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Effect of microwave irradiation on the molecular and structural properties of hyaluronan

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Abstract

Hyaluronan (Na^+ salt of hyaluronic acid, HA) was extensively depolymerised by HCl-catalyzed hydrolysis at pH 3 for up to 500 min under temperature-controlled microwave irradiation. The effects of microwave heating on the hydrodynamic properties of the polysaccharide were determined by SEC-MALLS and viscometry. The weight-average molecular mass (M_w) of HA decreased from 1.44×10^6 to $\sim$5000, reaching the region of higher oligosaccharides. The scission of HA chains was found to proceed randomly during the whole degradation process. Treatment of the M_w and intrinsic viscosity data according to the Mark–Houwink equation, $[\eta] = k \times M_w^{\alpha}$ suggested three relationships with $\alpha_1 = 0.46$ for $M_w > 500{,}000$, $\alpha_2 = 0.84$ for M_w between 500,000 and 50,000, and $\alpha_3 = 1.13$ for $M_w < 50{,}000$. The results revealed that HA with $M_w > 10{,}000$ adopts a stiffish coil conformation in solution. As monitored by FT-IR and NMR spectroscopic techniques, the primary structure of the HA chains was maintained during the microwave-assisted hydrolysis at pH 3 at 105 °C. At reaction times larger than 240 min, uv spectroscopy suggested the depolymerisation of HA was accompanied by formation of by-products produced by side reaction.

Keywords: Microwave irradiation; Hyaluronan; Depolymerisation; SEC-MALLS; Viscometry; FT-IR, NMR and UV-spectroscopy

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1. Introduction

Hyaluronic acid (HA) is a natural linear polymer with a molecular mass up to 6 MDa, composed of disaccharide repeating units, namely, [1→4)-β-D-GlcpA-(1→3)-β-D-GlcpNAc-(1→]. HA plays an important role in many biological processes and has found many applications in medicine, pharmacy and cosmetics (Fraser, Laurent, & Laurent, 1997; Goa & Benfield, 1994; Lapčík, Lapčík, De Smedt, Demeester, & Chrabreček, 1998).

This interest is related to its unique physico-chemical properties, such as high intrinsic viscosity and chain stiffness and water-holding capacity. For insight into the relationship between structure and properties of HA studies on low molecular mass HA fractions are desirable (Mahoney, Aplin, Calabro, Hascall, & Day, 2001).

Although ultrasonic degradation can be considered as a very simple and efficient method to obtain HA fractions of lower molecular mass, the depolymerisation ceased on reaching a limiting molecular mass of approximately 100,000 g/mol (Dřímalová, Velebný, Sasinková, Hromádková, & Ebringerová, 2005; Miyazaki, Yomota, & Okada, 2001). Controlled enzymic digestion (Ernst, Langer, Cooney, & Sasisekharan, 1995; Mahoney et al., 2001; Turner, Lin, & Cowman, 1988 and references therein) has been used to prepare HA with M_w varying between 36,000 to 7600 g/mol. The disadvantage is that some lyases generate a modified unsaturated uronic acid moiety on the non-reducing termini, that might not be acceptable in certain applications. This can be avoided by depolymerisation of

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HA under acid and alkaline conditions (Cleland, 1984; Tokita & Okamoto, 1995) and by thermal treatment (Beaty, Tew, & Mello, 1985; Bothner, Waaler, & Wik, 1988). However, also under these conditions, the cleavage of glycosidic bonds is always accompanied with secondary reactions resulting in formation of degradation products and modification of the shortened macromolecular chains. In recent years, microwave (MW) irradiation has received increasing interest in organic chemistry due to the ability not only to drive chemical processes (primary and secondary reactions) but to perform them in reduced time scale in comparison to conventional heating (Bogdal, Penczek, Pielichowski, & Prociak, 2003; Galema, 1997). Only a few studies deal with the application of MW irradiation to depolymerise polysaccharides, such as in acid hydrolysis of cellulose and starch (Allan, Krieger, & Work, 1980) and seed gums (Singh, Sethi, Tewari, Srivastava, & Sanghi, 2003), and the preparation of water-soluble oligoglycosamines from chitosan (Shao, Yang, & Zhong, 2003).

