Since the dose–response plot obtained in the present assay did not pass through the origin, though the line obtained in the assay system without NAHase did pass through it, a dose–response line must be prepared from two doses, high and low, of lysozyme standard in the assay. The present assay may not be used, therefore, as an absolute assay system for lysozyme.

In Japan, the cell turbidimetric assay has been widely used for the estimation of hen egg-white lysozyme in pharmaceutical preparations. It was reconfirmed in the present study that the assay system without NAHase is not suitable for pharmaceutical preparation. However, addition of NAHase results in an effective lysozyme assay system for pharmaceutical preparations giving better reproducibility than the cell turbidimetric assay.

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References and Notes

1) A. L. N. Prasad and G. Litwack, *Anal. Biochem.*, **6**, 328 (1963).
2) R. M. Parry, Jr., R. C. Chandan, and K. M. Shahani, *Proc. Soc. Exptl. Biol. Med.*, **119**, 384 (1965).
3) G. Gorin, S.-F. Wang, and L. Papapavou, *Anal. Biochem.*, **39**, 113 (1971).

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4) R. C. Davies, A. Neuberger, and B. M. Wilson, *Biochim. Biophys. Acta*, **178**, 294 (1969).
5) J. Saint-Blancard, P. Chuzel, Y. Mathieu, J. Perrot, and P. Jollès, *Biochim. Biophys. Acta*, **220**, 300 (1970).
6) K. Y. Chang and C. W. Carr, *Biochim. Biophys. Acta*, **229**, 496 (1971).
7) S. K. Banerjee, I. Kregar, V. Turk, and J. A. Rupley, *J. Biol. Chem.*, **248**, 4786 (1973).
8) L. S. Johnston and F. C. Neuhaus, *Anal. Biochem.*, **85**, 56 (1978).
9) A. Masaki, T. Fukamizo, A. Otakara, T. Torikata, K. Hayashi, and T. Imoto, *J. Biochem.* (Tokyo), **90**, 527 (1981).
10) T. Fukamizo, T. Torikata, T. Nagayama, T. Minematsu, and K. Hayashi, *J. Biochem.* (Tokyo), **94**, 115 (1983).
11) T. Tanimoto, H. Fukuda, and J. Kawamura, *Iyakuhin Kenkyu*, **15**, 942 (1984).
12) T. Inaba, T. Ohgushi, Y. Iga, and E. Hasegawa, *Chem. Pharm. Bull.*, **32**, 1597 (1984).
13) A. Masaki, T. Fukamizo, A. Otakara, T. Torikata, K. Hayashi, and T. Imoto, *J. Biochem.* (Tokyo), **90**, 1167 (1981).
14) T. Inaba, personal communication.
15) S.-C. Li and Y.-T. Li, *J. Biol. Chem.*, **245**, 5153 (1970).

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