Carbohydrate Polymers 85 (2011) 303–311

Contents lists available at ScienceDirect

Carbohydrate Polymers

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

pH effects on the hyaluronan hydrolysis catalysed by hyaluronidase in the presence of proteins: Part II. The electrostatic hyaluronan – Protein complexes

Hélène Lenormand, Jean-Claude Vincent*

Laboratoire "Polymères, Biopolymères, Surfaces", CNRS UMR 6270 & FR 3038 – Université de Rouen, 76821 Mont-Saint-Aignan Cedex, France

ARTICLE INFO

Article history:
Received 31 July 2010
Received in revised form 6 December 2010
Accepted 4 February 2011
Available online 22 February 2011

Keywords:
Hyaluronan
Hyaluronidase
Bovine serum albumin
Lysozyme
Polysaccharide–protein complex
pH effect

ABSTRACT

Hyaluronan (HA) hydrolysis catalysed by hyaluronidase (HAase) is enhanced when bovine serum albumin (BSA) is present and competes with HAase to form electrostatic complexes with HA. At 1 g L^{-1} HA and BSA concentrations, BSA is able to form three types of complexes with HA depending on pH ranging from 2.5 to 6: insoluble neutral complexes at low pH values, sedimentable slightly charged complexes at pH near 4 and soluble highly charged complexes at pH near 5. The BSA content, charge and solubility of the HA–BSA complexes increase when pH is increased up to the pI of BSA. The normalised charge excess does not exceed 20% for the sedimentable complexes and 40% for the soluble complexes. It has been shown that the sedimentable slightly charged HA–BSA complexes are the most efficient to compete with HAase and release it. All the HA–BSA complexes are hydrolysable by HAase. The HA–BSA binding site shows that one BSA molecule is associated with 85–170 HA carboxyl groups, depending on pH. Similar results have been obtained for lysozyme over an extended pH domain, including the neutrality.

1. Introduction

Hyaluronan (HA) is a linear high-molar-mass polysaccharide composed of repeating D-glucuronic acid-β(1,3)-N-acetyl-D-glucosamine disaccharide units linked together through β(1,4) glycosidic bonds. Since its discovery in the vitreous humor by Meyer in 1936, it has been well established that HA is widely distributed in the extra-cellular matrix (ECM) of vertebrate tissues where it is involved in cellular adhesion, mobility, proliferation and differentiation (Catterall, 1995; Delpech et al., 1997; Girish & Kemparaju, 2007; Kennedy, Phillips, & Williams, 2002; Laurent, 1987; Rooney, Kumar, Ponting, & Wang, 1995). HA is the substrate of hyaluronidase (HAase). HAases are present in mammals, insects, parasites and bacteria (Csóka, Frost, & Stern, 1997) and catalyse the cleavage of HA into oligosaccharides. Recently, it has been demonstrated that properties and functions of HA strongly depend on the chain size (David-Raoudi et al., 2008; Deschrevel, Tranchepain, & Vincent, 2008; Stern, Asari, & Sugahara, 2006) and that HA fragments and native HA may have opposite roles, especially in regards to angiogenesis (Deed et al., 1997; West & Kumar, 1989; West, Hampson, Arnold, & Kumar, 1985). It is now established that the action of HAase plays a role in cancer development (Lokeshwar, Lokeshwar, Pham, & Block, 1996; Mio & Stern, 2002; Rooney et al., 1995; Stern, 2008). More generally, the action of HAases is very important because their presence in the ECM often results in the degradation of the polysaccharide, altering its structure and consequently its function. Understanding the interactions between polysaccharides and proteins, or polysaccharides and enzymes, are thus of fundamental importance. Crystal structure and molecular modeling of such specific assemblies (with short oligosaccharides) have recently been reviewed (Imberty, Lortat-Jacob, & Perez, 2007), offering very important progresses in that field.

At pH 4, HA and HAase can form non-specific electrostatic HA–protein complexes (Deschrevel, Lenormand, et al., 2008; Lenormand, Deschrevel, Tranchepain, & Vincent, 2008) because HA is negative and HAase is positive. Moreover, it has been shown that HAase is no longer catalytically active when electrostatically complexed with HA (Astériou, Vincent, Tranchepain, & Deschrevel, 2006; Deschrevel, Lenormand, et al., 2008; Lenormand, Deschrevel, & Vincent, 2007). In fact, various proteins, like bovine serum albumin (BSA) or lysozyme (LYS), can also form electrostatic complexes with HA (Gacesa, Savitsky, Dodgson, & Olavesen, 1981; Moss, Van Damme, Murphy, & Preston, 1997; Xu, Yamanaka, Sato, Miyama, & Yonese, 2000). Moreover, Gold (1982) observed that BSA is able to activate both human liver HAase and bovine testicular HAase at pH 4. Maingonnat et al. (1999) showed that BSA and other proteins such as hyaluronectin, hemoglobin and immunoglobulins are able to enhance HAase activity. However, the enhancement depends on the protein concentration. It is thus very important to understand how proteins, simultaneously present in the tissue, may modulate the HAase action.

* Corresponding author. Tel.: +33 2 35 14 67 42; fax: +33 2 35 14 67 04.
E-mail address: jean-claude.vincent@univ-rouen.fr (J.-C. Vincent).

0144-8617/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2011.02.007

We have shown that BSA is able to form complexes with HA at pH 4 and is able to compete with HAase when the two proteins are simultaneously present (Deschrevel, Lenormand, et al., 2008; Lenormand et al., 2008). At pH 4, we have shown that HAase activity depends on the BSA concentration in a very atypical manner. BSA can enhance or suppress HAase activity. At low concentrations, BSA forms complexes with HA and prevents the complexation of HAase. At high concentrations, BSA forms dense complexes with HA hindering a great number of hydrolysable sites on the HA molecule. At pH 5.25, the action of BSA is similar but less efficient than at pH 4 (Lenormand, Tranchepain, Deschrevel, & Vincent, 2009) because, pH being close to the isoelectric pH (pI) of BSA (pI = 5.2 (Xu et al., 2000)), the number of positive charges borne by the BSA molecule and required to form electrostatic complexes with the polyanionic HA molecule is low.

The question of the BSA ability to form electrostatic complexes with HA under physiological pH conditions is of importance for the HAase modulation in the ECM, and we are now engaged in a detailed study of the HAase activity as a function of pH (Lenormand, Deschrevel, & Vincent, 2010a). 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 a precise 3D arrangement 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 (Deschrevel, Lenormand, et al., 2008; Lenormand et al., 2007, 2008) through electrostatic interactions. The actual pH-dependence is thus the result of the two following pH-dependences: the intrinsic pH-dependence of the enzyme action and the pH-dependence of the formation of the non-specific electrostatic HA–protein complexes (Lenormand et al., 2010a). The enhancement of the HAase activity in the presence of non-catalytic proteins thus requires two conditions: (i) the protein has to be able to form a complex with HA and (ii) this complex has to be more stable than the electrostatic complex formed between HA and HAase.

