Analysis of Urinary *N*-Acetyl- β -D-Glucosaminidase Using 2,4-Dinitrophenyl-1-Thio *N*-Acetyl- β -D-Glucosaminide as the Substrate

Magohei Yamada¹ and Toshio Fujita^{2*}

¹International Reagents Company, Ltd., Kobe, Japan

²EMIL Project, Kyoto, Japan

2,4-Dinitrophenyl-1-thio *N*-acetyl- β -D-glucosaminide was examined as a new substrate for analyzing the level of *N*-acetyl- β -D-glucosaminidase in the urine of patients suffering from renal diseases. The analysis is based on the fact that the substrate, when hydrolyzed in the presence of *N*-acetyl- β -D-glucosaminidase, liberates 2,4-dinitrothiophenol as the chromogen. The optimum pH for the enzyme reaction is 4.6, which is covered by the optimal range for the UV absorbance of the

chromogen. The first-order rate of increase of the absorbance at this pH was linear to the enzyme concentration up to 600 U/L, with a high sensitivity. Analytical reagents with glucosaminides of 2,4-dinitrophenol and 2-chloro-4-nitrophenol are less stable than the reagent with glucosaminide of 2,4-dinitrothiophenol. The optimum pH for the absorbance of *p*-nitrophenol and 2-chloro-4-nitrophenol does not match that for the enzyme reaction. J. Clin. Lab. Anal. 17:127–131, 2003. © 2003 Wiley-Liss, Inc.

Key words: *N*-acetyl-glucosaminidase; 2,4-dinitrophenylthio *N*-acetyl-glucosaminide; enzyme substrate; urine diagnosis; diagnosis of renal disorders

INTRODUCTION

N-Acetyl- β -D-glucosaminidase (NAG-ase, EC 3.2.1.30; MW = ~140,000) is an enzyme that exists in the lysosomes of the renal proximal tubules (1–4). The urinary concentration of this enzyme is used as a marker for the diagnosis and follow-up of various renal diseases and lesions (5–7).

Various substrates are currently used for the NAG-ase assay, including 2,4-dinitrophenyl 1-*N*-acetyl- β -D-glucosaminide (DNP-O-NAG) (8) and 2-chloro-4-nitrophenyl 1-*N*-acetyl- β -D-glucosaminide (CNP-O-NAG) (9). 4-Nitrophenyl 1-*N*-acetyl- β -D-glucosaminide (PNP-O-NAG) has also been used (10). However, these substrates may not necessarily be appropriate in terms of stability and analytical sensitivity. In this study, we examined a new substrate, 2,4-dinitrophenyl-1-thio *N*-acetyl- β -D-glucosaminide (DNP-S-NAG). This substrate is highly stable, and the optimum pH range for the UV absorbance of the chromogen covers the optimum pH for the enzyme reaction.

MATERIALS AND METHODS

Materials

The standard NAG-ase preparations (from human urine and serum, and bovine kidney) were purchased

from Sigma Chemical Co. (St. Louis, MO). Other reagents were obtained from Wako Pure Chemical Industry Ltd., Osaka, Japan. The test kits for the measurement of NAG-ase reactivity with PNP-O-NAG and CNP-O-NAG as substrates were from Sanko Junyaku Co., Ltd. (Tokyo, Japan). DNP-O-NAG was synthesized by a previously described method (8), and DNP-S-NAG was newly synthesized for this study.

Urine Specimens

Urine specimens were collected from inpatients and outpatients of the Osaka University Hospital, Suita, Japan. Normal urine specimens were obtained from healthy employees of the hospital.

