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Heterogeneous hydrolysis of hyaluronic acid in ethanolic HCl slurry

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ABSTRACT

In this work, a novel procedure for hydrolysis of hyaluronic acid (HA) is presented. HA was hydrolysed in ethanol with added HCl during heterogeneous conditions using varying temperatures (40, 60 and 80 °C) and acid concentrations (0.01, 0.05, and 0.1 M HCl). The degradation process was monitored by determination of weight average molecular weight (M_w) by size-exclusion chromatography with online multi-angle laser light, refractive index and intrinsic viscosity detectors (SEC-MALLS-RI-visc) on samples withdrawn continuously during the hydrolysis. SEC-MALLS-RI-visc showed that the degradation gave molecular weight distributions with polydispersity indexes (M_w/M_n) of 1.3–1.8. Kinetic plots of $(1/M_w - 1/M_{w,0})$ vs. time gave linear plots, showing that heterogeneous acid hydrolysis of HA is a random process and that it follows a first order kinetics making it possible to determine the kinetic rate constant (k_h) from the slope. During the hydrolysis in heterogeneous conditions it was found that k_h depended on the water concentration in the slurry (increases with increasing water concentration). Additionally, for heterogeneous hydrolysis in ethanol/HCl at 40, 60 and 80 °C, it was shown that k_h depended linearly on the acid concentration, but not to the same extent as previously seen in hydrolysis under aqueous, homogeneous conditions. Further, the dependence of temperature on the hydrolysis in 0.05 and 0.1 M ethanol/HCl was found to follow the Arrhenius kinetics (linear plot of $\ln k_h$ vs. $1/T$). For hydrolysis in 0.05 and 0.1 M ethanolic HCl, the activation energy (E_a) was determined to 107 and 104 kJ/mol and the Arrhenius constant (A) was determined to 1.2×10^{10} and 8.8×10^9 h^{-1}, respectively. Both E_a and A were significantly lower compared to those determined for hydrolysis performed under aqueous, homogeneous conditions at the same acid concentration. It was shown that the availability of water was rate limiting for the hydrolysis process in ethanol/HCl slurry. ^{1}H NMR was used to characterize the product of extensive hydrolysis. No indication of de-*N*-acetylation of the GlcNAc units, indication of ethanolysis or other by-products were seen. The new heterogeneous hydrolysis procedure provides several advantages compared to homogenous hydrolysis such as lower ethanol and water consumption and the possibility to hydrolyse a larger amount of material in the same volume.

1. Introduction

Hyaluronic acid (HA) is a linear biopolymer naturally abundant in mammalian tissues. A human adult contain approximately 15 g HA where it mainly occurs in the eyes, skin (both in the epidermis and dermis) and synovial fluid (Lepperdinger, Fehrer, & Reitinger, 2004). Further, HA makes up the backbone of the proteoglycan aggregates being a main component of tendons and cartilage (Heinegård, Björnsson, Mörgelin, & Sommarin, 1998). The polysaccharide has a high turnover in the body, 7 g/day, i.e., half of all HA in the body is exchanged every day (Lepperdinger et al., 2004).

The molecular weight of HA strongly depends on its biological origin (Heinegård et al., 1998). When produced by microbial fermentation the molecular weight ranges from just below 1×10^6 up to 4×10^6 g/mol (Armstrong & Johns, 1997). When prepared by extraction, molecular weights up to 6×10^6 g/mol have been reported (Lee & Cowman, 1994).

The chemical structure of HA consists of two alternating monosaccharide units, D-*N*-acetyl glucosamine (GlcNAc) and D-glucuronic acid (GlcA), connected by β-(1→3) and β-(1→4) glycosidic bonds, respectively. It is the negative charge on the repeating disaccharide which is the cause of many of the unique properties of this polyacid, i.e., HA produces highly viscous solution in water (Gibbs, Merrill, Smith, & Balazs, 1968; Lapcik Jr., Lapcik, De, Demeester, & Chabrecek, 1998). Due to the combination of having a flexible alternating β-backbone and being a charged polymer, the viscosity of HA is strongly dependant on ionic strength and pH. As the concentrations of salts increase, the repulsion between negatively charged disaccharide units decrease and the conformation change from the rod-like towards that of the flexible random coil (Furla, La Penna, & Perico, 2005; Hayashi, Tsutsumi, & Teramoto, 1996; Smidsrød & Haug, 1971; Tanford, 1961).

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Today, two main microbial sources are exploited for HA production, *Streptococcus* strains (Sutherland, 1990) and recombinant *Bacillus subtilis* (Widner et al., 2005). In *Streptococci*, HA is produced as part of the capsule bound to the cell membrane, making it challenging to isolate. HA from *Bacillus*, on the other hand, is excreted extracellularly and can therefore conveniently be isolated as a high purity product (Widner et al., 2005).