In a previous paper, we compared the effects of ultrasonication, microwave irradiation and conventional heating on HA in various aqueous media (Dřímalová et al., 2005). The aim of the present study was to investigate the temperature controlled MW-assisted depolymerisation of HA at pH 3 to very low molecular masses, and to characterise changes in the molecular and structural properties of this biopolymer.

2. Experimental

2.1. Materials

The hyaluronan sample (HA) with weight-average molecular mass, $M_w = 1\ 221 \times 10^3$ g/mol was a commercial product from CPN spol. s r.o. (Dolní Dobrouč, Czech Republic) prepared by fermentation. All other chemicals used were of analytical grade.

2.2. Analytical methods

^{1}H-NMR spectra were recorded in D_2O at 25 °C on an FT NMR Bruker AVANCE DPX 300 spectrometer (^{1}H at 300.13 MHz and ^{13}C at 75.46 MHz) equipped with a selective unit and gradient enhanced spectroscopy kit (GRASP) for generation of z-gradients up to 50 Gauss cm^{-1} in a 5 mm inverse probe kit. Chemical shifts of signals were referenced to internal acetone (2.225 and 31.07 ppm for ^{1}H and ^{13}C, respectively). UV-spectra were measured using the spectrophotometer Shimadzu UV-2410PC. The sample for the analysis was prepared by adding 0.1 g of the polysaccharide into 50 ml of boiling distilled water and allowing the solution to stay overnight at 70 °C.

2.3. Microwave-irradiation treatment

The HA sample (4.5 g) dissolved in 450 ml of water was stirred for 10 min using an Ultraturax mixer, and the pH was adjusted to 3 with 5% HCl. The solution was poured into a 500 ml boiling flask equipped with a cooler. The irradiation was performed in the microwave reactor RM 2001 (Plazmatronika, Poland). Microwave frequency was 2.45 GHz and the maximum microwave power 800 W. The solution was stirred with a magnetic stirrer. The heating temperature adjusted to 105 °C was monitored by a digital thermometer. The reaction mixture was irradiated with microwave power level of 800 W for a given time.

After irradiation, the solution pH was adjusted to pH 7 with 5% NaOH and the degraded HA sample was precipitated from the cooled solution in the presence of 8 g l^{-1} NaCl with isopropanol (1:2–8, v/v) or ethanol (1:3–5, v/v) depending on the expected M_w, then dewatered with the respective alcohol and dried at 60 °C to constant weight.

2.4. Determination of absolute molar mass by size exclusion chromatography/multi-angle laser light scattering (SEC-MALLS)

Samples were dissolved overnight in the mobile phase and mechanical stirring 30 min. Concentration of samples degraded for 20–120 min was 2 mg ml^{-1}, 4 mg ml^{-1} (150–240 min) and 10 mg ml^{-1} (300–500 min). SEC was performed using a Shimadzu LC-10ADVP pump equipped with Rheodyne 7125 injection valve. The injection volume was 100 μl. The instrument set-up consisted of PL aquagel-OH 60 (300 × 7.5 mm; Agilent Technologies, England) and HEMA BIO 1000 (250 × 8 mm; Tessek, Czech Republic) columns in series thermostated at 40 °C, combined with a miniDAWN MALLS detector (Wyatt Technology Corporation, USA) followed by a RI detector (Shimadzu RID-10A, Japan). The system was also equipped with a guard column (Waters 40 × 8 mm column GMB 200 × 5 μm). The mobile phase was 0.1 M sodium phosphate buffer (pH adjusted to 7.5) + 0.05% NaN_3 at a flow rate 0.8 ml min^{-1}. Data acquisition and molecular weight calculations were performed using the ASTRA software, Version 4.90.07 (Wyatt Technologies Corporation, USA). The specific refractive index increment $dn/dc = 0.155$ ml/g was used for sodium hyaluronate, which is in the range of published values (0.150 and 0.176 (Theisen, Johann, Deacon, & Harding, 2000; Šoltés, Mendichi, Lath, Mach, & Bakoš, 2002)).