We have shown (Lenormand et al., 2010a) that BSA is able to control the enhancement/suppression of the HAase activity at pH values ranging from 3.5 to 5.25. For an HA concentration of 1 g L^{-1} and an HAase concentration of 0.5 g L^{-1}, the optimal BSA concentration varies from 0.6 to 2 g L^{-1} according to pH. At pH higher than 4.5, BSA can still form complexes with HA, but the release of HAase requires more BSA molecules, corresponding to BSA concentrations higher than 4 g L^{-1}. The fact that BSA can enhance the HAase activity on a pH domain ranging from 3.5 to 5.25 means that: (i) BSA is able to compete with HAase to form complexes with HA, (ii) HAase is able to form complexes with HA over this pH domain, and (iii) HAase is catalytically active over this pH domain when BSA limits its complexation with HA. All of this have been considered in a theoretical model (Vincent & Lenormand, 2009) which clearly explains the functioning of the HA–HAase–BSA system. Nevertheless, BSA is not able to enhance HAase activity at physiological pH. We have shown (Lenormand et al., 2010a) that LYS, which has a pI value higher than that of BSA, is able to enhance HAase activity at physiological pH. That means that HA–HAase complexes can be formed at physiological pH. It should thus be important to study the HA–BSA, HA–LYS and HA–HAase complexes as a function of pH, in order to see which type of complex is concerned.

Most of the work on protein–polyelectrolyte complexes is reviewed by Schmitt, Sanchez, Desobry-Banon, and Hardy (1998), Cooper, Dubin, Kayimazer, and Turksen (2005) and Imberty et al. (2007). Two main classes can be distinguished for the electrostatic complexes: (i) neutral insoluble complexes at the phase separation when the net positive charge of one of the biomacromolecules exactly compensates the net negative charge of the other, and (ii) charged complexes, when an excess of charge exists, which are maintained in suspension by solvatation and can produce turbidity. Numerous studies have been devoted to the complexes formed between HA and proteins (for details, see Lenormand et al., 2010a). Xu et al. (2000) studied HA/BSA mixtures and showed the electrostatic nature of the interactions between the two molecules and the strong influence of pH on solubility of the HA–BSA complex. They determined the stoichiometry, viscosity and size of the HA–BSA complexes at pH near 5 and proposed an interesting model for HA–BSA complex formation showing the change from a worm-like structure to spherical particules by intra-polymer then interpolymer interactions when BSA concentration is increased. Grymonpré, Staggemeier, Dubin, and Mattison (2001) studied the effects of both pH and ionic strength on the existence and solubility of the HA–BSA complexes. They showed that the electrostatic HA–BSA complexes can be formed even at a pH higher than the pI of the protein thanks to the presence of charge patches.

Other papers reported pH effects on complexes formed between another anionic polysaccharide (heparin) and BSA, between another protein (silk fibroin) and HA (Malay, Bayraktar, & Batigün, 2007), or between a cationic polyelectrolyte and BSA (Kaibara, Okazaki, Bohidar, & Dubin, 2000; Mattison, Dubin, & Brittain, 1998). In our group, we have studied the HA–BSA complexes formed at pH 4 and low ionic strength (Lenormand et al., 2008). We have determined the conditions for existence, the nature, the stoichiometry and the size of the complexes, with respect to the BSA over HA ratio. We have shown that HA and BSA can form three types of electrostatic complexes: highly charged soluble complexes, slightly charged sedimentable complexes and neutral insoluble complexes at the phase separation.

Existence and nature of HA–BSA complexes being related to the charge borne by the two macromolecules, they are pH dependent. In complement to the important work of Grymonpré et al. (2001) and Xu et al. (2000), and to our own work (Deschrevel, Lenormand, et al., 2008; Lenormand et al., 2008, 2009, 2010a), the present paper deals with the detailed study of the HA–protein (BSA or LYS) complexes as a function of pH and presents their nature, solubility, stoichiometry, size and composition, in order to establish a relationship between the HAase activity (Lenormand et al., 2010a) and the existence of the electrostatic nonspecific HA–protein complexes. In addition, one of the objectives of the paper is to determine among these complexes, which type is the most efficient to compete with the HA–HAase complex and to release HAase?

2. Experimental

2.1. Materials

Bovine testicular HAase (H 3884, lot 38H7026), BSA (A 3675, lot 78H1399), LYS (L 6876, lot 051K7028) 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 BSA was 69,000 g mol^{-1}. HA, BSA, LYS 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. Measurements of hydrodynamic diameters of HA–protein complexes were carried out using a Zeta Sizer (Nano ZS) from Malvern Instruments equipped with a laser source ($\lambda = 632$ nm). This apparatus was able to measure diameters of suspended particles ranging from 1 nm to 1 μm. Diameters of insoluble particles were not available.

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 the dissociation of the electrostatic complex at pH near 12.

2.2.2. N-acetyl-D-glucosamine reducing end assay

Measurement of the concentration of N-acetyl-D-glucosamine reducing ends enabled the determination of the HA chain concentration. It was performed according to the method described by Reissig, Strominger, and Leloir (1955). Because of the presence of proteins in the samples, turbidity and colour were simultaneously present and we used the improvement of the Reissig method described by Astériou et al. (2001). The experimental procedure using DMAB is detailed in our previous papers (Astériou et al., 2001; Vincent et al., 2003). N-acetyl-D-glucosamine was used for calibration.

2.2.3. Kinetics of the HA hydrolysis

An adequate volume of the HA mother solution was placed in a reactor, diluted to the desired concentration with milli-Q water, adjusted to the desired pH with HCl (or KOH), stirred and maintained at 37 °C. If necessary, before pH adjustment, an appropriate volume of a concentrated BSA solution (10 g L^{-1}) was added. After 2 min, the reaction was started by adding an adequate volume of a concentrated HAase solution (10 g L^{-1} in milli-Q water). At each time point, a 200 μL aliquot of the reaction mixture was removed from the reactor and assayed by using the N-acetyl-D-glucosamine reducing end assay. For each kinetics, the hydrolysis reaction was followed for 3 h and the concentration of the HA reducing ends was plotted against time. The method thus gave the time evolution of the HA chain concentration; the slope of the tangent to that curve directly represented the reaction rate. The kinetic curve was then fitted by the bi-exponential model developed in Vincent et al. (2003) and the initial reaction rate was calculated as being equal to the value of the first derivative of that function at time zero.

2.2.4. Turbidity measurements

The solutions of HA and BSA prepared in Milli-Q water were first adjusted to a pH value higher than 8 in order to limit complexation when mixed. The HA/BSA mixture was prepared by diluting adequate volumes of HA and BSA solutions in Milli-Q water so that the final concentrations were 1 g L^{-1} for both HA and BSA. The mixture was placed in the reactor maintained at 37 °C. Then pH was progressively decreased and turbidity variation in time was measured at 400 nm in a spectrophotometer thermostated (37 °C) sample cell with a magnetic stirrer.

2.2.5. Analysis of the HA–protein complexes

The HA/BSA mixture prepared in a reactor was incubated at 37 °C 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 BSA concentration at 280 nm by using the calibration curve previously established with BSA 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 BSA and HA in the obtained solution were then measured using the same methods as above.

2.2.6. Measurement of the HA–protein complexes' size

Size measurements were performed with the HA/BSA mixtures at four pH values. The mixture was introduced into an appropriate plastic cuvette of 1 cm pathlength and placed in the Zeta Sizer. Three runs were performed per experiment. Results were expressed in percentage of light intensity scattered by the solution as a function of the diameter of the particles responsible for this scattering (Mattison & Kaszuba, 2004).