Acid hydrolysis of polysaccharides is a well-known process both in manufacturing of low-molecular weight (LMW) polysaccharides and oligosaccharide products. Acid hydrolysis has been studied for several polysaccharide materials: starch (BeMiller, 2009), chitosan and chitin (Einbu, Grasdalen, & Varum, 2007; Varum, Myhr, Hjerde, & Smidsrod, 1997), alginate (Holtan, Zhang, Strand, & Skjak-Braek, 2006; Smidsrød, Haug, & Larsen, 1966; Smidsrød, Larsen, Painter, & Haug, 1969) and cellulose derivatives (Sjöström, 1993; Smidsrød et al., 1969). Most industrial hydrolysis processes are performed in aqueous, homogeneous, conditions (BeMiller, 2009; Einbu et al., 2007; Holtan et al., 2006; Sjöström, 1993; Smidsrød et al., 1966, 1969; Varum et al., 1997) followed by a recovery step including solvent-based precipitation. For crystalline polymer such as cellulose and chitin, heterogeneous hydrolysis has also been reported (Einbu et al., 2007; Sjöström, 1993; Smidsrød et al., 1969; Varum et al., 1997). Reduction of the molecular weight is generally performed in order to obtain materials of different physical and chemical characteristics (lower viscosity in aqueous solutions).

Hydrolysis of glucosides generally involves three steps (see Fig. 1): (1) the glucosidic oxygen atom becomes protonated (i.e., less basic) giving the conjugate acid; this step is rapid and the acid will exist in its equilibrium concentration; (2) an unimolecular heterolysis of the conjugate acid with the formation of a non-reducing end group and a carbonium-oxonium ion; this step is slow and rate-determining; and (3) a rapid addition of water to the carbonium-oxonium ion with the formation of a reducing end group and a proton. In general, the rate of hydrolysis in acidic solution will depend on the ability of the solution to protonate the glycosidic oxygen, but also substituents on the new reducing end will influence the hydrolysis rate (Collins & Ferrier, 1995; Sharon, 1975; Sjöström, 1993). It has been shown that presence of a 2-amino group ($-NH_3^+$) reduces the hydrolysis rate due to its basic properties drawing electrons away from the glucosidic oxygen (Einbu et al., 2007; Varum et al., 1997). A carboxylic acid group (–COOH) on the C-5 on the aglycon increases the hydrolysis rate of a β-(1→4) linkage due to its electron donating properties (Holtan et al., 2006; Sjöström, 1993; Smidsrød et al., 1966, 1969). Further, the size of the sugar ring and the configuration of the glucosidic linkage influence the stability of the carbonium-oxonium ion (Collins & Ferrier, 1995; Sharon, 1975; Sjöström, 1993). Acid hydrolysis of HA in 10 mM HCl has been studied as a way for producing oligosaccharides by Inoue and Nagasawa (1985). A study on homogeneous acid hydrolysis of HA has been made by the authors evaluating the influence of pH and temperature on the kinetics (Tømmeraas & Melander, 2008). In this work we have studied the kinetics and chemistry of heterogeneous acid hydrolysis of HA in EtOH and compared it with homogeneous hydrolysis (aqueous). The effect of slurry composition (%w/w solid), acid concentration and temperature was studied. To the authors' knowledge, no previous studies of heterogeneous hydrolysis of HA have been published.

2. Experimental

2.1. Materials

Experimental sample of sodium hyaluronate (M_w = 1.6 MDa, M_w/M_n = 1.2) produced by fermentation of *Streptococcus equi* was used in this study. All HCl solutions were prepared by diluting 4 M HCl obtained from Bie and Berntsen (Denmark) with deionized water (Milli-Q). All NaOH solutions were prepared by dilution of 4 M NaOH (obtained from Bie and Berntsen, Denmark) with deionized water. All other chemicals were obtained from Sigma–Aldrich in puriss p.a. quality if not otherwise stated.

2.2. Acid hydrolysis using hydrochloric acid in EtOH

HA (0.5 g unless other stated) was put into sealable vials and 15 ml of 93% EtOH was added. The solutions were pre-warmed to 40, 60 or 80 °C before 1 M HCl was added and the vial was vigorously shaken for approximately 10 s to give acid concentrations of 0, 0.01, 0.05, and 0.1 M. The vials were left without agitation at 40, 60, or 80 °C (in an oil-filled thermostated water bath) up to 30 h. Vials where withdrawn at intervals during the 30 h time period. Each sample was immediately cooled in an ice-bath and neutralised with equimolar amounts of NaOH (1 M solution) before recovery of the hydrolysed HA by filtration with Whatman 4 filter paper. Each filter paper was scaled prior to filtration. In order to remove excess of salt and water, the samples were washed three times with 15 ml of 93% EtOH, dried at room temperature, and finally weighed whereby recovery was measured. For samples where the effect of HCl/EtOH vs. HA ratio was investigated, a 0.01 M HCl solution was added of 5, 7.5, 10 and 15 ml to a constant amount of HA (0.5 g) and the samples were hydrolysed for 19 h at 80 °C. Molecular weights were analysed by use of SEC-MALLS-RI-visc.

