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

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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 D₃-binding protein leads to immunosuppression in cancer patients. Cancer Res. 56, 2827–2831).

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

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, $3500 \times g$) 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 $1.233 \text{ g} \cdot \text{mL}^{-1}$, 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 $k = 9.684 \times 10^{-7} \text{ s}^{-1}$. Invariably, a 2 mM solution showed a DNP production rate of $0.291 \text{ nmol} \cdot \text{min}^{-1}$ in an assay volume of 2.5 mL. Conversion of absorbance changes (dA/dt) to $\text{mol} \cdot \text{min}^{-1}$, 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 $1.6 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mL}^{-1}$). 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. $0.35 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mL}^{-1}$), 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 $2.2 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mL}^{-1}$, a minimum at a pH 6.5 and a prominent peak again at pH 8 (up to $1.5 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mL}^{-1}$).

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
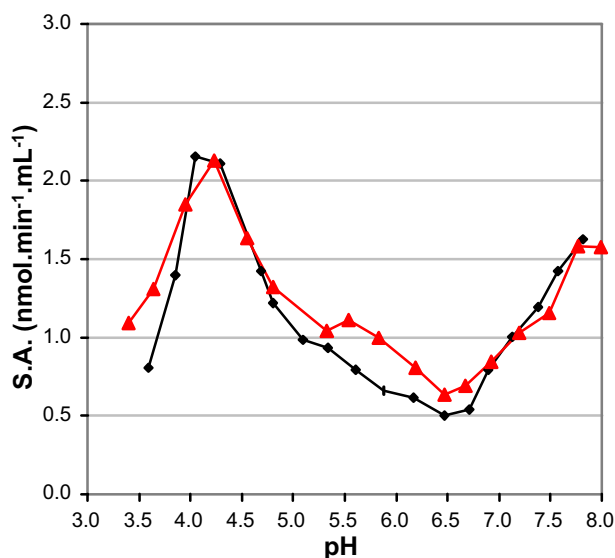
Fig. 1. pH profiles of the Naga reaction in TM and routine serum. The Naga reaction was measured in buffer (50 mM citric acid, 70 mM Na_2HPO_4) of varying pH with 2 mM DNP-α-GalNAc at 37 °C. Black curve, activities of pooled routine serum (250 μL serum in assay, i.e. 10%). Red curve, activities of pooled TM serum (250 μL serum in assay). S.A., specific activity in nmol per min per mL serum (nmol · min^{-1} · mL^{-1}). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

increasing serum concentrations (Fig. 2B).

3.3. Treatment of serum with 70% sat. $(NH_4)_2SO_4$ and subsequent removal of that salt did not stimulate the Naga6 activity

According to Yamamoto et al. [7,8], the inhibitor of the Naga6 activity could be removed by precipitation of the serum protein with 70% saturated $(NH_4)_2SO_4$ in 50 mM Na-citrate buffer (pH 6) and subsequent dialysis of the re-dissolved pellet against this buffer for ca. 2 h [3].

We have followed this procedure using pooled TM serum. If an inhibitor can indeed be removed by $(NH_4)_2SO_4$ precipitation of the protein, and if the inhibitor remains in the supernatant, it is reasonable to assume that sufficient dilution of the serum sample prior to the precipitation step will minimize remnants of the inhibitor in the pellet. It was found that dilution of serum with buffer (at pH 5.8), plus
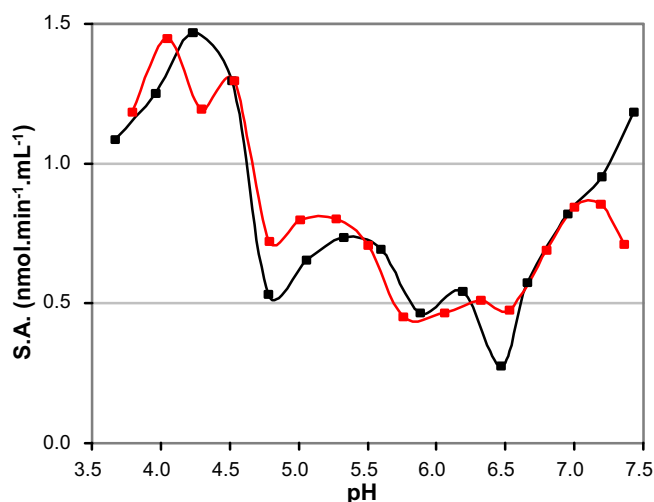

