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pH effects on the hyaluronan hydrolysis catalysed by hyaluronidase in the presence of proteins. Part III. The electrostatic non-specific hyaluronan–hyaluronidase complex

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ABSTRACT

The existence of electrostatic non-specific complexes between hyaluronan (HA) and hyaluronidase (HAase) has been demonstrated over a very large pH domain ranging from 3 to 10. At 1 g L^{-1} HA and HAase concentrations and 6 °C, two types of electrostatic HA–HAase complexes have been pointed out: sedimentable slightly charged complexes at pH ranging from 3 to 5 and soluble highly charged complexes at pH ranging from 5 to 10. The HAase over HA mass stoichiometric ratio varies from 2 to 6 for the sedimentable complexes and is maximum at pH ranging from 5 to 6. This characterises the size of the non-specific HA–HAase binding site: one HAase molecule is associated with 23–71 HA carboxyl groups, depending on pH. The dissociation constant of the electrostatic HA–HAase complex has been estimated to 3.8×10^{-5} mol L^{-1}. The pI value for HAase has been estimated to 5.3 by Zeta potential measurements and is consistent with the theoretical predictions based on the charge state of the HAase molecule as a function of pH. The numbers of basic and acidic amino acids of HAase are quite equal and constant over the 5–9 pH domain, including pH 7. This favours the existence of positive patches on the surface of the HAase molecule, consistent with the formation of electrostatic HA–HAase complexes over this pH domain. This supports that HAase may be present under an inactive bound form in the extracellular matrix together with HA.

1. Introduction

We have shown that hyaluronidase (HAase) has an atypical kinetic behaviour at acidic pH and low ionic strength due to the formation of electrostatic non-specific complexes between hyaluronan (HA) and proteins. The electrostatic complexes formed between HA and bovine serum albumin (BSA) have been characterised, firstly with respect to the HA and BSA concentrations (Lenormand, Deschrevel, Tranchepain, & Vincent, 2008), then with respect to pH (Lenormand & Vincent, 2011). It has been shown that three types of complex exist: neutral insoluble complexes, sedimentable slightly charged complexes and soluble highly charged complexes. It is likely that similar electrostatic non-specific complexes can be formed between HA and HAase, because HAase is a protein. These complexes have never been described in the literature. In fact, they are more difficult to study, because the HA/HAase mixture is obviously reactive, HA being the natural substrate of HAase. Nevertheless, it has been shown by turbidimetric measurements at pH 4 that these complexes exist (Deschrevel et al., 2008) and that the complexed HAase loses its catalytic activity (Astériou, Vincent, Tranchepain, & Deschrevel, 2006; Deschrevel et al., 2008). Thus, at high HA concentrations, the whole HAase forms complexes with HA and the enzyme is not active any more, resulting in the total inhibition of the catalytic system. When the HA concentration is medium, a part of the HAase molecules cannot form complexes with HA and remains free and catalytically active; the hydrolytic activity is high. When the HA concentration is low, only a small part of the HAase molecules forms complexes with HA, but the low substrate concentration induces a weak hydrolytic activity. This characterises the atypical kinetic behaviour of HAase, its substrate-dependence is similar to the substrate-dependence produced by an enzyme with inhibition by an excess of substrate. Modelling of the system has shown that the atypical substrate-dependence of HAase can be interpreted as the combination of the increasing Michaelis–Menten type behaviour of HAase with the decreasing effect of its complexation (Lenormand, Deschrevel, & Vincent, 2007; Vincent & Lenormand, 2009).

We then have shown that the addition of a non-catalytic protein, such as BSA, allows to restore and enhance the HAase activity, even at high HA concentrations (Lenormand et al., 2008). In that situation, the enhancer protein preferably forms complexes with HA releasing HAase which recovers its catalytic activity. Activa-

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tion by proteins of the HA hydrolysis catalysed by HAase has been previously reported by many authors (Gacesa, Savitsky, Dodgson, & Olavesen, 1981; Gold, 1982; Maingonnat et al., 1999) without any mention of this mechanism of complexation. In fact, activation of HAase results from the competition between HAase and the enhancer protein to form complexes with HA (Deschrevel et al., 2008; Lenormand et al., 2008; Lenormand, Tranchepain, Deschrevel, & Vincent, 2009). The non-catalytic protein can thus be considered as an activator of HAase. However, when the protein concentration is too high, the protein and HA form very dense complexes which cannot be hydrolysed by HAase any more; the protein is thus regarded as an inhibitor of the HAase action. This double action, activation/inhibition, of proteins leads to the existence of an optimal protein concentration for the HAase activation. This behaviour exists at pH 4, but also at any pH value for which HA is able to form complexes with both HAase and the enhancer protein. It has been recently shown that HAase is activated by BSA over the pH-domain ranging from 3 to 5.25 (Lenormand, Deschrevel, & Vincent, 2010). In the presence of lysozyme (LYS), which has a pI value much higher than that of BSA, we have shown that the hydrolytic activity of HAase is enhanced over a larger pH-domain ranging from 3 to 9 (Lenormand et al., 2010a). Modulation of the HAase activity by proteins might thus occur at the pH of the extracellular matrix (ECM), and especially of the ECM of cancer cells and be implicated in the development of cancer tumours because of the angiogenic action of the short HA oligosaccharides (see Lenormand et al., 2010a).

The possible modulation of HAase by BSA and LYS over the pH-domain ranging from 3 to 9 signifies that HA is able to form complexes both with BSA or LYS, and with HAase. In fact, HA forms two types of complex with HAase: the first one is the classical catalytic enzyme–substrate complex which involves hydrogen bonds, electrostatic and Van der Waals interactions in the precise 3D arrangement of the catalytic site and the second one is the non-specific complex that HA is able to form with a great variety of proteins, such as BSA, LYS, and HAase (Astériou et al., 2006; Deschrevel et al., 2008; Lenormand et al., 2008), mainly through electrostatic interactions. The actual pH-dependence of HAase is thus the result of the two contributions: the intrinsic pH-dependence of the enzyme action and the pH-dependence of the formation of the electrostatic HA–protein complexes (Lenormand et al., 2010a). The enhancement of HAase activity in the presence of enhancer proteins requires three conditions: (i) HAase has to be able to form a complex with HA, (ii) the enhancer protein has to be able to form a complex with HA and (iii) this complex has to be more stable than the electrostatic complex formed between HA and HAase. As LYS is able to enhance HAase activity over the large pH-domain ranging from 3 to 9, it is expected that HAase can form complexes with HA over this pH domain and thus the pI of the bovine testicular HAase is higher than 9. To our knowledge, there is no answer to this question in the literature and the present study can bring interesting information about the pI of HAase. Another question concerns the dissociation constant of the electrostatic non-specific HA–HAase complex. The fact that LYS is able to prevent or reverse the formation of electrostatic HA–HAase complexes means that the dissociation constant of the HA–HAase complex is higher than the dissociation constant of the HA–LYS complex which has been estimated to 10^{-7}–10^{-8} mol L^{-1} (Moss, Van Damme, Murphy, & Preston, 1997).

