Glycoconj J (2008) 25:101–109
DOI 10.1007/s10719-007-9058-8

Isoenzyme-specific differences in the degradation of hyaluronic acid by mammalian-type hyaluronidases

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Received: 11 May 2007 / Revised: 12 June 2007 / Accepted: 15 June 2007 / Published online: 10 July 2007

Abstract Bovine testicular hyaluronidase (BTH) has been used as a spreading factor for many years and was primarily characterized by its enzymatic activity. As recombinant human hyaluronidases are now available the bovine preparations can be replaced by the human enzymes. However, data on the pH-dependent activity of hyaluronidases reported in literature are inconsistent in part or even contradictory. Detection of the pH-dependent activity of PH-20 type hyaluronidases, *i.e.* recombinant human PH-20 (rhPH-20) and BTH, showed a shift of the pH optimum from acidic pH values in a colorimetric activity assay to higher pH values in a turbidimetric activity assay. Contrarily, recombinant human Hyal-1 (rhHyal-1) and bee venom hyaluronidase (BVH) exhibited nearly identical pH profiles in both commonly used types of activity assays. Analysis of the hyaluronic acid (HA) degradation products by capillary zone electrophoresis showed that hyaluronan was catabolized by rhHyal-1 continuously into HA oligosaccharides. BTH and, to a less extent, rhPH-20 exhibited a different mode of action: at acidic pH (pH 4.5) HA was degraded as described for rhHyal-1, while at elevated pH (pH 5.5) small oligosaccharides were produced in addition to HA fragments of medium molecular weight, thus explaining the pH-dependent discrepancies in the activity assays. Our results suggest a sub-classification of mammalian-type hyaluronidases into a PH-20/BTH and a Hyal-1/BVH subtype. As the biological effects of HA fragments are reported to depend on the size of the molecules it can be speculated that different pH values at the site of hyaluronan degradation may result in different biological responses.

Keywords Bovine testicular hyaluronidase · Human Hyal-1 · Human PH-20 · Bee venom hyaluronidase · Capillary zone electrophoresis

Abbreviations

BSA	bovine serum albumin
BTH	bovine testicular hyaluronidase
BVH	bee venom hyaluronidase
CZE	capillary zone electrophoresis
GlcNAc	*N*-acetylglucosamine
GlcUAc	glucuronic acid
HA	hyaluronic acid
n	hyaluronic acid disaccharide unit
rhHyal-1	recombinant human Hyal-1
rhPH-20	recombinant human PH-20
SEC	size exclusion chromatography

Introduction

Hyaluronidases represent a group of enzymes catalyzing the depolymerization of certain acidic glycosaminoglycans with hyaluronic acid (HA) as the preferred substrate. According to their hydrolytic mechanisms hyaluronidases were classified into three main families [1]. The hyaluronidases of the first group, endo-β-*N*-acetylglucosaminidases (mammalian-type hyaluronidases, *e.g.* testicular, lysosomal and bee venom hyaluronidase, EC 3.2.1.35) catalyze the hydrolysis of the β-1,4 glycosidic bond. The second group, termed β-eliminases

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or lyases (EC 4.2.2.1), include bacterial hyaluronidases and catabolize HA in a β-elimination of the β1-4 glycosidic bond. The third group of hyaluronidases comprises endo-β-glucuronidases (leech hyaluronidase, EC 3.2.1.36).

The best characterized mammalian hyaluronidase is bovine testicular hyaluronidase (BTH; Hylase® "Dessau," Neopermease® and Wydase®), an enzyme preparation that has been therapeutically applied as spreading factor, degrading the glycosaminoglycans of the extracellular matrix [2, 3], for many years. The human hyaluronidases, however, have been available in only very small amounts until recombinant protein production systems enabled the production and purification of larger quantities [4–6]. As the first engineered human hyaluronidases, Hylenex (Baxter, Deerfield, IL, USA) and Cumulase (Halozyme, San Diego, CA, USA), are now commercially available on the market and also used in clinical trials, the capability of the methods for the characterization of these enzyme preparations is of major importance. Hylenex and Cumulase are, according to the suppliers, soluble recombinant variants of human PH-20. This subtype of hyaluronidase is mainly located on the sperm-head, facilitating fertilization by degradation of the HA-rich cumulus extracellular matrix of the oocyte [7]. Hyaluronidase preparations from mammalian testes, such as BTH, contain different hyaluronidase isoforms of the PH-20 subtype [8].

According to different pharmacopoeiae, hyaluronidase preparations from mammalian testes are exclusively characterized by their enzymatic activity. However, a problem in the determination of the hydrolytic activity of these enzymes arises from the fact that BTH, and presumably all mammalian-type hyaluronidases, additionally have transglycosylase activity [9, 10]. Data on the pH-dependent activity of BTH reported in the literature are inconsistent in part or even contradictory: optimum activity of BTH was found at acidic pH (pH 3.5–4.0) [11–13] as well as at weakly acidic pH (pH 5.0–6.0) [14–16] and at neutral pH [17, 18]. However, the pH optimum of human Hyal-1, located in lysosomes of the liver, in plasma and in urine, was reported consistently to be at acidic pH (pH 3.5–3.8) [19–21] by several authors.

