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Degradation of hyaluronan by ultrasonication in comparison to microwave and conventional heating

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Abstract

Hyaluronan (hyaluronic acid, HA) was depolymerised by ultrasonication (US), microwave irradiation (MW) and conventional heating (CH), and the effect of pH and oxidants was investigated. The degradation was followed by viscometry and size exclusion chromatography coupled with low-angle light scattering. The results demonstrated that depolymerisation of HA by US leveled off to a limiting molecular mass, and the degradation was significantly enhanced by acidic and alkaline pH only in the presence of oxidants. In contrast to US, the course of depolymerisation by MW was strongly pH-dependent, and the degradation rate increased with decreasing pH. The expected enhancement of depolymerisation by MW in comparison to CH was marked only at very short heating time at pH <4. The NMR and FTIR spectral analyses indicated that HA in the whole M_w-range studied retained almost the backbone of the parent polysaccharide independently on the degradation method used. At harsh degradation conditions (long-term treatments, particularly at acidic pH or alkaline pH and in presence of oxidants) the depolymerisation was accompanied by destruction of both constituent sugar residues and formation of unsaturated structures detectable by UV-absorption at 230–240 and 260–270 nm. US-assisted oxidative degradation under mild reaction conditions was shown to be the most appropriate procedure to reduce the molecular mass of HA to ~100 kDa without significant chemical modification of the polysaccharide.

Keywords: Hyaluronan; Ultrasonicaton; Microwave irradiation; Conventional heating; Depolymerisation

1. Introduction

Hyaluronan (hyaluronic acid, HA) is a linear glycosaminoglycan composed of disaccharide repeating units, namely, [→4)-β-D-Glc*p*A-(1→3)-β-D-Glc*p*NAc-(1→] (Weissmann & Meyer, 1954). The high molecular mass HA, which plays an important role in many biological processes such as in tissue hydration, proteoglycan organisation in the extracellular matrix, and tissue repair, has found application in several clinical treatments and cosmetic use (Arshinoff, 1995; Goa & Benfield, 1994; Lapčík, Lapčík, De Smedt, Demeester, & Chrabreček, 1998; Laurent, 1998). Of interest are also HA of lower molecular mass, which were reported to promote angiogenesis (West, Hampson, Arnold, & Kumar, 1985), and to possess biological effects of the high molecular mass HA (Underhill, 1992) as well as activities not associated with the parent molecule (Horton, Shapiro, Bao, Loewenstein, & Noble, 1999; McKee et al., 1996). HA oligosaccharides served also as models for hyaluronan–protein interaction studies (Sicińska & Lerner, 1996) and analytical purposes (Gilli, Kačuráková, Mathlouti, Navarini, & Paoletti, 1994; Toffanin et al, 1993). For certain applications in the field of medical treatments and cosmetics, polysaccharides of lower molecular mass have selected advantages over the high molecular mass candidates. However, it is of importance to have depolymerised HA preparations structurally and chemically well-characterized for biological studies.

Many methods have been applied to depolymerise biopolymers into lower molecular mass fragments. Among them ultrasonic degradation is a well established procedure (Mason & Lorimer, 2002), which has been applied on HA

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by various authors (Gura, Hückel, & Müller, 1998; Kubo, Nakamura, Takagaki, Yoshida, & Endo, 1993; Orviský, Šoltés, Chabreček, Novák, & Stančiková, 1993). Recently, HA of a broad range of molecular masses have been prepared by ultrasonication in neutral aqueous solutions containing various cations (Miyazaki, Yomota, & Okada, 2001) as well as by oxidative degradation using the ascorbic acid/H_2O_2 system (Hokputsa, Jumel, Alexander, & Harding, 2003).

Depolymerisation of HA has been performed also by exposure to high temperature in an autoclave (Bothner, Waaler, & Wik, 1988) and by acid hydrolysis (Tokita & Okamoto, 1995). A promising degradation method seems to be microwave irradiation (Galema, 1997), which has received increasing interest in hydrolytic procedures due to remarkable enhancement of the reaction rates and significant effects over conventional heating, such as less formation of side reaction products (Singh, Sethi, Tewari, Srivastava, & Sanghi, 2003). In any case, the essential requirement is to preserve the fundamental structure of the polymer during depolymerisation, and to avoid or minimise the formation of new functional groups and/or contamination with side reaction products, which both might affect the biological response of the degraded polymer.