Synthesis of 2,4-Dinitrophenyl-1-Thio *N*-Acetyl- β -D-Glucosaminide (DNP-S-NAG)

2-Acetamido-2-deoxy-3,4,6-tri-*O*-acetyl- α -D-glucopyranosyl chloride and 2,4-dinitrophenylthiol (DNP-SH)

*Correspondence to: Toshio Fujita, EMIL Project, #305 Heights Kyogoshō, Fuyacho-Nishikikoji-agaru, Nakagyoku, Kyoto 604-8057, Japan. E-mail: ped01545@nifty.com

Received 1 November 2002; Accepted 21 February 2003

DOI 10.1002/jcla.10080

Published online in Wiley InterScience (www.interscience.wiley.com).

were synthesized according to reported procedures (10,11), and mixed in a 1:2 molar ratio in acetone in the presence of aq. NaOH. After the usual operations (10), crystals (m.p. 168°C) of 2,4-dinitrophenyl-1-thio *N*-acetyl-3,4,6-tri-*O*-acetyl- α -D-glucopyranosaminide were isolated. Deacetylation of the tri-*O*-acetyl compound with NaOCH₃ in anhydrous methanol, and recrystallization from methanol produced colorless needle-like crystals of DNP-S-NAG (0.8g; yield: 45%). The procedure included the following parameters: melting point: 121–124°C; elemental analysis calculated for C₁₄H₁₇N₃O₉S: C; 41.69, H; 4.22, N; 10.42, O; 35.73, and S; 7.94; found: C; 40.97, H; 4.53, N; 10.25, O; 36.13 and S; 8.12.

Measurement of NAG-ase Reactivity

Unless otherwise noted, 0.2 mL of urine specimens or aqueous solutions of standard NAG-ase preparations were mixed with 2.6 mL of the citrate buffer solution and 0.7 mL of the aqueous solution of substrates. The enzyme reactivity was measured spectrophotometrically in terms of the rate of increase in the absorbance (Δ abs/min) of the chromogen. The reaction mixture was prepared so that it included 10 mmol/L of MgCl₂, 8% of β -cyclodextrin, and 100 mM/L of the buffer agent in the final concentration (see below). The measurements were carried out with a Shimadzu UV-160A spectrophotometer.

Analysis of Creatinine in Urine Specimens, and Correction for the Urine Volume

The urinary concentration of creatinine was measured in terms of the absorbance of its complex with picric acid at 515 nm under alkaline conditions (12). The correction of the NAG-ase concentration in urine specimens was made using 1 g of creatinine/L of urine as the reference point, and was expressed as U/L(urine)/g(creatinine) (12).

Stability of the Substrates in the Reagent Solution

The storage stability of substrate reagents was examined by analyzing the Arrhenius relationship of the spontaneous hydrolytic rate to the reciprocal of the temperature, according to the method of Garrett and Carper (13).

RESULTS

pH Dependence of the Enzyme Activity

The aqueous solution of the standard human urine NAG-ase preparation (500 U/L) was mixed with the citrate buffer solution (100 mmol/L), covering a pH

range of 4–6. The aqueous DNP-S-NAG solution (15 mmol/L) was added to this buffered solution. The rate of increase of absorbance per minute (Δ abs/min) was measured at 400 nm for 10 min at 37°C. As shown in Fig. 1, the optimum pH for the enzyme reaction was about 4.6.

pH Dependence of the Absorbance of Chromogens

Chromogens were dissolved in the citrate buffer solutions (at pH 4–5) with 8% β -cyclodextrin. The UV absorbance of solutions was measured at 400 nm. DNP-SH showed the highest molecular absorption coefficient (ϵ) of $16 \times 10^3 \text{ L} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ at pH 4.6–5.0, which covered the optimum pH for the enzyme reaction. The ϵ value of DNP-OH was slightly higher and that of CNP-OH was much lower than that of DNP-SH. PNP-OH did not show absorbance at the acidic region, as shown in Fig. 2.

Effect of the Addition of Magnesium Chloride and β -Cyclodextrin

The effect of MgCl₂ on NAG-ase reactivity, which has been described previously (9), was examined up to 20 mmol/L using the standard human urine NAG-ase preparation (500 U/L). The results showed that 10 mmol/L was optimal, with an approximately 9% increase in the reactivity of the control at pH 4.6. The addition of β -cyclodextrin (8%) increased the aqueous solubility of the substrates and stabilized the reaction.

Fig. 1. Effect of pH on NAG-ase reactivity with DNP-S-NAG as the substrate; ordinate: rate of increase of absorbance at 400 nm for 10 min at 37°C.