Therefore, we investigated the pH-dependent activity of BTH, recombinant human PH-20 (rhPH-20), recombinant human Hyal-1 (rhHyal-1) and bee venom hyaluronidase (BVH) in two different hyaluronidase activity assays. These assays, the colorimetric (Morgan–Elson) assay and the turbidimetric assay, are widely accepted hyaluronidase activity assays, used for the comparison of hyaluronidase activities of different enzyme preparations.

In the colorimetric assay (Morgan–Elson assay) product formation is detected by derivatization of *N*-acetylglucosamine (GlcNAc) residues at the reducing end of HA fragments generated by HA hydrolysis and transglycosylation. According to the International Union of Biochemistry 1 μmol of reducing GlcNAc end groups liberated per min under specified assay conditions is used for the definition of one hyaluronidase catalytic unit (1 U) [23]. The turbidimetric assay is based on the precipitation of HA fragments with a molecular mass >6–8 kDa with quaternary ammonium salts. Thus, degradation of high molecular weight HA causes a decrease in turbidity [24, 25]. The turbidity assay was used for the definition of the "International Standard for Hyaluronidase" and the International Unit (IU) [26].

It is generally accepted that HA fragments exert different biological effects depending on their molecular weight (for review see [22]). The size of the HA fragments produced by hyaluronidases and the effect on processes such as angiogenesis, apoptosis and immune response is of particular interest with respect to tumorigenesis, invasion and metastasis.

In the present study we demonstrate that the mammalian-type hyaluronidases, *i.e.* the hyaluronate 4-glycanohydrolases, can be divided with respect to their pH-dependent activity in the colorimetric and in the turbidimetric activity assay into two groups: those hyaluronidases of the Hyal-1/BVH type exhibiting identical pH-profiles in both assays and those of the PH-20 type exhibiting a shift in the pH activity profile in the two different assay systems. Analysis of the HA degradation products of BTH, rhPH-20 and rhHyal-1 by capillary zone electrophoresis revealed the formation of HA fragments of distinct size depending on the pH and on the type of the hyaluronidase used.

Materials and methods

Chemicals

BTH (Neopermease®) was a gift from Sanabo (Vienna, Austria). Recombinant human Hyal-1 (rhHyal-1) and PH-20 (rhPH-20) were expressed in *Drosophila* Schneider-2 cells and purified as described [4, 27]. Purified bee venom hyaluronidase (BVH) was a gift from Z. Markouvic–Housley, University of Basel, Switzerland [28]. Hyaluronic acid (HA) from *Streptococcus zooepidemicus* was purchased from Aqua Biochem GmbH (Dessau, Germany). Bovine serum albumin (BSA) was purchased from Serva (Heidelberg, Germany). Cetyltrimethylammonium bromide (CTAB) was from Roth (Karlsruhe, Germany). All other chemicals were of analytical grade and were obtained from Merck (Darmstadt, Germany). Water was purified by a Milli-Q system (Millipore, Eschborn, Germany).

Colorimetric hyaluronidase activity assay (Morgan–Elson assay)

The colorimetric determination of the hyaluronidase activity was based on the method of Reissig *et al.* [29] and

performed as described previously [12]. For determination of the pH profiles the incubation mixtures consisted of McIlvaine's buffer (citrate/phosphate 44 mM/88 mM), containing 44 mM NaCl, 0.56 mg/ml HA and 44 μg/ml BSA. The reaction was started by addition of the respective enzyme solubilized in 0.2 mg/ml BSA (BTH and BVH) and in 50 mM sodium phosphate, 0.5 M NaCl, 0.1% Triton X-100, 1 mM EDTA, pH 6.0 (rhPH-20 and rhHyal-1), respectively. The enzyme solution comprised 11% of the total incubation volume. McIlvaine's buffer was prepared by mixing the citric acid and the Na_2HPO_4 solution to adjust the required pH. For variation of the HA concentration the incubation mixture described above was prepared with 0.23 mg/ml HA instead of 0.56 mg/ml HA. The incubation mixtures were incubated at 37°C for 1 h.

For the determination of the enzymatic activity during CZE analysis CZE incubation mixtures were prepared and treated as described above after incubation for a defined time period. The absorbance of the Morgan–Elson reaction product was measured at 585 nm (A_{585}).

Turbidimetric hyaluronidase activity assay

The turbidimetric determination of the hyaluronidase activity was based on the method of Di Ferrante [30] and performed as described [4].

Incubation mixtures were identical to those described in the colorimetric activity assay, but contained 0.22 mg/ml HA instead of 0.56 mg/ml HA.