2.3. Polymer characterisation using SEC-MALLS-RI-visc

The hydrolysed HA samples were analysed using SEC-MALLS-RI-visc system consisting of a Waters Alliance HPLC with Wyatt Optilab rex RI detector, Wyatt EON MALLS detector and Wyatt Viscostar viscosity detector. Four TSK columns (2500, 4000, 5000, 6000 PW_{XL}) were eluted with a buffer of 50 mM HaH_2PO_4 and 150 mM NaCl at a flow rate of 0.5 ml/min and 30 °C. Samples were prepared

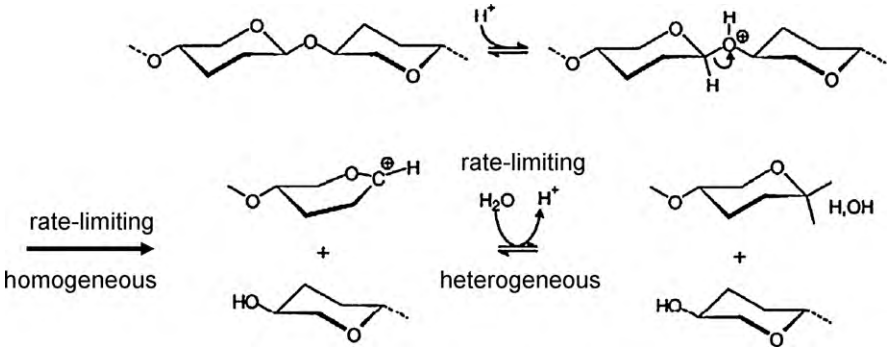

Fig. 1. Hydrolysis reaction of carbohydrates (Einbu et al., 2007).

of approximately 0.1% (w/v) for high molecular weight samples (above 100,000 g/mol), smaller molecular weight samples were analysed at higher concentrations (up to 1%) due to weaker light-scattering signal for smaller polymers. An injection loop of 500 μl was used. Refractive index increment (dn/dc) of 0.153 and second virial coefficient (A_2) of 2.3×10^{-3} was used in the processing of the data. All data were calculated in Astra software v.5.1.3.0 and Microsoft Excel.

2.4. Determination of kinetic rate constants

For polymers degraded by random scission, the rate at which bonds are broken is proportional to the total number of intact bonds (Tanford, 1961):

$$-\frac{d(N_0p)}{dt} = kN_0p, \quad (1)$$

where N_0p is the number of bonds at time t and k is the rate constant. For non-random scission, there will be several classes in which each will be ruptured at a different rate. The solution of (1) for a single strand linear polymer with random degradation gives the following equations (Einbu, Naess, Elgsaeter, & Varum, 2004; Hjerde, Smidsrød, & Christensen, 1996; Tanford, 1961):

$$\frac{1}{DP_{n,t}} \propto kt, \quad (2)$$

$$\frac{1}{M_{w,t}} \propto k't. \quad (3)$$

From (3) we get the expression $1/M_{w,t}(t) = k_h t + 1/M_{w,t=0}$, where k_h is the kinetic rate constant of the hydrolysis process and $M_{w,t=0}$ is the molecular weight at the beginning of the hydrolysis. To obtain information about the kinetics of the hydrolysis processes, the change in the inverse value of the weight-average molecular weight ($1/M_w - 1/M_{w,0}$). The rate constant (k_h) is found by determining the slope of the curve.

2.5. Structural characterisation using ^{1}H NMR spectroscopy

HA samples from the acid hydrolysis (0.1 M HCl) were evaluated by ^{1}H NMR spectroscopy. Samples were prepared by dissolving HA samples (10 mg/ml) in D_2O with 5 μl from a 1% stock solution in D_2O of (trimethylsilyl) propionate-d_4 (TSP) added as internal standard. The solution (0.7 ml) was transferred to a 5 mm NMR tube before analysis. Spectra were acquired at a Varian Mercury 400 MHz and at 80 °C. Spectra were acquired using a 45° pulse angle, 256 scans, SW 6389.8 Hz, 2 s acquisition time and 1 s relaxation delay.

3. Results and discussion

3.1. Structural characterization of hydrolysed material

When subjecting polysaccharide materials towards acidic alcohol at low water content one could expect that the alcohol replaces water in the reaction with the carbonium-oxonium ion (step 3 in the hydrolysis mechanism of glucosides, Fig. 1). This is well known in structural elucidation of polysaccharides (e.g. methanolysis) (Toshihiko, Guoning, Takuya, Yoshinori, & Toshio, 2006).