The aim of this study was to compare the degradation of HA by ultrasound (US), microwave irradiation (MW) and conventional heating (CH) at different pH, and in presence or absence of oxidants in view of the achieved decrease of the molecular mass as well as in relation to changes in the structural integrity of the polysaccharide.

2. Experimental

2.1. Materials

The starting HA samples were prepared by a fermentation process in CPN spol. s r.o. (Dolní Dobrouč, Czech Republic) and designated as HA1, HA2, and HA3. Their weight-average M_w determined by SEC/LALS were 1.72×10^6, 1.44×10^6, and 1.16×10^6, respectively. All used reagents were of analytical grade.

2.2. Analytical methods

Fourier-transform infrared (FT-IR) spectra of samples (2 mg) in KBr pellets (200 mg) were obtained on the NICOLET Magna 750 spectrometer with DTGS detector and OMNIC 3.2 software using 128 scans at a resolution of 4 cm^{-1}. The ^1H- and ^{13}C-NMR spectra (in D_2O) were recorded in the inverse gated decoupling mode at 25 °C on a Bruker DPX AVANCE-300 spectrometer operating at 300 MHz for ^1H and 75.46 MHz for ^{13}C. 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 in a thermostat at 70 °C.

The relative molecular mass of HA (expressed as M_r) was derived from the kinematic viscosity of the 0.05% (w/v) solution of the degraded samples in phosphate buffer pH 7, measured at 25 ± 0.2 °C using the Ubbelohde viscometer U I-0,636 ($K=0.01$ mm^2 s^{-1}). Each determination was carried out twice and the mean value was taken as the result. A series of HA standards of different molecular mass was prepared by γ-irradiation of HA (performed by Artim, Prague). Their absolute molecular mass, M_w, was determined at the Institute of Macromolecular Chemistry, CAS (Prague) by HP SEC chromatography coupled with a low-angle LS unit (Chromatix KMX-6). From the kinematic viscosity and M_w data of these standards, a calibration curve was constructed and used to calculate the M_r of the HA samples degraded by various methods.

2.3. Depolymerisation of HA

All degradation experiments were performed at polysaccharide concentration 1% (w/v).

2.3.1. Degradation by ultrasound

Irradiation was carried out with the aid of the Ultrasonic processor U 200 S control (24 kHz, IKA, Germany) at 200 W ultrasound power and ultrasonic intensity of 3.5 W cm^{-2}. The HA sample (4.5 g) was immersed into distilled water (450 ml), kept overnight, and then intensively stirred for 15 min. The acidic and alkaline pH of the solutions was adjusted with 5% HCl and 5% NaOH, respectively. The solution was poured into a glass vessel surrounded by ice and the cylindric sonotrode US 200-14 (108 mm × 14 mm, IKA) was immersed into the solution to a depth of 72 mm. The solution constantly circulated through an externally ice-cooled stainless steel coil in order to maintain the temperature at 15 ± 3 °C and agitation during sonication. After a defined irradiation time, the HA sample was filtered using the filtration desk HS 800 or Millipore TCMF to remove solid particles released from the sonotrode. After adjusting the pH to 7, the degraded HA was precipitated in the presence of 8 g/l NaCl with IPA (1: 2–8, v/v) or EtOH (1: 3–5, v/v) in dependence on the expected molecular mass, then dewatered with the respective alcohol and dried at 60 °C to constant mass. Under these conditions, oligosaccharides and low molecular mass degradation products were separated from the polymeric HA samples.