Capillary zone electrophoresis (CZE)

High molecular weight HA was incubated for different time periods in the following incubation mixtures:

- BTH, pH 4.5: In a total volume of 450 μl 44 μg/ml BSA, 22 mM HOAc/NaOAc, pH 4.5, 22 mM NaCl, pH 4.5, 0.56 mg/ml HA and 200 IU of BTH (according to the supplier) were incubated for a defined time period.
- BTH, pH 5.5: analogous to BTH, pH 4.5, but using 22 mM HOAc/NaOAc, pH 5.5 and 460 IU of BTH (according to the supplier).
- rhPH-20, pH 4.5: analogous to BTH, pH 4.5, but containing rhPH-20 with an activity 0.08 nmol GlcNAc min^{-1} (determined by the colorimetric assay at pH 5.0).
- rhPH-20, pH 5.5: analogous to BTH, pH 5.5, but using rhPH-20 with an activity of 0.08 nmol GlcNAc min^{-1} (determined by the colorimetric assay at pH 5.0).
- rhHyal-1: analogous to BTH, pH 4.5, but using 22 mM formic acid/Na-formate, pH 3.5 and 6.3 nmol GlcNAc min^{-1} of rhHyal-1 (determined by the colorimetric assay at pH 3.5).

After incubation at 37°C for various time periods the enzymatic reaction was stopped by addition of 900 μl of cold acetonitrile. The samples were dried in the Speed Vac (Savant speed-vac Plus SC110A, Divebid, Cambridge, USA), dissolved in 225 μl of tenfold diluted CZE operating buffer and centrifuged for 10 min at 12,000×*g*. The supernatant was used for CZE analysis.

CZE conditions were adapted from Grimshaw [31]. CZE analysis was carried out in the normal mode on a Biofocus 3000 capillary electrophoresis system (Biorad, München, Germany) equipped with an uncoated fused silica capillary (75 cm×50 μm; Chrompack). Operating buffer was 50 mM Na_2HPO_4, 20 mM $Na_2B_4O_7$, pH 9 and sample buffer was operating buffer diluted tenfold with Milli-Q water. The sample was injected by pressure (20 psi · s) and analysis was performed at a capillary temperature of 15°C applying a voltage of 15 kV. The detector wavelength was 200 nm. Between the individual analyses the capillary was conditioned with 0.1 M NaOH (200 s), Milli-Q water (200 s) and finally with the operating buffer (300 s).

Identification of low molecular weight oligosaccharides in the CZE

Low molecular weight oligosaccharides (n_1–n_4) were identified by the mean differences in the migration times between peaks of HA oligosaccharides. The mean differences in the migration times were measured in 14 independent experiments for mixtures of pure HA di-(n_1), tetra-(n_2), hexa-(n_3) and octasaccharides (n_4). Oligosaccharides were produced and identified as described previously [27].

Results

pH activity profiles of BTH, rhPH-20, rhHyal-1 and BVH

The enzymatic activity of the hyaluronidases was determined in the turbidimetric and the colorimetric hyaluronidase activity assay under very similar conditions.

For BTH (Neopermease®) and rhPH-20 the pH profiles measured with the two different assays yielded inverse curves (Fig. 1). In the colorimetric assay maximum activity was found at acidic pH (pH 3.5 for BTH, pH 4.5 for rhPH-20), whereas the turbidimetric assay showed the maximum activity at higher pH values (pH 5.5–6.0 for BTH and rhPH-20). Compared to rhPH-20 the pH profiles of BTH exhibited more asymmetric curves with a larger difference in the pH optima (Fig. 1). Furthermore, a second small maximum with 60% of the maximal activity was detected in the turbidimetric assay at pH 3.5 (Fig. 1a). In contrast to PH-20, which consists of a single enzymatically active protein fraction of 56 kDa [27], Neopermease® comprises

three enzymatically active protein fractions (33, 58 and 77 kDa) [8] with the 58 kDa fraction showing the highest activity in both assays. While the 33 and 77 kDa fractions exhibited maximum enzymatic activity around pH 6.0 in both assays, the highly active 58 kDa fraction exhibited the inverse pH activity profile, typical for the whole preparation (Fig. 2). Since the 58 kDa fraction exhibits the highest enzymatic activity [8] the pH profile of the whole BTH preparation is dominated by the activity of this fraction.

From previous investigations it is known that changes in the composition of the incubation mixture with respect to the type of buffer, NaCl concentration and BSA concentration, cause a shift of the pH optimum of BTH [32, 33]. Therefore, both assays were performed under identical incubation conditions, except for the concentration of HA,

