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Multiple *exo*-glycosidases in human serum as detected with the substrate DNP-α-GalNAc. I. A new assay for lysosomal α-*N*-acetylgalactosaminidase

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ABSTRACT

This paper presents a new assay to determine the activity of the lysosomal enzyme α-*N*-acetylgalactosaminidase (Naga, EC 3.2.1.49) in human serum. It is based on the use of a new chromogenic substrate, DNP-α-GalNAc (2,4-dinitrophenyl-*N*-acetyl-α-D-galactosaminide) and is performed at pH 4.3 and 37 °C. This allows continuous monitoring of the absorbance of the released DNP. The assay can be performed with a standard spectrophotometer. Compared to established methods using an endpoint assay with MU-α-GalNAc (4-methylumbelliferyl-GalNAc), the present method gives a ca. 3-fold higher specific activity, while only one tenth of the serum concentration in the assay is required. Hence, the assay is at least 30-fold more sensitive than that with MU-α-GalNAc. The pH dependence of the reaction with DNP-α-GalNAc in the pH 3.5 to 6.5 region, while using 4% serum in the assay, shows only one peak around pH 4. This pH optimum is similar to that reported with MU-α-GalNAc. In the accompanying paper (S.P.J Albracht and J. Van Pelt (2017) Multiple *exo*-glycosidases in human serum as detected with the substrate DNP-α-GalNAc. II. Three α-*N*-acetylgalactosaminidase-like activities in the pH 5 to 8 region. BBA Clin. 8 (2017) 90-96), the method is used to show that, under special assay conditions, three more Naga-like activities can be uncovered in human serum.

1. Introduction

The lysosomal enzyme α-*N*-acetylgalactosaminidase (Naga, EC 3.2.1.49) is an *exo*-glycosidase which can hydrolyse R-α-GalNAc molecules, where R can be a polypeptide, a polysaccharide or an artificial organic compound, and α-GalNAc is *N*-acetyl-α-D-galactosaminide. Naga is one of the many lysosomal glycosidases involved in the removal of sugar units from glycoproteins, to enable further degradation by peptidases. Its occurrence in mammals has first been described in 1966 [1], using phenyl-α-GalNAc as substrate. Reports on partial purification of the enzyme from pig and beef liver soon followed, and experimental evidence suggested that it was a lysosomal enzyme [2].

The catalytic properties of the partly purified enzyme from human liver were first characterized in 1973, using 4-nitrophenyl-α-GalNAc (*para*-nitrophenyl-α-GalNAc, pNP-α-GalNAc) as substrate. The pH optimum of this enzyme was determined as 4.3 [3].

During a study of two apparent isoenzymes of human α-galactosidase (at that time termed α-galactosidase A and B) Schram et al. discovered that in the liver from patients with Fabry disease, a lysosomal storage disease caused by the absence of intact lysosomal α-galactosidase A (Gla, EC 3.2.1.22), the residual activity, until then ascribed to α-galactosidase B, was a distinct protein and not an isoenzyme [4]. Its substrate specificity characterized it as an α-*N*-acetylgalactosaminidase rather than Gla [5]. It had an apparent molecular mass of 110 ± 5 kDa [6]. Independently, Dean et al. had purified the α-galactosidase B from human liver and demonstrated its high specificity for *ortho*-nitrophenyl-α-GalNAc and 4-methylumbelliferyl-α-GalNAc (MU-α-GalNAc) [7].

The first patients, deficient in Naga activity, were described in the late 1980s [8,9]. Later, the phenotype of this deficiency became known as Schindler disease [10].

The amino-acid sequence of Naga was published in 1990 and showed a remarkable homology with that of Gla, but not with that of any other protein. It was suggested that both enzymes have evolved from a common ancestral gene [11].

Comparison of the X-ray structures of the human α-galactosidase

Abbreviations: A_{380}, optical absorbance at 380 nm; α-GalNAc, *N*-acetyl-α-D-galactosaminide; DMSO, dimethylsulphoxide; DMF, dimethylformamide; DNP^-, 2,4-dinitrophenolate; DNPH, 2,4-dinitrophenol; DNP-α-GalNAc, 2,4-dinitrophenyl-*N*-acetyl-α-D-galactosaminide; Gla, α-galactosidase A; Naga, α-*N*-acetylgalactosaminidase; MU, 4-methylumbelliferone; pNP-α-GalNAc, *para*-nitrophenyl-α-GalNAc; RT, room temperature; S.A., specific activity in nmol substrate per min per mL serum ($nmol \cdot min^{-1} \cdot mL^{-1}$), using 2 mM DNP-α-GalNAc