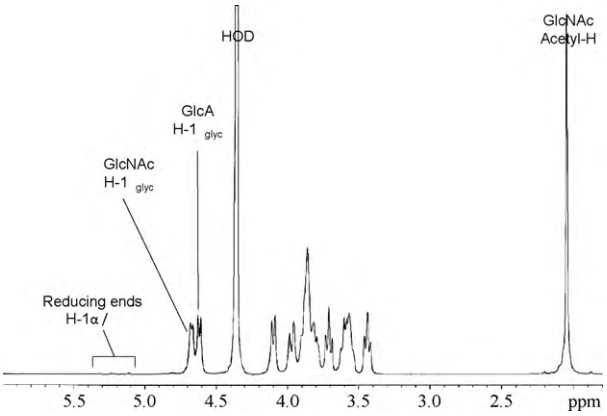

Fig. 2. ^{1}H NMR spectrum of hyaluronic acid sample (50,000 g/mol) made by hydrolysis in 0.1 M HCl at 80 °C for 24 h. Acquisition conditions: 400 MHz, 10 mg/ml in D_2O, 256 scans, 80 °C.

Further, it is known that de-*N*-acetylation reactions can occur in acidic solution at high temperature (Einbu et al., 2007). In order to investigate if HA degraded by heterogeneous hydrolysis maintains its structural integrity, HA was subjected to extensive hydrolytic conditions (0.1 M EtOH/HCl (3.3%, w/w) for 25 h at 80 °C). The degraded HA was analysed by ^{1}H NMR spectroscopy and the spectrum is given in Fig. 2. The α-/β-anomeric protons of the reducing end of GlcNAc were seen as very weak peaks at chemical shifts 5.18 ppm (H-1α) and 4.78 ppm (H-1β) (Sicinska, Adams, & Lerner, 1993; Tawada et al., 2002). No indication of suspected by-products such as de-*N*-acetylation or ethanolysis at the reducing end was observed. This is in agreement with the previous observation that de-*N*-acetylation of HA does not occur until after extensive depolymerisation (Tømmeraas & Melander, 2008).

3.2. Effect of HCl concentrations and temperature on hydrolysis rate

The hydrolysis of HA in dilute HCl/EtOH was studied over time at various conditions by taking out samples and analysing them using SEC-MALLS-RI-visc.

To obtain information about the kinetics of the hydrolysis process, the change in the inverse value of the weight-average molecular weight ($1/M_w - 1/M_{w,0}$) was plotted as function of time as shown in Fig. 3a–c. For a random degradation process, this plot gives a linear relationship where the kinetic constant (k_h) is given by the slope. The k_h determined for HA is a combined value of the hydrolysis rate of the linkage GlcA-GlcNAc ($k_{GlcA\text{-}GlcNAc}$) and GlcNAc-GlcA ($k_{GlcNAc\text{-}GlcA}$): $k_h = k_{GlcA\text{-}GlcNAc} + k_{GlcNAc\text{-}GlcA}$. For most polysaccharides, hydrolysis under homogeneous conditions is expected to be of first-order for breaking of glucosidic bonds. A constant hydrolysis rate (slope) was observed for all experiments using HCl/EtOH at 40, 60 and 80 °C (see Fig. 3a–c). The kinetic rate constants (k_h) were determined by taking the slope of a fitted linear regression curve and the resulting constants are listed in Table 1. Furthermore, increasing the temperature from 60 to 80 °C speeded up the hydrolysis process by more than 20 times. At neutral conditions, the hydrolysis of HA was negligible, at least at up to 80 °C

Table 1
Kinetic rate constants, k_h (h^{-1}) determined from slope of kinetic plots (see Fig. 3 and Tømmeraas & Melander, 2008).

Temperature (°C)	Concentration (heterogeneous)			Concentration (homogenous)	
	0.01 M	0.05 M	0.1 M	0.01 M	0.1 M
40	n.d.	1.3E−8	4.1E−8	n.d.	1.0E−7
60	n.d.	2.2E−7	1.0E−6	n.d.	1.0E−6
80	1.7E−7	1.2E−6	3.4E−6	2.0E−6	4.0E−5