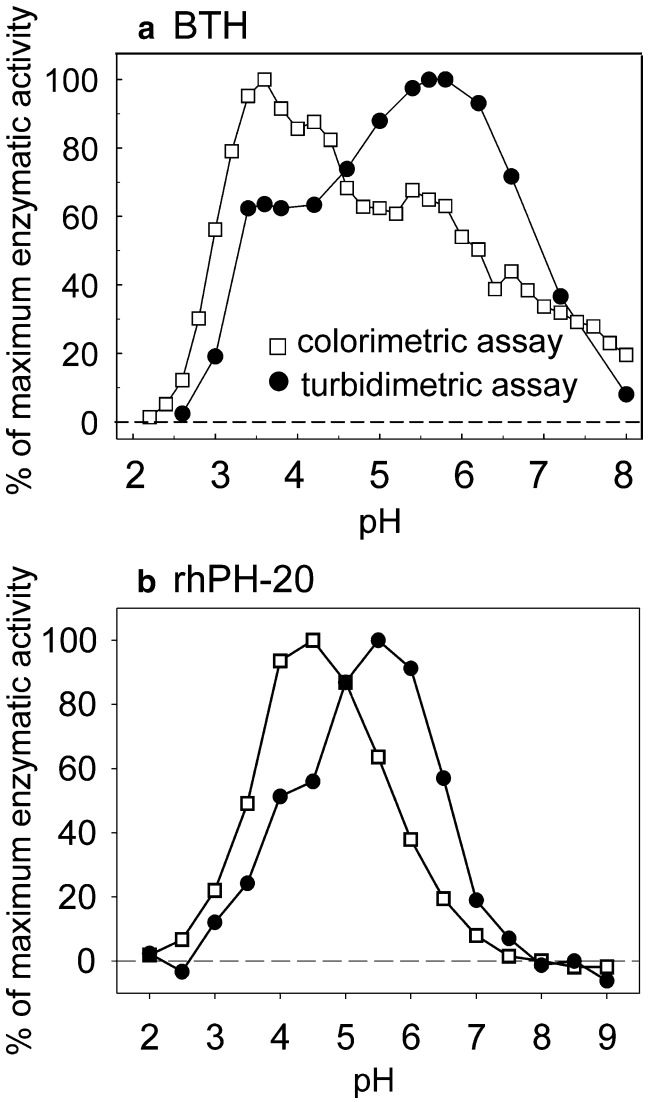

Fig. 1 Effect of pH on the activity of BTH (**a**) and rhPH-20 (**b**) determined by the colorimetric method (*white squares*) and the turbidimetric assay (*black circles*). Maximum enzymatic activity was set 100% for each individual curve

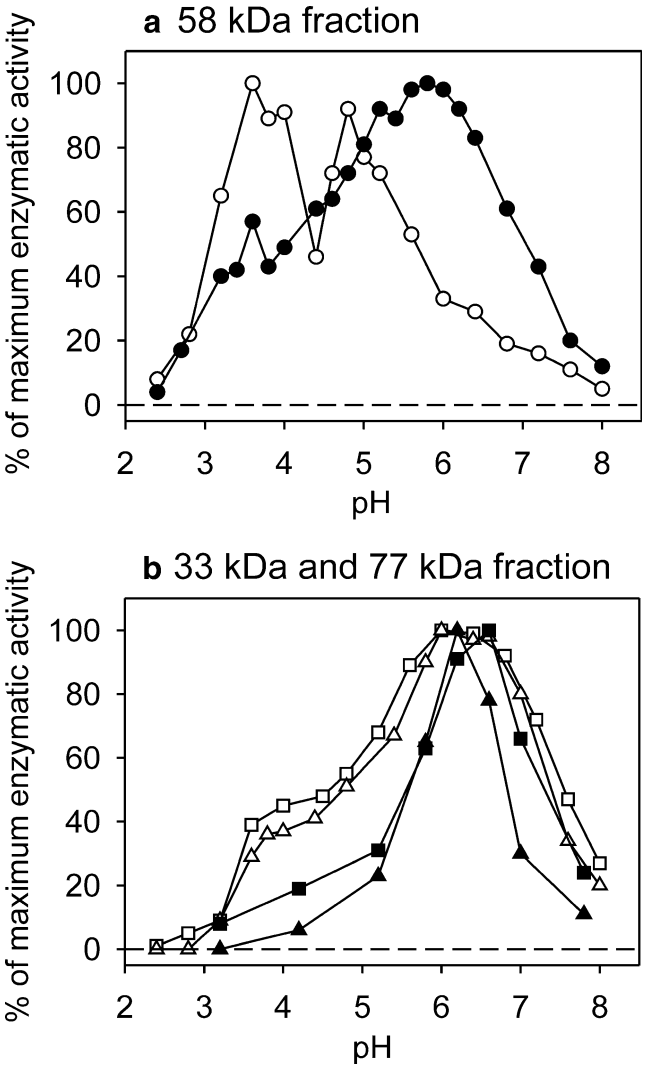

Fig. 2 Effect of pH on the activity of the SEC fractions, isolated from BTH as described in [8]. BTH contained three enzymatically active fractions: a 33 kDa fraction (*squares*), a 58 kDa fraction (*circles*) and a 77 kDa fraction (*triangles*). pH profiles for the 58 kDa (**a**) and the 33 and 77 kDa fraction (**b**) were detected in both, the colorimetric (*white symbols*) and the turbidimetric activity assay (*black symbols*). Maximum enzymatic activity was set 100% for each curve. For comparison activity data measured in the colorimetric activity assay in a previous study [8] are included

which was chosen by a factor of 2.5 lower in the turbidimetric assay to guarantee a linear relationship between the HA concentration and the induced turbidity. However, variations of the HA concentration (0.22 and 0.56 mg/ml) in the colorimetric assay did not affect the pH profile obtained with this assay (data not shown).