2.4. Degradation by microwave heating

A microwave reactor RM 2001 (Plazmatronika, Poland) with microwave frequency 2.45 GHz and maximum microwave power 800 W was used. After adjusting the pH to the required level, the HA solution was poured into a 500 ml boiling flask equipped with a cooler and placed into the reactor. The solution was stirred with a magnetic stirrer

and boiled under reflux for the given time. The bulk temperature, controlled with a digital thermometer installed in the reactor, varied between 100 and 110 °C, what is in accord with the published 'superheating effect' (Kingston & Haswell, 1997). After heating had been stopped, the solution was cooled to room temperature and the degraded HA recovered as described in Section 2.3.

2.5. Degradation by conventional heating

Degradation by CH under reflux of the 1% aqueous solution of HA, adjusted to pH 3 with 5% H_2SO_4, was carried out in a 500 ml boiling flask fitted with a cooler and placed in a heating mantle. The reaction ran without stirring. After cooling the solution to room temperature, the degraded samples were recovered as described in Section 2.3.

3. Results and discussion

3.1. Depolymerisation of HA

Ultrasonic irradiation produced permanent reduction in M_r as illustrated in Fig. 1 for experiments carried out in neutral, acidic and alkaline media with and without addition of oxidants. As seen, the depolymerisation proceeded rapidly in the early stage of the reaction and leveled off with prolonged sonication to a limiting M_r, similarly as has been reported in previous studies (Gura et al., 1998; Kubo et al., 1993; Miyazaki et al., 2001; Orviský et al., 1993). US of HA1 at neutral and alkaline pH reduced the M_r to 107 kDa after 120 min. The degradation was slower at acidic pH and the M_r of ~100 kDa was obtained after 480 min (not shown). Increasing the temperature to 40–65 °C accelerated the degradation. As further seen, the course of depolymerisation is less dependent on pH in the studied pH-range, but strongly affected by the presence of oxidants. US in the pH 4/0.4%H_2O_2 medium yielded after 120 min HA with M_r of 42 kDa. However, the most effective enhancement of HA1 depolymerisation was achieved in the alkaline solution containing 0.09% NaOCl. After 15 min sonication the M_r was found to be reduced to 133 kDa, i.e. by more than 90%.

The depolymerisation by US has been generally accepted to proceed by mechanical force (Basedow & Ebert, 1978; Mason & Lorimer, 2002) causing breakage roughly at the centre of the molecular chains and with larger molecules degrading fastest, and a limiting molecular mass below which degradation does not take place. The observed weak pH-dependence of HA depolymerisation rate, shown in Fig. 1, is in accord with this suggestion. Sonochemical effects have been supposed to play a minor role in ultrasonic depolymerisation (Miyazaki et al., 2001). However, such reactions as carboxyl group formation, ring-opening and further transformations leading to unsaturated structures on both the GlcA and GlcNAc units, and chain cleavage, are known to proceed in the presence of oxidants (Uchiyama, Dobashi, Ohkouchi, & Nagasawa, 1990), and were related to reactions of oxygen-derived radicals.

The depolymerisation of HA3 by MW is illustrated in Fig. 2. In contrast to US in the absence of oxidants, the effect of MW was strongly pH-dependent. At pH <4, a pronounced decrease of the molecular mass was observed. In the present study, experiments at pH lower