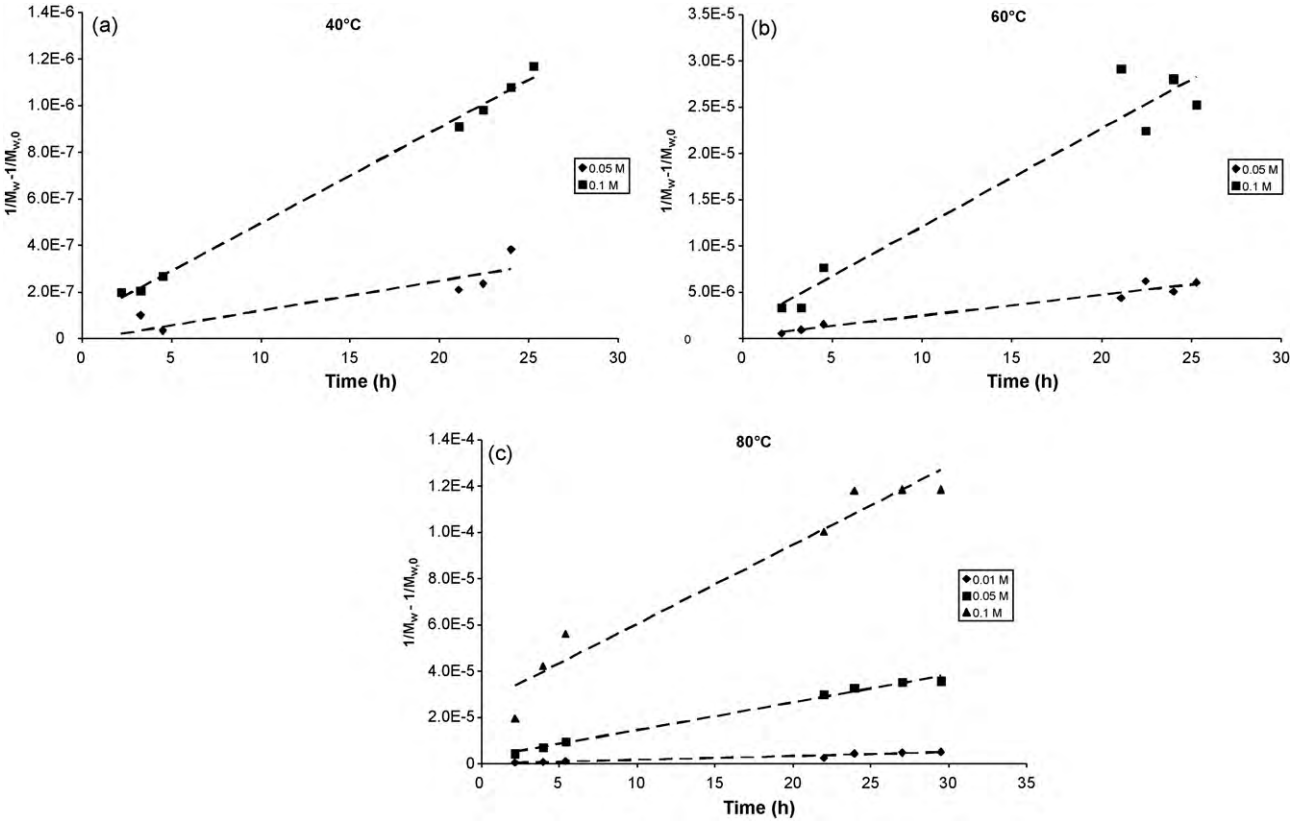

Fig. 3. Plot of $1/M_w - 1/M_{w,0}$ as a function of hydrolysis time and acid concentration gives information about hydrolysis kinetics: (A) 40 °C, (B) 60 °C and (C) 80 °C.

(Tømmeraas & Melander, 2008). The kinetic rate constants (k_h) at 80 °C were determined from the slopes of the kinetic plots and plotted in Fig. 4 as a function of HCl concentration. For comparison, homogeneous hydrolysis data taken from Tømmeraas and Melander (2008) are included. For most polysaccharides a linear dependence of the hydrolysis rate on the proton concentration is expected (Holtan et al., 2006; Smidsrød et al., 1966, 1969) as the hydrolysis process is directly proportional to the amount of oxonium ions (H_3O^+) in the solution. The slope is significantly lower for the heterogeneous hydrolysis and as can be seen, the kinetic rate constant is less affected by changes in acid concentration. Thus, the effect of the lower amount of water significantly influences the hydrolysis reaction (a factor 10) by limiting the presence of water that is required during the formation of a new reducing end. The

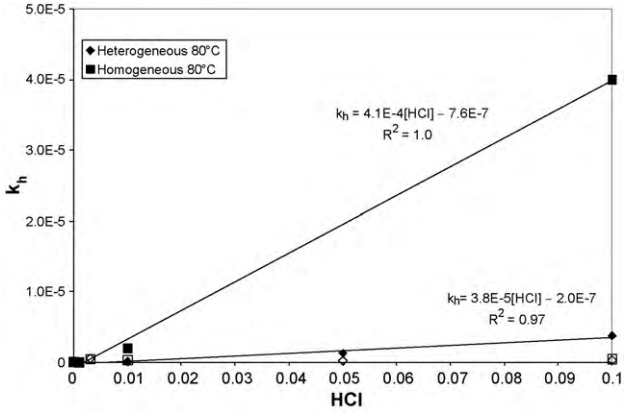

Fig. 4. Plot of the hydrolysis constant as a function of acid concentration at 80 °C for homogeneous (Tømmeraas & Melander, 2008) and heterogeneous acid hydrolysis.

rate constant determined for heterogeneous conditions in 0.1 M HCl at 60 °C were determined to 1.0E−6, the same value as for homogeneous condition at same temperature and acid concentration (see Table 1). This is an improbable result, as one would expect a lower value for the heterogeneous system since this was found for all other conditions studied. The reason for this is probably that the rate at this low acid concentration the rate is slow so that it is difficult to measure accurately.