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–BSA complexes

Existence of electrostatic complexes formed between HA and BSA was first studied using spectrophotometric measurements. The HA/BSA mixture, at 1 g L^{-1} HA concentration and 1 g L^{-1} BSA concentration, was first adjusted to pH 10, then pH was slowly decreased and the turbidity of the mixture was measured as a function of pH. In that experiment, pH was the only adjustable parameter and the system was theoretically monitored by the charges of the two biomacromolecules. The pKa of HA being equal to 2.9 (Cleland, Wang, & Detweiler, 1982) and the pI of BSA being equal to 5.2 (Wang, Gao, & Dubin, 1996; Xu et al., 2000), the theoretical pH domain for complexation is between 2.9 and 5.2, in a first approximation. Fig. 1 shows the turbidity of the HA/BSA mixture at 400 nm as a function of pH. Turbidity existed at pH values between 2.3 and 6.5 and was maximum between 3.3 and 4. According to Xu et al. (2000) and Lenormand et al. (2010a), five pH domains were identified: (i) from pH 8 to 6.5, absorbance was nil corresponding to soluble and individual HA and BSA molecules, (ii) from pH 6.5 to 5.2, absorbance increased slightly corresponding to soluble highly charged HA–BSA complexes, (iii) from pH 5.2 to 3.9, absorbance increased strongly corresponding to sedimentable slightly charged HA–BSA complexes, (iv) from pH 3.9 to 3.3, absorbance was constant at a high level corresponding to a maximum of sedimentable HA–BSA complexes, and (v) at pH lower than 3.3, absorbance decreased because of the phase separation of the HA–BSA complexes and of the apparition of insoluble complexes.

Because HA solubility was higher than 10 g L^{-1} and BSA solubility was higher than 20 g L^{-1}, the presence of turbidity showed the existence of HA–BSA coacervates. Indeed, the light scattered by the HA–BSA coacervates (or complexes) whose solubility depends on the charge excess at the surface of the complex and the existence of the electrostatic HA–BSA complexes were related to the charges brought by the two macromolecules. At high pH values, HA was negatively charged; when pH was close to 3.9 (i.e. pKa of HA + 1),

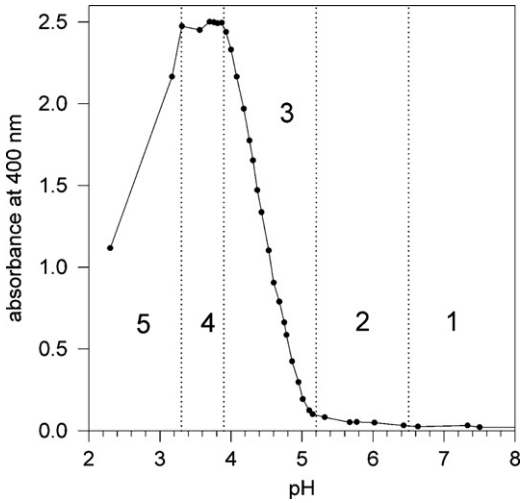

Fig. 1. Absorbance at 400 nm of mixtures composed of HA at 1 g L^{-1} and BSA at 1 g L^{-1}, at low ionic strength and at different pH values ranging from 2.3 to 7.5. Five domains were distiguished.

HA started to decrease its negative charge; when pH was lower than 1.9 (i.e. pKa of HA – 1), HA was neutral. For BSA, the scheme was similar: at high pH values, BSA was negatively charged; when pH was close to 6.2 (i.e. pI of BSA + 1), BSA started to decrease its net negative charge; when pH was close to 5.2 (i.e. pI of BSA), BSA started to increase its net positive charge; when pH was lower than 4.2 (i.e. pI of BSA – 1), BSA was positively charged. The theoretical pH domain for HA–BSA complexes can thus be extended from 1.9 to 6.2.

3.2. The theoretical charge of the two macromolecules

As HA is alternately composed of glucuronic acid and N-acetyl-glucosamine, one carboxyl group, with a defined pKa 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. The variations of the charge state of HA, expressed in milli-equivalents per gram of HA, as a function of pH, are given in Fig. 2. For BSA, 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

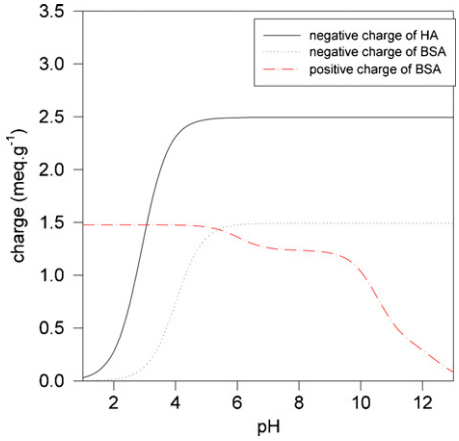

Fig. 2. pH-dependence of the charge state of the two biomacromolecules. Milli-equivalents brought per gram of HA and BSA as a function of pH.

Table 1
pK values of the ionisable amino acids.

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

aqueous solvent. Thanks to the high level of sequence homology between BSA and Human serum albumin (HSA), the 3D structure of HSA and according to Lenormand et al. (2008) and Grymonpré et al. (2001), we may assume such a conformation for BSA in water and calculate the number of mole of positive and negative charges brought per gram of BSA as a function of pH. The number, n_i, of each ionisable amino acid (i.e. aspartate (D), glutamate (Q), arginine (R), histidine (H) and lysine (K)) was calculated from the primary sequence of BSA. By assuming that the pKa of any ionisable amino acid remains constant and independent of its neighbourhood, the charge brought by each ionisable amino acid can be estimated by multiplying the number of this ionisable amino acid by its charge fraction, calculated from its pKa (Table 1) and the pH of the HA/BSA mixture, according to Eqs. (1) and (2). The net charge of the protein is finally estimated by algebraically summing all the partial charges of the amino acid residues and by adding the charges brought by the two end amino acids.

$$\text{negative charge fraction of BSA} = \sum_{i=d,q} n_i \times \frac{1}{1+10^{\text{pKi}-\text{pH}}} \quad (1)$$

$$\text{positive charge fraction of BSA} = \sum_{i=r,h,k} n_i \times \frac{1}{1+10^{\text{pH}-\text{pKi}}} \quad (2)$$

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 over a large pH domain. Fig. 2 shows the theoretical charges of the two macromolecules as a function of pH.

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

An HA/BSA mixture with 1 g L^{-1} HA concentration and 1 g L^{-1} BSA concentration was placed in a reactor maintained at 37 °C under magnetic stirring. The pH was progressively lowered and 1 mL aliquots of the mixture were removed over time and analysed. After centrifugation of the liquid phase, the HA and BSA assays gave the HA and BSA concentrations in the supernatant and in the pellet for each pH value, as described in the Section 2. Fig. 3 shows the distribution of the BSA and HA concentrations between the supernatant, $[BSA]_s$ and $[HA]_s$, and the pellet, $[BSA]_p$ and $[HA]_p$, as a function of pH. In addition to these curves, the $[BSA]_i$ and $[HA]_i$, corresponding to the parts of the total concentrations of BSA and HA implicated in the insoluble complexes, were simply deduced by subtracting the concentrations corresponding to BSA and HA in the supernatant and in the pellet from the total BSA and HA concentrations.