2.5. Intrinsic viscosity

The intrinsic viscosities [η] were measured using two dilution Ubbelohde viscometers with different capillaries for the high and low-molecular weight samples. Measurements were performed in aqueous 0.1 M NaCl on sample concentrations ranging from ~0.3 to ~0.08 g/100 ml at 25 ± 0.2 °C maintained by thermostating. Each determination was carried out at least three times and the mean value was given as the result. The intrinsic viscosity $[\eta]$ was calculated by linearly extrapolating the reduced viscosity (η_{red}) to zero concentration.

3. Results and discussion

Based on the results of previous investigations (Dřímalová et al., 2005), the acid hydrolysis of HA using microwave (MW) irradiation as heating source was performed at pH 3 in order to preserve the primary structural features of HA and minimise secondary reactions known to accompany cleavage of glycosidic bonds at acidic conditions (Deguine et al., 1998; Tokita et al., 1995). Fig. 1 illustrates the MALLS (a) and RI (b) chromatograms of HA (in 0.1 M sodium phosphate buffer) as a function of the MW-irradiation time. The elution profiles of both series of chromatograms appeared as single peaks. As the irradiation time increased, peak elution volumes by the MALLS detector became smaller and shifted towards lower molecular masses, what in accord with shiftening of peak elution volumes by the RI-detector to higher values indicate decrease in hydrodynamic volume and increasing extent of depolymerisation.

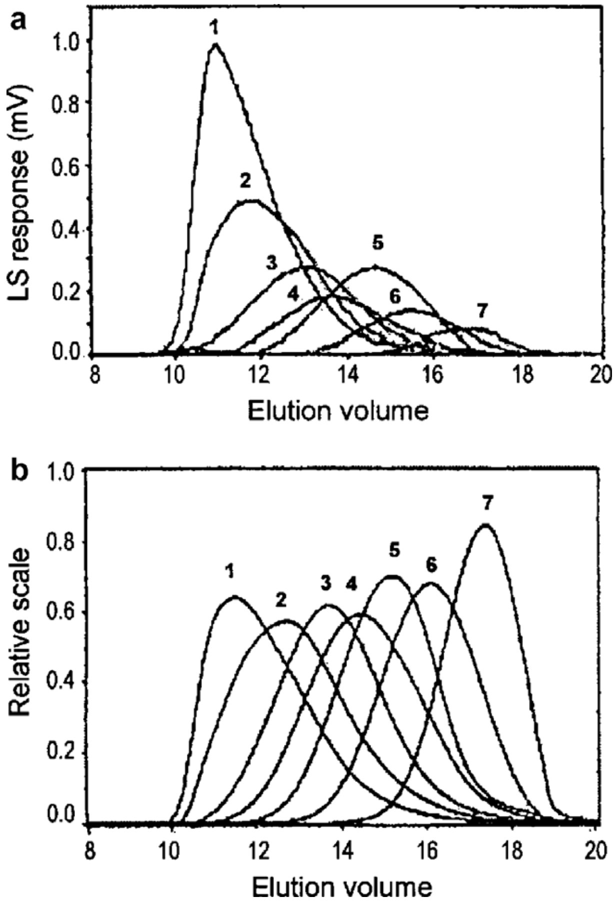

Fig. 1. MALLS (a) and RI (b) chromatograms of HA before and after microwave treatments. The aqueous solution of HA (10 g/L) adjusted to pH 3 was irradiated at 105 °C. Numbers in the figure indicate the irradiation time in min: (1) 20, (2) 30, (3) 60, (4) 120, (5) 150, (6) 180, and (7) 240.