In contrast to BTH and rhPH-20, both belonging to the subtype of PH-20, BVH and rhHyal-1 exhibited nearly identical pH activity profiles in the colorimetric and in the turbidimetric hyaluronidase activity assay (Fig. 3). Both enzymes showed maximum enzymatic activity at pH 3.5–4.0 and extremely low or no activity at neutral pH.

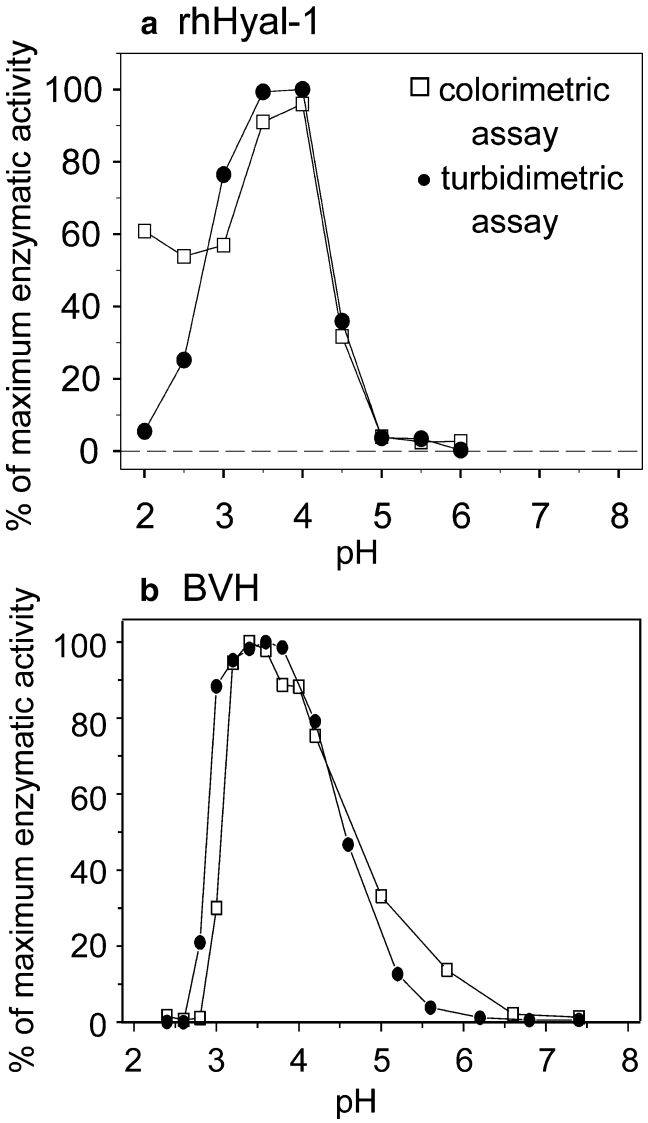

Fig. 3 Effect of pH on the activity of rhHyal-1 (**a**) and BVH (**b**) determined by the colorimetric method (*white squares*) and the turbidimetric assay (*black circles*). Maximum enzymatic activity was set 100% for each curve

Hyaluronate digestion by BTH

To gain more detailed insight into the degradation processes, the HA digestion products were analyzed by capillary zone electrophoresis (CZE). The negatively charged HA chains consisting of repeating (βGlcUAc1–3βGlcNAc1-4) disaccharide units (in the following designated with *n*) were separated showing increasing migration times with increasing chain length [31, 34, 35]. As described previously [35], high molecular weight HA ($n_{2,500}$–$n_{5,000}$, $1–2 \cdot 10^6$ Da, according to the supplier) formed a single peak in the CZE due to the small differences in the charge to mass ratio of the high molecular weight HA chains.

To enable a direct comparison of the degradation products produced by BTH and by rhPH-20 both PH-20 type enzymes were allowed to degrade HA at pH 4.5 and 5.5, the pH optima of rhPH-20 in the turbidimetric and in the colorimetric assay, respectively.