Fig. 3. pH profiles of TM serum before and after precipitation with 70% sat. $(NH_4)_2SO_4$ plus subsequent removal of this salt from the re-dissolved pellet. The activities were determined in 50 mM citric acid, 70 mM Na_2HPO_4 adjusted to the indicated pH. Black curve, activities of untreated serum (250 μL, i.e. 10%). Red curve, activities of treated serum (188 μL serum equivalents, i.e. 7.5%). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

subsequent addition of solid $(NH_4)_2SO_4$ to 70% saturation gave the same results as dilution of serum with buffer containing 75% sat. $(NH_4)_2SO_4$ (i.e. a dilution to approximately the same final high concentration of $(NH_4)_2SO_4$ where all serum proteins precipitate). As an example, the pH profiles of TM serum, before and after $(NH_4)_2SO_4$ precipitation and subsequent removal of this salt, are shown in Fig. 3. With the untreated, pooled TM serum (250 μL serum in assay, i.e. 10% serum), a small but notable, broad peak was observed in the pH 5 to 6 region. However, this activity (Naga6) was hardly changed, after the treatment described by Yamamoto. The activity at pH 6.5–7.5 (Naga8) did not increase either. The treatment did not affect the Naga activity at pH 4. Thus, we could not confirm the claim of Yamamoto et al. that $(NH_4)_2SO_4$ precipitation plus dialysis could remove an inhibitor of the Naga6 activity in serum.

3.4. The Naga6 activity was affected by $(NH_4)_2SO_4$ in the assay

We noticed, that the specific activity at pH 5.8 of the re-dissolved pellet of the $(NH_4)_2SO_4$ precipitation step was often 2- to 4-fold higher
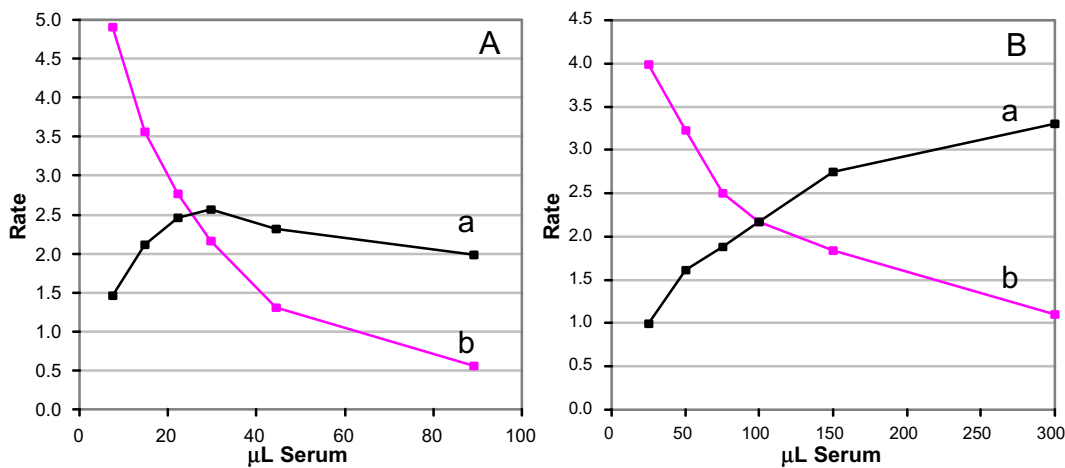

Fig. 2. Dependence of the Naga6 and Naga8 activities on the serum concentration. (A) Pooled TM serum assayed in 50 mM Na-citrate buffer (pH 6.1). Trace a: activity (nmol · min^{-1}) multiplied by 40; trace b: S.A. (nmol · min^{-1} · mL^{-1}). (B) Pooled routine serum assayed in 50 mM citric acid, 70 mM Na_2HPO_4 (pH 7.7). Trace a: activity (nmol · min^{-1}) multiplied by 10; trace b: S.A. (nmol · min^{-1} · mL^{-1}).

than that of the original serum. However, complete removal of the $(NH_4)_2SO_4$ (as in Fig. 3) reduced this activity again to values equal to, or less than, those of the original serum. This did not depend on the method (see Section 2.1.) to remove the $(NH_4)_2SO_4$. The combined results suggested that remnant of $(NH_4)_2SO_4$ in the solution of the re-dissolved pellet from the 70% sat. $(NH_4)_2SO_4$ precipitation step might be responsible for the increase in Naga6 activity.