Fig. 2. Effect of pH on the absorbance (ϵ) of chromogen; ○ PNP-OH, ● DNP-OH, □ CNP-OH, and ■ DNP-SH.

Fig. 3. Effect of the concentration of the citrate buffer (pH 4.6) on the reactivity of the NAG-ase preparation from ● human urine, ▲ human serum, and ■ bovine kidney.

In a range of 4–15% of the addition, the effect was observed to be nearly constant.

Effect of Buffer Concentration

The effect of the concentration of the citrate buffer at pH 4.6 on the enzyme reactivity was examined for 50–500 mmol/L using standard urine NAG-ase preparations (500 U/L). The NAG-ase reactivity was lower, but stabilized with increasing concentration of the buffer above 100 mmol/L (Fig. 3).

Effect of the Substrate Concentration

The effect of concentration was examined up to 6 mmol/L. As shown in Fig. 4, the enzyme reactivity was close to maximum above 3 mmol/L. The Lineweaver-Burk plots in Fig. 5 show that the K_m values of the DNP-S-NAG were 1.33 mmol/L for human urine, 1.22 mmol/L for human serum, and 1.45 mmol/L for the bovine kidney enzyme preparations.

Fig. 4. Effect of the substrate (DNP-S-NAG) concentration on the reactivity of the NAG-ase preparation from ● human urine, △ human serum, and ■ bovine kidney.

Determination of the NAG-ase Concentration

Before the reaction with the substrate was initiated, 0.2 mL of the urine specimens (as defined above) was added to the citrate buffer solution (100 mmol/L, pH 4.6) in the cell. The aqueous DNP-S-NAG solution (15 mmol/L) was then added to the buffered mixture,

and the increase of the absorbance at 400 nm was followed under a constant stirring for 10 min at 37°C. The final substrate concentration in the reaction mixture was 3 mmol/L, which is considerably higher than the Michaelis constant value, K_m , shown above. Under the

Fig. 5. Double reciprocal plots of NAG-reactivity and substrate concentration. S: substrate concentration. V: velocity of the reaction of enzyme preparation from ● human urine, ▲ human serum, and ■ bovine kidney.

present conditions, the increase was linear for at least the first 15–20 min. From the slope of the increase, $\Delta\text{Abs}/\text{min}$, the concentration of urinary NAG-ase (U/L) was evaluated as follows:

$$\text{U/L} = [(\Delta\text{Abs}/\text{min})/\epsilon] \times [\text{Total Volume of the Reaction Medium}/\text{Volume of the Urine}] \times 10^6$$
, where $\epsilon = 16 \times 10^3 \text{ L} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$, and the volume ratio = 3.5/0.2. Using the standard human urine enzyme preparation, the value of $\Delta\text{Abs}/\text{min}$ was confirmed to be linear with the concentration (correlation coefficient (r) = 0.999) of the enzyme up to 600 U/L.

Effect of Possible Contaminants in Urine Specimens

The interference of urinary contaminants with the NAG-ase reactivity was examined by adding contaminants to the urine specimens and the standard human urine enzyme solution. There was no effect on NAG-ase reactivity with the addition of up to 20 g/L glucose, 5 g/L ascorbic acid, or 160 mg/L bilirubin. There was also no effect from bromosulphophthalein and phenolsulphonphthalein. Anticholinesterase agents, such as neostigmine bromide and phenylmethanesulfonyl fluoride, were added to eliminate possible hydrolyses by cholinesterase (EC 3.1.1.8) and other esterases in the urine specimens. There was no significant change in NAG-ase reactivity with the addition of these agents.

Precision of the Measurements

The precision for the within- and between-run measurements was estimated with two differently pooled urine specimens. For the lower NAG-level specimen, the within-run analytical result was 41.3 U/L/g creatinine

($n = 10$, $\text{SD} = 0.87$, $\text{CV} = 2.1\%$), and the between-run result was 39.8 U/L/g creatinine ($n = 20$, $\text{SD} = 1.15$, $\text{CV} = 2.9\%$). For the higher-level specimen, the within-run value was 232.5 U/L/g creatinine ($n = 10$, $\text{SD} = 4.42$, $\text{CV} = 1.9\%$), while the between-run value was 228.9 U/L/g creatinine ($n = 20$, $\text{SD} = 4.58$, $\text{CV} = 2.0\%$).