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[12] and the human α-*N*-acetylgalactosaminidase [13] revealed that the active sites of these enzymes were amazingly similar; 11 of the 13 amino acids forming the active-site pocket in both enzymes were identical. In Gla a glutamate (E) and a leucine (L) residue are positioned close to the 2-OH group of the substrate. In Naga these residues are serine (S) and alanine (A), respectively. These smaller residues allow the substrate with the extra *N*-acetyl group to enter the active-site pocket. This was elegantly demonstrated by mutation studies. The Gla mutant protein (E203S, L206A) now preferred pNP-α-GalNAc as substrate. Conversely, the Naga mutant protein (S188E, A191L) could no longer react with pNP-α-GalNAc, but only with pNP-α-Gal (pNP-α-D-galactose). The knowledge of the X-ray structures also enabled a better understanding of the many phenotypes described for patients with defective Gla (Fabry disease) or defective Naga (Schindler disease) [12–14].

At pH 4.3 the yellow colour of *p*-nitrophenol (pK_a = 7.15 [15]) released from pNP-α-GalNAc by Naga, cannot be detected. Hence, to evoke the full colour of the chromophore, the pH is raised to 10.6. This high pH also stops the reaction. The absorbance of the formed chromophore is then measured at 400 nm (ε_{400} = 10,300 $M^{-1}{\cdot}cm^{-1}$ [16]).

A more sensitive assay has been described with the fluorescent compound 4-methylumbelliferone (MU) as the leaving group in the substrate MU-α-GalNAc [7,17]. Also with this substrate, optimal fluorescence (λ_{ex} 348 nm, λ_{em} 460 nm) is only obtained after adjusting the pH to 10.7. In 1996 this substrate was first used to determine the Naga activity in human serum and plasma at pH 4.5 [18]. The disadvantage of these indirect assays may be overcome by using a substrate with the more appropriate leaving group 2,4-dinitrophenol (DNP, pK_a = 4.06 [15]), enabling continuous monitoring of the catalytic reaction at pH 4.5. Glycosides with DNP as leaving group have been introduced as substrates for glycosidases already in 1979 [19]. However, the synthesis of DNP-α-*N*-acetylhexosaminides appeared unsuccessful [20]. Only in 2007, a convenient synthesis of a number of 2,4-dinitrophenyl α-D-glycopyranosides, including DNP-α-GalNAc, has been described [21]. DNP-α-GalNAc has already been of use in a study on *endo*-α-*N*-acetylgalactosaminidase from *Streptococcus pneumoniae* R6 [22]. To our knowledge, this substrate has not yet been used to quantify Naga activity in human serum.

The objective at the start of this investigation was, to develop a direct assay method for Naga, that can also be used to verify reports on a Naga-like activity at pH 6 in human serum. The latter activity was claimed to be elevated in serum from patients with a wide range of cancers [23–25]. The present paper describes some essential properties of the new substrate DNP-α-GalNAc and its use to directly determine the lysosomal Naga activity at pH 4.3 in human serum. In the accompanying paper [26] the new substrate has been used to show that, under special assay conditions, three more Naga-like activities can be revealed in human serum.

2. Materials and methods

2.1. Substrate

DNP-α-GalNAc (2,4-dinitrophenyl 2-acetamido-2-deoxy-α-D-galactopyranoside, MW = 387.30) was not commercially available at the start of this research. Therefore we requested Dr. J. van Wiltenburg (Syncom, Groningen, The Netherlands) to synthesise the substrate according to the method of Chen and Withers [21]. The delivered solid was 99.1% pure. DNP (2,4-dinitrophenol) was from Aldrich. Other chemicals were from Merck.

2.2. Serum and buffer

Serum was obtained after clotting of blood (in BD vacutainers, SST II Advance, 3.5 mL, red-brown cap) and centrifugation (BD vacutainers instructions). Most experiments were performed with pooled serum samples obtained from capped tubes stored at 4 °C for 7 to 10 days. This serum is here referred to 'routine serum' and had earlier been used for a variety of routine analyses in the laboratory. It originated from anonymous individuals (females, males, all ages) who had given their consent that after use for the intended analyses, the serum could be used for other purposes, like scientific research. Serum from apparently healthy male blood donors (here referred to as 'donor serum'), kept at 4 °C in capped tubes for one day, was obtained from Sanquin, Blood Transfusion Department Northern Holland, Amsterdam, The Netherlands. All serum originating from the stored, capped tubes was acidified by addition of 11 μL 5 M acetic acid per mL (final pH ca. 5.2, final acetate concentration 54 mM) and was either used directly or stored at − 20 °C [18,27]. Before use, the acidified serum samples were centrifuged at room temperature (RT) for 10 min at 16,000 x*g* (Eppendorf Microcentrifuge, type 5424). Unless specified otherwise, activity assays were carried out at 37 °C in Na-citrate buffer (50 mM citric acid adjusted at RT to pH 4.3 with 5 M NaOH) plus 100 mM NaCl.