Indeed, a clear stimulating effect of $(NH_4)_2SO_4$ on the activity at pH 6 of routine serum was observed (Fig. S2A). A maximal Naga6 activity (2.8 nmol·min^{-1}·mL^{-1}) was obtained with 10% sat. $(NH_4)_2SO_4$ in the assay medium. Higher concentrations caused inhibition. With 7.5% of the salt, a plot of the Naga6 rate against the serum concentration was still curved (Fig. S2B, trace a). At low serum concentrations the curve could be extrapolated to the substrate-control rate, measured in the absence of $(NH_4)_2SO_4$. This corroborated the finding that the decomposition rate of the substrate is independent of the salt concentration. Trace b in Fig. S2B shows that specific activities of Naga6 up to 4.2 nmol·min^{-1}·mL^{-1} were obtained at low serum concentrations.

The stimulating effect of $(NH_4)_2SO_4$ on the Naga6 activity was also found with donor serum (Fig. S3, trace a). In that case a nearly 2.5-fold increase was already observed with 4% sat. $(NH_4)_2SO_4$. Like in Fig. S2A, the activity decreased at higher concentrations of the salt. A treatment with 70% sat. $(NH_4)_2SO_4$ precipitation plus subsequent removal of this salt did not change this behaviour (Fig. S3, trace b), although a slight decrease of all activities was observed. Again, this experiment did not confirm the reports of Yamamoto et al. [7,8] that $(NH_4)_2SO_4$ precipitation plus dialysis results in removal of a Naga6 inhibitor.

Figs. S2A and S3 showed that maximal stimulation of the Naga6 activity by $(NH_4)_2SO_4$ in routine and donor sera, occurred at different concentrations of the salt. In Fig. S4 these results were combined with the behaviour of three more sera and are plotted in more detail. The figure shows that the effects of $(NH_4)_2SO_4$ on the Naga6 activity strongly varied with the serum source. However, in all cases a concentration of 1% sat. $(NH_4)_2SO_4$ induced a significant stimulation (1.3 to 1.8 fold, see red dashed line in Fig. S4B). At higher concentrations all activities showed a (variable) maximum, above which a slight inhibition was observed. With 300 μL serum (Fig. S4, trace g) lower activities were found than with 75 μL of the same serum (Fig. S4, trace f), in line with the findings in Section 3.2.

3.5. Optimisation of the assay conditions for the Naga6 activity

In Fig. S4 the effect of $(NH_4)_2SO_4$ on the Naga6 activity was shown for different serum dilutions (73 to 300 μL serum in a final assay volume of 2.5 mL). As shown in Figs. 2 and S2B, optimal Naga6 activities could only be obtained at much lower serum concentrations. Fig. 4 shows the Naga activity in the pH 3.5 to 6.5 region assayed with only 15 μL TM serum, i.e. 0.6% serum. With this low serum concentration a peak in the pH 4.8 to 5.8 region became very prominent (Fig. 4, trace a). Addition of 1% sat. $(NH_4)_2SO_4$ hardly affected the activity at pH 4.1. However, at this low serum concentration a pronounced stimulating effect was observed in the pH 4.8 to 5.8 region (Fig. 4, trace b). Note that with the pooled TM serum the maximum Naga6 activity in the presence of 1% sat. $(NH_4)_2SO_4$ (at pH 5.44) was ca. 1.7-fold higher than the Naga activity at pH 4.1.

3.6. Detailed pH profiles of the Naga6 activity in donor and TM sera

In Fig. 5 detailed pH profiles are shown for six donor sera (panel A) and six TM sera (from patients with an elevated PSA-marker value; panel B), using 20 μL serum (0.8% serum) in 2.5 mL buffer with 1% sat. $(NH_4)_2SO_4$. Four of the donor sera showed apparent maxima at ca. pH 5.1 and 5.8. These maxima were less prominent in the other two samples. The six TM sera showed apparent maxima at pH 5.2 and 5.6.
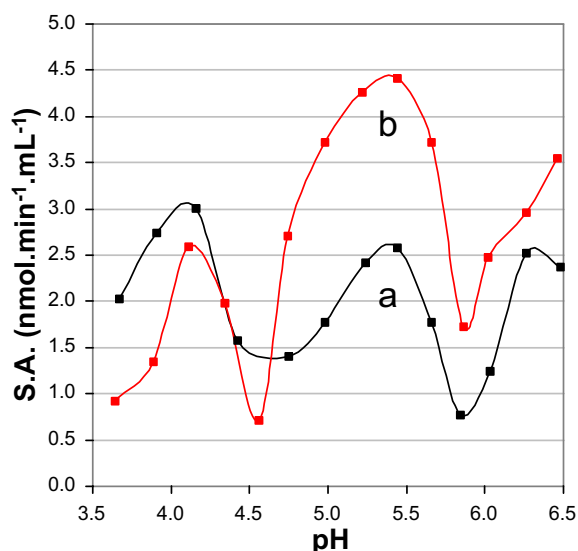