As shown in Fig. 4a, high molecular weight HA was degraded by BTH at pH 4.5 sequentially into smaller fragments with di-(n_1) to octasaccharides (n_4) accumulating as final products, the hexasaccharide (n_3) being the main degradation product. However, at pH 5.5 HA fragments of a different sizes were observed compared to pH 4.5: high molecular weight HA was degraded to small oligosaccharides (n_1–n_4), but also fragments of higher molecular

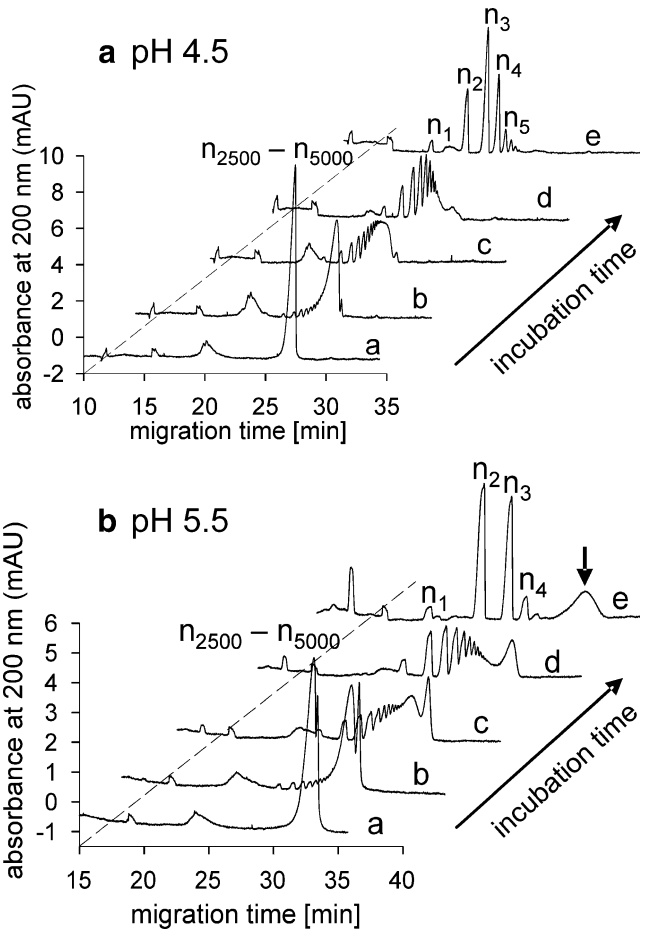

Fig. 4 CZE analysis of HA digestion mixtures produced by the action of BTH at pH 4.5 (**a**) and pH 5.5 (**b**). The degree of polymerization (*n*) is given as multiples of (βGlcUAc1–3βGlcNAc1-4) disaccharide units. The activity of BTH was determined by the colorimetric activity assay with the absorbance of the reaction product at 585 nm, in the following designated as A_{585}. Enzymatic activity was stopped by addition of acetonitrile after *a*, 0 min (0.0 A_{585}); *b*, 10 min (0.2 A_{585}); *c*, 20 min (0.4 A_{585}); *d*, 90 min (0.6 A_{585}); and *e*, 16 h (1.1 A_{585}) at pH 4.5 (**a**) and 17 h (1.3 A_{585}) at pH 5.5 (**b**). The fraction of high molecular weight HA fragments occurring at pH 5.5 is indicated with an *arrow*

weight were still present in the incubation mixture (arrow in Fig. 4b). The exact size of the higher molecular weight HA fragments could not be determined since no separation of the fragments was achieved at these charge to mass ratios. A very small fraction of the higher molecular weight HA fragments could also be observed during degradation of HA by BTH at pH 4.5 (Fig. 4a).

At the respective pH used for the CZE analysis the enzymatic activity of BTH produced a comparable amount of GlcNAc ends per time as determined in the colorimetric hyaluronidase activity assay. The absorbance of the Morgan–Elson product detected at 585 nm (A_{585}) confirmed that the amount of reducing GlcNAc ends at pH 4.5 and 5.5 was nearly identical for each incubation period (Fig. 4).

Hyaluronate digestion by rhPH-20

The degradation of high molecular weight HA by rhPH-20 was monitored at the pH optimum of the enzyme in the turbidimetric (pH 5.5) and the colorimetric assay (pH 4.5; Fig. 5). Due to instability problems during storage the enzymatic activity of PH-20 available for the CZE experiments was significantly lower than the activity used for the degradation of HA by BTH and Hyal-1. The low activity was compensated by extended incubation periods in order to gain similar amounts of reducing GlcNAc groups for each of the time points analyzed with CZE. As shown in Fig. 5 the HA degradation products formed at pH 4.5 and at pH 5.5 were very similar. The formation of small oligosaccharides in the presence of HA fragments of higher molecular weight, as observed with BTH at pH 5.5, was detected at both pH values. However, only a small fraction of higher molecular weight HA fragments was present in the mixture (indicated by arrows in Fig. 5). The fraction of the residual high molecular weight HA seemed to be only slightly increased at pH 5.5 compared to pH 4.5.

Hyaluronate digestion by rhHyal-1

In parallel to the experiments performed with the PH-20 type hyaluronidases, the HA fragments produced by rhHyal-1 after different times of incubation were analyzed by CZE. Digestion of high molecular weight HA was performed at pH 3.5, *i.e.* at the pH activity optimum of rhHyal-1 in both, the turbidimetric and the colorimetric hyaluronidase assay.

rhHyal-1 degraded high molecular weight HA continuously into smaller fragments until tetra-(n_2), hexa-(n_3) and octasaccharides (n_4) with traces of disaccharide (n_1) accumulated as final products (Fig. 6).