The detailed study of the HA–BSA and HA–LYS complexes has been performed at 37 °C over a large pH domain (Lenormand & Vincent, 2011). It has been shown that the sedimentable form of the HA–protein complexes is the most efficient to compete with the HA–HAase complexes and to restore the catalytic activity of HAase. The present paper is the last of the series and deals with the characterisation of the electrostatic HA–HAase complexes with respect to pH and the determination of their nature, solubility, charge and stoichiometry. Discussions about the binding site and the charge of the complexes have been also included. However, in order to avoid the hydrolysis of the HA molecule by HAase during the study of the electrostatic HA–HAase complexes, the experiments were performed at 6 °C instead of at 37 °C for the complexes of HA with the non-catalytic proteins (Lenormand & Vincent, 2011).

2. Experimental

2.1. Material

Bovine testicular HAase (H 3884, lot 38H7026) and sodium hyaluronate from human umbilical cord (H 1876, lot 127H0482) were obtained from Sigma. The molar mass of HA was close to 1 MDa. More precisely, the number-average molar mass (M_n) of HA was 0.967×10^6 g mol^{-1} and its polydispersity index, which represents its degree of homogeneity, was 1.45. The molar mass of HAase was 57,000 g mol^{-1}. HA and HAase were used without any further purification. The chemicals used in the assays were: sodium tetraborate (Prolabo 27 727-297), sulfuric acid (Sigma S 1526), carbazole (Sigma C 5132), boric acid (Sigma B 7660), p-dimethylaminobenzaldehyde (DMAB) (Sigma D 8904), glacial acetic acid (Sigma A 6283).

Absorbance was measured using a Uvikon 860 KONTRON spectrophotometer equipped with a temperature-controlled chamber and connected to a computer. pH adjustments were carried out using a Metrohm 632 pH-meter equipped with a Radiometer Analytical XC161 pH electrode. Centrifugation of the HA–protein mixtures was performed with a MiniSpain Plus centrifuge from Eppendorf. Zeta potential measurements were carried out using a Zeta Sizer (Nano ZS) from Malvern Instruments equipped with a laser source (λ = 632 nm).

2.2. Methods

2.2.1. Uronic acid assay

The HA mother solution (weighed at 10 g L^{-1}) was prepared in Milli-Q water and assayed by the method described by Dische (1947) and modified by Bitter and Muir (1962). The experimental procedure using carbazole was extensively described in our previous papers (Astériou et al., 2001; Vincent, Asteriou, & Deschrevel, 2003). Sodium D-glucuronate was used for calibration. All the HA assays were performed using this method which is suitable for both freely soluble HA and complexed HA after dissociation of the electrostatic complex at pH near 12.

2.2.2. Turbidity measurements

The solutions of HA and HAase prepared in Milli-Q water were first adjusted to a pH value higher than 10 in order to limit complexation when mixed. The HA/HAase mixture was prepared by diluting adequate volumes of HA and HAase solutions in Milli-Q water so that the final concentrations were 1 g L^{-1} for both HA and HAase. The mixture was placed in the reactor maintained at 6 °C. Then pH was progressively decreased and turbidity was measured at 400 nm over time by placing the plastic cuvette containing the mixture in the thermostated (6 °C) chamber of the spectrophotometer equipped with a magnetic stirrer.

2.2.3. Analysis of the HA–protein complexes

The HA/HAase mixture was incubated at 6 °C in a reactor under magnetic stirring for 30 min. As long as pH was decreased, aliquots of 1 mL were removed, transferred to Eppendorf cones and centrifuged at 10,000 rpm for 20 min. The supernatant was removed and placed in a quartz cuvette in order to measure the HAase

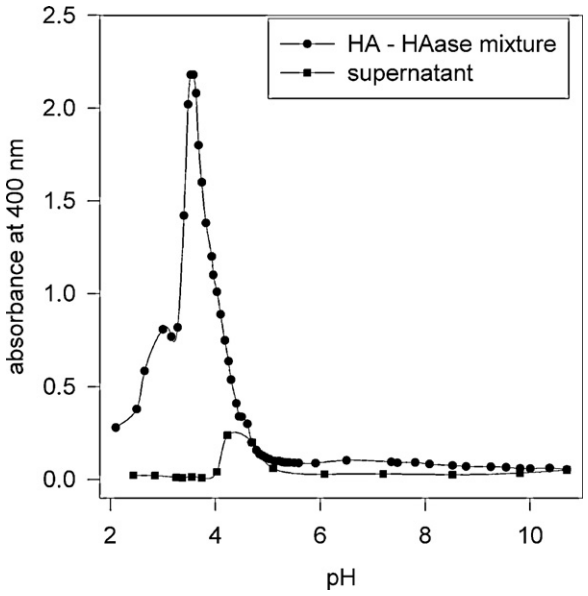

Fig. 1. Adsorbance at 400 nm of mixtures composed of HA at 1 g L^{-1} and HAase at 1 g L^{-1}, at low ionic strength, 6 °C and pH values ranging from 2.3 to 10.7. Adsorbance at 400 nm of the supernatant obtained after centrifugation of these mixtures.

concentration at 280 nm by using the calibration curve previously established with HAase solutions of known concentrations. A 200 μL aliquot of the supernatant was then analysed using the uronic acid assay method in order to measure its HA content. Then, 1 mL of water containing 20 μL of KOH 2 mol L^{-1} was added to the pellet to obtain its complete dissolution. The concentrations of HAase and HA in the obtained solution were then measured using the same methods as above.

3. Results

In all the experiments, no salt was added in the solution. The ionic strength was thus very low and only due to the presence of the counterions of the biopolymers and pH adjustments. It was of the order of magnitude of 10^{-3} mol L^{-1} and thus very different from the physiological level. For sake of simplicity, the term "low ionic strength" was used throughout the text.

3.1. Existence of the HA–HAase complexes

Existence of the electrostatic complexes formed between HA and HAase was first studied using spectrophotometric measurements. The HA/HAase mixture, at 1 g L^{-1} HA concentration and 1 g L^{-1} HAase concentration, was first adjusted to pH 10.7, then pH was slowly decreased and the turbidity of the mixture was measured as a function of pH. Fig. 1 shows the turbidity at 400 nm of the HA/HAase mixture as a function of pH ranging from 2 to 10.7. According to Xu, Yamanaka, Sato, Miyama, and Yonese (2000) and Lenormand et al. (2010a), three pH domains were identified: (i) from pH 10.7 to 5, absorbance slightly increased and remained low corresponding to a mixture of soluble HA–HAase complexes and individual HA and HAase molecules, (ii) from pH 5 to 3.5, absorbance strongly increased up to 2.2 corresponding to sedimentable HA–HAase complexes, and (iii) at pH lower than 3.5, absorbance strongly decreased because of the phase separation of the HA–HAase complexes occurring at pH 3.5.

Because HA solubility was higher than 10 g L^{-1} and HAase solubility was higher than 50 g L^{-1}, the presence of turbidity demonstrated the existence of HA–HAase complexes. Turbidity corresponds to the light scattered by the complexes whose solubility depends on the charge excess at their surface. After centrifugation at 10,000 rpm for 20 min, turbidity of the supernatant was very low, but not zero (Fig. 1). This means that the major part of the HA–HAase complexes were formed between pH 2 and 5 and corresponded to sedimentable slightly charged complexes. Fig. 1 also shows that soluble highly charged HA–HAase complexes existed between pH 5 and 10.7. As existence of the electrostatic HA–protein complexes was related to the charges borne by the two macromolecules, this suggests that HAase could be positively charged at pH ranged from 2 to 10.7.

