ELSEVIER

0144-8617(94)00084-0

Carbohydrate Polymers 26 (1995) 69–73

0144-8617/95/$09.50+.00

Hydration of hyaluronic acid as a function of the counterion type and relative humidity

N. Jouon*, M. Rinaudo, M. Milas & J. Desbrières

Centre de Recherches sur les Macromolécules Végétales (CERMAV), CNRS, associé à l'Université Joseph Fourier, B.P. 53 X, 38041 Grenoble-cedex, France

(Received 29 July 1993; revised version received 7 February 1994; accepted 21 February 1994)

The form of water associated with different ionic and physical forms of hyaluronic acid was investigated primarily using differential scanning calorimetry. The amount of non-freezable water associated with the polysaccharide was about 0·7 g H_2O/g dry solids which corresponded to 15 water molecules per disaccharide unit. This value depended slightly on the physical form of the material (powder, film or freeze-dried material) and also on the ionic form. The physical properties of a hyaluronic acid film showed a transition around a relative humidity of 88% when freezable water starts to be observed.

INTRODUCTION

Hyaluronic acid is a glycosaminoglycan consisting of alternating βD(1 → 4) linked 2-acetamido-2-deoxy-D-glucose and βD(1 → 3) linked D-glucuronic acid. It is a water-soluble polysaccharide giving viscoelastic fluids. The solution properties have been previously discussed (Fouissac *et al.*, 1992) but the question of hydration in the powder form is important to clarify. It may have a role in cosmetic applications, particularly to control skin hydration (Davies *et al.*, 1982). These authors found that 9 water molecules are located on each disaccharide unit. Different types of water can be differentiated (Mauritz & Fu, 1988; Wise & Pfeffer, 1987): non-freezable bound water, freezable bound water and free water. Extensive work has been performed on the hydration of polysaccharides (Hatakeyama *et al.*, 1987*a*, 1989*a*, 1989*b*; Yoshida *et al.*, 1987, 1992). Different techniques were used to differentiate between these water molecules: NMR (Froix & Nelson, 1975; Hatakeyama *et al.*, 1987*b*, 1987*c*), DSC (Hatakeyama *et al.*, 1988; Nakamura *et al.*, 1987; Yoshida *et al.*, 1990), thermogravimetry or IR.

Recently, a phase transition has been demonstrated by Brillouin spectroscopy (Rajamohanan *et al.*, 1991; Lee *et al.*, 1993). From this paper, it appears that there is a strong influence of relative humidity in the solid state on physical properties. It is therefore of importance to relate the 3D structure to the degree and nature of hydration of hyaluronic acid (HA) (or salt). In this paper, we investigate some physical properties of HA in different physical forms as a function of the relative humidity using different techniques.

EXPERIMENTAL

A hyaluronic acid sample (Na salt form) was investigated. It was obtained from a bacterial strain (*Streptococcus zooepidemicus*) by ARD (France). Different ionic forms were prepared by addition of 1 N XCl_n salts (X = Na, K, Ca, Mg, n = 1 or 2) in an aqueous 5 g/l HA solution to exchange the initial counterions; the polymer was then reprecipitated in the X form by ethanol. The acidic form of HA is obtained by dispersion of the salt form in EtOH (70% v/v) aqueous HCl 0·1 N solution, then washed and dried using EtOH. The films were prepared by casting a 20 g/l aqueous solution of the corresponding polymer onto a Teflon plate and drying under ambient conditions (15 days).