Fig. 4. Optimising the pH profile of TM serum by the use of a low serum concentration and the addition of 1% sat. $(NH_4)_2SO_4$. Pooled TM serum was first diluted 10-fold in 50 mM tri-sodium citrate (pH 6.0) and then 150 μL sample (final serum concentration 0.6%) was assayed in 50 mM tri-sodium citrate adjusted to the indicated (measured) pH with 0.6 M HCl (trace a), or in the same buffer plus 1% sat. $(NH_4)_2SO_4$ (trace b). Because 1% sat. $(NH_4)_2SO_4$ decreases the pH reading by ca. 0.1 pH unit (see Fig. S1), trace b was shifted by 0.1 pH unit to higher values. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

When comparing both figures, it can be noted that the variability in the data points with the donor sera was greater than that with the TM sera. In particular, the range in the Naga activities at pH 5.8 (encircled) in the donor sera (1.8 to 3.5 nmol·min^{-1}·mL^{-1}) was larger than that in the TM sera (2.1 to 2.7 nmol·min^{-1}·mL^{-1}). The absolute activities of the TM sera were similar to or lower than, but not higher than those observed in the donor sera.

3.7. Variability of the Naga activity at pH 4.3 in TM sera

Fig. 5A showed that the Naga6 activity at pH 5.8 in the six donor sera displayed a larger variability than the same activity in six TM sera (Fig. 5B). To examine whether this also holds for the Naga activity at pH 4.3, a set of 30 TM sera was investigated (Fig. 6A) and compared to the set of 18 donor sera used in the preceding paper [1] (Fig. 6B). Whereas the variability in the data points of the 18 donor sera was 13%, it was considerably larger (48%) with the 30 TM sera. Moreover, the average activity of the TM sera (1.42 ± 0.69 nmol·min^{-1}·mL^{-1}) was lower than that of donor sera (1.77 ± 0.23 nmol·min^{-1}·mL^{-1}). In addition, the Naga activity in the TM sera seemed to increase from 1.0 to 2.0 nmol·min^{-1}·mL^{-1} with decreasing values of the tumour markers.

3.8. Substrate inhibition of the Naga6 activity

All the above mentioned assays were performed with 2 mM substrate. To obtain an impression of the K_m value of DNP-α-GalNAc for the Naga6 activity, the substrate concentration was varied between 1 and 6 mM, using 25 μL of TM serum. However, the resulting plots (Fig. S5) were not like those expected for normal Michaelis-Menten kinetics. Instead, they were indicative for substrate inhibition [9]. A similar result was also found with other sera. Thus, the substrate apparently binds to the Naga6 enzyme at a secondary site where it causes inhibition.
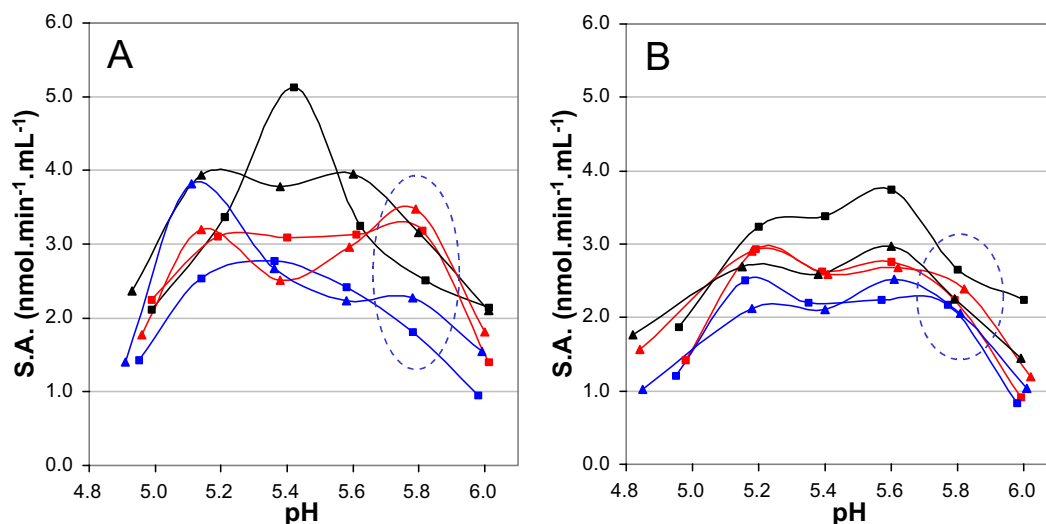
Fig. 5. Detailed pH profiles (pH 5 to 6) of the reaction of DNP-α-GalNAc with donor and TM sera in buffer plus 1% sat. $(NH_4)_2SO_4$. (A) pH profiles of sera from six apparently healthy females. (B) pH profiles of sera from six patients with elevated PSA values (11.9 to 38.1 ng · mL^{-1}). In all cases, serum was first diluted 10-fold in 50 mM tri-sodium citrate (pH 6.0) and then 200 μL sample (final serum concentration 0.8%) was assayed in 50 mM tri-sodium citrate (adjusted to the indicated (measured) pH with 0.6 M HCl) plus 1% sat. $(NH_4)_2SO_4$.