3.2. The theoretical charge of the two macromolecules

As HA is alternately composed of glucuronic acid and N-acetylglucosamine, one carboxyl group, with a defined pK_a value, exists for each disaccharide unit, and thus one molar fraction of negative charge exists per mole of disaccharide unit depending on the pH value. The molar mass of the sodium salt of the disaccharide unit being equal to 401 g mol^{-1}, there is a 1/401th molar fraction of negative charge per gram of sodium HA.

For HAase, we may assume that, as for any soluble protein, its tri-dimensional conformation in water is a globular molecule with almost all the charged amino acids located at the surface of the molecule, in contact with the aqueous solvent. Thanks to the high level of sequence homology between bovine testicular HAase and bee venom HAase, the 3D structure of bee venom HAase and according to Lenormand et al. (2008) and Grymonpré, Staggemeier, Dubin, and Mattison (2001), we may assume such a conformation for bovine testicular HAase in water. We thus used the technique described in our previous papers (Lenormand & Vincent, 2011; Lenormand et al., 2008) to calculate the charge of the molecule from its primary sequence (Table 1) and the pK values of its ionisable amino acids (Table 2) (assumed to be constant and independent of their neighbourhood). Even if this procedure requires several assumptions which are not rigorously true, we think that it is the only one which allows a quantitative estimation of the charge over a large pH domain.

Fig. 2 shows the theoretical charges of the two macromolecules, HA and HAase, expressed in milli-equivalents per gram, as a function of pH. The figure also indicates that the pI of HAase is near 6, and that a large pH domain, ranging from 5.6 to 9, exists where