Three pH domains were distiguished: (i) From pH 6 to 5.3, the HA and BSA contents did not change; most of the HA and BSA were in the supernatant and only a little amount of each biomolecule was present in the pellet; no insoluble complexes were present, (ii) from pH 5.3 to 3.2, $[BSA]_p$ and $[HA]_p$ strongly increased from values close to zero to values close to the total HA and BSA concentrations; in parallel, $[BSA]_s$ and $[HA]_s$ decreased down to values close to zero and $[BSA]_i$ and $[HA]_i$ remained nil; this characterised an exchange between soluble HA–BSA complexes and sedimentable HA–BSA complexes, (iii) from pH 3.2 to 2.3, $[BSA]_s$ and $[HA]_s$ remainded very low, $[BSA]_p$ and $[HA]_p$ slightly decreased and

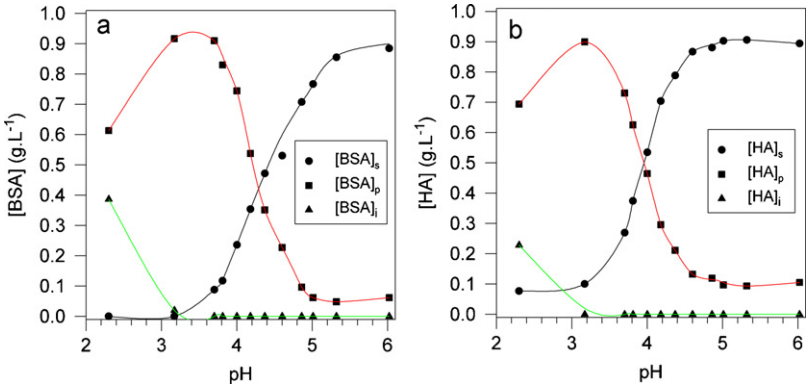

Fig. 3. Analysis of the composition of the HA–BSA complexes as a function of pH. Distribution of BSA (a) and HA (b) after centrifugation of mixtures composed of HA at 1 g L^{-1} and BSA at 1 g L^{-1}, at low ionic strength: in the pellet (■), in the supernatant (●), and in the insoluble HA–BSA complexes (▲).

$[BSA]_i$ and $[HA]_i$ slightly increased; this characterised an exchange between sedimentable HA–BSA complexes and insoluble HA–BSA complexes.

When BSA was assayed in the supernatant, the UV–Visible spectra showed that turbidity was still present signifying that solubilized HA–BSA complexes were present in the supernatant. Fig. 4 shows the absorbance of the supernatant at 400 nm as a function of pH. In order to quantify these soluble complexes, the supernatant was submitted to filtration through 0.45 μm PTFE filters. $[BSA]_{sf}$ and $[HA]_{sf}$ were the BSA and HA concentrations recovered in the filtrates and the difference between $[BSA]_s$ and $[HA]_s$ on one hand, and $[BSA]_{sf}$ and $[HA]_{sf}$ on the other hand, gave $[BSA]_{s-sf}$ and $[HA]_{s-sf}$, the BSA and HA concentrations retained on the filters, i.e. involved in the soluble HA–BSA complexes larger than 450 nm. Fig. 5 shows that HA–BSA complexes were formed up to pH 6; these complexes contained a very small quantity of HA associated to many BSA molecules which were very slightly positively charged around pH 5 or even, slightly negatively charged and containing positive patches around pH 6 (Mattison et al., 1998; Park, Muhoberac, Dubin, & Xia, 1992). Fig. 5 (part a) clearly shows the exchange between the soluble highly charged HA–BSA complexes which were preponderant at pH lower than 5 and the very soluble highly charged HA–BSA complexes and/or individual molecules which were preponderant at pH higher than 5.

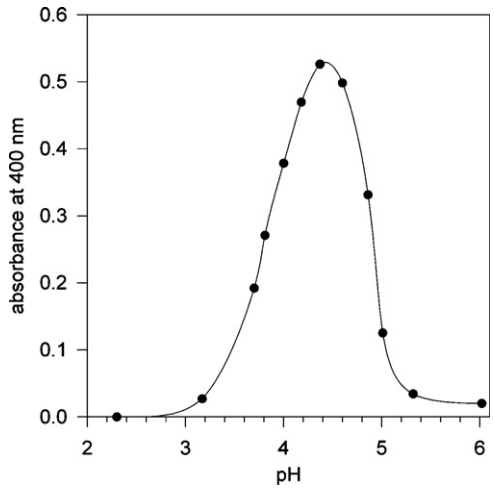

Fig. 4. Absorbance at 400 nm of the supernatant after centrifugation of mixtures composed of HA at 1 g L^{-1} and BSA at 1 g L^{-1}, at low ionic strength and at different pH values ranging from 2.3 to 6.

3.4. Size of the HA–BSA complexes as a function of pH

Fig. 6 represents the superimposition of the turbidity and of the HA and BSA contents of the different types of HA–BSA complexes. The turbidity due to the sedimentable HA–BSA complexes was obtained by subtracting the turbidity of the supernatant from that of the HA/BSA mixture. The figure shows that the sedimentable slightly charged complexes had a higher contribution to the turbidity than the soluble highly charged complexes. It is known that large particles scatter much more light than small particles. The highest contribution to the turbidity of the slightly charged complexes is thus due to: (i) the highly charged HA–BSA complexes were smaller than the slightly charged complexes, and/or (ii) the slightly charged complexes were much more numerous than the highly charged complexes. Size measurements using a zeta sizer (Fig. 7) confirmed that the diameter of the predominant population of the HA–BSA complexes decreased when pH was increased, ranging from 200 nm at pH 3 to 10 nm at pH 5 and only a few nanometers at pH 6. It means that size of the HA–BSA complexes decreased when the charge of the complexes increased. This is in agreement with our previous observations (Lenormand et al., 2008).

3.5. Stoichiometry of the HA–BSA complexes as a function of pH

Stoichiometry of the HA–BSA complexes can be calculated from their HA and BSA contents shown in Figs. 1 and 5. Fig. 8 represents the BSA over HA mass stoichiometry of the different HA–BSA complexes. For each type of complex, the stoichiometry increased when pH was increased. For the slightly charged complexes (p indice), it increased from 0.9 at pH 2.3 to 1.7 at pH close to 4.5; for the highly charged complexes (s–sf indice), it increased from 1 at pH 3.7 to 1.7 at pH 4.8. For each type of complex, the mass stoichiometry increased when pH reached a value close to 5, because complexation required more and more BSA molecules as the net positive charge of BSA decreased near its pI. Roughly, Fig. 8 shows that the mass stoichiometry of the complexes remained between 1 and 2.

The HA and BSA contents shown in Figs. 1 and 5 can also be analysed in terms of charge excess. The net charge of the HA–BSA complexes was normalised with respect to the total charge of the complexes. Fig. 9 shows the normalised charge of the HA–BSA complexes as a function of pH. For the slightly charged complexes (p indice), the normalised charge excess was around 20%, for the highly charged complexes (s–sf indice) it remained under 40%; for the soluble complexes or individual molecules (sf indice), it reached 100% at pH lower than 4.2.