Controlled relative humidity (RH) was obtained in closed desiccators at room temperature using saturated salt conditions: P_2O_5 (0); KCH_3CO_2 (22); $K_2CO_3, 2H_2O$ (44); NaCl (76); $BaCl_2, 2H_2O$ (88); KNO_3 (94); $CuSO_4, 5H_2O$ (98); H_2O (100). The polymeric samples were stabilized in each atmosphere at ambient temperature for 3 weeks. Total water content W_T was determined by thermogravimetry using a SETARAM G70 equipment (g/g) = m_{H_2O}/m_O where m_{H_2O}, is the weight of water evaporated and m_O, the mass of the dried material. The

---

*Author to whom correspondence should be addressed.

freezing water was determined using a DSC7 from Perkin Elmer; all the ΔH values are taken on the heating curve with a heating rate of 1°C/min from −50°C to +20°C. The ΔH signal was calibrated by comparison with the ΔH of melting of a well defined quantity of double distilled water. The weight of freezable water in the sample was calculated assuming that the enthalpy of melting of the water absorbed on the polymer sample is the same as that of the bulk water. This is certainly a crude hypothesis which underestimates W_{Fb}, the freezable water content adsorbed by 1 g of dried hyaluronate. The difference $W_b = W_T - W_{Fb}$ represents the weight of water which strongly interacts with 1 g of dried material.

The mechanical properties of the films were determined with an Instron 4301 in a tension test which allows the determination of the elastic modulus and the breaking strain. The conductivity of the film was a relative measurement obtained within a platinum electrode specially made in our laboratory. The relaxation characteristics of water adsorbed on the samples were determined by proton NMR using an AC 300 Bruker machine at 25°C directly on the hydrated powders.

RESULTS AND DISCUSSION

Hydration as a function of relative humidity

The sodium form of HA was equilibrated in different controlled atmospheres and total water retention (W_T) determined by thermogravimetry. The results concerning the reprecipitated form are shown in Fig. 1. The water interaction is large for this very polar polysaccharide due to H-bonds with -OH groups and also electrostatic interactions with the carboxylic sites.

The water retention remains small until RH = 75% then increases quickly to saturation at RH = 100%. On the same samples, DSC allows the freezable interacting water (W_{Fb}) to be quantified assuming a ΔH of melting equal to that of the free water used to calibrate the signal. Examples of thermograms are given in Fig. 2 for increasing RH. The difference between W_T and W_{Fb} gives the content of bound non-freezable water W_b.

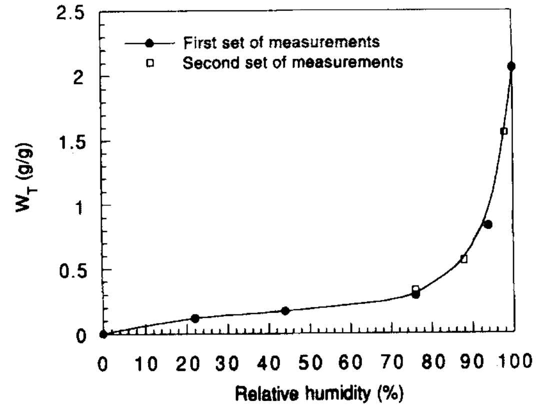

Fig. 1. Total water retention (g/g) adsorbed on HA-2 (sodium form) as a function of relative humidity.

Peak I corresponds to the bound freezable water; the peak is large and the melting temperature of the water is lower than that of free water (from −25 to −10°C) reflecting the presence of a series of water molecules with different interaction energies. Peak II is narrower and corresponds to water molecules having nearly the same characteristics as bulk water. When the RH is lower than 94%,this latter peak is not seen. The sum of the two peaks is considered to be an estimate of W_{Fb}. The data are given in Fig. 3. The non-freezable water (W_b) goes to a limit of 0·7 g H_2O/g dry material. This number corresponds to an average of 15 water molecules firmly bound per disaccharide unit. Very recently, it was found that 13 water molecules could be bound to the disaccharide unit of hyalan, a crosslinked hyaluronan (Takigami *et al.*, in press). Using the same techniques as described in this paper, an average of 1·3 H_2O per -OH sites and 5 H_2O per -COONa site were determined for cellulose and cellulose derivatives (Berthold *et al.*, 1994). The sum of these numbers gives 10·2 H_2O molecules per disaccharide unit without taking into account the *N*-acetyl group. Our experimental values are in good agreement with these data. For hyaluronic acid Yoshida *et al.* (1989) found 30 moles of water per tetrasaccharide unit. The freezable bound water content (W_{Fb}) increases quickly when the bound non-freezable water reaches its limit (Fig. 3). This water is less stable than the bulk water and must be a partially H-bound disordered water located between the layer of strongly interacting water and the bulk water.