2.3. Instruments and data analysis

Optical measurements were performed with a Jasco V-650 spectrophotometer, equipped with a Jasco PAC-743 thermostatted sample changer for six cuvettes. Disposable polystyrene cuvettes with a 2.5 mL assay volume and a path length of 1 cm were used. Cuvettes with an assay volume of 1.5 mL were not suited; their shape hindered a proper thermal equilibrium between the cuvette contents and the thermostatted sample changer. The absorbance was recorded every minute and the data were processed with Microsoft Excel. Measurements of the pH were performed with a MetrOhm 691 pH meter equipped with a 6.0234.100 probe head.

2.4. Choice of the monitoring wavelength for the assay

In enzyme assays with pNP or DNP as leaving groups, an observing wavelength in the 400 to 420 nm region has commonly been used. The anion of DNP has an absorbance peak at 360 and a shoulder at 400 nm (Fig. S1). The extinction coefficient at 360 nm for a solution of DNP prepared with analytical precision, is 14,800 $M^{-1}{\cdot}cm^{-1}$ (deduced from Fig. 1 in [28]). The acidic form (DNPH) does not absorb above 400 nm (Fig. S1).

Serum has a faint yellow-brown colour with a prominent absorption peak at 419 nm (the Soret band of heme, Fig. S2). At lower pH the peak broadened considerably and shifted to 407 to 410 nm. In view of this, and considering the spectra in Fig. S1, it was decided to choose 380 nm as the observing wavelength in the present paper, unless specified otherwise. At 380 nm, DNPH has an absorbance of only 5% of that of the anion; at lower wavelengths this contribution increases (isosbestic point at 324 nm). The minor (pH dependent) correction for the contribution of DNPH at 380 nm has been neglected in this paper.

2.5. Serum treatments

The optimum pH of the Naga activity in human plasma has been reported as pH 4.5 [18]. As anticipated, it was found that when acidified, routine serum was added to some conventional buffers used in established endpoint assays (citrate-phosphate, citrate or acetate) of pH 4 to 5.5, a steadily-increasing turbidity appeared due to proteins that are no longer soluble at low pH. For an optically monitored assay, any change in turbidity is undesirable. With donor serum, which was acidified, frozen and stored at − 20 °C, only a tiny yellow-brown precipitate appeared upon thawing and centrifugation (10 min at 16,000 x*g*). Thus, hardly any protein was removed and the supernatant was still a concentrated protein solution (serum contains ca. 62 to 84 mg protein per mL). Addition of this serum to buffers of pH 4.0 again resulted in an increasing turbidity. Addition of 100 mM NaCl to a 50 mM Na-citrate buffer (pH 4.0) greatly diminished or even abolished

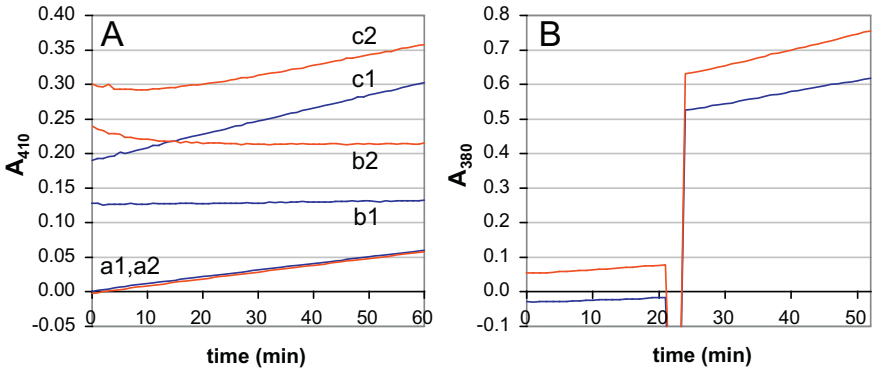

Fig. 1. Examples of absorbance traces. (A) Method A. The six traces represent recordings of the A_{410} of two different serum samples (150 µL in assay, i.e. 6% serum), sample 1 (blue traces) and sample 2 (red traces). Traces a1 and a2 are from the substrate controls. Traces b1 and b2 are from the enzyme controls; a stabilization of the change in turbidity can be observed after ca. 35 min. Traces c1 and c2 are from the reaction cuvettes. (B) Method B. Two of six traces recorded at A_{380} are shown (250 µL serum in assay, i.e. 10%). During the first 20 min the A_{380} (turbidity) steadily increased (enzyme control). The addition of substrate (spikes) caused a jump in A_{380} due to the small amount of DNP in the substrate stock solution. Thereafter, the increase in A_{380} was caused by a summation of the Naga reaction, the spontaneous decomposition of the substrate (0.291 nmol·min^{-1}) and the increase in turbidity. Rates were deduced from the last 10 min before the substrate addition and from the last 10 min of the recording.