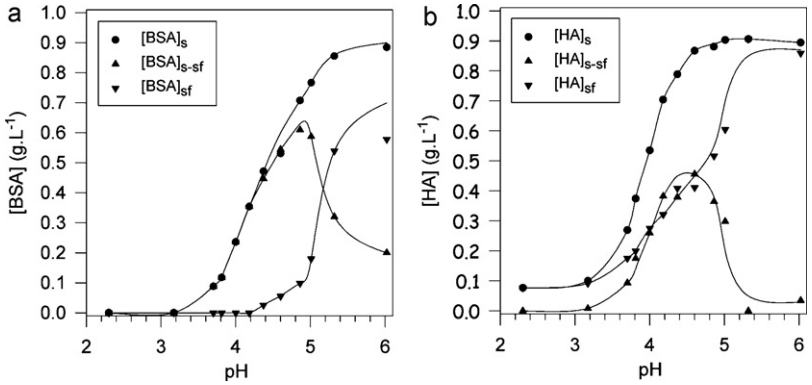

Fig. 5. Analysis of the composition of the HA–BSA complexes as a function of pH. Distribution of BSA (a) and HA (b) after centrifugation of mixtures composed of HA at 1 g L^{-1} and BSA at 1 g L^{-1} at low ionic strength, and filtration of the supernatant: in the supernatant (●, s indice), in the filtrate (▼, sf indice) and in the complexes retained on the filter (▲, s-sf indice).

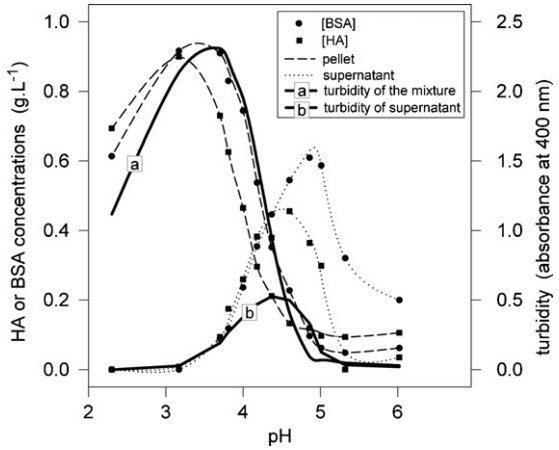

Fig. 6. Superimposition of the HA and BSA contents of the complexes formed between HA and BSA after centrifugation of mixtures composed of HA at 1 g L^{-1} and BSA at 1 g L^{-1} at low ionic strength, and the turbidity of the HA/BSA mixtures, as a function of pH.

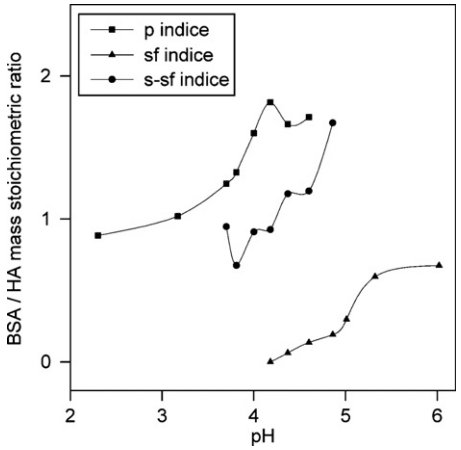

Fig. 8. Stoichiometry of the HA–BSA complexes expressed in mass ratio. BSA over HA mass ratio of the complexes formed in mixtures composed of HA at 1 g L^{-1} and BSA at 1 g L^{-1}, at low ionic strength and at different pH values ranging from 2.3 to 6. Ratios were calculated from the results in the pellet (■), for the filtrated supernatant (▲) and for the complexes retained on the filter (●).

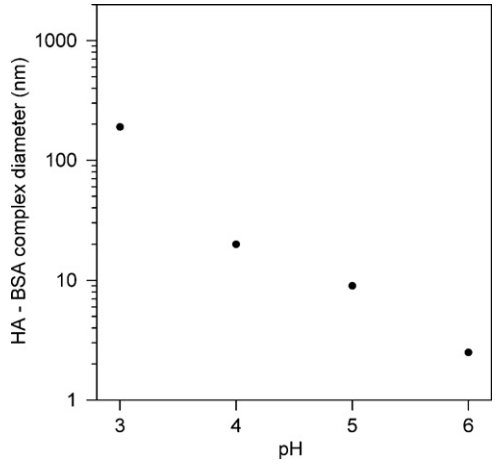

Fig. 7. Diameter of the HA–BSA complexes as a function of pH. Diameters of the particles present in the mixtures composed of HA at 1 g L^{-1} and BSA at 1 g L^{-1}, at low ionic strength and at different pH values ranging from 3 to 6.

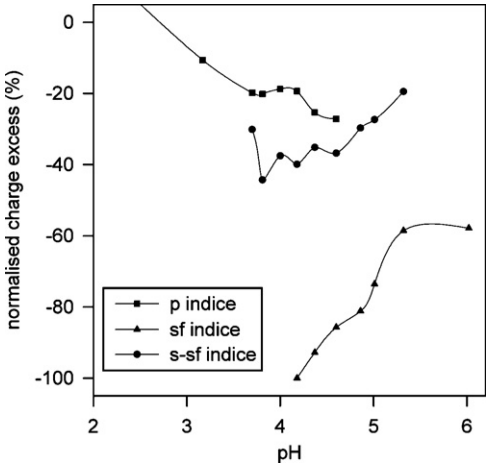

Fig. 9. Stoichiometry of the HA–BSA complexes expressed in normalised charge excess. BSA over HA mass ratio of the complexes formed in mixtures composed of HA at 1 g L^{-1} and BSA at 1 g L^{-1}, at low ionic strength and at different pH values ranging from 2.3 to 6. Ratios were calculated from the results in the pellet (■), for the filtrated supernatant (▲) and for the complexes retained on the filter (●).

3.6. Size of the binding site

A value of the mass stoechiometric ratio between 1 and 2 means that 0.967 g of HA was bound to 1–2 g of BSA, i.e. 10^{-6} moles of HA were bound to 15 10^{-6} to 30 10^{-6} moles of BSA. This corresponds to 15–30 BSA molecules per HA molecule of 1 MDa containing approximately 2500 disaccharide units of 401 g mol^{-1}. One BSA molecule was thus associated with 170–85 carboxyl groups when pH was increased from 2.3 to 4.5. This constitutes the size of the HA-BSA binding site. When pH was increased up to a value close to the pI of BSA, the positive charge of BSA decreased and the number of BSA molecules bound to one HA molecule increased. The number of carboxyl groups involved in the binding site thus decreased.

3.7. Relation between the HA–BSA complexes and the HAase activity as a function of pH

When HA and HAase were present together in the reaction medium, HA formed two types of complexes with HAase: the first one was the classical catalytic enzyme–substrate complex and the second one was the electrostatic non-catalytic and non-specific HA–protein complex (Lenormand et al., 2010a). HA was able to form the latter type of complex with a great variety of proteins, such as BSA, LYS and HAase. When HA, HAase and BSA were simultaneously present, BSA competed with HAase to form complexes with HA, resulting to the release of HAase which recovers its activity. The question is: which type of HA–BSA complex is the most efficient to compete with HAase? Fig. 10 shows the amplification factor of the HAase activity due to the addition of BSA at 1 g L^{-1} in the reaction medium (data were extracted from (Lenormand et al., 2010a)). We also represented in that figure the HA and BSA concentrations recovered in the two types of complexes, the slightly and the highly charged complexes, as a function of pH. Fig. 10 shows that the presence of sedimentable slightly charged HA–BSA complexes and the amplification of the HAase activity were closely related. It is thus clear that only the slightly charged HA–BSA complexes were able to release HAase and to induce an increase in the HAase activity over the 3.75–5.2 pH domain. However, at pH below 3.75, the HA–BSA complexes were not able to restore the HAase activity. It is likely that the HA–BSA complexes were able to release the HAase molecules from the HA–HAase complexes, but HAase was much less active at these low pH values because of its conformation. It means that (i) BSA has to form slightly charged complexes with HA to be able to positively compete with HAase and thus to release HAase from complexes, and (ii) the slightly charged HA–BSA complexes were hydrolysable by HAase.

