BBA Clinical 8 (2017) 90–96

Contents lists available at ScienceDirect

BBA Clinical

journal homepage: www.elsevier.com/locate/bbaclin

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

Simon P.J. Albracht[a], Johannes van Pelt[b,*]

[a] Retired from the Swammerdam Institute for Life Sciences, University of Amsterdam, Amsterdam, The Netherlands
[b] Noordwest Ziekenhuisgroep, Laboratory of Clinical Chemistry, Hematology and Immunology, Alkmaar, The Netherlands

ARTICLE INFO

Keywords:
α-*N*-Acetylgalactosaminidase
Human serum
DNP-α-GalNAc
General tumour marker

ABSTRACT

With the substrate DNP-α-GalNAc (2,4-dinitrophenyl-*N*-acetyl-α-D-galactosaminide) three α-*N*-acetylgalactosaminidase-like activities could be distinguished in serum, in addition to the classical lysosomal enzyme (Naga, EC 3.2.1.49, pH optimum at 4). Two activities had optima in the pH 5 to 6 region and one peaked around pH 8. Like the Naga activity at pH 4, the activity at pH 8 was detectable under standard assay conditions. However, the two activities in the pH 5 to 6 range were not readily apparent in such assays. They could be unmasked as separate activities only when low serum concentrations were used. Addition of 1% saturated ammonium sulphate to the assay medium stimulated these activities. All activities in the pH 5 to 8 range decreased with increasing serum concentration in the assay, suggesting the presence of endogenous inhibitors. The activities between pH 5 and 6 might be similar to an activity described in 1996, which was considerably elevated in serum of patients with great variety of cancers (N. Yamamoto, V.R. Naraparaju, and S.O. Asbell (1996). Deglycosylation of serum vitamin D_3-binding protein leads to immunosuppression in cancer patients. Cancer Res. 56, 2827–2811).

1. Introduction

In the preceding paper [1] we have described a new assay to determine the activity of the classical lysosomal enzyme α-*N*-acetylgalactosaminidase (Naga, EC 3.2.1.49) in serum at pH 4.3, using 2 mM of the chromogenic substrate 2,4-dinitrophenyl-*N*-acetyl-α-D-galactosiminide (DNP-α-GalNAc). In the present paper, using this substrate, we have tried to verify reports from Yamamoto and co-workers, that serum contains an additional Naga-like activity with a pH optimum at 6. Yamamoto et al. employed a different substrate, *para*-nitrophenyl-α-GalNAc (pNP-α-GalNAc). They showed that this activity (an *exo*-glycosidase reaction) was often elevated in serum from patients with a wide range of cancers [2–4]. They named this pH 6 activity 'NaGalase' [4]. However, we were not able to reproduce their result on the basis of their reported experimental details, because they were by no means accurate enough.

In the preceding paper [1], using 2 mM DNP-α-GalNAc, we found no evidence for Naga-like activities in sera, other than that from the classical lysosomal enzyme at pH 4. In the present study, using a modification of our Naga assay, we have unmasked three additional Naga-like activities. One or two of these could be the activity described by Yamamoto and co-workers [4].

2. Materials and methods

2.1. General methods and treatment of serum

Most of the used materials and methods was as described in the preceding paper [1]. Serum samples earlier used for tumour-marker analyses, here termed 'TM serum' (TM, tumour marker), came from capped tubes stored for up to 3 days at 4 °C. These sera originated from anonymous individuals (females, males, all ages, unless specified otherwise), who had given their consent that after use for the intended analyses, the serum could be used for other purposes, like scientific

Abbreviations: A_{380}, optical absorbance at 380 nm; α-GalNAc, *N*-acetyl-α-D-galactosaminide; DMF, dimethylformamide; DNP, 2,4-dinitrophenol; DNP-α-GalNAc, 2,4-dinitrophenyl-*N*-acetyl-α-D-galactosaminide; MU, 4-methylumbelliferone; Naga, α-*N*-acetylgalactosaminidase (EC 3.2.1.49); Naga6, the collective Naga-like activities in the pH 5 to 6 region; Naga8, the Naga-like activity in the pH 6.5 to 8 region; pNP-α-GalNAc, *para*-nitrophenyl-α-GalNAc; RT, room temperature; S.A., specific activity in nmol per min per mL serum (nmol min^{-1} mL^{-1}), using 2 mM DNP-α-GalNAc; TM, tumour marker