Table 1
Physicochemical data for HA before and MW-irradiation at pH 3 (105 °C)

Degradation time (min)	$M_{\mathrm{w}} \times 10^{-3}$ (g/mol)	$M_{\mathrm{n}} \times 10^{-3}$ (g/mol)	$M_{\mathrm{w}}/M_{\mathrm{n}}$	$[\eta]$ (ml/g)
0	1212.0	730.56	1.66	1030
3	590.61	387.79	1.52	770
5	411.62	278.67	1.48	620
7	310.40	211.59	1.47	520
10	167.61	120.49	1.39	354
20	89.43	62.54	1.43	206
30	62.00	42.35	1.46	150
60	38.44	28.18	1.36	93.5
80	26.21	18.91	1.39	61.5
100	24.13	18.09	1.33	60.8
120	19.26	14.11	1.36	46.5
150	15.73	12.24	1.29	39.5
180	13.63	11.35	1.21	29.0
240	10.25	8.51	1.20	23.5
300	7.89	5.68	1.39	Nd
350	6.68	4.82	1.38	Nd
400	5.99	4.43	1.35	Nd
500	4.97	3.73	1.33	Nd

Nd, not determined.

As shown in Table 1, the depolymerisation was very fast during the first 10 min and slowed down with further MW-irradiation, whereupon the M_{w} values converged to about 5000. The degradation time-dependence of the polydispersity ($M_{\mathrm{w}}/M_{\mathrm{n}}$), which is assumed to give information about the degradation mechanism, showed a peculiar course. First it declined from 1.66 to a minimum value 1.20 at 180–240 min (i.e. at $M_{\mathrm{w}} \sim 12{,}000$), then it increased again up to 1.39–1.33. Such variation in polydispersity, however, with an opposite trend was observed during the ultrasonic degradation of HA (Miyazaki et al., 2001). It was explained by the sonomechanical effect causing rupture at regions close to the midpoint of the chains and, thus, broadening of the distribution curve at the beginning of degradation.

The rate of the hydrolytic degradation was followed by changes of the M_{n} values, which were converted into the number-average degree of polymerization (DP) using the molecular mass of the repeating HA disaccharide unit, 400 g/mol. The values were plotted in Fig. 2 according to a random degradation mechanism (Tanford, 1961), i.e. a first-order reaction, where the number of glycosidic bonds broken is proportional to the degradation time, t:

$$1/[\mathrm{DP}]_n - 1/[\mathrm{DP}]_{n0} = k_{\mathrm{b}} \times t \qquad (1)$$

where DP_n and DP_{n0} correspond to the number of main chain scissions at irradiation time $t = \mathrm{n}$ and $t = 0$, respectively.

The obtained linear relationship is evidently indicating a random scission mechanism. However, the M_{w} data after 60 min of degradation (M_{w} decreased from 1212×10^3 to 38,400) are more dispersed. Similar uncertainty in the lower M_{w}-region was observed for HA by Bothner et al. (1988) and explained by the uncertainty in the $[\eta]$ values and therefore the estimated M_{w}. However, in our study SEC/MALLS was used to determine the molecular masses.

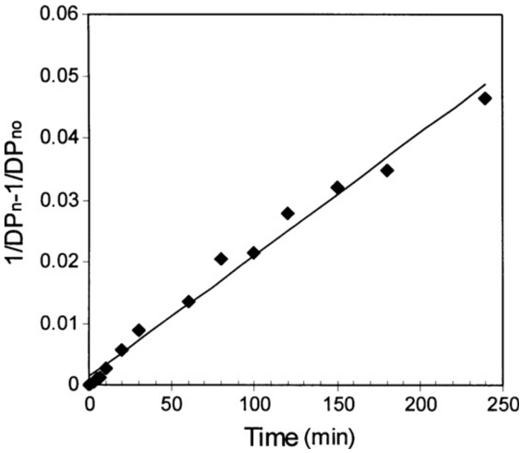

Fig. 2. Degradation of HA at 105 °C plotted as $1/\text{DP}_n - 1/\text{DP}_{no} = k_b \times t$ according to Tokita et al. (1995).