Table 1
Primary structure of bovine testicular HAase (PH-20, P38567).

```
MGVLKFKHIF FRSFVKSSGV SQIVFTFLLI PCCLTLNFRA PPVIPNVPFL WAWNAPSEFC
LGKFDEPLDM SLFSFIGSPR INATGQGVTI FYVDRLGYYP YIDSITGVTV NGGIPQKISL
QDHLDKAKKD ITFYMPVDNL GMAVIDWEEW RPTWARNWKP KDVYKNRSIE LVQQQNVQLS
LTEATEKAKQ EFEKAGKDFL VETIKLGKLL RPNHLWGYYL FPDCYNHHYK KPGYNGSCFN
VEIKRNDDLS WLWNESTALY PSIYLNTQQS PVAATLYVRN RVREAIRVSK IPDAKSPLPV
FAYTRIVFTD QVLKFLSQDE LVYTFGETVA LGASGIVIWG TLSIMRSMKS CLLLDNYMET
ILNPYIINVT LAAKMCSQVL CQEQGVCIRK NWNSSDYLHL NPDNFAIQLE KGGKFTVRGK
PTLEDLEQFS EKFYCSCYST LSCKEKADVK DTDAVDVCIA DGVCIDAFLK PPMETEEPQI
                FYNASPSTLS ATMFIVSILF LIISSVASL
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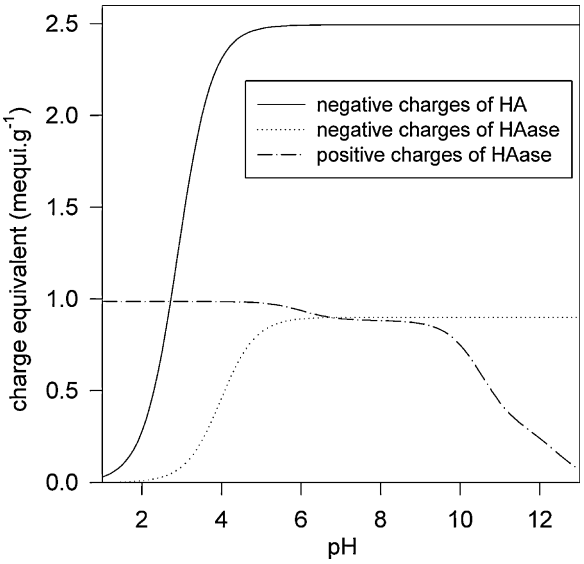

Fig. 2. Theoretical pH-dependence of the charge state of the two biomacromolecules. Milli-equivalents brought per gram of HA and HAase as a function of pH.

the negative charges and the positive charges of HAase are almost equivalent. The presence of soluble HA–HAase complexes over the pH domain ranging from 5 to 10.7 (Fig. 1) can thus be attributed to a pI value close to 6 associated with positive patches (Mattison, Dubin, & Brittain, 1998) over the pH region ranging from 6 to 9.

3.3. Content of the HA–HAase complexes as a function of pH

A calibration curve was performed by measuring the absorbance at 280 nm of solutions containing HAase at different concentrations ranging from 0 to 1 g L^{-1}. Then, an HA/HAase mixture with 1 g L^{-1} HA concentration and 1 g L^{-1} HAase concentration was placed in a reactor maintained at 6 °C under magnetic stirring. pH, initially adjusted to 10.7, was progressively lowered and 1 mL aliquots of the mixture were removed over time and analysed as described in Section 2. The HAase and HA assays gave the HAase and HA concentrations in the supernatant, $[HAase]_s$ and $[HA]_s$, and in the pellet, $[HAase]_p$ and $[HA]_p$, as a function of pH (Fig. 3). Two pH domains were distinguished: (i) From pH 10 to 5, HA and HAase were mostly present in the supernatant where they were free or associated in soluble complexes; the supernatant did not show any turbidity. In the pellet, the very low HA concentration (at the limit of sensitivity of the assay) was associated with an increasing HAase concentration corresponding to a small quantity of sedimentable HA–HAase complexes. These complexes contain a large excess of HAase but only a small charge excess due to the quasi-equivalence of the positive and negative charges on the HAase molecule and (ii) from pH 5 to 3, $[HAase]_p$ and $[HA]_p$ strongly increased and $[HAase]_s$ and $[HA]_s$ decreased, characterising an exchange between soluble and sedimentable HA–HAase complexes. At pH 3.8, 80% of the present HAase formed sedimentable complexes with only 20% of the present HA. As for the soluble complexes, the sedimentable complexes were rich in HAase.

Table 2
p*K* values of the ionisable amino acids.

Amino-acid		p*K*
Arg	R	12.48
Asp	D	3.9
Glu	Q	4.07
His	H	6.04
Lys	K	10.54

3.4. Stoichiometry of the HA–HAase complexes as a function of pH

The same data were analysed in terms of HAase over HA mass stoichiometric ratio in the complexes (Fig. 4). At pH higher than 5, the major part of HA and HAase was recovered in the supernatant. The HAase over HA mass ratio slightly increased from 0.8 to 1 signifying that the predominant soluble HA–HAase complexes were progressively dissociated into free HA and HAase when pH was increased from 5 to 10.7. In the pellet, there was only a small quantity of HA–HAase complex which was rich in HAase: The HAase over HA mass ratio was close to 6 at pH 5 and decreased down to 2 at pH 10.7. At pH lower than 5, there was an exchange between the soluble HA–HAase complexes predominant at pH 5, and the sedimentable HA–HAase complexes predominant at pH 3. As for the HA–BSA complexes (Lenormand & Vincent, 2011), the HAase over HA mass stoichiometric ratio for the HA–HAase complexes

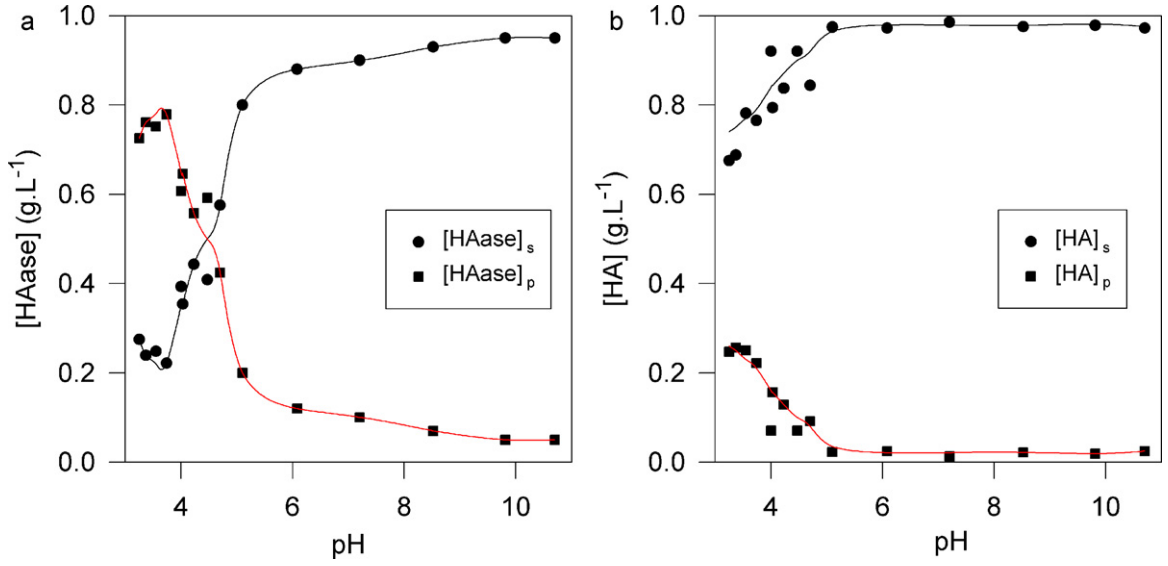

Fig. 3. Analysis of the cosmposition of the HA–HAase complexes as a function of pH. Distribution of HAase (a) and HA (b) after centrifugation of mixtures composed of HA at 1 g L^{-1} and HAase at 1 g L^{-1}, at 6 °C, low ionic strength, and pH values ranging from 3 to 10.7 in the pellet (■), and in the supernatant (●).

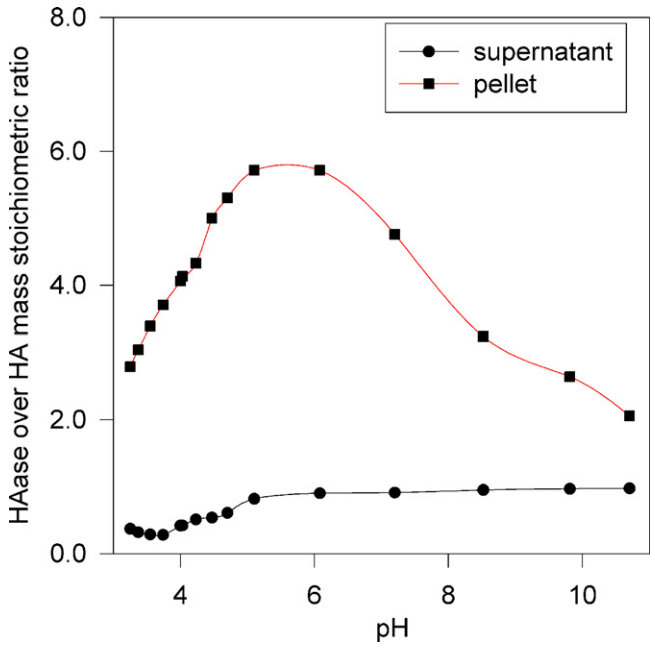

Fig. 4. Stoichiometry of the HA–HAase complexes. HAase over HA mass ratio of mixtures composed of HA at 1 g L^{-1} and HAase at 1 g L^{-1}, at 6 °C, low ionic strength and pH values ranging from 3 to 10.7. Ratios were calculated from the results presented in Fig. 3 for the pellet (■) and the supernatant (●).

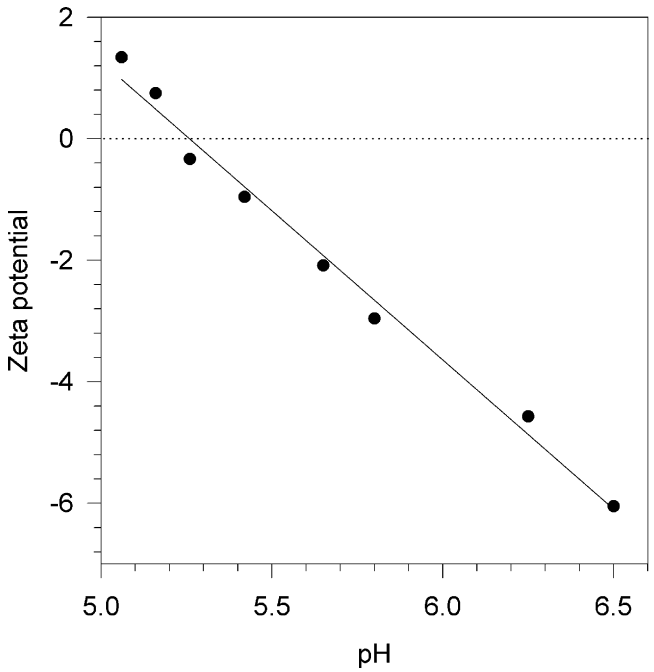

Fig. 5. Zeta potential of the HAase molecule as a function of pH.

increased when pH was increased because the complexes required more HAase molecules, less positively charged at pH 5 than at pH 3. This is generally observed. For the sedimentable complexes, the mass stoichiometric ratio increased from 2.8 at pH 3 to 6 at pH 5–5.3. At pH 4, the mass stoichiometric ratio was equal to 4 signifying that one HAase molecule formed complexes with an equivalent of 36 HA disaccharides. For the soluble complexes, the mass stoichiometric ratio increased from 0.37 at pH 3 to 0.8 at pH 5 and was equal to 0.42 at pH 4. The fact that the mass stoichiometric ratio of the sedimentable HA–HAase complexes increased with pH and was maximum at pH 5–5.3 supports the idea that the pI value of HAase is close to 5.

3.5. Zeta potential of HAase as a function of pH

The Malvern Zetameter used to measure the diameter of the HA–protein complex particles (Lenormand et al., 2008) was also suitable for measuring the Zeta potential at the surface of macromolecules. A series of experiments was performed to measure the Zeta potential at the surface of HAase at different pH values ranged from 5.05 to 6.5. Fig. 5 shows that the Zeta potential of HAase at 5 g L^{-1} was a quasi linear function of pH over that domain. The zero charge was obtained for pH 5.3 that can be considered as being the pI value of HAase. At pH lower than 5.3 the Zeta potential of HAase was positive; at pH higher than 5.3 the Zeta potential of HAase was negative. This is in agreement with the fact that the mass stoichiometric ratio of the HA–HAase complexes, both for sedimentable and soluble complexes, increased with pH up to pH 5–5.3 (Fig. 4). This strongly suggests that the HA–HAase complexes formed at pH higher than 5.3 were due to the existence of positive patches at the surface of the HAase molecule.

3.6. Dissociation constant of the HA–HAase complexes as a function of pH

Contrary to the HA–BSA complexes, the sedimentable HA–HAase complexes were simultaneously present with the free HA and HAase molecules, at least over the 5–9 pH domain. The equilibrium between the free forms of the macromolecules and the complex was thus not entirely displaced and the determination of the dissociation constant was possible. For that, we may consider the equilibrium between the HA–HAase complex on one hand, and both the HAase molecule and the HA fragment able to form a complex with that HAase molecule on the other hand (Vincent & Lenormand, 2009):