this. Moreover, the rate of the increase in turbidity, measured at A_{380}, stabilized after ca. 45 min at 37 °C. This rate tended to increase with serum samples stored in capped tubes for more than 3 days at 4 °C.

2.6. Assay methods

2.6.1. Method A

In experiments with varying amounts of substrate and/or serum, the following assay method was used. The substrate-control cuvette contained assay buffer plus substrate (2 mM DNP-α-GalNAc, from a 200 mM stock solution in ice-cold dimethylformamide (DMF)). The enzyme control held buffer plus serum and the third cuvette, the reaction cuvette, contained buffer, serum and substrate. All cuvettes contained the same amount of DMF. The cuvettes were capped with parafilm, their contents were mixed by hand and they were then inserted into the thermostatted (39 °C) cuvette holder of the spectrophotometer. After 15 min the temperature of the cuvette contents stabilized at 37 °C (as measured with a thermocouple). Alternatively, the capped cuvettes were first incubated in a water bath at 35 °C for ca. 45 min, after which the A_{380} was recorded during 20 to 60 min (Fig. 1A). After cooling to RT, the pH was measured in each cuvette.

2.6.2. Method B

For routine activity assays, six cuvettes were each filled with 2000 µL assay buffer (62.5 mM Na-citrate buffer, 125 mM NaCl, pH 4.0), 100 to 250 µL serum and water up to a total volume of 2475 µL. The cuvettes were capped with parafilm, their contents were mixed by hand and they were placed in a water bath of 35 °C for ca. 45 min. Subsequently, the A_{380} was recorded during ca. 20 min. This provided the rate of turbidity change, if any, in each of the cuvettes (enzyme control). The parafilm cover was then removed and 25 µL substrate (from an ice-cold 200 mM stock in DMF) was added (final concentration 2 mM), after which the A_{380} was recorded for another 20 min (Fig. 1B). After cooling to RT, the pH was measured in each cuvette. As discussed later on (Section 3.1), the substrate control rate was determined as 0.291 nmol·min^{-1} in a volume of 2.5 mL.

2.7. The pK_a value of DNPH under assay conditions

Twenty five µL of a DNP solution in DMF (0.63 mM) was added to cuvettes containing 2500 µL buffer (62.5 mM citric acid, 125 mM NaCl) adjusted with 5 M NaOH to pH values between 3.0 and 6.5. The cuvettes were capped with parafilm, inserted into a 35 °C water bath for 30 min and then placed in the thermostatted cuvette holder. After temperature equilibration (to 37 °C) for 15 min, the A_{380} and A_{410} of each cuvette were measured (average of three readings) against water. These values were plotted against the pH and a theoretical curve for the equilibrium $DNPH \Leftrightarrow DNP^- + H^+$, i.e. $pH = pK_a + \log([DNP^-]/[DNPH])$, where DNP^- is the DNP anion, was fitted to the data. A best fit was obtained for $pK_a = 3.67$ (Fig. S3). As described below (Section 2.8), the sodium concentration in the medium slightly affected the pH reading, causing an apparent decrease of ca. 0.15 pH units. Hence, the true pK_a value, corrected for this Na^+ effect, will be ca. 3.8. The ε_{380} and ε_{410} values of DNP^- were determined as 11,900 and 9700 $M^{-1}\,cm^{-1}$, respectively. These were 96% and 103%, respectively, of the values 12,400 and 9400 $M^{-1}\cdot cm^{-1}$, deduced from Fig. 1 of [28]. The latter values have been used in the present paper.

From the theoretical equation mentioned above, the ε_M values of DNP^- and the pK_a of DNPH, one can derive the equation $\varepsilon_{M,app} = ((10^p)/(1 + 10^p))\varepsilon_M$, where $\varepsilon_{M,app}$ is the apparent ε_M value of a DNP solution at any pH and $p = pH - pK_a$. This enabled the conversion of absorbance changes (dA/dt) to mol·min^{-1}, using the volume of the assay medium, the pH and Lambert-Beer's law.