The slope of the line (up to 20 min) suggested the rate constant $k_b = 4.0 \times 10^{-6}$ ($R^2 = 0.9859$), which is in the range of the data (4–6 × 10^{-6}) reported for the depolymerisation of HA in an autoclave at neutral pH and 128 °C (Bothner et al., 1988). The rate constants, k_b, reported by Tokita et al. (1995) for the classical acid hydrolysis of HA at pH 3 at 40 °C and 60 °C, were 5.0×10^{-10} and 6.0×10^{-8}, respectively, i.e. lower but in accord with the lower reaction temperatures used by the authors.

In our previous paper on the tamarind seed xyloglucan degradation by various methods (Dřímalová et al., 2005), a more rapid decrease of the relative molecular mass during microwave heating than during conventional heating was observable only in the initial phases. Comparing the above mentioned rate constants, a moderate acceleration of HA hydrolysis under microwave heating might be presumed. This would indicate rather a thermal effect and not a special non-thermal effect of microwaves supposed when unusually high reaction rate values were found (Galema, 1997). It has to be mentioned that, although, initially both thermal and non-thermal microwave dielectric heating effects have been claimed, it is now generally accepted that a different temperature regime caused by microwave heating is the main contributing factor to any acceleration observed (Bogdal et al., 2003). However, the mechanism of interaction of microwave radiation with materials is still not well known.

As the HA sample formed a homologous series, the Mark–Houwink relationship, $[\eta] = k'M^{\alpha}$, was used to characterise the gross conformation of the polysaccharide (Harding, 1997). In Fig. 3, the double logarithmic plot of intrinsic viscosity, $[\eta]$, versus molecular mass, M_w, is depicted. In the graph were included M_w and $[\eta]$ values of samples obtained by conventional heating of HA at pH 3 in a previous study (Dřímalová et al., 2005). Based on linear regression analyses, three linear regions with different α parameters were suggested from the graph indicating a dependence on the molecular mass. For HA samples with $M_w > 500{,}000$ $\alpha_1 = 0.46$, with M_w ranging between 500,000 and 50,000 $\alpha_2 = 0.84$, and with $M_w < 50{,}000$ $\alpha_3 = 1.13$.

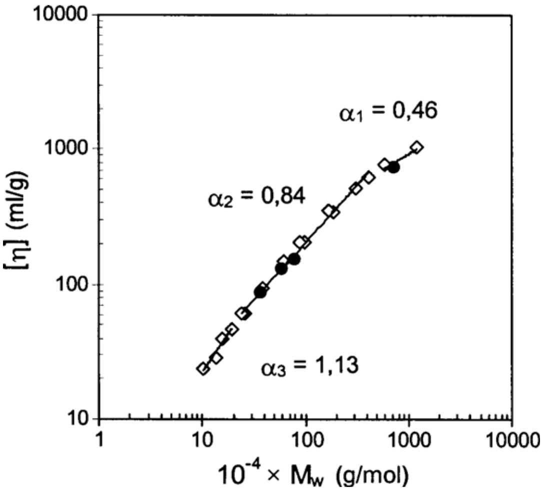

Fig. 3. Double logarithmic plot of intrinsic viscosity $[\eta]$ of HA measured in 0.1 M NaCl versus weight average molar mass, M_w. The values (full symbols) from hydrolysis of HA by conventional heating at pH 3 (Dřímalová et al., 2005) are included. The solid lines represent the best fit from power type regression.

Most of the authors investigating the Mark–Houwink relationship for HA preparations reported one value of the α parameter for HA, ranging between 0.70 and 0.84 (Table 2 and references therein) and indicating the gross conformation of HA in aqueous solution to be a random coil (Harding, 1992). A molecular mass dependence of the α parameter was suggested by Bothner et al. (1988) for HA degraded by autoclaving at 128 °C at neutral pH, and, recently, by Hokputsa, Jumel, Alexander, and Harding (2003) for chemically degraded HA using ascorbic acid/hydrogen peroxide treatments. As seen in Table 1, our data are consistant with the last mentioned report, although some differences are in the corresponding M_w regions. It is to be mentioned that apart from differences in methods used to prepare the HA samples and determination of M_w, factors such as ionic strength of the solvent and shear rate in the capillary viscometer strongly affect the hydrodynamic volume in the region of very high molar masses, in particular. Moreover, uncertainties in $[\eta]$ determination of HA with very low molar masses must be taken into consideration as well (Bothner et al., 1988; Šoltés et al., 2002). The presented values of α parameters confirmed the previous statement (Hokputsa et al., 2003) that HA adopts a 'stiffish' coil conformation in aqueous solution in the studied molecular mass range of HA fractions.