$$\text{HA–HAase complex} \rightleftarrows \text{free HA fragment} + \text{free HAase}$$

Fig. 3 gives the concentrations of each species involved in the equilibrium: at pH 4, the concentration of HAase involved in the sedimentable complex was 0.6 g L^{-1}, the concentration of HA involved in the sedimentable complex was 0.15 g L^{-1}, corresponding to a mass stoichiometric ratio of 4, the concentration of HAase not involved in the sedimentable complex was 0.4 g L^{-1} and the concentration of HA not involved in the sedimentable complex was 0.85 g L^{-1}. Therefore, the molar concentration of the non-complexed HA fragments of 36 disaccharides (see Section 3.4) was 5.75×10^{-5} mol L^{-1}. The molar concentration of the non-complexed HAase was 0.69×10^{-5} mol L^{-1}. The molar concentration of the complexed HAase was 1.03×10^{-5} mol L^{-1}. Under these conditions the dissociation constant, K_d, at pH 4 was estimated to 3.8×10^{-5} mol L^{-1}.

3.7. Stoichiometry of the HA–HAase complexes at pH 4 as a function of the HAase concentration

As for the HA–BSA complexes (Lenormand & Vincent, 2011), the protein over HA stoichiometric ratio not only depends on pH but also depends on the total HA and protein concentrations. A new series of experiments were performed at pH 4 and 6 °C for a unique HA concentration of 0.2 g L^{-1} and different HAase concentrations ranging from 0 to 5.5 g L^{-1}. HA and HAase were mixed in Eppendorf tubes; after centrifugation at 10,000 rpm for 20 min, the supernatant and the pellet were analysed for their HA and HAase contents as described in Section 2. Fig. 6 shows the distribution of the HAase and HA concentrations between the supernatant,

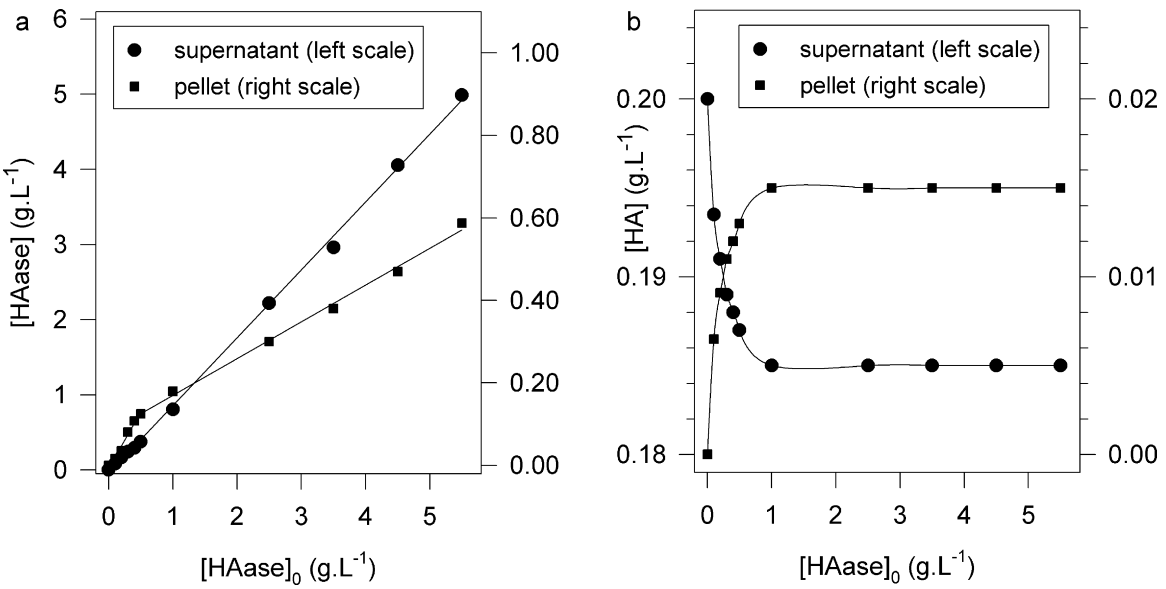

Fig. 6. Analysis of the composition of the HA–HAase complexes as a function of the total HAase concentration. Distribution of HAase (a) and HA (b) after centrifugation of mixtures composed of HA at 0.2 g L^{-1} and different HAase concentrations ranging from 0 to 5.5 g L^{-1}, at 6 °C, low ionic strength and pH 4 in the pellet (■), and in the supernatant (●).

$[HAase]_s$ and $[HA]_s$, and the pellet, $[HAase]_p$ and $[HA]_p$, as a function of the total HAase concentration $[HAase]_o$. The first remark is that there was about 20 times less of HA and HAase in the pellet than in the supernatant. Second, the changes in HA and HAase as a function of the total HAase concentration show very different shapes: while the HA concentration in the supernatant and in the pellet were stabilised at $[HAase]_o$ higher than 1 g L^{-1}, the HAase concentration continuously increased. Third, the HAase concentration in the pellet increased rapidly in the first domain corresponding to an $[HAase]_o$ ranging from 0 to 1 g L^{-1}, then it increased linearly with a lower slope of 0.5.

As the HA concentration remained stable and the HAase concentration continuously increased in the sedimentable HA–HAase complexes when $[HAase]_o$ was increased, the mass stochiometric ratio increased from 10 for $[HAase]_o = 0.5$ g L^{-1}, to 40 for $[HAase]_o = 5.5$ g L^{-1} (Fig. 7). It means that HA was able to fix a very high quantity of HAase in the electrostatic non-specific complexes. The two different shapes of the curve representing the changes in the HAase over HA mass ratio as a function of the total HAase concentration suggest two different modes for the formation

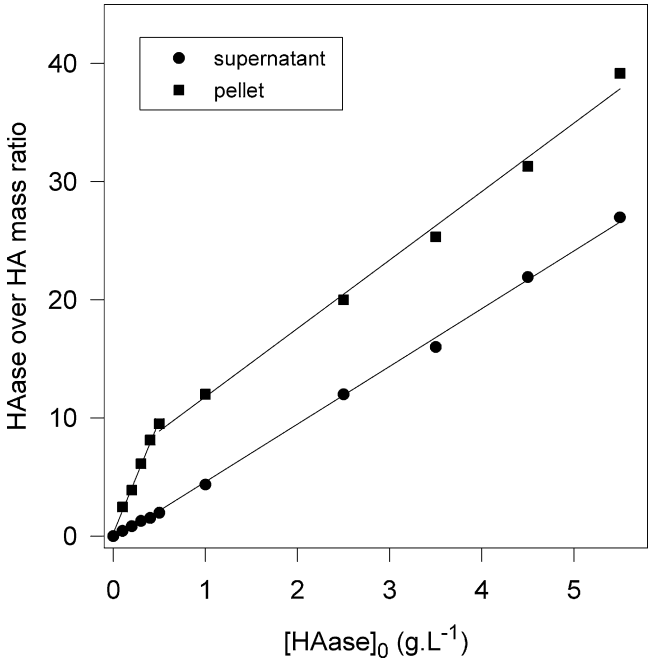

Fig. 7. Stoichiometry of the HA–HAase complexes. HAase over HA mass ratio of mixtures composed of HA at 0.2 g L^{-1} and different HAase concentrations ranging from 0 to 5.5 g L^{-1}, at 6 °C, low ionic strength and pH 4. Ratios were calculated from the data presented in Fig. 6 for the pellet (■) and the supernatant (●).

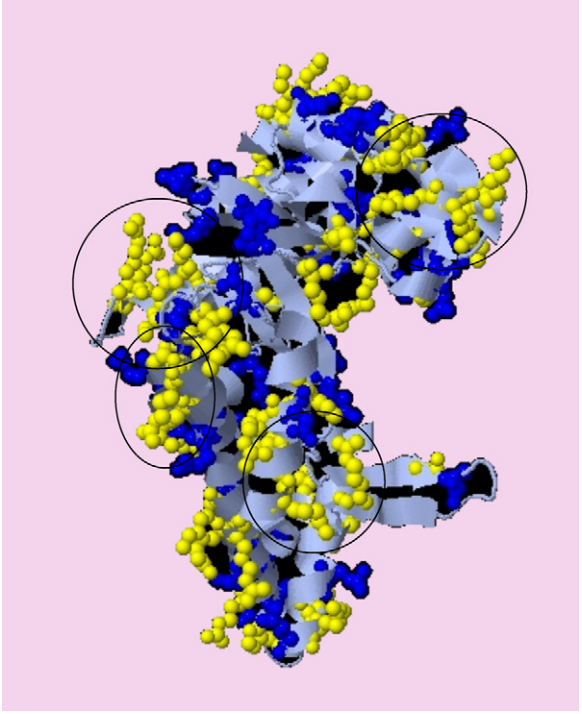

Fig. 8. Three-dimensional structure of the bee venom HAase molecule (www.rcsb.org/: 1FCU), an HAase of the same family than Bovine testicular HAase, which shows positive amino-acids in clear and negative amino-acids in dark at its surface. Possible positive patches were drawn by circles on its surface.

of the HA–HAase complexes: the first step could concern electrostatic HA–HAase interactions whilst the second could concern electrostatic HAase–HAase interactions through the charge patches leading to multi-layer complexes.

3.8. Size of the binding site

Values of mass stoichiometric ratio ranging from 2 to 6 mean that 1 g of HA was bound to 2–6 g of HAase, i.e., 10^{-6} moles of HA were bound to 35×10^{-6} to 105×10^{-6} moles of HAase. This corresponds to 35–105 HAase molecules per HA molecule of 1 Mda containing 2500 disaccharide units of 401 g mol^{-1}. One HAase molecule was thus associated with 71–23 carboxyl groups depending on pH. This constitutes the size of the non-specific HA–HAase binding site.

However, at pH 4, 6 °C and increasing quantities of HAase, the stoichiometric ratio can reach 40, signifying that one HA molecule was bound to up to 700 HAase molecules. Each HAase molecule was thus associated with 3–4 carboxyl groups. It is evident that, in this case, the structure of the complex cannot be stabilised by only 3 or 4 electrostatic bonds between HA and HAase. It is likely that a great part of the HAase molecules were associated in a multilayer mode through interactions between negative and positive patches.

4. Discussion and conclusion

HA is a very important member of the glycosaminoglycan family which is an important class of the biological polysaccharides; it is a major component of the ECM. HAase is its hydrolytic enzyme and is now known to play an important role in cancer development (Maingonnat et al., 1999). By using the bovine testicular HAase as a model, we have studied the HA hydrolysis catalysed by HAase, and more generally the interactions between HA and HAase, for several years. The kinetic behaviour of the hydrolytic system has shown that the interactions between HA and HAase are much more complex than the interactions occurring between the components of most of simple enzyme/substrate systems. We have published several papers dealing with the HAase activity and its control by the substrate concentration (Astériou et al., 2006), the chain length of the substrate (Lenormand, Deschrevel, & Vincent, 2010), the ionic strength (Lenormand et al., 2008) and the presence of non-catalytic proteins (Lenormand et al., 2009). The study of the HA/HAase reactive system at low ionic strength has shown a very atypical kinetic behaviour (Astériou et al., 2006), characterised by an inhibition of the enzyme activity at high substrate concentrations. The only hypothesis consistent with all the experimental results is based on the formation of non-specific electrostatic complexes between HA and HAase (Astériou et al., 2006), in addition to the catalytic enzyme–substrate complex which involves hydrogen bonds, electrostatic and Van der Waals interactions in a precise 3D arrangement. In fact, the main distinction between the two complexes probably consists in the orientation of the HAase molecule as regards HA. Many different orientations can only lead to electrostatic interactions defining a non-specific electrostatic complex, whilst only one specific orientation can lead to the catalytic complex.