Influence of the hyaluronate physical form

The sodium form of HA was examined as the commercial sample (HA-1), a proportion of this as repurified on a laboratory scale and reprecipitated with ethanol (HA-2); a part was further freeze-dried from a solution of HA-1 (HA-3) or dried as a thin film (HA-4). The total water contents were determined for different relative humidities; the results are given in Table 1.

Up to RH = 76%, the total water content depends on the accessibility of the solid materials (increasing as the surface area). It follows this sequence (Table 1):

film	< reprecipitated	< commercial	< freeze-dried ,
HA-4	HA-2	HA-1	HA-3

and for higher RH, the sequence becomes:

HA-4 ≈ HA-2 < HA-1 ≈ HA-3.

The number of water molecules firmly bound (determined exactly when DSC peak appears i.e. for RH = 88%) are respectively (Table 2):

12 (HA-4) = 12 (HA-2) < 15 (HA-3) < 17 (HA-1).

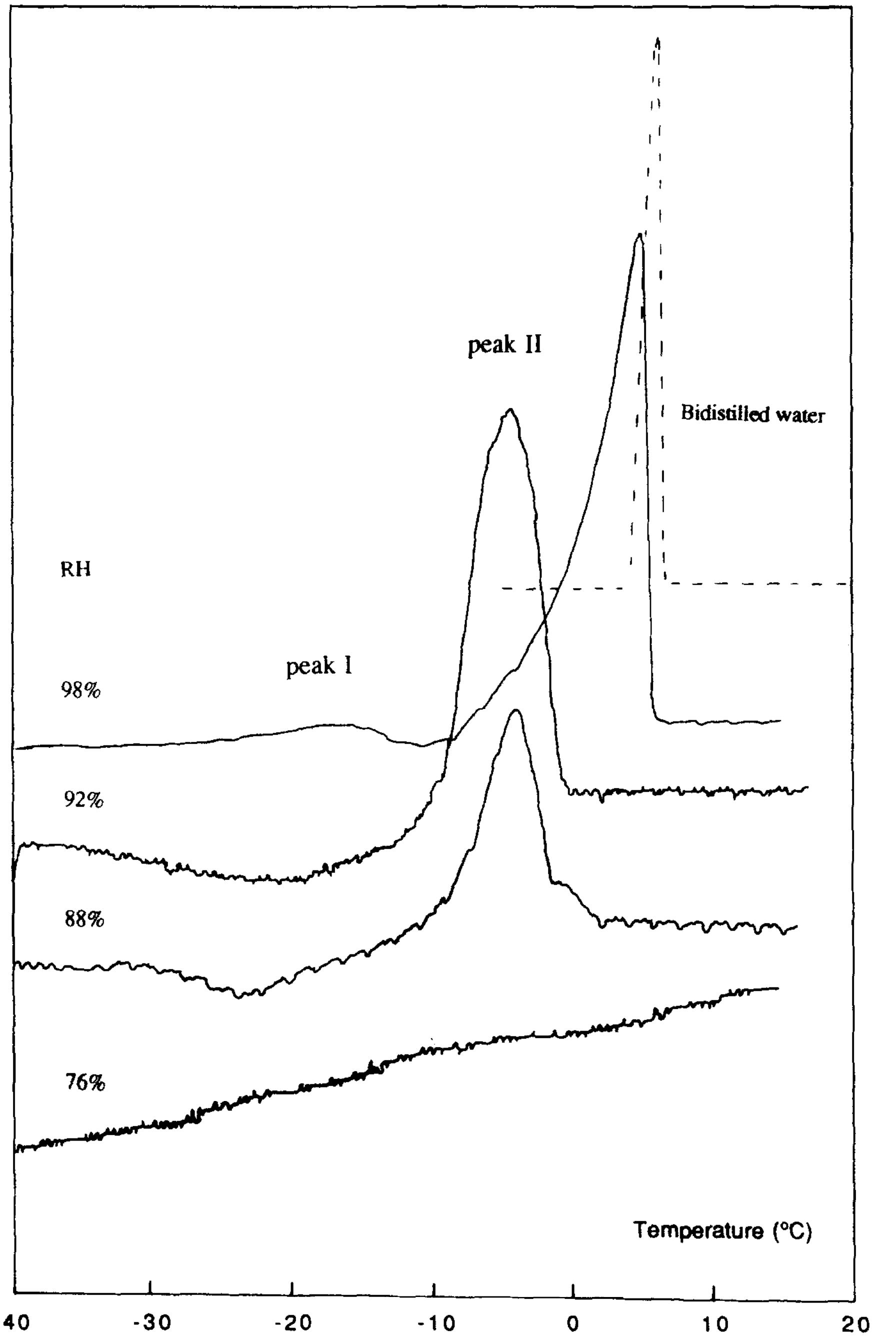

Fig. 2. DSC thermograms obtained on heating HA-2 (sodium form) stabilized at different RH. Rate of temperature increases 1°/min.

Table 1. Total water content (W_T, g/g) of the different sodium hyaluronate samples as a function of the external relative humidity (RH, %)

RH%	HA-1	HA-2	HA-3	HA-4
22	0·179	0·121	0·294	0·053
44	0·244	0·173	0·386	0·109
76	0·35	0·311	0·426	0·206
88	0·803	0·562	0·751	0·548
94	1·119	0·83	1·163	1·18
98	1·738	1·558	1·777	1·562
100	4·125	2·057	5·812	2·017