4. Discussion

4.1. Presence in serum of inhibitors of the Naga6 and Naga8 reactions

The decrease of the specific Naga6 activity with increasing serum concentration (Figs. 2 and S2B) is consistent with the notion [2,6], that an endogenous inhibitor is affecting its activity in serum. At higher serum concentrations, with more inhibitor present, the activity becomes nearly completely inhibited.

We note, however, that with untreated, pooled TM serum (10% serum in assay), an enhanced activity between pH 5 and 6 was observed in the pH profile which was not observed with routine serum (10% serum in assay, Fig. 1). This leaves the possibility open that among the TM-serum samples used to prepare the pooled serum for this experiment, there may have been one or more samples with a strongly enhanced Naga6 activity.

In the assays reported by Yamamoto et al. the medium contained 9% [2] to 15% [3] serum. With cancer patients, before the start of their medical treatment, they found Naga6 activities up to ca. 25 times the

values observed in apparently healthy individuals. Such high values have not been observed in the present study. However, in the present study all TM sera were from cancer patients who presumably already received treatment. Therefore, it remains to be firmly established whether the Naga6 activity in serum from cancer patients prior to the start of their treatment is indeed elevated and whether it can serve as a general marker for cancer as proposed by Yamamoto and co-workers [2–4].

4.2. Effects of $(NH_4)_2SO_4$ on the Naga and Naga6 activities

A concentration of 1% sat. $(NH_4)_2SO_4$ hardly affected the Naga activity at pH 4.3 (Fig. 4). However, it did stimulate the Naga6 activity in all tested samples (Fig. S4B), but at higher concentrations the activity decreased again. The concentration required for optimal stimulation of the Naga6 activity was highly dependent on the serum source. The ionic strength of the used buffer (50 mM tri-sodium citrate adjusted with a known amount of 0.6 M HCl to pH 5.86; calculated ionic strength 310 mM) increased by the addition of 1% sat. $(NH_4)_2SO_4$ (38.3 mM,
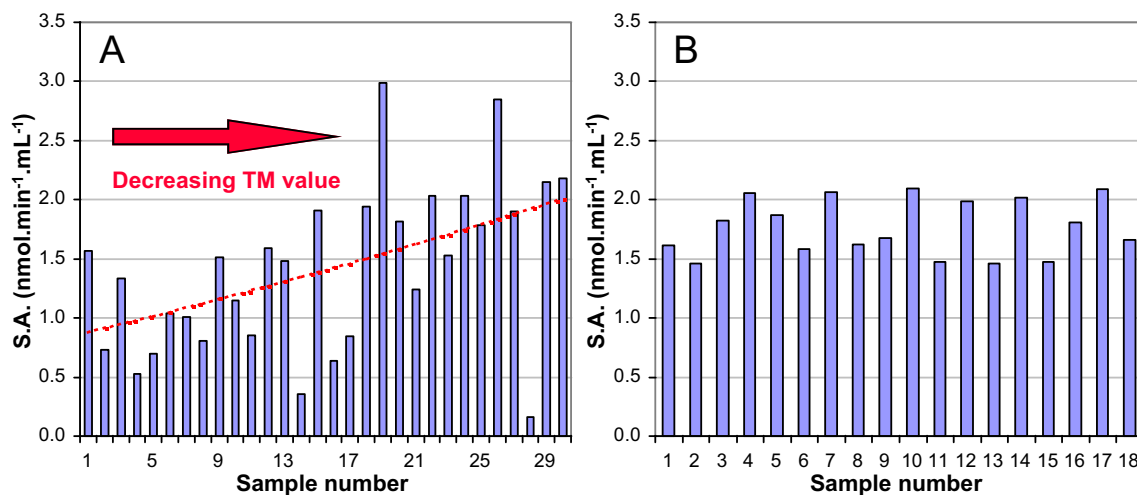