Our studies have shown that the non-specifically complexed HAase is no longer catalytically active. However, the complexed HA remains a potential substrate for HAase. This constitutes the main reason for the inhibition of the reactive system at high substrate concentrations, because all the HAase molecules are complexed in excess of HA. The HA–HAase complex is controlled by the laws of the electrostatic polycation–polyanion complexes (Cousin, Gummel, Ung, & Boué, 2005; Schmitt, Sanchez, Desobry-Banon, & Hardy, 1998; Turgeon, Schmitt, & Sanchez, 2007). This also means that another protein showing interactions with HA stronger than HAase can compete with HAase and preferably form complexes with HA inducing the release of HAase which recovers its catalytic activity. In that case, the system is activated. Nevertheless, at very high protein concentrations, the high density of the complexed protein on the surface of the HA molecule does not allow any HAase action. The system is thus inhibited. The added protein can thus be considered as an inhibitor and/or an enhancer. Because of the great importance of HA and HAase in biology and especially in cancer research, we have developed a detailed study on pH effects in order to examine if this type of interactions can occur at the pH of the ECM. The whole study is composed of three inter-dependent papers.

The first paper (Lenormand et al., 2010a) highlights the HAase activity and its enhancement/suppression control by proteins as a function of pH. This has been demonstrated for BSA at pH values ranging from 2.3 to 5.3 and for LYS at pH ranging from 3 to 9. In these pH ranges, the protein-dependences are similar whatever the pH value. Roughly, two domains can be distinguished: (i) at low protein concentrations, the HAase activity increases when the protein concentration is increased, due to the predominant complexation of HA by the protein releasing HAase and (ii) at high protein concentrations, the HAase activity decreases when the protein concentration is increased, due to the tight complexation of HA by the protein. The fact that a protein, like LYS, can enhance the HAase activity on a wide pH domain ranging from 3 to 9, means that: (i) HAase is able to form complexes with HA on the whole pH domain ranging from 3 to 9, (ii) LYS is able to compete with HAase to form complexes with HA, (iii) HAase is catalytically active on the whole pH domain as long as LYS limits its complexation with HA, and (iv) the pI of HAase is high or positive patches on the surface of the HAase molecule favour the formation of electrostatic HA–HAase complexes at high pH values. To our knowledge, this study is the first demonstration that HAase has a hydrolytic activity at pH as high as 9. In the literature, different pH-domains (De Salegui, Plonska, & Pigman, 1967; Gacesa et al., 1981; Krishnapillai, Taylor, Morris, & Quantick, 1999) and different optimal pHs (Gacesa et al., 1981; Gorham, Olavesen, & Dodgson, 1975) have been reported. However, they do not correspond to similar experimental conditions and similar assay methods. Overall, most of the authors did not control the possible complexation of the enzyme with HA and their results may correspond to a non-constant active enzyme concentration at the different pH values. Conversely, we have tried to distinguish the catalytic action from the complexation of the enzyme, and thus to measure the HAase activity at constant reactive enzyme concentration. Our study shows the dramatic influence of the complexation on the HAase activity and explains why so many different results have been reported in the literature.

Another important conclusion concerns the pH-dependence of HAase. We have shown that the experimental pH-dependence of the HAase activity is the combination of two pH-dependences: the intrinsic pH-dependence of the enzyme action and the pH-dependence of the non-specific HA–HAase complex formation. Both of them control the ability of HAase to catalyse the HA hydrolysis. The first one depends on HAase and is a function of the enzyme conformation which mainly varies with ionic strength and pH. The second one depends on ionic strength, pH and HAase over HA stoichiometry, but can also integrate the presence of other non-catalytic proteins and thus be a function of stoichiometry, pI, size and charge density of these proteins, resulting in the HAase complexation or not. The experimentally observed pH-dependence and the maxima in catalysis are thus greatly dependent on ionic strength, pH, protein over HA stoichiometries, together with pI, size and charge density of the non catalytic proteins present in the medium, characterising the experimental conditions. This explains

why the pH-dependence of HAase varies according to the experimental conditions, such as the pH buffer (Gacesa et al., 1981).

The second paper (Lenormand & Vincent, 2011) highlights the nature, solubility, charge and stoichiometry of the complexes formed between HA and non-catalytic proteins as a function of pH. Concerning BSA, when equal concentrations of HA and protein ($1\,g\,L^{-1}$) and low ionic strength are used, three types of electrostatic complexes are formed: insoluble neutral complexes at pH values lower than 3.2, sedimentable slightly charged complexes at pH near 4 and soluble highly charged complexes at pH near 5. The HA–BSA complexes are more charged and soluble when pH is increased up to 5.2, i.e. the pI of BSA, and the BSA content of these complexes also increases. For the sedimentable HA–BSA complexes, the normalised charge excess does not exceed 20%, while it reaches 40% for the soluble highly charged complexes. Finally, concerning the stoichiometry of the HA–protein complexes, we have shown that the BSA over HA mass stoichiometric ratio for the insoluble and/or sedimentable HA–BSA complexes remains between 1 and 2 over the whole 2.3–5.3 pH-domain. This means that one BSA molecule is associated with 85–170 HA carboxyl groups, depending on pH. This defines the size of the binding site of the HA–BSA complex. Similar results were obtained for LYS. Very few studies concerning the stoichiometry of that type of complex have been reported in the literature (Van Damme, Moss, Murphy, & Preston, 1994; Xu et al., 2000). Their results were quantitatively different from ours but their experimental conditions were also different, especially in terms of pH and concentrations in HA and proteins.