Table 2. Freezable bound water content (W_{Fb}), bound water content (W_b) and number of bound water molecules per repeating unit (n_b) for the different sodium hyaluronate samples at RH = 88% (W_T see Table 1)

Sample	W_{Fb} (g/g)	W_b (g/g)	n_b
HA-1	0·05	0·753	17
HA-2	0·036	0·526	12
HA-3	0·062	0·689	15
HA-4	0·025	0·523	12

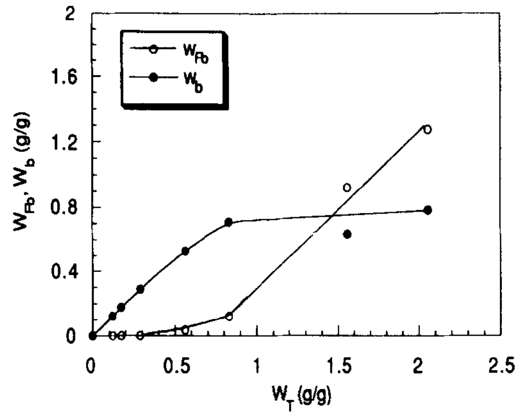

Fig. 3. Freezing water (W_{Fb}) and bound water (W_b) contents (g/g) as a function of the total water retention (W_T) on HA-2 (sodium form).

For cellulose, bound water was confined to the amorphous zone (Berthold *et al.*, 1994). This can explain some differences between the different HA samples.

Influence of the counterion

Figure 4 shows the content of non-freezable bound and freezable bound water for different RH and different counterion forms. Up to RH = 76%, there is no significant influence of the counterion. For RH ≥ 94%, freezable water appears depending on the counterion; at 94%, no free water exists for the H form but a low content in the Ca form. The following order is obtained for W_{Fb}.

$$H^+ \ll Ca^{+2} < Mg^{+2} < Na^+ < K^+ \quad (RH = 94\%)$$

and at RH = 100% (Table 3):

$$H^+ < Mg^{+2} = Ca^{+2} < Na^+ = K^+ \quad (RH = 100\%).$$

The same sequences are also observed for W_T but not for W_b.

It would be expected that with an ion exchanger, the net charge is lower for divalent counterions and H forms with a carboxylic site. This sequence is similar to that for the dissociation of the carboxylic sites with respect to the type of counterion.