2.8. Effect of cations (Na^+, K^+ and NH_4^+) on the pH reading

Addition of 100 mM NaCl to 50 mM Na-citrate buffers of pH 3.63, 4.70 or 6.64 decreased the pH reading by 0.16, 0.15 and 0.12, respectively. Addition of 100 mM KCl or NH_4Cl likewise reduced the pH reading, although somewhat less (Nicolsky-Eisenman equation [29]). At a very high protein concentration (e.g. 60 mg bovine serum albumin per mL) this effect was quenched. Under the conditions used here, the deviations were not more than ca. 0.15. The pH values reported hereafter refer only to the measured pH values.

3. Results

3.1. Substrate: properties and optimisation of the stock solution

As the properties of the new substrate DNP-α-GalNAc have not been described before, we shortly summarize our findings. The solid, colourless substrate (99.1% pure) was delivered at ambient temperature in a brown glass bottle. When stored at − 20 °C it was stable for at least two years. In a closed vial it did not change colour after heating to 100 °C for 20 min; this treatment did not affect its substrate properties in the Naga reaction with serum.

Like the pNP-α-GalNAc and MU-α-GalNAc substrates, DNP-α-GalNAc had only a limited solubility in aqueous media. At room temperature the maximally obtainable concentration in pure water was ca. 12 mM. The pH of such a solution (ca. pH 6.2 to 7.7) was similar to that of the used water (ca. pH 6.5). Also in buffer (pH 4.5) the substrate could be dissolved up to 12 mM. Overnight storage of these solutions at 4 °C resulted in the appearance of white needles. These crystals readily dissolved again at RT. In a 4 mM solution in buffer (pH 4.5), the substrate remained soluble at 4 °C.

The solubility in solvents like ethanol or methanol was also limited; e.g. a concentration of 50 mM could not be obtained in ethanol. The substrate readily dissolved in dimethylsulphoxide (DMSO, melting point 19 °C) or DMF (melting point − 60 °C) up to concentrations of 200 mM. However, these concentrated solutions slowly turned yellow

at RT and therefore ice-cold DMF was chosen as the most appropriate solvent.

Thus, for routine use, a stock solution of DNP-α-GalNAc was prepared in ice cold DMF (200 mM, 387.3 mg + 5 mL DMF) and stored as 0.5 mL aliquots at − 18 °C. Its faint yellow colour did not change in time under these storage conditions. Remarkably, as judged by eye, the substrate remained soluble at this low temperature. However, it was repeatedly noticed during activity assays, that a tiny part of the substrate probably crystallized at this low temperature (invisible to the eye) resulting in activities lower than expected. Placing the stock solution in ice (or a refrigerator) for about 1 h prior to the measurements resolved that problem. The stock solution was added directly to the assay medium in the cuvette (2 mM final substrate concentration). DMF from this solution (1% final concentration in the assay, equivalent to 130 mM DMF), did not disturb the assays. It was earlier reported that 5% DMF did not affect the Naga activity in serum or plasma [18].

As shown in Fig. 1A, a slow, spontaneous decomposition of the substrate was observed in buffer of pH 4.3 and 37 °C. The rate of this reaction, monitored at 380 nm, was proportional to the substrate concentration (Fig. S4). The first-order rate constant (equation - $d[S]/dt = k[S]$) derived from this plot was $k = 9.684 \times 10^{-7}\ s^{-1}$, i.e. at 37 °C a 2 mM solution gave a DNP-production rate of 0.291 nmol·min^{-1} in an assay volume of 2.5 mL. The spontaneous decomposition reaction was strongly temperature dependent (Fig. S5A, trace a). The estimated Arrhenius activation energy in 50 mM Na-citrate buffer (pH 4.5) was 32.6 kcal·mol^{-1} (136.4 kJ·mol^{-1}, Fig. S5B, trace a). The absorption spectrum of the resulting yellow product was identical to that of the anion of DNP. The temperature dependence of the Naga activity was as expected (Fig. S5A, trace b). At 37 °C the rate was 5.7 times that at 20 °C. The Arrhenius activation energy of the Naga reaction (Fig. S5B, trace b) was 19.4 to 20.7 kcal·mol^{-1} (81.2 to 86.6 kJ·mol^{-1}, respectively).

At 4 °C the DNP production of a 4 mM substrate solution in buffer was equivalent to a loss of 0.1% of substrate per week. The decomposition rate was not affected by light and was independent of pH in buffers in the range of pH 3.0 to 8.0 (see Section 3.3). At 37 °C the loss of substrate was ca. 0.006% per min, i.e. 9% per 24 h.