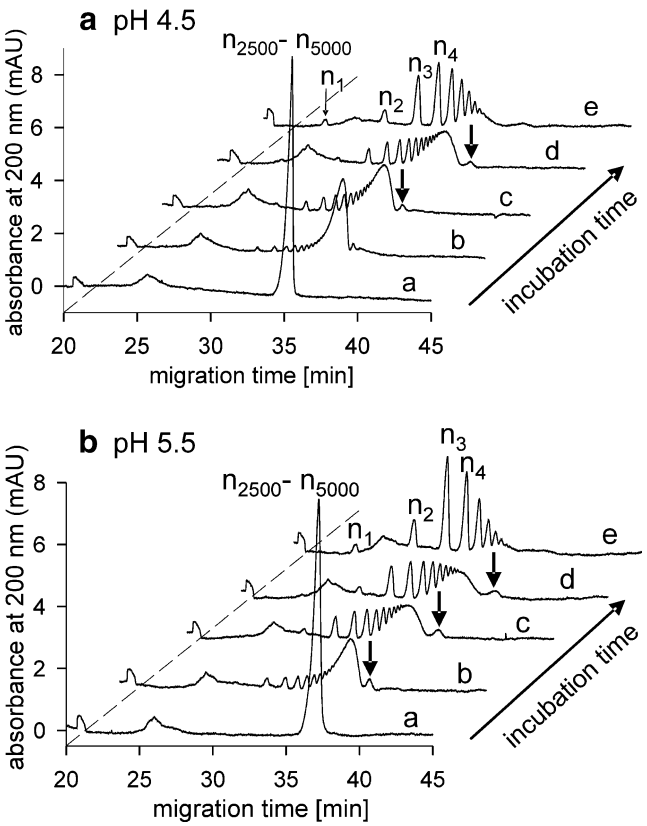

Fig. 5 CZE analysis of HA digestion mixtures produced by the action of rhPH-20 at pH 4.5 (**a**) and pH 5.5 (**b**). The degree of polymerization (n) is given as multiples of (βGlcUAc1–3βGlcNAc1-4) disaccharide units. The activity of rhPH-20 was determined after each period of incubation by the colorimetric activity assay with the absorbance of the reaction product at 585 nm given in the following as A_{585}. The enzyme reaction was stopped by addition of acetonitrile after *a*, 0 min (0.0 A_{585}); *b*, 2 h (0.2 A_{585}); *c*, 4 h [0.4 A_{585} at pH 4.5 (**a**) and 0.3 A_{585} at pH 5.5 (**b**)]; *d*, 6 h [0.5 A_{585} at pH 4.5 (**a**) and 0.4 A_{585} at pH 5.5(**b**)] and *e*, 27 h [0.9 A_{585} at pH 4.5 (**a**) and 0.8 A_{585} at pH 5.5 (**b**)]. The fractions of high molecular weight HA fragments occurring at pH 5.5 are indicated with *arrows*

As no sufficient amount of enzyme was available, CZE analysis of the reaction products of BVH could not be performed.

Discussion

For BVH and rhHyal-1 the pH activity profiles detected in the two different assay systems were in good agreement, with maximum enzymatic activity detected at acidic pH (pH 3.5–4.0). CZE analysis of the HA fragments produced by rhHyal-1 revealed a continuous degradation of high molecular weight HA to smaller fragments and finally to oligosaccharides. This simple mode of action is in good agreement with the biological function of Hyal-1, degrading hyaluronan chains within the lysosomes in a concerted action with exoglycosidases to monosaccharides.

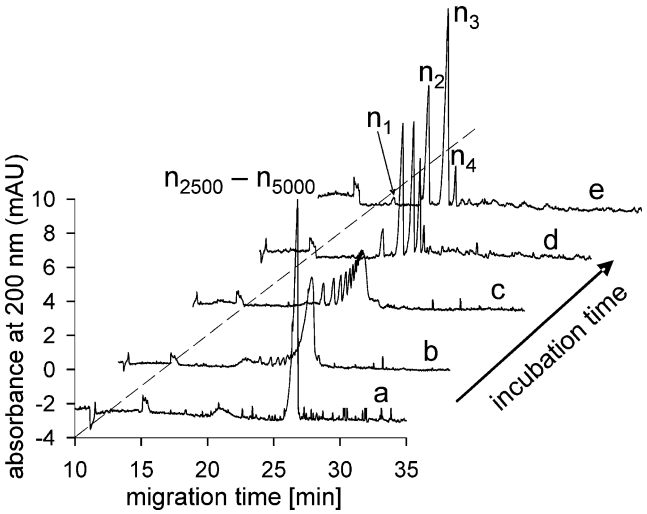

Fig. 6 CZE analysis of HA digestion mixtures produced by the action of rhHyal-1 at pH 3.5. The degree of polymerization (*n*) is given as multiples of (βGlcUAc1–3βGlcNAc1-4) disaccharide units. The activity of rhHyal-1 was determined after each period of incubation in the colorimetric activity assay with the absorbance of the reaction product at 585 nm given in the following as A_{585}. Enzyme activity was stopped by addition of acetonitrile after *a*, 0 min (0.0 A_{585}); *b*, 10 min (0.2 A_{585}); *c*, 40 min (0.4 A_{585}); *d*, 90 min (0.9 A_{585}) and *e*, 23 h (1.2 A_{585})

However, the pH activity profile of BTH and rhPH-20 obtained in the turbidimetric assay was almost inverted to the pH profile obtained by the colorimetric (Morgan–Elson) method. It can be ruled out that the discrepancy between the colorimetrically and the turbidimetrically determined pH activity curves originates from different HA concentrations or from differences in the incubation conditions at varying pH [36].