Physical properties

The conductance of films and their mechanical properties were determined for the different hydration states (Table 4). A large transition occurs between RH = 76 and 88% for the Na salt form. This domain of relative humidity also corresponds to the appearance of freezable bound water. This RH domain also corresponds to the transition observed by Brillouin spectroscopy which

Table 3. Freezable bound water content (W_{Fb}), bound water content (W_b) and number of bound water molecules per repeating unit (n_b) as a function of counterions for HA-2 at RH = 100%

Counterion	W_{Fb} (g/g)	W_b (g/g)	n_b
Na^+	1·27	0·79	17
K^+	1·40	0·71	16
Mg^{2+}	0·66	1·16	26
Ca^{2+}	0·72	1·06	25
H^+	0·20	0·70	15

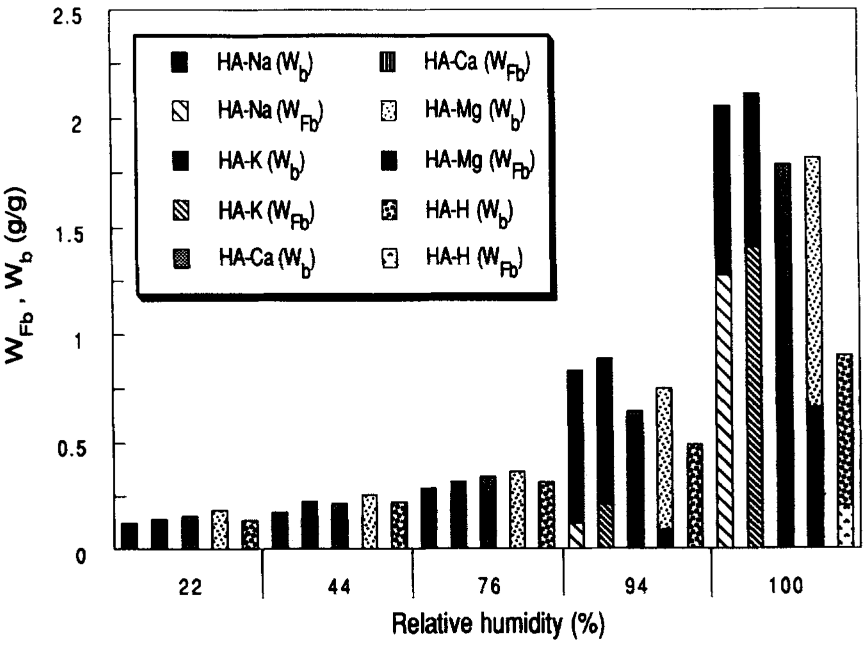

Fig. 4. Influence of the counterions on the freezing (W_{Fb}) and bound water (W_b) contents (g/g) as a function of relative humidity.

Table 4. Conductance, Young modulus (E) and number of freezable bound water molecules per repeating unit (n_{Fb}) for hyaluronate samples (HA-4) in the sodium or acid forms

RH (%)	Counterion	Conductance ($\mu\Omega^{-1}$)	$E \times 10^{-6}$ (Pa)	n_{Fb}
44	Na^+	0·018	3500	0
76	Na^+	0·205	1150	0
88	Na^+	50·3	3	0–1
98	Na^+	311	<0·1	18
76	H^+	0·5	1100	0
88	H^+	—	500	0–1

is independent of the degree of crystallinity (Lee *et al.*, 1993). It corresponds to the plastification of the material by H_2O molecules. All the data are in favour of a transition corresponding to the presence of a fraction of freezable water. On the same type of samples but in the powder form, the relaxation of water molecules was investigated by ^{1}[H] NMR. Two degrees of hydration were investigated: 76 and 98%, the average values of T_1 are respectively 0·46 and 1·02 s.

T_1 and T_2 of water molecules were extensively studied by ^{1}[H] NMR (Hatakeyama *et al.*, 1987*c*); they investigated the influence of temperature and degree of hydration; at 25°C, T_1 (s) for $W_T = 0{\cdot}83$ is around 0·5 s for our samples.