3.2. The enzyme assay

When the amount of serum was varied, the Naga reaction rate changed as expected for a standard first-order enzymatic reaction (Fig. 2). The slope of each line in Fig. 2 provides the rate (in nmol per min per volume serum) and is independent of the underlying substrate-control rate. The intercept of each line with the *y*-axis provides the rate of the substrate-control reaction (here 0.291 ± 0.021 nmol·min^{-1} in an assay volume of 2.5 mL). This was equal to the value of 0.291 nmol·min^{-1} obtained from Fig. S4.

When a vial with serum was inserted in boiling water for 20 min and subsequently centrifuged (10 min, 16,000 xg), the activity of the clear supernatant was identical to that of the substrate control.

Plasma, prepared from blood plus EDTA, was not suited for the present activity assay, because the solubility of EDTA is limited at acidic conditions, resulting in an unacceptable turbidity.

3.3. The pH dependence of the Naga reaction

The pH dependence of the Naga activity of serum in assay buffer of varying pH is depicted in Fig. 3. It shows that the pH optimum of the Naga reaction with serum is around pH 4. An earlier report, with MU-α-GalNAc as substrate, reported a pH_{opt} value of 4.5 in both serum and plasma, although no pH profile was shown [18]. Fig. 3 also shows that the spontaneous-decomposition rate of the substrate was independent of pH.

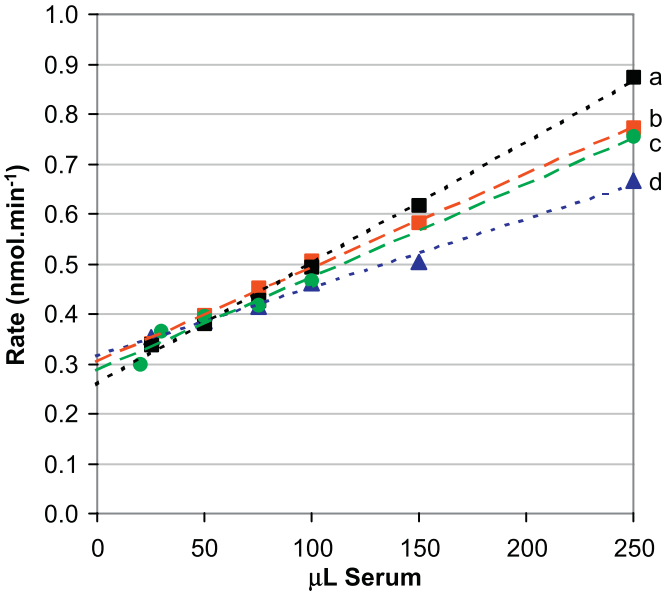

Fig. 2. Plots of the observed reaction rate against the amount of serum. The reaction rate, observed at 380 nm, was measured for increasing amounts of serum from four different sources (Method B, assay volume 2.5 mL, 2 mM substrate). The slope of the linear least-square fits gave the rates of the Naga reaction (range 1.38 to 2.43 nmol·min^{-1}·mL^{-1}), while the intercept with the *y*-axis provided the decomposition rate of the substrate (substrate blank, range 0.260 to 0.315 nmol·min^{-1}; average 0.291 ± 0.021). The type of serum (indicated at the top-right) was routine sample 1 (a), donor sample 1 (b), donor sample 2 (c) and routine sample 2 (d).