Chemical and structural changes of HA during MW irradiation were monitored by FT-IR, NMR and UV-spectroscopy techniques. The FT-IR spectra of the original HA and the degraded samples (Fig. 4) indicate no substantial changes of the spectral pattern in the region typical of absorption bands corresponding to vibrations of the acetamido and carboxylate groups and the pyranose ring (Gilli, Kačuráková, Mathlouti, Navarini, & Paoletti, 1994), even in the case of the most degraded sample (e). Only the band

Table 2
Values of the α parameter in the Mark–Houwink equation for hyaluronan from various references and the present study

Reference	α	Molar mass (g/mol)	Solvent
Laurent, Ryan, and Pietruszkiewicz (1960)	0.78	$7.7 \times 10^{4-}.7 \times 10^6$	0.2 M NaCl
Cleland and Wang (1970)	0.816	$>10^5$	0.2 M NaCl
	0.777	$>10^5$	0.5 M NaCl
Shimada and Matsumura (1975)	0.76		0.2 M Na phosphate
Balazs, Briller, and Denlinger (1981)	0.8		0.2 M NaCl
Terbojevich, Cosani, Palumbo, and Pregnolato (1986)	0.75		0.15 M NaCl
Turner et al. (1988)	1.16	$7–36 \times 10^3$	0.15 M NaCl
Bothner et al. (1988)	0.601	$\geqslant 1 \times 10^6$	0.15 M NaCl
	0.779	$<1 \times 10^6$	0.15 M NaCl
Gamini, Paoletti, and Zanetti (1992)	0.81		0.15 M NaCl
Fouissac, Milas, and Rinaudo (1993)	0.841	$>2.4 \times 10^6$	0.1 M NaCl
Yanaki and Yamaguchi (1994)	0.829[a]	$>40 \times 10^4$	0.2 M NaCl
Li, Rosenfeld, Vilar, and Cowman (1997)	0.80		0.15 M NaCl
Gura, Hückel, and Müller (1998)	0.716	$10^5–10^6$	0.2 M NaCl
	0.770[a]	$10^5–10^6$	
Šoltés et al. (2002)	0.601	$\geqslant 1 \times 10^6$	0.15 M NaCl
	0.779	$<1 \times 10^6$	
Hokputsa et al. (2003)	0.69	$>5 \times 10^5$	PBS
	0.73	$<1 \times 10^6$	
	1.10	$<1 \times 10^5$	
This study	0.46	$>5 \times 10^5$	0.1 M NaCl
	0.84	$5 \times 105–5 \times 10^4$	
	1.13	$<5 \times 10^4$	

[a] Measured in a shear-rate viscometer; PBS, 1% solution in phosphate buffered saline.