The polydispersity index (M_w/M_n) of the acid degraded samples showed more or less no change compared to the starting HA (1.3–1.8). Following from the theory on random degradation, one should according to the Kuhn distribution expect an M_w/M_n of around 2.0 for extensively degraded samples (Hjerde et al., 1996; Inoue & Nagasawa, 1985). The reason for the low value is probably that determination of the number average molecular weight (M_n) becomes more uncertain and overestimated as the hydrolysis proceeds, giving a somewhat underestimated polydispersity index. Since HA is a linear polymer of alternating GlcNAc and GlcA, one would expect a total random degradation if either (i) the reaction rate is equal for the cleavage of the β-(1→4) between GlcNAc-GlcA and the β-(1→3) between GlcA-GlcNAc or (ii) the reaction is predominantly occurring on one of these linkages.

The effect of temperature on acid hydrolysis was evaluated by plotting the natural logarithm of the kinetic rate constants ($\ln k_h$) at 40, 60 and 80 °C as function of the inverse absolute temperature (Arrhenius plot), giving a linear curve (Fig. 5). Similar tendencies have been observed for acid hydrolysis of other linear single-strand polysaccharides (Einbu et al., 2007; Holtan et al., 2006; Smidsrød et al., 1966, 1969). From the plot it is possible to determine the parameters in equation: $k_h = A\,e^{-E_a/RT}$, where A is the Arrhenius constant (also called the pre-exponential factor or frequency factor) and E_a is the activation energy of the reaction (Atkins, 1994). From linear

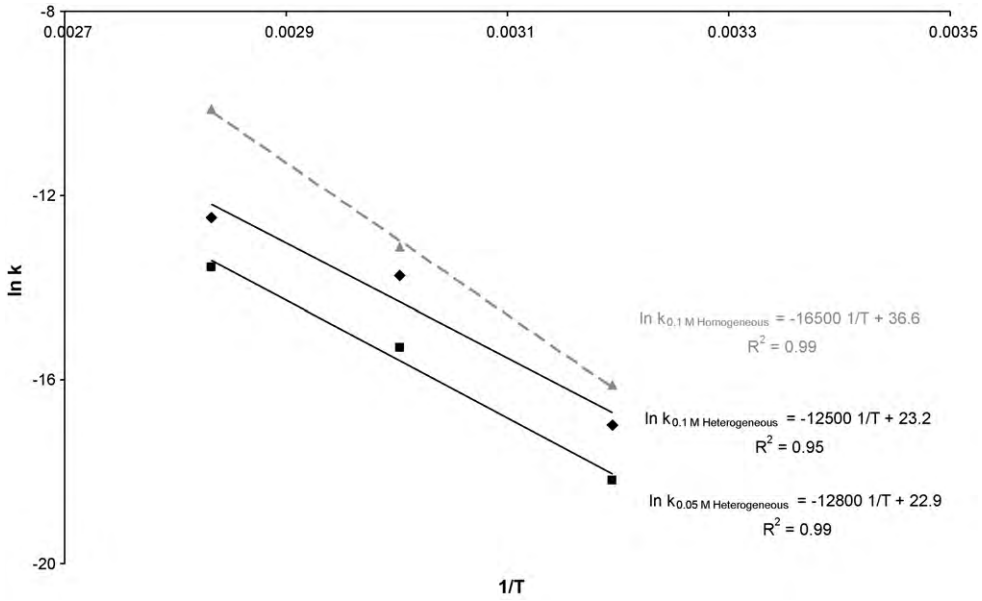

Fig. 5. Arrhenius plots of homogeneous (Tømmeraas & Melander, 2008) (0.1 M HCl) and heterogonous hydrolysis (0.1 and 0.05 M HCl).

regression on the Arrhenius plot, it was possible to determine A from the intercept and the activation energy (E_a) from the slope for the heterogeneous and homogeneous hydrolysis of HA in 0.1 M HCl and for heterogeneous hydrolysis at 0.05 M HCl (data for homogeneous hydrolysis taken from Tømmeraas and Melander (2008)). The results are compared in Table 2. The explanation for the difference in E_a between heterogeneous and homogeneous hydrolysis, is mainly explained by the availability of water that is involved in the rate-limiting step for the hydrolysis in heterogeneous conditions (see Fig. 1). As can be seen, a change in acid concentration leads to a parallel shift in the Arrhenius plot. This shows that a change in the acid concentration gives the same affect on the hydrolytic constant at all temperatures explored. See next section for further discussion.