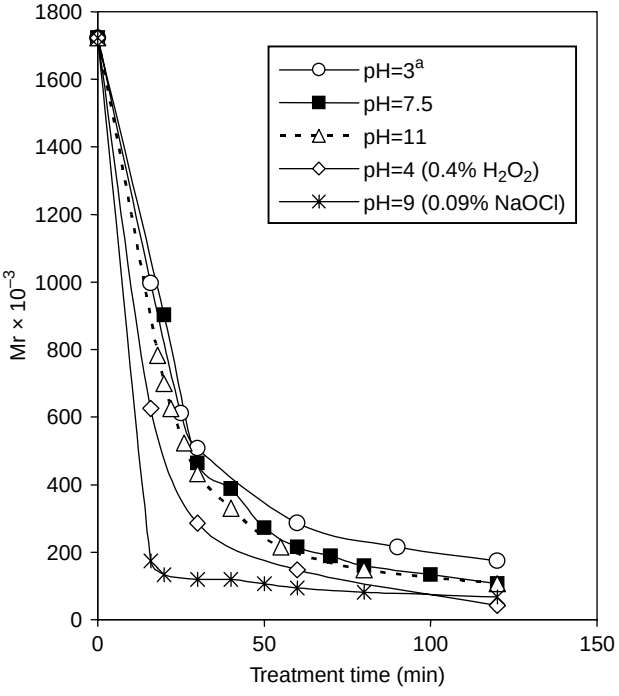

Fig. 1. Effect of pH and oxidants on the depolymerisation of HA1 by ultrasound at 15±3 °C. (a) Sonication temperature: 40–60 °C.

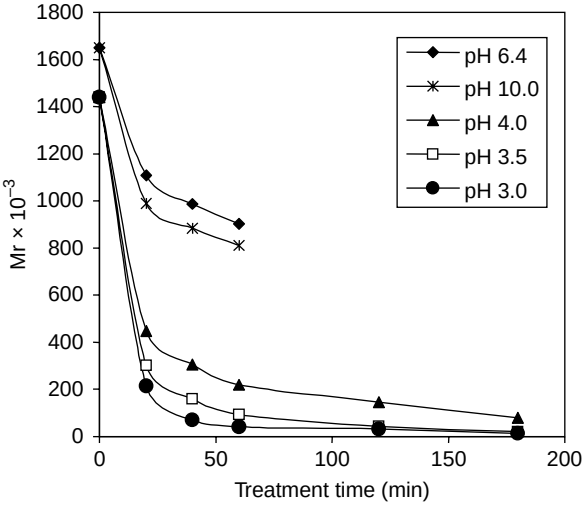

Fig. 2. Effect of pH on the depolymerisation of HA by microwave irradiation. HA1 (pH 6.4 and 10.0); HA3 (pH 3.0–4.0).

than 3 were not performed in order to avoid possible deacetylation of the GlcNAc sugar constituent and decomposition (Bothner et al., 1988). By decreasing the pH from 4 to 3, the M_r decreased to 215 kDa after 20 min and to 12.7 kDa after 180 min.

According to Galema (1997), microwave heating is occurring within molecules and results in polarisation of polar bonds (such as the C–O–C glycosidic linkages), increasing their reactivity. Therefore, MW is able to enhance the hydrolytic cleavage of macromolecular chains. As seen in Fig. 2, there was a weak but distinct M_r-decrease in the neutral and, even, in alkaline reaction medium, indicating that additional reactions were initiated by MW irradiation as well.

The effect of MW-assisted depolymerisation of HA was compared to that of CH in dependence on pH ranging between 3 and 6. As shown in Fig. 3, the reduction of M_r proceeded more rapidly by MW irradiation. However, the expected enhancement of the depolymerisation rate by MW in comparison to CH was marked only at pH <4 and very short heating time. This was indicated by the difference in the M_r obtained after 10 min treatment, which increased with decreasing pH. The differences faded with prolonging the heating time and the limiting M_r reached the same value of $\sim$21 kDa after 120 min by both methods. However, it is to mention that the reported (Singh et al., 2003) fast depolymerisation of plant gum to monosaccharide constituents by extremely short MW heating (<2 min) was achieved at a substantially higher microwave power (1200 W).