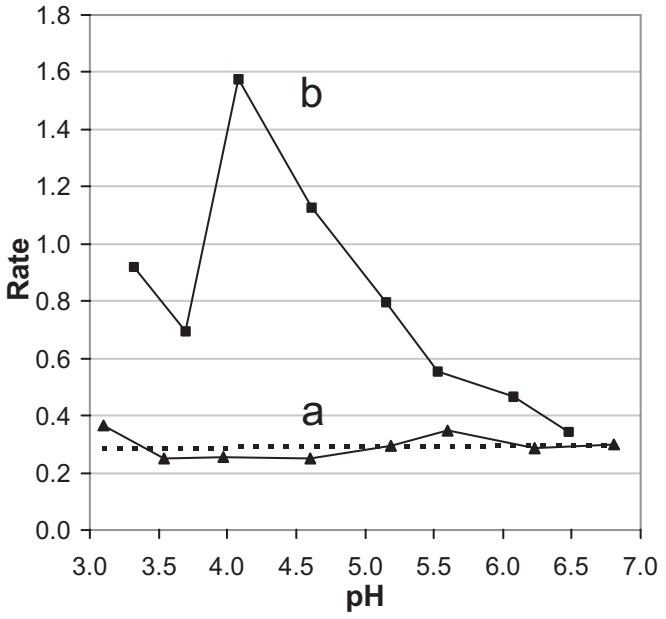

Fig. 3. Effect of pH on the Naga reaction rate and on the spontaneous decomposition reaction of DNP-α-GalNAc. The Naga activity (using 200 µL serum and 2 mM substrate) and substrate-control reaction rates were measured in 50 mM citric acid, 100 mM NaH_2PO_4, adjusted with (pre-determined amounts of) 5 M NaOH to the desired pH (Method A). Using the $\varepsilon_{M,app}$ (Section 2.7.), the rates of the substrate controls (nmol·min^{-1}) and the enzyme reaction (nmol·min^{-1}·mL^{-1}) were calculated. The average substrate-control rate (trace a), here 0.294 ± 0.044 nmol·min^{-1}, was independent of the pH. The dotted line is a linear least-square fit. The enzyme-control rates and the standard substrate-control rate of 0.291 nmol·min^{-1} were used to obtain the correct Naga rates (trace b).

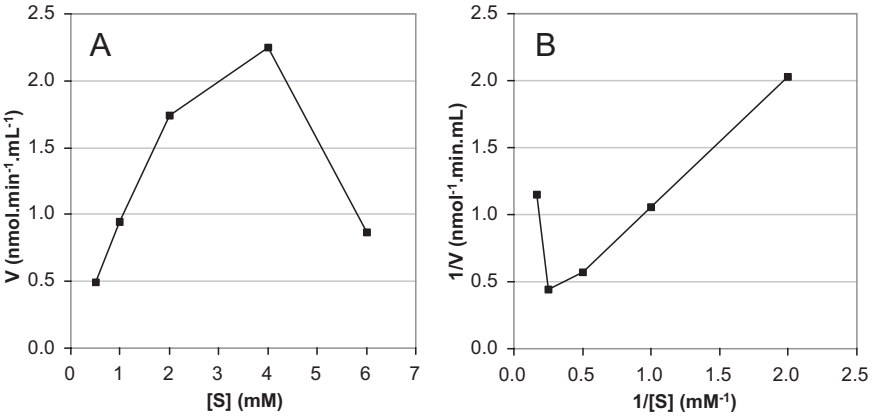

Fig. 4. Plot of the Naga reaction rate at five different substrate concentrations. For this experiment 100 µL pooled donor serum (4% serum in cuvette) was used (Method A). The reaction was carried out in 50 mM Na-citrate buffer, 100 mM NaCl (pH 4.2). The substrate concentration ranged from 0.5 to 6 mM. (A) Plot of the rate V against the substrate concentration. (B) Double-reciprocal plot (Lineweaver-Burk plot).

3.4. The dependence of the Naga reaction on the substrate concentration

With donor serum, we have tried to estimate the apparent K_m for DNP-α-GalNAc at pH 4.3 by varying the substrate concentration between 0.2 and 6 mM. However, no satisfactory results were obtained. Unlike predicted by the Michaelis-Menten equation, the reaction rate showed a maximum after which the rate declined (Fig. 4A). This unexpected behaviour was also obvious in a Lineweaver-Burk plot (Fig. 4B) and was observed repeatedly.

3.5. The Naga activity in serum from healthy, male individuals

To compare the present assay with those reported with pNP-α-GalNAc or MU-α-GalNAc as substrate, the serum activities from 18 healthy male blood donors were measured. With 2 mM substrate an activity of 1.77 ± 0.23 nmol·min^{-1}·mL^{-1} was found. In Table 1 this value is compared with literature values. With 1 mM substrate, the Naga specific activity measured with DNP-α-GalNAc is 3-fold higher than that measured with MU-α-GalNAc as substrate.

4. Discussion

4.1. Substrate stability in buffer at pH 4.3 and 4 °C or 37 °C

The stability of a DNP-α-GalNAc solution in buffer at pH 4.3 and 4 °C is good. Under these conditions, the released DNP was equivalent to a loss of only ca. 0.1% of substrate per week. At 37 °C the loss was ca. 0.006% per min, i.e. 9% per day.

Table 1
Comparison of the present assay with published procedures.