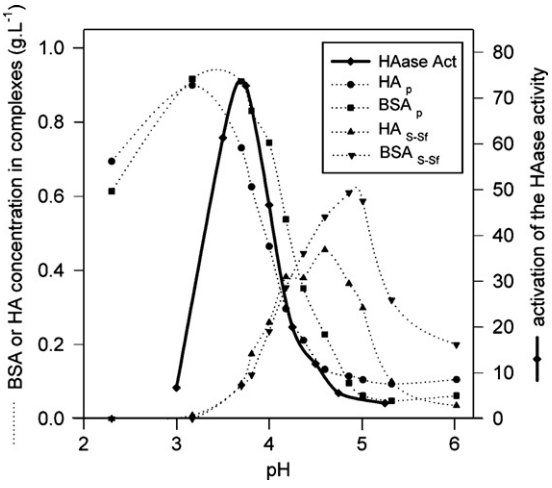

Fig. 10. Superimposition of the HA and BSA contents of the complexes formed between HA and BSA after centrifugation of mixtures composed of HA at 1 g L^{-1} and BSA at 1 g L^{-1} at low ionic strength, and the amplification of the HAase activity due to BSA (HAase activity with 1 g L^{-1} BSA divided by HAase activity without BSA), as a function of pH.

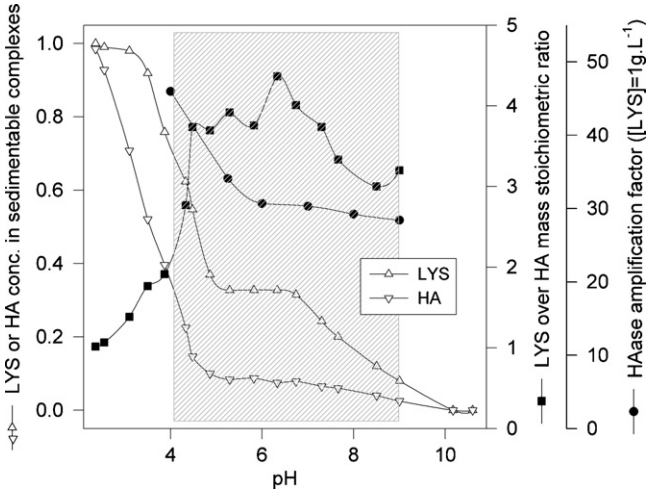

Fig. 11. Superimposition of the HA and LYS contents of the complexes formed between HA and LYS after centrifugation of mixtures composed of HA at 1 g L^{-1} and LYS at 1 g L^{-1} at low ionic strength as a function of pH. The figure also shows the mass stoichiometric ratio and the amplification of the HAase activity due to LYS (HAase activity with 1 g L^{-1} LYS divided by HAase activity without LYS) as a function of pH.

3.8. Relation between the HA–LYS complexes and the HAase activity as a function of pH

In our previous paper (Lenormand et al., 2010a), two proteins, BSA and LYS, were used to modulate the HAase activity. BSA and LYS are thus two enhancer proteins for HAase. When LYS was added in an HA/HAase system, it competed with HAase to form complexes with HA resulting in the release of HAase which recovered its activity. Although BSA was able to enhance the HAase activity from pH 3 to pH 5.25, LYS was able to reveal and enhance the HAase activity up to pH 9 (Lenormand et al., 2010a). As for BSA, we investigated the HA–LYS complexes in order to determine the different types of complexes and which type of HA–LYS complex is the most efficient to compete with HAase? The three types of complexes also exist for LYS: insoluble neutral complexes at pH lower than 3, sedimentable slightly charged complexes at pH from 3 to 10 and soluble highly charged complexes at pH higher than 5. Fig. 11 shows the amplification factor of the HAase activity due to the addition of LYS at 1 g L^{-1} in the reaction medium (data extracted from Lenormand et al., 2010a). The figure also shows the HA and LYS concentrations found in the sedimentable slightly charged complexes and the LYS over HA mass stoichiometric ratio, as a function of pH. As for BSA, the amplification factor of the HAase activity was high as long as the sedimentable HA–LYS complex concentrations were significant. It means that these sedimentable complexes, with a quite constant LYS over HA mass stoichiometric ratio equal to 4, prevents HAase to form electrostatic complexes with HA over the large pH domain ranging from 4 to 9, thus including pH 7.

4. Discussion and conclusion

At 1 g L^{-1} HA and BSA concentrations and low ionic strength, BSA is able, depending on pH, to form three types of electrostatic complexes with HA: insoluble neutral complexes at pH values lower than 3.2, sedimentable slightly charged complexes at pH near 4 and decreasing from pH 3.2 to 5.3 and soluble highly charged

complexes at pH near 4.5 and increasing from pH 3.2 to 5.3. The HA–BSA complexes are more charged and soluble when pH is increased up to 5.2, i.e. the pI of BSA, because the BSA content of the complexes increases when pH is increased. The wide region of the HA–BSA complex existence shows that the phase separation is more broad than the precise electroneutrality domain. This aspect has been studied in detail by Cousin, Gummel, Clemens, Grillo, and Boué (2010) in the electrostatic complexes. Very interesting studies have also shown that the charge ratio and the size of the polysaccharide chain influence the multiple scale structure of the polyelectrolyte–protein complexes (Cousin, Gummel, Ung, & Boué, 2005; Turgeon, Schmitt, & Sanchez, 2007). The BSA over HA mass stoichiometric ratio of the insoluble or sedimentable HA–BSA complexes remains between 1 and 2; it means that 15–30 BSA molecules form a complex with one HA molecule of number-average molar mass of $0.967 \times 10^6\,g\,mol^{-1}$. It also means that one BSA molecule is associated with 85–170 HA carboxyl groups, depending on pH. This constitutes the size of the HA–BSA binding site. Sedimentable HA–BSA complexes are slightly charged complexes for which the normalised charge excess does not exceed 20%, while it reaches 40% for the soluble highly charged complexes. There is only a very few studies concerning the stoichiometry and the binding site of the HA–protein complexes reported in the literature. Xu et al. have determined that one BSA molecule is associated with 15 HA carboxyl groups for soluble negatively charged complexes at pH between 5.1 and 5.6 (Xu et al., 2000). This corresponds to experiments performed at BSA over HA ratio and pH much higher than we used. Their pH value is also higher than the pI of BSA. It is thus expected that the number of carboxyl groups associated to one BSA molecule is much lower than in our case. Moreover, their results were obtained by a different method, i.e. the measurement of the Na^+ ions released from sodium HA during the formation of the HA–BSA coacervate. The very small number of carboxyl groups associated to one BSA molecule even suggests that multi-layer HA–BSA complexes may be formed with an excess of BSA (BSA–BSA interactions) and an excess of negative charges. Using a LYS over HA ratio equal to 2.5, Van Damme, Moss, Murphy, and Preston (1994) showed that one LYS molecule, which is five times smaller than BSA links to 14 HA carboxyl groups at pH 7.5. Our values are thus consistent with the literature.