Fig. 6. Naga activity at pH 4.3 in 30 TM sera compared to the activity in 18 donor sera. (A) Naga activity at pH 4.3 in samples from 30 TM sera (average 1.42 ± 0.69 nmol · min^{-1} · mL^{-1} (48%); range: 0.16 to 2.99). The samples were sorted from left to right by descending tumour-marker values (range 10,000 down to 2 units; markers CEA, CA19.9, CA15.3, CA125). The red dotted line is a second-order polynomial fit to the data. (B) Naga activity (data from [1]) in serum from 18 apparently healthy male individuals (average 1.77 ± 0.23 nmol · min^{-1} · mL^{-1} (13%); range 1.46 to 2.10). The assays were performed at 37 °C in 50 mM Na-citrate, 100 mM NaCl (pH 4.3) and 2 mM DNP-α-GalNAc, while using 10% serum. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

calculated ionic strength 115 mM) and this could be a possible reason for the enhanced activity. However, addition to the buffer of NaCl, NH_4Cl or Na_2SO_4 with an ionic strength of 115 mM did not give the same stimulation as that with 1% sat. $(NH_4)_2SO_4$. Hence, this stimulation is apparently not solely an effect of the increased ionic strength.

4.3. Inhibitors of Naga6 could not be removed by 70% sat. $(NH_4)_2SO_4$ precipitation and subsequent removal of this salt

We could not detect any increase of the Naga6 activity in serum by 70% sat. $(NH_4)_2SO_4$ precipitation plus subsequent removal of this salt from the re-dissolved pellet (Figs. 3 and S4). We note that any remaining $(NH_4)_2SO_4$ in the re-dissolved pellet after the $(NH_4)_2SO_4$ precipitation step will enhance this activity. The stimulating effect of this salt is amply shown in the present paper. Therefore, we are inclined to assume that the dialysis procedure of Yamamoto et al. [3] did not remove all $(NH_4)_2SO_4$. The remnants $(NH_4)_2SO_4$ caused a higher Naga6 activity than that of untreated serum.

4.4. Possible origin of the Naga6 and Naga8 activities

Figs. 4 and 5 confirm the reports of Yamamoto and co-workers, that serum contains an extra Naga-like activity that can be measured at pH 6 [2–4]. In fact, our data indicate that there are (presumably) two extra activities in the pH 5 to 6 region with slightly different pH optima. Note that 1% sat. $(NH_4)_2SO_4$ will shift the pH reading by ca. 0.1 pH unit (Fig. S1), so the pH of the maxima in Fig. 5 should be increased by 0.1 pH unit.

We can only speculate on the possible origin of the Naga6 and Naga8 activities. Den Tandt has described the activities of 14 lysosomal glycohydrolases in blood [10]. One possibility is that the Naga6 and Naga8 activities are side activities of one or more of these enzymes.

Yamamoto and co-workers proposed that the elevated Naga6 activity (Nagalase, an *exo*-glycosidase activity at pH 6) was an *endo*-glycosidase excreted by cancer cells [2–4]. When examining their data, that is a real possibility. They reported that the elevated Naga6 activity drastically decreased one day after removal of the primary tumour or of metastasized lymph nodes [3]. Another group [11] has described similar observations. This is consistent with the proposal that the tumours constantly excrete the enzyme responsible for this activity. The level of the serum activity would then depend on the rate of its formation by the tumours and the rate of its removal from the blood circulation by the patient.