CZE analysis of the composition of the oligosaccharide mixtures produced by the action of the hyaluronidases revealed the formation of different HA fragments by hyaluronidases of the PH-20 subtype (BTH and rhPH-20) dependent on the pH. While at pH 4.5 the high molecular weight HA was continually degraded to shorter fragments, HA was degraded at pH 5.5 to low molecular weight oligosaccharides in the presence of a residual fraction of higher molecular weight HA fragments.

The pH-dependent differences between the HA fragments was much more pronounced for BTH than for rhPH20, which is also reflected in the pH activity profiles. A possible reason for this might be the presence of two additional enzymatically active protein fractions present in BTH [8] with yet unknown influence on the pattern of HA fragments.

The discrepancy between the pH profiles determined in the turbidimetric and in the colorimetric assay can be explained by the formation of HA fragments of different sizes at varying pH. In the turbidimetric assay hyaluronidase activity is quantified according to the ability of the enzyme to reduce the molecular mass of substrate chains, while the colorimetric assay detects the liberation of GlcNAc groups at the reducing end of the hyaluronic acid. When high molecular weight HA was degraded continuously, the highest number of GlcNAc residues was produced per time. Thus, the pH optimum determined in the colorimetric assay was located at acidic pH (pH 3.5–4.5).

In the turbidimetric assay HA fragments of a size >8 kDa, *i.e.* comprising more than 20 disaccharide units, cause turbidity. Apparently, high molecular weight HA (>8 kDa) was degraded by BTH and rhPH-20 faster at pH 5.5, the pH optimum of the turbidimetric assay, than at pH 4.5. Although the number of reducing GlcNAc ends liberated at pH 5.5 was lower than at pH 4.5 due to the unusual distribution of HA fragment sizes, the reduction of high molecular weight HA causing turbidity proceeded faster at pH 5.5.

The formation of the unusual HA fragment size distribution at pH 5.5 is very likely due to transglycosylase activity, since it is well-known that transglycosylation by BTH prevails around neutral pH values [33, 37]. Transglycosylation by PH-20 type hyaluronidases has been studied by several authors using mainly low molecular weight oligosaccharides [9, 10, 16, 33, 37]. However, the biological role of this unusual type of reaction observed under *in vitro* conditions is far from being understood.

Intriguingly, pH values >5.3 were found during mammalian fertilization in the environment of the sperm before and after the acrosome reaction [38]. Considering the results obtained for hyaluronidases of the PH-20 type at this pH *in vitro*, it can be speculated that the sperm hyaluronidase clears the way for penetration through the cumulus extracellular matrix of the oocyte without complete destruction of the HA network. Posttranslationally modified variants of PH-20, which occur at various stages during sperm maturation [7], might enhance the effect; such modified proteins might be present in the BTH preparation but not in rhPH-20.

Analysis of the conversion of low molecular weight HA oligosaccharides by BTH, rhPH-20 and rhHyal-1 [27] showed that rhHyal-1 is also able to catalyze transglycosylation reactions. However, these occurred only at high substrate concentrations in addition to hydrolysis reactions. Thus, the intermediate size HA fragments seem to be formed in a process in which transglycosylation significantly prevails hydrolysis.

In conclusion, the apparent differences in the pH-dependent degradation of high molecular weight HA by different enzymes of the same hyaluronidase family (EC 3.2.1.35) suggest the existence of two subclasses of mammalian-type hyaluronidases: the PH-20 subtype exhibiting pH-dependent differences in the degradation of high molecular weight HA and the Hyal-1 subtype degrading HA exclusively at acidic pH values in a simple mode of

action. Obviously, the mammalian hyaluronidase isozymes have evolved into very specialized enzymes with respect to their catalytic mechanism.

As the size of the hyaluronan fragments plays a crucial role in the biological activity of the polysaccharide [39] it can be speculated that the specialized properties of the mammalian hyaluronidases cause different biological responses in dependence on the pH value at the site of action.

Acknowledgements The authors thank S. Bollwein for technical assistance and S. Weitensteiner for assistance. Support of this work by the Graduate Training Program (Graduiertenkolleg) GRK 760 "Medicinal Chemistry: Molecular Recognition—Ligand–Receptor Interactions" of the Deutsche Forschungsgemeinschaft is gratefully acknowledged.

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