* Corresponding author at: Noordwest Ziekenhuisgroep, Laboratory of Clinical Chemistry, Hematology and Immunology, Juliana van Stolberglaan 13, NL-1814 HB Alkmaar, The Netherlands.
E-mail address: j.van.pelt@nwz.nl (J. van Pelt).

http://dx.doi.org/10.1016/j.bbacli.2017.09.002
Received 9 August 2017; Received in revised form 12 September 2017; Accepted 12 September 2017
Available online 22 September 2017

research. Serum samples were either pooled or assayed individually. Serum referred to as 'routine serum' and 'donor serum', as well as acidification, long-term storage and centrifugation were as described before [1].

Where indicated, serum was treated with 70% saturated $(NH_4)_2SO_4$ in 50 mM Na-citrate buffer (citric acid adjusted to pH 5.8 with 5 M NaOH) plus subsequent dialysis of the re-dissolver pellet against the same buffer, exactly as described by Yamamoto and co-workers [4]. In addition, we have also applied this procedure with some minor modifications, i.e. serum was 10- to 50-fold diluted with a solution of 75% sat. $(NH_4)_2SO_4$ in 50 mM Na-citrate buffer (pH 5.8). After 30 min the suspension was centrifuged (60 min, 3500 × *g*) and the pellet was completely dissolved in 50 mM Na-citrate buffer (pH 5.8). If indicated, remaining $(NH_4)_2SO_4$ was removed by one of the following three methods: (a) By dialysis in a Slide-A-Lyser dialysis cassette with 10 MWCO membranes (Pierce) for 2 h in 300 vol 50 mM Na-citrate buffer (pH 5.8) with replacement of the buffer after 30 min and 1 h; (b) By dialysis for 2 h in a normal dialysis bag in 300 vol of buffer with the same intermittent replacements of the buffer as under (a); (c) By a rapid procedure with a size-exclusion column, originally described by Penefsky [5], using Zeba Desalt Spin Columns (2 mL) from Thermo Scientific (Pierce). These procedures were performed at RT. All dilution factors were taken into account. The dilution due to the acidification of the original serum with 5 M acetic acid (11 µL per mL serum, i.e. 0.989) was the same for all samples. For calculation of the dilution during the $(NH_4)_2SO_4$ precipitation treatment and subsequent removal of salt, all output volumes were determined by weighing and determination of the specific weight.

A saturated solution of $(NH_4)_2SO_4$ (MW 132.1) was prepared by adding 69.7 g to 100 mL water at room temperature (RT). The specific weight of this solution was 1.233 g mL^{-1}, so its concentration was 3.83 M.

2.2. Data treatment

As described in the preceding paper [1], a slow, spontaneous decomposition of the substrate DNP-α-GalNAc was observed. The rate of this reaction, monitored at 380 nm, was only dependent on temperature and substrate concentration, but not on pH, buffer or salt concentration. At 37 °C it followed the equation $-d[S]/dt = k[S]$, where $k = 9.684 \times 10^{-7}\ s^{-1}$. Invariably, a 2 mM solution showed a DNP production rate of 0.291 nmol min^{-1} in an assay volume of 2.5 mL. Conversion of absorbance changes (dA/dt) to mol min^{-1}, using the volume of the assay medium, the pH and Lambert-Beer's law, was carried out as described in the preceding paper [1]. Data handling and line fitting were performed with Microsoft Excel.