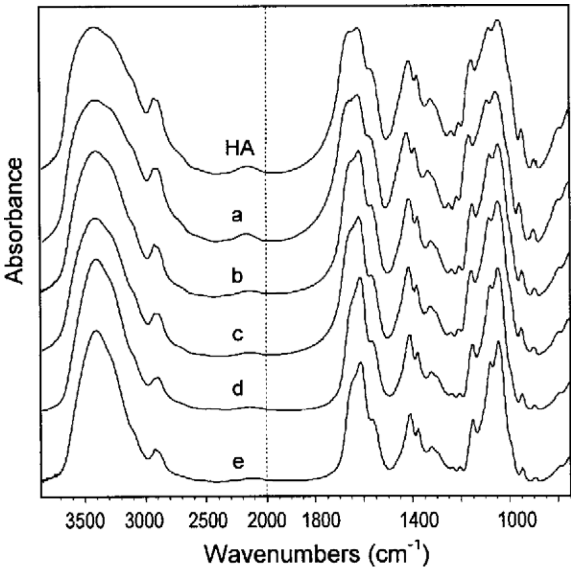

Fig. 4. FT-IR spectra (in KBr) of a series of hyaluronic acid (HA) before and after MW treatment for (a) 60 min, $M_w = 38{,}400$, (b) 180 min, $M_w = 13{,}600$, (c) 240 min, $M_w = 10{,}200$, (d) 350 min, $M_w = 6700$, and (e) 500 min, $M_w = 5000$; N, GlcpNAc unit; A, GlcpA unit.

heights of amide I and $\nu_{as}(COO^-)$ vibrations at ~1660 and 1618 cm^{-1}, respectively, showed subtle change with increasing depolymerisation. However, a narrowing of the originally broad asymmetric band at 3450–3100 cm^{-1}, corresponding to stretching vibration of the hydroxyl groups and partially to ν(NH) stretching vibration of the *N*-acetyl side chains involved in hydrogen bond systems of the supramolecular structure of HA (Gilli et al., 1994), can be observed after degradation times >60 min. This indicates decrease of hydrogen-bonds strength. The observed self-aggregation tendency of HA in solution by forming stable tertiary structures was reported to decrease substantially at DP < 20, i.e. at $M_w < 8000$ (Scott & Heatley, 1999). However, the effect of the hydrogen bonds and their strength on the depolymerisation rate is not clarified.

The ^1H and ^{13}C NMR spectra of the original and degraded HA are shown in Fig. 5. Their spectral pattern resembled that of the hitherto published spectra of HA and hyaluro-oligosaccharides (Chai, Beeson, Kogelberg, Brown, & Lawson, 2001; Scott & Heatley, 1999; Toffanin et al., 1993). After MW- irradiation for 150 min (M_w 15,700) weak C-1/H-1 resonances of the α- and β-anomers of the Glc*p*NAc reducing end residues are visible at δ 91.9/5.18 and 95.3/4.74. Their intensity increased with further decrease of the molar mass. The very weak signals at δ 92.9 and 97.0 in the ^{13}C NMR spectra of HA with $M_w < 10{,}000$ were assigned to C1 of α- and β-anomers of Glc*p*A reducing end residues. The results confirmed the suggestions of Tokita et al. (1995) that hydrolysis at C4 in comparison to C1of the Glc*p*A residues is preferred. Moreover, there were no convincing indications in the FTIR and NMR spectra about cleavage of acetyl groups and ring-opening reactions of both HA monomer units even with the most degraded HA sample. No signals typical of 4,5-unsaturated glucuronic acid moieties at $\delta \sim 108$/5.84 (C-4/H-4) and $\delta \sim 145$ (C5) (Uchiyama, Dobashi, Ohkouchi, & Nagasawa, 1990) were seen in the respective NMR spectra.

In the UV spectra (Fig. 6), the absorbance at ~210 nm attributed to carboxyl groups increased with increasing irradiation time, and an expanding absorption band at