Storage Stability of Substrates

Aqueous solutions of DNP-S-NAG (4 mmol/L), DNP-O-NAG (4 mmol/L), and CNP-O-NAG (2.5 mmol/L) were prepared. They were kept at 25°, 37°, and 50°C, in vials of an appropriate volume. An aliquot of the solution of each substrate was withdrawn from the vial at certain time intervals. After the solution was brought to room temperature, 0.01 mL of the solution was mixed with 2.6 mL of the Tris buffer solution (100 mmol/L, pH 8.0) and 0.2 mL of water. The absorbance of the solution was quickly measured at 400 nm. This absorbance (A_s) corresponds to the concentration of chromogen formed non-enzymatically.

At the same time, 0.01 mL of the aliquot was added to a mixture of 2.6 mL of the same Tris buffer solution as above and 0.2 mL of the bovine-kidney NAG-ase preparation (0.5 U/mL). The mixed solution was kept at 37°C for 1 hr to hydrolyze the substrate completely, and the absorbance of the chromogen was measured at 400 nm. This absorbance (A_t) value corresponds to the total of chromogen liberated by the spontaneous hydrolysis and that produced by the forcible enzyme reaction. The rate of the increase of the absorbance in terms of A_s was followed for 31 days. The first-order rate constant, k , at each of the temperatures was estimated as the slope of the plot of $\log(A_t - A_s)$ for the remaining substrate vs. time (hour). The hydrolytic rate constants of each substrate at various temperatures are listed in Table 1.

The values of $\log k$ were then subjected to the Arrhenius plot against the corresponding $1/T$ (T is the absolute temperature). By extrapolating the linear Arrhenius relationship formulated by the regression analysis, the rate constant at 5°C was estimated. The stability of the aqueous reagent solution in terms of $t_{0.8}$, or the 20% loss time, was calculated from this estimation, as shown in Table 1.

NAG-ase-Level in Urine Specimens From Healthy Volunteers

The reference interval of the NAG-ase concentration (corrected by the creatinine content) was evaluated using urine specimens from 84 employees (42 males and 42 females) of the Osaka University Hospital, whose hepatic and renal functions were assessed as normal

TABLE 1. Rate of spontaneous hydrolysis of substrates

Substrates	Log k (1 st order, hr ⁻¹)			5°C (calc.) ^a	t _{0.8} (5°C)	
	25°C	37°C	50°C		× 10 ⁴ hrs	Years
CNP-O-NAG	-4.83	-4.52	-4.21	-5.42	2.63	3.0
DNP-O-NAG	-4.39	-4.04	-3.69	-5.04	1.3	1.3
DNT-S-NAG	-4.91	-4.57	-4.23	-5.54	3.50	4.0

^aLog k value at 5°C was estimated from those at 25°, 37°, and 50°C by the Arrhenius plot.

by clinical tests. The mean NAG-ase content was 15.6 U/L per g of creatinine, and the mean $\pm$ 2 SD was 5.4–36.6 U/L.

DISCUSSION

Although the DNP-O-NAG reagent is currently used for assays of urinary NAG-ase, its stability is about one-third that of DNT-S-NAG in terms of the $t_{0.8}$ value with constant storage at 5°C, as shown in Table 1. We observed that DNP-O-NAG was not very stable when it was frequently removed from the refrigerator for the urine tests. The temperature in the refrigerator varied from 2–8°C. Within 2 weeks, a significant hydrolysis occurred spontaneously. CNP-O-NAG is more stable than DNP-O-NAG, but the absorbance of its chromogen at pH 4.6 is much lower than that of DNP-S-NAG. The optimal pH range for the UV absorption of CNP-O-NAG does not coincide with the optimum pH for the enzyme reaction. The chromogen of PNP-O-NAG, *p*-nitrophenol, shows no UV absorption in the acidic region. CNP-O-NAG was reported to cover the range of 2–500 U/L of NAG-ase (9), whereas the present substrate, DNP-S-NAG, covered the range of higher concentrations up to 600 U/L.