In 2009 it has been reported [12] that recombinant *endo-α-N*-acetylgalactosaminidase from *Streptococcus pneumoniae* R6 showed an activity at pH 6.5 with DNP-α-GalNAc (K_m 2.4 mM, k_{cat} 1.6 s^{-1}, an *exo*-glycosidase activity), which was ca. 0.3% of the activity with DNP-α-GalNAc-3,1-β-Gal (K_m 34 μM, k_{cat} 541 s^{-1}, the *endo*-glycosidase activity). Unfortunately, Nagalase has never been purified and/or characterized, so its origin in human serum remains to be investigated.

4.5. Units of the Naga6 activity

We have expressed all Naga activities in nmol per min per mL serum ($nmol^{-1} \cdot min^{-1} \cdot mL^{-1}$). However, Yamamoto and co-workers always expressed the Naga6 activity in "$nmol \cdot min^{-1}$ per mg of serum protein" [4] and serum activities ranged from 0.23 to 0.39 $nmol \cdot min^{-1} \cdot mg^{-1}$ in apparently healthy individuals and up to 8 $nmol \cdot min^{-1} \cdot mg^{-1}$ in cancer patients [2,3]. Similar units were also reported by two other groups [11,13].

Serum contains a large amount of protein (62 to 84 $mg \cdot mL^{-1}$). Yamamoto et al. used the substrate pNP-α-GalNAc. Assuming an average protein content of 73 $mg \cdot mL^{-1}$ and using Yamamoto's Naga6-activity values for serum from healthy individuals of 0.3 $nmol \cdot min^{-1} \cdot mg^{-1}$ and up to 8 $nmol \cdot min^{-1} \cdot mg^{-1}$ for TM serum, this would be equivalent to Naga6 activities of 22 to 580 $nmol \cdot min^{-1} \cdot mL^{-1}$. Such values are in

sharp contrast to the activity measured in the present paper with the substrate DNP-α-GalNAc with Naga6 activities of 0.5 to 5 $nmol \cdot min^{-1} \cdot mL^{-1}$. There is consensus that the Naga activity at pH 4.3 in serum from healthy individuals, measured with 1 mM MU-α-GalNAc, is ca. 0.3 $nmol \cdot min^{-1} \cdot mL^{-1}$ [14,15]. The Naga6 activity for serum from healthy individuals reported by Yamamoto would then be two-orders of magnitude greater than the Naga activity. In view of the pH profiles determined in the present paper, this is totally unrealistic. Likewise, based on the assay conditions reported by Yamamoto and co-workers, one can calculate that the substrate pNP-α-GalNAc must have been used up during the 1 h assay. We do not understand these discrepancies. If, by some co-incidence, the units $nmol \cdot min^{-1} \cdot mg^{-1}$ [4] should have been $nmol \cdot min^{-1} \cdot mL^{-1}$, then the Naga6 activities in serum from healthy individuals would closely match those in the present paper.

4.6. Importance of the serum Naga6 activity for early detection of cancer

Yamamoto and co-workers suggested that the serum Naga6 activity of individual patients might serve as a general diagnostic tumour marker for a broad range of cancers [2–4]. We note that it cannot be ruled out that the serum Naga6 activity in individuals affected by beginning cancers may increase even before any symptoms of the disease would appear. Thus, we support Yamamoto's suggestion [2] that a regular clinical test for the Naga6 activity may be a valuable tool to help in the detection of upcoming tumours. It would also be interesting to measure whether the Naga activities (at pH 4.3) in potential patients deviate from normal, or do so upon treatment of such patients. Fig. 6 suggests that such anomalies might occur.

5. Conclusions

We conclude that sera from apparently healthy individuals and from cancer patients contain extra Naga-like activities with pH optima in the pH 5 to 6 region, Naga6, in addition to the classical Naga with an optimum at pH 4. This Naga6 activity is presumably the activity ('Nagalase') that Yamamoto and co-workers have reported on two decades ago [2,4]. The reason that the Naga6 activity has escaped detection in all earlier studies on the classical lysosomal Naga enzyme in serum, is the fact that high serum concentrations (30 to 50%; see preceding paper [1]) were used in the enzyme assay. These high concentrations suppress the Naga6 activity. With low serum concentrations in the assay (0.6 to 1%) the Naga6 activity is clearly detectable. Addition of 1% sat. $(NH_4)_2SO_4$ further enhances this activity. The assay can easily be adapted for use with a plate reader or a spectrophotometer for semi-automatic analysis.

Conflict of interest

Transparency document

Acknowledgements

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbacli.2017.09.002.

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