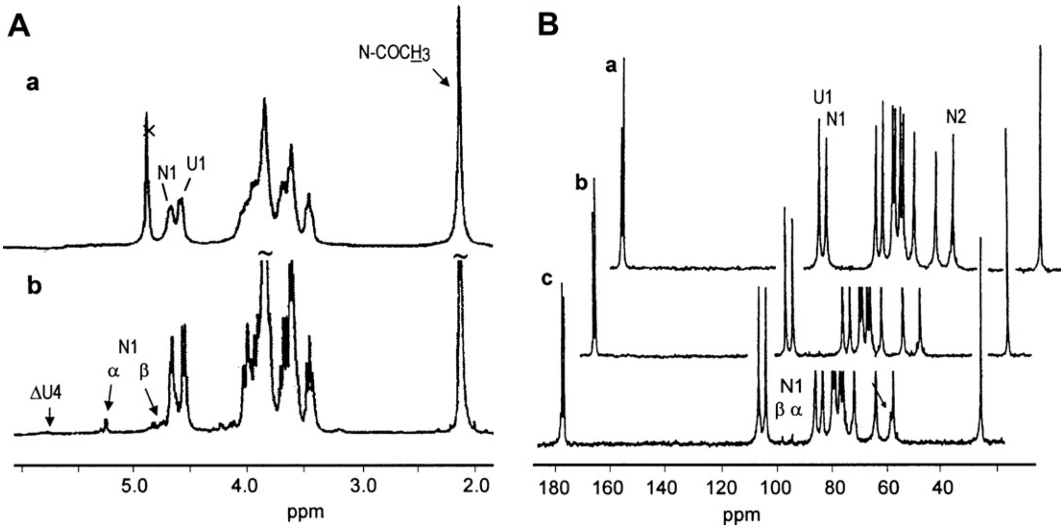

Fig. 5. (A) ^{1}H NMR NMR (a) before and (b) after 500 min of microwave irradiation; (B) ^{13}C NMR spectra of HA (a) before and after microwave irradiation. for (b) 240 min and (c) 500 min. The numbers refer to proton and carbon resonances of the GlcpNAc (N), GlcpA (U) and unsaturated GlcpA (ΔU) residues.

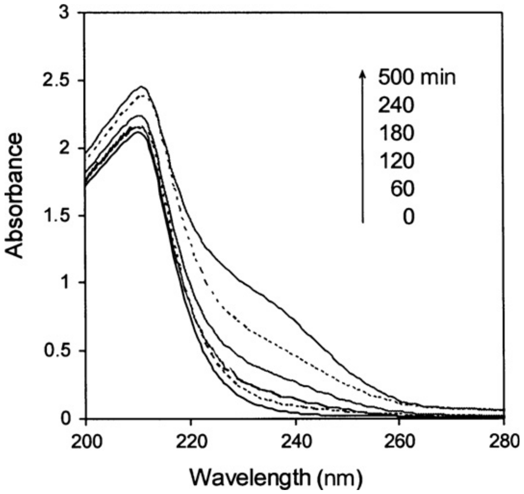

Fig. 6. UV spectra of HA solutions measured at various time of microwave-assisted hydrolysis at pH 3: (a) HA, 0 min; (b) HA, 60 min; (c) HA, 120 min; (d) HA, 180 min; (e) HA, 250 min; and (f) HA, 500 min.

~240 nm appeared after 180 min irradiation indicating the presence of unsaturated structures, such as the 4,5-unsaturated glucuronic acid moieties absorbing at ~232 nm (Uchiyama et al., 1990). As the reaction was not performed under oxygen-free conditions, formation of degradation products due to radical reactions on the reducing end groups of the short chains cannot be ruled out. However, their content is too low to be evidenced in the FTIR and NMR spectra.

4. Conclusions

MW irradiation at mild acidic conditions yielded a homologous series of HA with definite molar mass up to 5000 g/mol and preserved primary structural features. Evaluation of the intrinsic viscosity/M_w relationship by the Mark–Houwink equation yielded three molecular mass regions with different α parameters. The results suggest that the overall solution conformation of HA samples obtained by the MW-assisted hydrolysis at pH 3, similarly as reported for the oxidatively degraded HA (Hokputsa et al., 2003), is between random coil and rigid rod, designated as a 'stiffish' coil.

The acid hydrolysis of HA under microwave irradiation at pH 3 and 105 °C is a random process with the rate constant k_b of 4×10^{-6}, which is similar to that reported for autoclaving of HYA solutions at 128 °C (Bothner et al., 1988). Although microwave energy is more expensive than electrical energy, the efficiency of MW under appropriate processing conditions is much higher and might compensate for the higher energy cost in comparison to the electrical energy (Bogdal et al., 2003; Galema, 1997). An advantage of the hydrolysis under temperature-controlled microwave heating is the fact that side reactions can be suppressed even for long irradiation times, and HA preparations of acceptable purity can be prepared.

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