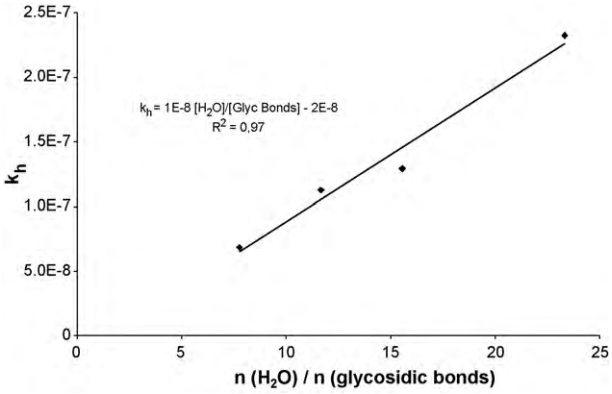

Fig. 6. Kinetic rate constant as a function of ratio between available water per glucosidic bond.

3.3. Effect of water concentration on hydrolysis rate

During initial experiments, the solid content of HA in relation to amount of hydrolysis media was studied. A 0.01 M HCl solution in 93% EtOH was prepared and put into four different vials at volumes corresponding to 5, 7.5, 10, and 15 ml to a constant amount of HA (0.5 g). The samples were hydrolysed at 80 °C for 19 h, cooled, neutralized, filtered, and air dried. At all experiments, the HA was completely covered with the HCl/EtOH solution.

As shown earlier, there is a linear relationship between $(1/M_w - 1/M_{w,0})$ vs. time. Therefore, the kinetic rate constant could be estimated and the results are presented in Fig. 6 as a function of relative molar amounts of water per glucosidic bonds.

As can be seen, there was an influence from the water concentration on the kinetics (Fig. 6). A linear relationship between the amounts of hydrolysed glucosidic linkages was obtained with a polydispersity during the reaction at 1.4. When the HCl/EtOH amount decreases (high %w/w HA), the molecular weight increases and the hydrolysis rate (k_h) decreases. This is again explained by the rate limiting step of acid catalysed hydrolysis for the glucosidic bond in polysaccharides is related to the addition of water to the carbonium-oxonium ion (see Fig. 2). Thus, the low amount of water limits the reaction and determines the rate constant. This is in contrast to homogenous hydrolysis where the rate limiting step is the formation of carbonium-oxonium ion (Einbu et al., 2007).

3.4. Industrial relevance

It is known that the solubility of polysaccharides in an ethanol/water media decreases with chain length (Gelders, Bijnens, Loosveld, Vidts, & Delcour, 2003). This is used industrially for fractionation of extracted HA from biological sources (e.g. ros-

Table 2
Table of slope, E_a, intercept and Arrhenius constant for heterogeneous and homogeneous hydrolysis.

	Slope	E_a (kJ/mol)	Intercept	Arrhenius constant (h^{-1})
0.1 M HCl	16,530	137	37	8.1E+15
0.1 M HCl	12,494	104	23	1.2E+10
0.05 M HCl	12,817	107	23	8.8E+9

ter comb). During the manufacturing of low molecular weight (LMW) HA, there are several different methods proposed (Lapcik Jr., Chabrecek, & Stasko, 1991; Tømmeraas & Melander, 2008). However, homogeneous acid hydrolysis is probably the most widely used, due to the low manufacturing cost and the high controllability of the hydrolysis process. Heterogeneous hydrolysis offers several advantages and involves a process where the HA is not dissolved prior to the hydrolysis making it possible to hydrolyse large amount of HA in limited volumes. Aqueous homogeneous hydrolysis of HA, followed by precipitation of the LMW HA require significant amount of ethanol (EtOH) in order to reach the level at which low molecular weight HA precipitates. Thus, the suggested heterogeneous hydrolysis requires much less ethanol. Also, the recovery of the material during homogeneous hydrolysis is limited to the added amount of ethanol (more ethanol – higher recovery). During the experiments conducted throughout this study, recoveries of HA material was generally above 94% (no data presented). Also, due to the high viscosity of HA in water the amount of material possible to process in a given volume during homogeneous hydrolysis is limited to approximately 1%. The suggested method makes it possible to hydrolyse several times this amount (up to 5% investigated – no data presented). Additionally, in order to obtain solid state HA, the heterogeneous process does not require any additional unit operations such as precipitation using large volumes of EtOH or spray drying. A simple filtration followed by a washing of the excess acid is sufficient. Also, the hydrolysis could easily be controlled to obtain molecular weights within a narrow range (generally below 10% variation – no data presented).

4. Conclusion

Heterogeneous hydrolysis of hyaluronic acid (HA) in ethanolic hydrochloric acid is a novel approach to hydrolyse HA to low molecular weight (LMW) material. The suggested method was shown to be a random degradation process for HA at all acid concentrations and temperatures explored giving no detectable side reactions. The hydrolysis was found to follow a first order kinetics with a linear Arrhenius dependence on the inverse absolute temperature as previously seen for the hydrolysis of HA and other single chain polysaccharides in aqueous solution. The kinetic rate constant could be controlled by varying the water content, temperature, and acid concentration in the heterogeneous EtOH/HCl hydrolysis. This process offers several advantages compared to conventional homogeneous hydrolysis due to less water and ethanol consumption, high recovery of LMW material, and no need for handling of viscous solutions.