By comparing the HAase activity and the presence of the different types of HA–protein complexes as a function of pH, we have shown that the sedimentable slightly charged HA–protein complexes are the most efficient complexes for competing with the complexes formed between HA and HAase. It seems to be the best compromise for both the release of HAase and the accessibility of the hydrolysable sites on the HA molecule. Several other proteins, such as immunoglobulins and hyaluronectin, can be considered as enhancer proteins for HAase (Maingonnat et al., 1999) and it is likely that the main results concerning BSA and LYS are valid for these proteins.

Finally, the present paper is the third and last contribution to this study. Here, we have demonstrated the existence of electrostatic non-specific HA–HAase complexes over the very large pH domain ranging from 3 to 10. Mainly two types of non-specific complexes exist: sedimentable slightly charged complexes at pH ranging from 3 to 5 and soluble highly charged complexes associated with free HA and/or HAase molecules at pH ranging from 5 to 10. Under our experimental conditions, i.e., for HA and HAase concentrations equal to $1\,g\,L^{-1}$, low ionic strength and 6 °C, no insoluble HA–HAase complex exists and all HA–HAase complexes are very rich in HAase since the HAase over HA mass stoichiometric ratio remains between 2 and 6 with a maximum at pH 5. This characterises the size of the non-specific HA–HAase binding site: one HAase molecule is associated with 23–71 HA carboxyl groups, depending on pH. At pH 4, the mass stoichiometric ratio is equal to 4. For an HA concentration equal to $0.2\,g\,L^{-1}$ and varying HAase concentrations, the ratio can even reach higher values, close to 40, characterising an over-complexation of HA by the HAase molecules, as it is the case with non-catalytic proteins (Lenormand et al., 2009).

Under our experimental conditions, pH 4 and 6 °C, the dissociation constant, K_d, has been estimated to be equal to $3.8 \times 10^{-5}\,mol\,L^{-1}$. This value is much higher than the dissociation constant for the HA–LYS complex ($K_d = 10^{-8}$–$10^{-7}\,mol\,L^{-1}$ (Moss et al., 1997)). This is in agreement with the possible enhancement of the HAase activity induced by the presence of LYS at pH lower than 9 (Lenormand et al., 2010a). For BSA, there is no data concerning K_d in the literature. However, our modelling of the HA/HAase/BSA system has shown that K_d values of $10^{-7}\,mol\,L^{-1}$ for the HA–BSA complex and $10^{-5}\,mol\,L^{-1}$ for the HA–HAase complex lead to a very good fitting of the experimental results (Vincent & Lenormand, 2009). The K_d value, determined in the present paper, is thus in good agreement with these suggestions. The K_d value for the HA–HAase complex is definitely higher than the K_d value for the HA–enhancer protein complex. These enhancer proteins, by forming stronger complexes with HA, are thus able to positively compete with HAase in the HA–protein complex formation and are thus able to avoid and/or reverse the complexation and the inactivation of HAase. It is likely that the ratio between the dissociation constants of the HA–HAase complex and the HA–protein complex could control the HAase activity, even in biology. The dissociation constant, determined here, concerns the electrostatic non-specific HA–HAase complex at 6 °C. It does not concern the catalytic HA–HAase complex formed when HAase is in the right orientation as regards HA. For these specific interactions, two values have been reported in the literature: the first one concerns the HA–CD44 complex with a K_d value of 10^{-5}–$10^{-6}\,mol\,L^{-1}$ (Skelton, Zeng, Nocks, & Stamenkovic, 1998), and the second one concerns the HA–cell complex with a K_d value of $2 \times 10^{-7}\,mol\,L^{-1}$ (Frost, Raja, & Weigel, 1990). It seems that the dissociation constant for the specific HA–HAase complex is lower than the value determined here for the non-specific complex. Conversely, the probability for the orientation of the HAase molecule clearly favours the formation of the non-specific complex.

The enhancement/suppression of the HAase activity by LYS (Lenormand et al., 2010a) and the presence of sedimentable HA–HAase complexes over the 5–9 pH-domain suggest that the pI value of HAase is high and close to 9. However, this is inconsistent with the experimental measure of the Zeta potential of the HAase molecule as a function of pH which confirms a low pI value close to 5.3. The theoretical calculation of the positive and negative charges on the surface of the HAase molecule as a function of pH also suggests a pI value close to 6 for HAase. Although this calculation is quite naive and can only predict an acidic pI value, it shows that the net charge of the HAase molecule is very low over the large pH-domain ranging from 5.6 to 9 because the number of anionic amino acids of HAase remains very close to the number of its cationic amino acids (51 and 52, respectively). As a consequence, the change in pH does not significantly modify the net charge of the HAase molecule. In addition, the spatial distribution of the charges on the surface of a protein is not always homogeneous and regions with high densities of positive or negative charges may exist on the protein surface. These regions are called patches. Such positive patches have been shown to exist on the surface of BSA (Mattison et al., 1998; Park, Muhoberac, Dubin, & Xia, 1992) inducing complexation with polyanions at pH higher than its pI value. Computational studies performed by Grymonpré et al. (2001) by using the charge density at the protein surface have also shown the presence of positively charged patches on the BSA surface (as for example in the Lys199 region) which allows the complex formation although there is no global positive charge on BSA. This could also exist for the HAase molecule. Usually, the net charge of a protein is positive at pH lower than pI and negative at pH higher than pI. For HAase, the net charge is only very slightly negative at pH higher than 5.3 and thus an abnormally high quantity of positive charges exist over this pH domain. This can favour the formation of positive patches on the HAase surface. The presence of such patches could be observable on the three-dimensional structure of the bovine testicular HAase. Unfortunately, this 3D structure is not available in the data bases of the literature (www.rcsb.org), but the 3D structure of the bee venom HAase, another HAase which belongs to the same group as the bovine testicular HAase, is available (1FCU). The 3D structure of the bee venom HAase (Fig. 8) shows that the spatial distribution of the positive charges is not homogeneous and can definitely form patches. All of this suggests that HA can form