CONCLUSION

The hydration properties of hyaluronic acid and its sodium salt are recognized as being important for cosmetic applications. In this paper, the relation between water adsorption and the structure of the polymer (film or powder form) and particularly its ionic form is determined. It is shown that the ionic form and especially the pH at the precipitation of the polymer will influence the water retention. Under normal conditions (RH = 50–65%), only non-freezable water is adsorbed. The physical properties of a HA film show a transition around RH = 88% when freezable water appears.

REFERENCES

Berthold, J., Desbrières, J., Rinaudo, M. & Salmen, L. (1994). *Polymer*, **35**, 5729–5736..

Davies, A., Gormaly, J., Wyn-Jones, E., Wedlock, D.J. & Phillips, G.O. (1982). *Int. J. Biol. Macromol.*, **4**, 436–438.

Fouissac, E., Milas, M., Rinaudo, M. & Borsali, R. (1992). *Macromolecules*, **25**, 5613–5617.

Froix, M.F. & Nelson, R. (1995). *Macromolecules*, **8**, 726–730.

Hatakeyama, T., Ikeda, Y. & Hatakeyama, H. (1987*a*). *Makromol. Chem.*, **188**, 1875–1884.

Hatakeyama, H., Iwata, H. & Hatakeyama, T. (1987*c*). In *Wood and Cellulosics*, eds J.F. Kennedy & G.O. Phillips. Ellis Horwood, Chichester, UK, pp. 39–46.

Hatakeyama, T., Nakamura, K. & Hatakeyama, H. (1988). *Thermochimica Acta*, **123**, 153–161.

Hatakeyama, T., Nakamura, K., Yoshida, H. & Hatakeyama, H. (1989*a*). *Food Hydrocolloids*, **3**, 301–311.

Hatakeyama, T., Yamamoto, S., Hirose, S. & Hatakeyama, H. (1989*b*). In *Cellulose and Wood Chemistry and Technology*, ed. C. Schuerch. J. Wiley & Sons, New York, pp. 431–446.

Hatakeyama, H., Yoshida, H. & Hatakeyama, T. (1987*b*). In *Wood and Cellulosics*, eds J.F. Kennedy & G.O. Phillips. Ellis Horwood, Chichester, UK, pp. 255–262.

Lee, S.A., Flowers, M.R., Oliver, W.F., Rupprecht, A. & Lindsay, S.M. (1993). *Phys. Rew.*, **47**, 677–683.

Mauritz, K.A. & Fu, R.M. (1988). *Macromolecules*, **21**, 1324–1333.

Nakamura, K., Hatakeyama, T. & Hatakeyama, H. (1987). In *Wood and Cellulosics*, eds. J.F. Kennedy & G.O. Phillips. Ellis Horwood, Chichester, UK, pp. 97–103.

Rajamohanan, P.R., Badiger, M.V., Ganapathy, S. & Mashelkar, R.A. (1991). *Macromolecules*, **24**, 1423–1424.

Takigami, S., Takigami, M. & Phillips, G.O. (in press).

Wise, W.B. & Pfeffer, P.E. (1987). *Macromolecules*, **20**, 1550–1554.

Yoshida, H., Hatakeyama, T. & Hatakeyama, H. (1987). In *Wood and Cellulosics*, eds J.F. Kennedy & G.O. Phillips. Ellis Horwood, Chichester, UK, pp. 305–311.

Yoshida, H., Hatakeyama, T. & Hatakeyama, H. (1989). In *Cellulose: Structural and Functional Aspects*, eds J.F. Kennedy, G.O. Phillips, P.A. Williams. Ellis Horwood, Chichester, UK, pp. 305–316.

Yoshida, H., Hatakeyama, T. & Hatakeyama, H. (1990). *Polymer*, **31**, 693–698.

Yoshida, H., Hatakeyama, T. & Hatakeyama, H. (1992). In *Viscoelasticity of Biomaterials*, eds W.G. Glasser & H. Hatakeyama. ACS Symp. Ser. 489. American Chemical Society, New Orleans, pp. 217–230.