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References

Armstrong, D. C., & Johns, M. R. (1997). *Applied Environmental Microbiology*, *63*(7), 2759–2764.
Atkins, P. W. (1994). *Physical chemistry*. Oxford: Oxford Press.
BeMiller, J. N. (2009). *Journal of Agricultural Food Chemistry*, *57*(18), 8125–8129.
Collins, P. M., & Ferrier, R. J. (1995). *Monosacchardies. Their chemistry and their roles in natural products*. Chichester: John Wiley & Sons, Inc.
Einbu, A., Grasdalen, H., & Varum, K. M. (2007). *Carbohydrate Research*, *342*(8), 1055–1062.
Einbu, A., Naess, S. N., Elgsaeter, A., & Varum, K. M. (2004). *Biomacromolecules*, *5*(5), 2048–2054.
Furla, S., La Penna, G., & Perico, A. (2005). *Carbohydrate Research*, *340*, 959–970.
Gelders, G. G., Bijnens, L., Loosveld, A. M., Vidts, A., & Delcour, J. A. (2003). *Journal of Chromatography A*, *992*(1–2), 75–83.
Gibbs, D. A., Merrill, E. W., Smith, K. A., & Balazs, E. A. (1968). *Biopolymers*, *6*(6), 777–791.
Hayashi, K., Tsutsumi, K., & Teramoto, A. (1996). *Polymer Journal*, *28*, 922–928.
Heinegård, D., Björnsson, S., Mörgelin, M., & Sommarin, Y. (1998). Hyaluronan-binding proteins. In T. C. Laurent (Ed.), *The chemistry, biology and medical applications of hyaluronan and its derivatives* (pp. 113–122). London, UK: Portland Press.
Hjerde, T., Smidsrød, O., & Christensen, B. E. (1996). *Carbohydrate Research*, *5*, 175–187.
Holtan, S., Zhang, Q., Strand, W. I., & Skjak-Braek, G. (2006). *Biomacromolecules*, *7*(7), 2108–2121.
Inoue, Y., & Nagasawa K. (1985). *Carbohydrate Research*, *141*, 99–110.
Lapcik, L., Jr., Chabrecek, P., & Stasko, A. (1991). *Biopolymers*, *31*(12), 1429–1435.
Lapcik, L., Jr., Lapcik, L., De, S. S., Demeester, J., & Chabrecek, P. (1998). *Chemical Review*, *98*(8), 2663–2684.
Lee, H. G., & Cowman, M. K. (1994). *Analytical Biochemistry*, *219*(2), 278–287.
Lepperdinger, G., Fehrer, C., & Reitinger, S. (2004). Biodegradation of hyaluronan. In H. G. Garg, & C. A. Hales (Eds.), *Chemistry and biology of hyaluronan* (pp. 71–82). Oxford, UK: Elsevier Ltd.
Sharon, N. (1975). *Complex carbohydrates, their properties, biosynthesis and functions*. Reading, MA: Addison Wesley.
Sicinska, W., Adams, B., & Lerner, L. (1993). *Carbohydrate Research*, *242*, 29–51.
Sjöström, E. (1993). *Wood chemistry, fundamentals and applications*. San Diego: Academic Press Inc.
Smidsrød, O., & Haug, A. (1971). *Biopolymers*, *10*, 1213–1227.
Smidsrød, O., Haug, A., & Larsen, B. (1966). *Acta Chemica Scandinavica*, *20*(4), 1026–1034.
Smidsrød, O., Larsen, B., Painter, T., & Haug, T. (1969). *Acta Chemica Scandinavica*, *23*, 1573–1580.
Sutherland, I. W. (1990). *Biotechnology of exopolysaccharides*. Cambridge: Cambridge University Press.
Tanford, C. (1961). *Physical chemistry of macromolecules*. New York: John Wiley & Sons, Inc.
Tawada, A., Masa, T., Oonuki, Y., Watanabe, A., Matsuzaki, Y., & Asari, A. (2002). *Glycobiology*, *12*(7), 421–426.
Tømmeraas, K., & Melander, C. (2008). *Biomacromolecules*, *9*(6), 1535–1540.
Toshihiko, T., Guoning, Q., Takuya, M., Yoshinori, S., & Toshio, I. (2006). *Analytical Science*, *6*, 799–804.
Varum, K. M., Myhr, M. M., Hjerde, R. J., & Smidsrod, O. (1997). *Carbohydrate Research*, *299*(1–2), 99–101.
Widner, B., Behr, R., Von, D. S., Tang, M., Heu, T., Sloma, A., et al. (2005). *Applied Environmental Microbiology*, *71*(7), 3747–3752.