electrostatic complexes with HAase over a very large pH-domain ranging from 3 to 9. At pH lower than 5.3, the complexes are formed because HA is negative and HAase has a positive net charge; at pH higher than 5.3, the complexes are formed because the negative HA carboxyl groups interact with the positive patches on the HAase molecule. The progressive decrease in the HAase content of the HA–HAase complexes when pH is increased, may also be an argument in favour of the existence of positives patches on the surface of the HAase molecule, because a high pI value should produce an increase in the protein content of the complex instead of a decrease. It is also expected that variations in the micro-environmental conditions may modify the existence of these positive patches, simply through modifications of the HAase conformation. However, these interpretations are limited by the lack of availability of accurate three-dimensional data of the macromolecules.

It is now necessary to wonder about the physiological relevance of our results. It is right that the low ionic strength used maximises the electrostatic contribution compared to the other interactions, but it is also a mean to enhance the electrostatic interactions in order to properly understand how they can modulate the HAase activity. In addition, low ionic strengths exist in biology, especially in joints, where HA and LYS are present at high concentrations. In such biological compartments, the ionic strength has been estimated to be at 40 mM (Moss et al., 1997) because of the high concentration of charged macromolecules. These electrostatic interactions are of course lowered at physiological ionic strength, but we have shown that they still exist (Lenormand et al., 2008). Concerning the temperature of 6 °C used in the last series of experiments, there is no physiological significance. This temperature was chosen in order to dissociate the complex formation from the hydrolysis reaction. According to the enzyme kinetics, the enzymatic reaction rate is divided by a factor close to 10 when the temperature is decreased from 37 °C to 6 °C. For the dissociation constant of the HA–HAase complex formation, it is likely that the variations are lower. In that case, the complexes formed at 37 °C could be similar to those formed at 6 °C, at least in terms of stoichiometry and charge; only solubility is probably modified. These variations could also be estimated by using the Van't Hoff law and performing additional experiments at different temperatures.

The great interest of the present study is to know more about the HA–protein complexes in order to predict the exact quantity of the non-catalytic protein required for releasing all the HAase molecules and avoiding the complexation of HAase. This allows measurement of the enzyme activity without any complexation. The pH-dependence observed under these conditions is thus the true pH effect on the enzyme hydrolytic action. This is of great importance since the HAase level can be considered as a tumour indicator, as for example the urinary HAase level is a marker for bladder cancer (Lokeshwar & Selzer, 2008; Lokeshwar, Obek, Soloway, & Block, 1997). The present study also shows that the mechanism of enhancement/suppression of the HAase activity by proteins may occur at pH usually encountered in the ECM. As bovine testicular HAase belongs to the same class as human HAases, it can be expected that human HAases have characteristics close to those of bovine testicular HAase that we studied as a model. According to Girish and Kemparaju (2007), all mammalian HAases are acid active enzymes with an optimal pH between 3 and 4, consistent with a lysosomal location, except PH20 and Hyal-2 which are active over a broad optimal pH range. However, there is no opposition to the fact that an enzyme with an acidic optimal pH has a significant activity at neutral pH or at the pH of the ECM. Our results clearly show this possibility since the maximal HAase activity at pH 4 is associated with a significant activity over a broad pH domain ranging from 3 to 9. The fact that positive patches on the surface of the HAase molecule allow its complexation by HA, and maybe by some other negative polysaccharides, like chondroitin, over a very large pH domain including 7, is of great importance in biology in the sense that it shows that HAase may be present under an inactive form together with HA in the ECM. According to Mio and Stern (2002), regulation of the size and concentration of HA chains is kinetically and energetically more efficient through the catabolic rather than through the anabolic pathway. In fact, HAase could be present in tissues together with inhibitors, like negatively charged polysaccharides, which may allow HAase to be rapidly activated or inactivated. However, even though some HAase inhibition activities have been detected in vivo, very little is known about their molecular structure and the mechanism of action in vivo (Mio & Stern, 2002). We show here that HA, by forming complexes with HAase, is a potential candidate for the inhibition.

In biology, HAases are very interesting enzymes because they must have activity both inside and outside the cell. The HA degradation is mostly enzymatic and occurs in a stepwise fashion leading to discrete sizes of HA fragments (Stern, 2008, www.glycoforum.gr.jp/). Hyal-2 which is the GPI-anchored enzyme outside the cell cleaves HA to 20 kDa limit fragments, although Hyal-1 degrades these fragments to short oligosaccharides inside the cell lysosomes. There are a lot of differences between the two enzyme systems: (i) pH is very acidic in lysosomes and close to neutrality outside the cell, although H^+ pumps associated with CD44 may significantly acidify the micro-domains of ECM close to the membrane (Bourguignon, Singleton, Diedrich, Stern, & Gilad, 2004), (ii) the molecular folding of the HA molecule which is size-dependent, (iii) the complex formation between HA and non-specific proteins which is also size-dependent with a maximum for 10–50 kDa (Lenormand et al., 2010b), pH-dependent, protein-dependent (Lenormand et al., 2010a) and leads to free active enzyme, and (iv) the substrate of HAase which is likely the HA molecule bound to the proteins which is thus protein-dependent too. Consequently, (i) regulation of HAase is possible and different inside and outside the cell, and (ii) the binding properties of HA with proteins and/or hyaladherins could be at the origin of the predominant HA sizes inside and outside the cell.

Finally, the HA/HAase system, able to form two types of complex, is very similar to the nucleic acid binding protein/DNA system, extensively studied by the Record's group (Record & Mossing, 1987) in order to understand the fundamental mechanism of regulation of gene expression by RNA-polymerase at the level of transcription initiation. In this system, nucleic acid binding proteins generally exhibit two competitive modes of interaction with nucleic acids: specific and nonspecific (Tsodikov, Holbrook, Shkel, & Record, 2001).

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