The K_m value of DNP-S-NAG (1.3–1.4 mmol), as indicated above, is about 110–120 times higher than that of CNP-O-NAG (9). Thus, the “binding” affinity of DNP-S-NAG is considerably lower than that of CNP-O-NAG, which decreases the rate of hydrolysis. However, the absorbance of DNP-SH at 400 nm is about 10⁴ times higher (see Fig. 2) at pH 4.6 than that of CNP-OH. The lower reaction rate appears to be overcome, resulting in a higher sensitivity.

To summarize, DNP-S-NAG as an agent for the NAG-ase assay is superior to other substrates, for the following reasons: 1) It offers higher storage stability in terms of the $t_{0.8}$ value (20% loss time). 2) It has higher sensitivity in response to the higher absorbance of the chromogen formed at the optimum pH for the enzyme reaction. 3) It covers a wider range of enzyme concentrations. This reagent provides high analytic precision and speed, and promises to be especially useful for automatic analyses of NAG-ase levels.

ACKNOWLEDGMENTS

The authors thank Professor Chozo Hayashi of the Laboratory for Clinical Investigation, Osaka University Hospital, Suita, Japan, and Professor Shoji Takemura of the Faculty of Pharmaceutical Sciences, Kinki University, Higashi-Osaka, Japan, for their expertise and support.

REFERENCES

1. Pugh D, Leabach DH, Walter PG. Studies on glucosaminidase: *N*-acetyl- β -glucosaminidase in rat kidney. *Biochem J* 1957;65:464–469.
2. Hayashi M. Comparative histochemical localization of lysosomal enzymes in rat tissue. *J Histochem Cytochem* 1967;15:83–92.
3. Le Hir M, Dubach UC, Schmidt U. Quantitative distribution of lysosomal hydrolases in the rat nephron. *Histochemistry* 1979;63:245–254.
4. Shrivastava KS, Awasthi YC, Yoshida A. Studies on human β -D-*N*-acetyl-hexosaminidase. I. Purification and properties. *J Biol Chem* 1974;249:2043–2048.
5. Dance N, Price RG, Cattell WR. The excretion of *N*-acetyl- β -D-glucosaminidase and β -D-galactosidase by patients with renal disease. *Clin Chim Acta* 1970;27:87–92.
6. Wellwood JM, Ellis BG, Price RG. Urinary *N*-acetyl- β -D-glucosaminidase activities in patients with renal disease. *Br Med J* 1975;16:408–411.
7. Price RG. Urinary enzymes, nephrotoxicity and renal disease. *Toxicology* 1982;23:99–134.
8. Jones CS, Kosman DJ. Purification, properties, kinetics, and mechanism of β -*N*-acetyl-hexosaminidase from *Aspergillus niger*. *J Biol Chem* 1980;255:11861–11869.
9. Makise J, Saito E, Obuchi M. Kinetic rate assay of urinary *N*-acetyl- β -D-glucosaminidase with 2-chloro-4-nitrophenyl-*N*-acetyl- β -D-glucosaminide as the substrate. *Clin Chem* 1988;34:2140–2143.
10. Tanaka M, Kyosaka S, Sekiguchi Y. Effect of substituents on the hydrolysis of substituted phenyl β -acetylglucosaminides by bovine liver β -acetylglucosaminidase. *Chem Pharmacol Bull* 1975;24:3133–3148.
11. Kharash N, Parker AJ. Derivatives of sulfenic acids. XXIV. Synthesis of certain thiophenols by cleavage of unsymmetrical disulfides. *J Org Chem* 1959;24:1029–1030.
12. Heinegard D, Tiderstrom G. Determination of serum creatinine by a direct colorimetric method. *Clin Chim Acta* 1973;43:305–310.
13. Garrett ER, Carper RF. Prediction of stability in pharmaceutical preparations. I. Color stability in a liquid multisulfa preparation. *J Am Pharmacol Assoc* 1955;44:515–519.