Substrate	[S][a] mM	pH[a]	Activity range	Activity mean	n[b]	Ref.
			nmol·min^{-1}·mL^{-1}			
MU-α-GalNAc	0.8	(4.5)	0.24–0.50	0.34 ± 0.08	23	[33]
MU-α-GalNAc	1	4.7	0.042–0.33	0.15	61	[31]
MU-α-GalNAc	1	4.5	0.10–0.77	0.29 ± 0.08	108	[18]
MU-α-GalNAc	(1)	(4.5)	0.18–0.50	0.31 ± 0.09	104	[10]
DNP-α-GalNAc	2	4.3	1.46–2.10	1.77 ± 0.23	18	(This paper)[c]
DNP- α-GalNAc	1	4.3	0.79–1.10	0.96 ± 0.12	18	(This paper)[d]

[a] Values in parenthesis: assumed values; no specific information in paper.
[b] Number of healthy individuals tested.
[c] Measured values.
[d] Recalculated values on basis of Fig. 4.

4.2. The substrate contained no β-anomer

Human tissues contain β-hexosaminidases (EC 3.2.1.52) with an activity one order of magnitude greater than that of Naga [17]. Such an activity is also present in serum and plasma [18] and might interfere with the assay if the substrate would contain some DNP-β-GalNAc. However, NMR spectra of the used substrate DNP-α-GalNAc did not show any contamination with the β anomer (Dr. J. van Wiltenburg, Syncom, personal communication). In addition, there were no indications for a spontaneous α to β conversion. When the substrate was pre-incubated in buffer (50 mM citric acid, 100 mM NaH_2PO_4, pH 8.0) at 37 °C for 55 min, the absorbance changes upon addition of serum (jump followed by the initial rate of the DNP production) were exactly the same as those without this pre-incubation.

4.3. Importance of acidification and centrifugation of the serum samples

The measured pH of serum taken from a capped tube was ca. 7.4, but upon removal of the cap and standing in air the pH readily increased up to 9.0. This is due to the release of CO_2, since the buffer capacity of blood and serum relies mainly on the concentration of bicarbonate (24 to 27 mM [30]). This pH increase can lead to a possible loss of Naga activity (measured as 22% in 24 h at RT [27]), which is prevented by acidification of the serum [27]. It was also noticed that without this acidification plus subsequent centrifugation for 10 min at 16,000 xg, the turbidity increase in the assay was unacceptably high. Thus, for an optimally reliable Naga activity measurement with the new method, serum samples should be acidified immediately upon preparation [27].

4.4. Comparison with other assays

In Table 1 the Naga activities in serum or plasma with 1 mM pNP- or MU-α-GalNAc reported in literature are compared with the activities with DNP-α-GalNAc presented in this paper. The assay with pNP-α-GalNAc is apparently less sensitive and very long reaction times (up to 18 h at 37 °C [31]) were required. A greater sensitivity was obtained with MU-α-GalNAc and a reaction time of 30 min [18] to 2 h [31] sufficed. In addition, the serum/plasma concentration in these assays was rather high: 33% and 50%, respectively. In the present assay, 100 µL serum in 2500 µL assay volume (4% serum) gave reliable results within 1 h. With 1 mM substrate, the reported mean activities with MU-α-GalNAc were 0.15 [31], 0.29 [18] and 0.31 nmol·min^{-1}·mL^{-1} [10]. With DNP-α-GalNAc, this was 0.96 nmol·min^{-1}·mL^{-1} (this paper). Thus, a three-fold higher activity was obtained with serum concentrations 8- to 12-fold less than those used with MU-α-GalNAc. Another advantage of the present assay is that the observed change in absorbance is direct and continuous, while with the other methods it is indirect and discontinuous. The assay can easily be performed with a

normal spectrophotometer and can be adapted to a plate reader.

An unexpected observation was the behaviour of the activity with increasing substrate concentrations (Fig. 4). Plots like these are indicative for substrate inhibition [32]. This means that the substrate can bind to the Naga enzyme at a secondary site where it causes inhibition. As far as we know, this has not been reported in the Naga literature.

It has been reported that DNP is a better leaving group than pNP in R-α-*N*-acetylhexosaminides compounds [16,22]. This might be the reason that DNP-α-GalNAc gave a higher activity in the Naga assay than pNP-α-GalNAc. It also may explain the slight instability of DNP-α-GalNAc in aqueous media at 37 °C, as well as why the synthesis of DNP-α-GalNAc was more difficult than that of other DNP-α-*N*-acetylhexosaminides [20].

4.5. The pH profile of the Naga activity

The maximum of the observed pH dependence of the serum Naga activity with DNP-α-GalNAc is similar to the optimum pH mentioned for MU-α-GalNAc [18]. The plot in Fig. 3 indicates that, under the used conditions, there is only one Naga activity in serum. However, as described in the accompanying paper, three more activities with different optima in the pH region 5 to 8 could be uncovered under special assay conditions [26].

Conflict of interest

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors. There are no conflicts of interest.

Transparency document

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbacli.2017.10.001.

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