2.3. Determination of the pH dependence of the enzyme reaction with serum

For activities in the pH range of 3 to 8, a buffer solution of Na_2HPO_4 in citric acid was used, such that the final concentrations in the assay medium were 70 mM and 50 mM, respectively. The pH of this solution was 4.10. Once filled in a cuvette, the pH was adjusted to the desired pH with either 6 M HCl or 4 M NH_4OH. The required amounts of acid/base were pre-determined from a titration of 25 mL of this buffer solution. Just before and after addition of a serum sample, the cuvettes (six at a time) were capped with parafilm and their contents were mixed by hand (final volume 2475 µL). The capped cuvettes were then incubated in a water bath of 35 °C for ca. 20 min. After incubation the absorbance at 380 nm (A_{380}) was recorded during ca. 20 min at 37 °C. This provided the rate of turbidity change, if any, in each of the cuvettes (enzyme control). Below pH 5 a slight and steady increase at A_{380} was often observed, especially with serum that had been stored in the cold room for more than three days. When serum was diluted in buffer at pH values of 5.5 or higher, no such increase was apparent. After ca. 20 min, the parafilm cover was removed and 25 µL substrate (from an ice-cold 200 mM stock solution in DMF) was added, after which the A_{380} was recorded for another 30 to 50 min. After cooling to RT, the pH was measured in each individual cuvette.

2.4. The Naga6 assay: the Naga-like activity measured at pH 5.8

Routine Naga6 activity assays were carried out as described above, but in a different buffer. The cuvettes were filled with 1000 µL 125 mM tri-sodium citrate, 70 µL 0.6 M HCl, water and sample (total volume 2475 µL, pH 5.8) prior to incubation and 2 mM substrate addition (25 µL 200 mM in DMF). Note that we use the term Naga6 for the collective Naga-like activities in the pH 5 to 6 region.

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

As mentioned in the preceding paper [1], addition of Na^+, K^+ and NH_4^+ ions to buffers affected the pH reading with the used pH meter (691 pH Meter Metrohm; pH electrode: type 6.0234.100). For example, addition of 150 mM Na^+ (at a pH of ca. 4.3) caused the measured pH to be ca. 0.15 units too low. In the present study, where sometimes high concentrations of $(NH_4)_2SO_4$ were used, the shift in the pH readings was found to be dependent on both the $(NH_4)_2SO_4$ concentration (Fig. S1) and the pH (not shown). With pH paper (MColorPlast pH-indicator strips (non-bleeding) pH 0–14 (Merck, Darmstadt)), no shifts in pH could be observed.

Because the colour of DNP is dependent on the pH, these cation-induced shifts in the pH reading were neglected in the calculation of the amount of formed DNP in the enzyme assays.

3. Results

3.1. Comparison of the pH profile of the DNP production from DNP-α-GalNAc of TM serum with that of routine serum

In the preceding paper (Fig. 3 in [1]) a pH profile in 50 mM Na-citrate, 100 mM NaCl (pH range 3 to 6.5) of pooled serum showed one peak of the classical Naga (EC 3.2.1.49) activity around pH 4 (maximal specific activity 1.6 nmol min^{-1} mL^{-1}). There was no indication for any additional peak in the pH 3.5 to 6.5 region. However, although the activity at pH 6 was low (ca. 0.35 nmol min^{-1} mL^{-1}), it was not zero. In the present study a pH profile between pH 3.5 to 8.0 of pooled TM serum (Fig. 1, red curve) was compared with that of pooled, routine serum assayed under identical conditions (Fig. 1, black curve). The black curve is similar to that in Fig. 3 from the preceding paper, but contains more data points over a wider pH range. The pH profile of TM serum was very similar to that of the pooled routine serum, but for an apparent higher activity in the pH 5 to 6 region. The activity peak of the classical Naga in the pH 4 region was prominent in both curves. Thus, under normal assay conditions both profiles showed a maximum activity at around pH 4 of 2.2 nmol min^{-1} mL^{-1}, a minimum at a pH 6.5 and a prominent peak again at pH 8 (up to 1.5 nmol min^{-1} mL^{-1}).

3.2. The reaction velocities of Naga6 and the Naga-like activity at pH 8 (Naga8) decreased with increasing serum concentration

Yamamoto and co-workers claimed that the activity with pNP-α-GalNAc at pH 6 was suppressed by an unknown inhibitor in serum [2,6]. If inhibitors of the Naga6 activity would be present in serum, then, dependent on the nature of inhibition, this might show up in plots of the reaction rate against the serum concentration. Indeed, at pH 6 such a plot, here with TM serum (Fig. 2A), clearly deviated from a straight line, while the specific activity strongly decreased with increasing serum concentration. In contrast, at pH 4.3 such plots showed straight lines both with routine and donor serum (Fig. 2 [1]), as well as with TM serum (this study; not shown). Like the specific activity at pH 6, the specific activity at pH 8 (Naga8) also decreased with