The second result of this study concerns the HA/HAase/BSA system and the relationship between the complex that HA is able to form with BSA and the amplification of the HAase activity resulting from this complex formation. Under these conditions, HAase is catalytically active and thus not involved in complexes with HA; HA forms complexes with BSA only (Lenormand et al., 2009). We have shown that the sedimentable slightly charged HA–BSA complexes are the most efficient complexes for competing with the complexes formed between HA and HAase. Our results also confirm that the slightly charged HA–BSA complexes are hydrolysable by HAase. In fact, enhancement of HAase activity depends on two phenomena: (i) the release of HAase which is favoured when the electrostatic interactions between HA and BSA are maximum, i.e. when the HA–BSA complexes are slightly charged or neutral, and (ii) the accessibility of the hydrolysable sites on the HA molecule which is minimalized when the neutral HA–BSA complexes aggregate. Under these conditions, the best compromise seems to be the sedimentable slightly charged complexes for enhancing HAase activity.

Results obtained with LYS are similar to those obtained with BSA. The same three types of complexes exist and LYS is able to modulate the HAase activity over a large pH domain ranging from 3 to 9. For LYS also, the sedimentable slightly charged complexes, i.e. the slightly soluble complexes, are the most efficient for competing with the complexes formed between HA and HAase. The present study thus highlights the functioning of HAase in the presence of proteins as a function of pH (Lenormand et al., 2010a). Several other proteins, such as immunoglobulins, hyaluronectin (Maingonnat et al., 1999), are enhancer proteins for HAase and it is likely that the main results concerning BSA or LYS are valid for these proteins. The role of BSA or LYS in these experiments can also be assimilated to the protective role of proteins, including BSA, added to prevent enzyme denaturation or loss of enzyme onto the non-specific binding sites of plastic and glassware surfaces, although hydrophobic interactions are mainly concerned in that case. On the biological point of view, BSA has been essentially considered as a model for HSA. As HSA and LYS are present in the ECM of numerous tissues together with HAase or other enzymes, the present study brings a better understanding of a possible enzyme regulation mechanism. More generally, our study may constitute an approach for investigating the role of proteins and other polyelectrolytes in the regulation of enzyme systems in the ECM.

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, Deschrevel, & Vincent, 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. Consequently, (i) activity and regulation of HAase seem to be actual and largely independent 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.

To better understand the HA/HAase/protein system, we need to know more about the formation of the non-specific electrostatic HA–HAase complexes as a function of pH. However, an additional difficulty appears because HAase has a double role: It is a protein able to form electrostatic complexes with HA at pH below its pI value, and it is an enzyme able to hydrolyse HA, and thus to change the conformation and the size of the polysaccharidic ligand. In order to avoid the hydrolysis of the HA molecule by HAase during the study of the electrostatic HA–HAase complexes, the experiments will be performed at a temperature lower than 37 °C to reduce the enzyme activity. Results will be published in Part III of this series.

Acknowledgements

We thank Dr. Annie Steinchen-Sanfeld and Vishwas Purohit for critical readings of the manuscript.

References

Astériou, T., Deschrevel, B., Delpech, B., Bertrand, P., Bultelle, F., Merai, C., et al. (2001). An improved assay for the N-acetyl-D-glucosamine reducing ends of polysaccharides in the presence of proteins. *Analytical Biochemistry*, *293*, 53–59.

Astériou, T., Vincent, J.-C., Tranchepain, F., & Deschrevel, B. (2006). Inhibition of hyaluronan hydrolysis catalysed by hyaluronidase at high substrate concentration and low ionic strength. *Matrix Biology*, *25*, 166–174.

Bitter, T., & Muir, H. M. (1962). A modified uronic acid carbazole reaction. *Analytical Biochemistry*, *4*, 330–334.

Bourguignon, L. Y., Singleton, P. A., Diedrich, F., Stern, R., & Gilad, E. (2004). CD44 interaction with Na^+–H^+ exchanger (NHE1) creates acidic microenvironments leading to hyaluronidase-2 and cathepsin B activation and breast tumor cell invasion. *Journal of Biological Chemistry*, *279*, 26991–27007.

Catterall, J. B. (1995). Hyaluronic acid, cell adhesion and metastasis. *Cancer Journal*, *8*, 320–324.

Cleland, R. L., Wang, J. L., & Detweiler, D. M. (1982). Polyelectrolyte properties of sodium hyaluronate: 2. Potentiometric titration of hyaluronic acid. *Macromolecules*, *15*, 382–386.

Cooper, C. L., Dubin, P. L., Kayimazer, A. B., & Turksen, S. (2005). Polyelectrolyte–protein complexes. *Current Opinion in Colloid and Interface Science*, *10*, 52–78.

Cousin, F., Gummel, J., Ung, D., & Boué, F. (2005). Polyelectrolyte–protein complexes: Structure and conformation of each specie revealed by SANS. *Langmuir*, *21*, 9675–9688.

Cousin, F., Gummel, J., Clemens, D., Grillo, I., & Boué, F. (2010). Multiple scale reorganization of electrostatic complexes of poly(styrenesulfonate) and lysozyme. *Langmuir*, *26*, 7078–7085.

Csóka, T. B., Frost, G. I., & Stern, R. (1997). Hyaluronidases in tissue invasion. *Invasion & Metastasis*, *17*, 297–311.

David-Raoudi, M., Tranchepain, F., Deschrevel, B., Vincent, J. C., Bogdanowicz, P., Boumediene, K., et al. (2008). Differential effects of hyaluronan and its fragments on fibroblasts: Relation to wound healing. *Wound Repair and Regeneration*, *16*, 274–287.

Deed, R., Rooney, P., Kumar, P., Norton, J. D., Smith, J., Freemont, A. J., et al. (1997). Early response gene signaling is induced by angiogenic oligosaccharides of hyaluronan in endothelial cells. Inhibition by nonangiogenic, high-molecular-weight hyaluronan. *International Journal of Cancer*, *71*, 251–256.

Delpech, B., Girard, N., Bertrand, P., Courel, M.-N., Chauzy, C., & Delpech, A. (1997). Hyaluronan: Fundamental principles and applications in cancer. *Journal of Internal Medicine*, *242*, 41–48.

Deschrevel, B., Lenormand, H., Tranchepain, F., Levasseur, N., Astériou, T., & Vincent, J. C. (2008). Hyaluronidase activity is modulated by complexing with various polyelectrolytes including hyaluronan. *Matrix Biology*, *27*, 242–253.

Deschrevel, B., Tranchepain, F., & Vincent, J. C. (2008). Chain-length dependence of the kinetics of the hyaluronan hydrolysis catalyzed by bovine testicular hyaluronidase. *Matrix Biology*, *27*, 475–486.

Dische, Z. (1947). A new specific color reaction of hexuronic acid. *Journal of Biological Chemistry*, *167*, 189–198.

Gacesa, P., Savitsky, M. J., Dodgson, K. S., & Olavesen, A. H. (1981). A recommended procedure for the estimation of bovine testicular hyaluronidase in the presence of human serum. *Analytical Biochemistry*, *118*, 76–84.

Girish, K. S., & Kemparaju, K. (2007). The magic glue hyaluronan and its eraser hyaluronidase: A biological overview. *Life Sciences*, *80*, 1921–1943.

Gold, E. W. (1982). Purification and properties of hyaluronidase from human liver. Differences from and similarities to the testicular enzyme. *Biochemical Journal*, *205*, 69–74.

Grymonpré, K. R., Staggemeier, B. A., Dubin, P. L., & Mattison, K. W. (2001). Identification by integrated computer modeling and light scattering studies of an electrostatic serum albumin–hyaluronic acid binding site. *Biomacromolecules*, *2*, 422–429.

Imberty, A., Lortat-Jacob, H., & Perez, S. (2007). Structural view of glycosaminoglycan–protein interactions. *Carbohydrate Research*, *342*, 430–439.

Kaibara, K., Okazaki, T., Bohidar, H. B., & Dubin, P. L. (2000). pH-induced coacervation in complexes of bovine serum albumin and cationic polyelectrolytes. *Biomacromolecules*, *1*, 100–107.

Kennedy, J. F., Phillips, G. O., & Williams, P. A. (2002). *Hyaluronan*. Wrexham, Wales: Woodhead Publishing.

Laurent, T. C. (1987). Biochemistry of hyaluronan. *Acta Oto-laryngologica (Suppl) (Stockholm)*, *442*, 7–24.

Lenormand, H., Deschrevel, B., & Vincent, J. C. (2007). How electrostatic interactions can change the kinetic behaviour of a Michaelis–Menten enzyme. Application to the hyaluronane/hyaluronidase system. *Journal of Biological Physics and Chemistry*, *7*, 129–134.

Lenormand, H., Deschrevel, B., Tranchepain, F., & Vincent, J. C. (2008). Electrostatic interactions between hyaluronan and proteins at pH 4: How do they modulate hyaluronidase activity. *Biopolymers*, *89*, 1088–1103.

Lenormand, H., Tranchepain, F., Deschrevel, B., & Vincent, J. C. (2009). The hyaluronan–protein complexes at low ionic strength: How the hyaluronidase activity is controlled by the bovine serum albumin. *Matrix Biology*, *28*, 365–372.

Lenormand, H., Deschrevel, B., & Vincent, J. C. (2010a). pH effects on the hyaluronan hydrolysis catalysed by hyaluronidase in the presence of proteins: Part I. Dual aspect of the pH-dependence. *Matrix Biology*, *29*, 330–337.

Lenormand, H., Deschrevel, B., & Vincent, J. C. (2010b). Chain length effects on electrostatic interactions between hyaluronan fragments and albumin. *Carbohydrate Polymers*, *82*, 887–894.

Lokeshwar, V. B., Lokeshwar, B. L., Pham, H. T., & Block, N. L. (1996). Association of elevated levels of hyaluronidase, a matrix-degrading enzyme, with prostate cancer progression. *Cancer Research*, *56*, 651–657.

Maingonnat, C., Victor, R., Bertrand, P., Courel, M.-N., Maunoury, R., & Delpech, B. (1999). Activation and inhibition of human cancer cell hyaluronidase by proteins. *Analytical Biochemistry*, *268*, 30–34.

Malay, O., Bayraktar, O., & Batigün, A. (2007). Complex coacervation of silk fibroin and hyaluronic acid. *International Journal of Biological Macromolecules*, *40*, 387–393.

Mattison, K. W., & Kaszuba, M. (2004). Molecular weight measurements with the zetasizer nano sytem. *American Biotechnological Laboratory*, *23*, 1–6.

Mattison, K. W., Dubin, P. L., & Brittain, I. J. (1998). Complex formation between bovine serum albumin and strong polyelectrolytes: Effect of polymer charge density. *Journal of Physical Chemistry*, *102*, 3830–3836.

Mio, K., & Stern, R. (2002). Inhibitors of the hyaluronidases. *Matrix Biology*, *21*, 31–37.

Moss, J. M., Van Damme, M. P., Murphy, W. H., & Preston, B. N. (1997). Dependence of salt concentration on glycosaminoglycan–lysozyme interactions in cartilage. *Archives of Biochemistry and Biophysics*, *348*, 49–55.

Park, J. M., Muhoberac, B. B., Dubin, P. L., & Xia, J. (1992). Effect of protein charge heterogeneity in protein-polyelectrolyte complexation. *Macromolecules*, *25*, 290–295.

Reissig, J., Strominger, J., & Leloir, J. (1955). A modified colorimetric method for the estimation of N-acetylamino sugars. *Journal of Biological Chemistry*, *217*, 959–966.

Rooney, P., Kumar, S., Ponting, J., & Wang, M. (1995). The role of hyaluronan in tumour neovascularization. *International Journal of Cancer*, *60*, 632–636.

Schmitt, C., Sanchez, C., Desobry-Banon, S., & Hardy, J. (1998). Structure and technofunctional properties of protein–polysaccharide complexes: A review. *Critical Reviews in Food Science and Nutrition*, *38*, 689–753.

Stern, R. (2008). Hyaluronidases in cancer biology. *Seminars in Cancer Biology*, *18*, 275–280.

Stern, R., Asari, A. A., & Sugahara, K. N. (2006). Hyaluronan fragments: An information-rich system. *European Journal of Cell Biology*, *85*, 699–715.

Turgeon, S. L., Schmitt, C., & Sanchez, C. (2007). Protein–polysaccharide complexes and coacervates. *Current Opinion in Colloid & Interface Science*, *12*, 166–178.

Van Damme, M. P. I., Moss, J. M., Murphy, W. H., & Preston, B. N. (1994). Binding properties of glycoasminoglycans to lysozyme – Effect of salt and molecular weight. *Archives of Biochemistry and Biophysics*, *310*, 16–24.

Vincent, J. C., & Lenormand, H. (2009). How hyaluronan-protein complexes modulate the hyaluronidase activity: The model. *Biophysical Chemistry*, *145*, 126–134.

Vincent, J. C., Asteriou, T., & Deschrevel, B. (2003). Kinetics of hyaluronan hydrolysis catalysed by hyaluronidase. Determination of the initial reaction rate and the kinetic parameters. *Journal of Biological Physics and Chemistry*, *3*, 35–44.

Wang, Y. F., Gao, J. Y., & Dubin, P. L. (1996). Protein separation via polyelectrolyte coacervation: Selectivity and efficiency. *Biotechnology Progress*, *12*, 356–362.

West, D. C., & Kumar, S. (1989). Hyaluronan and angiogenesis. In D. Evered, & J. Whelan (Eds.), *The biology of hyaluronan* (pp. 187–207). Chichester: John Wiley and Sons.

West, D. C., Hampson, I. N., Arnold, F., & Kumar, S. (1985). Angiogenesis induced by degradation products of hyaluronic acid. *Science*, *228*, 1324–1326.

Xu, S., Yamanaka, J., Sato, S., Miyama, I., & Yonese, M. (2000). Characteristics of complexes composed of sodium hyaluronate and bovine serum albumin. *Chemical and Pharmaceutical Bulletin*, *48*, 779–783.