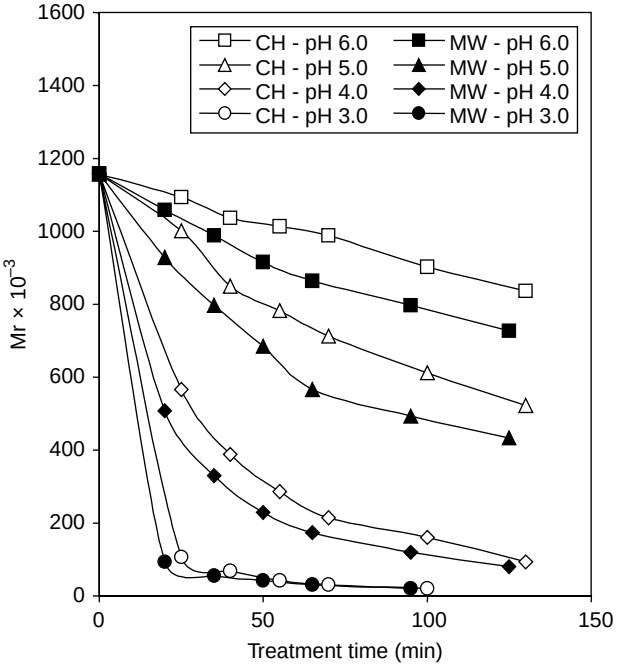

Fig. 3. Depolymerisation of HA3 by microwave (MW) and conventional heating (CH) in dependence on pH.

3.2. Chemical and structural changes

In Fig. 4, the FT–IR spectra of representative samples from the US, MW and CH degradation experiments are presented and compared to that of the parent HA. As seen, the overall spectral pattern has not changed by decreasing the molecular mass of HA what is in accord with a previous infrared study on HA (Gilli et al., 1994). Additional bands have not appeared even in the spectra of the most degraded samples. However, there were some differences in the height and shape of certain absorption bands such as narrowing or broadening of the absorption band at 3450–3100 cm^{-1}, which are assigned to ν(OH) stretching and partially to ν(NH) stretching vibration of *N*-acetyl side chains involved in hydrogen bonds of different strength (Gilli et al., 1994). This might be due to changes of the inter- and intramolecular hydrogen bond systems of the rigid supramolecular structure of HA (Scott & Heatley, 1999) as well as by creation of additional hydrogen bonds during the recovery of HA from solution by alcohol precipitation in presence of salt (Turner, Lin, & Cowman, 1988). Also the height proportion of the bands at $\sim$1660 cm^{-1} and 1618 cm^{-1}, assigned to the amide I mode of the GlcNAc unit and the antisymmetrical stretching mode of the carboxylate group of the GlcA unit, respectively, varied slightly with increasing depolymerisation. The relative increase of the carboxylate group vibration might result

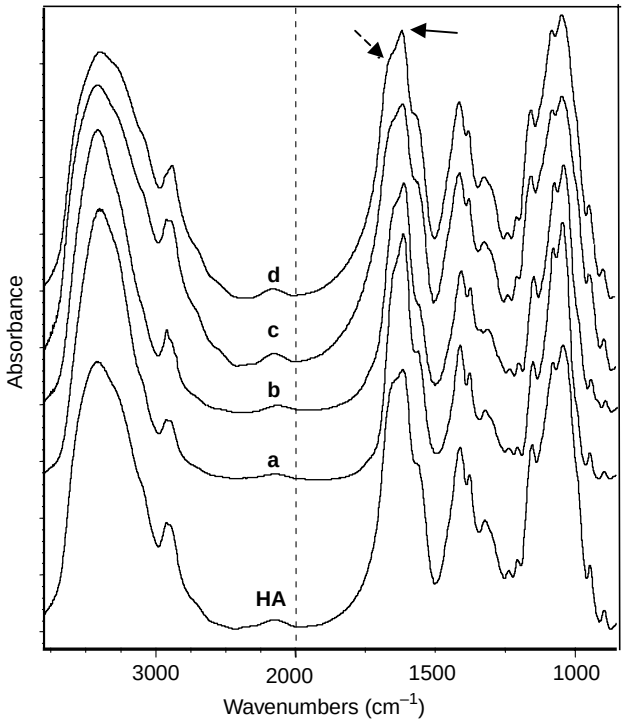

Fig. 4. FT-IR spectra of HA before and after 120 min treatment by US at (a) pH 3/0.4%H_2O_2 and (b) pH 9/0.09% NaOCl, by (c) MW at pH 3 for 240 min, and (d) CH at pH 3 for 480 min. The corresponding M_r values for *a–d* were as follows: 42, 68, 12.7, and 5.5 kDa. The arrows indicate absorption bands of (dotted line) amide I and (full line) $\nu_{as}(COO^-)$ groups.

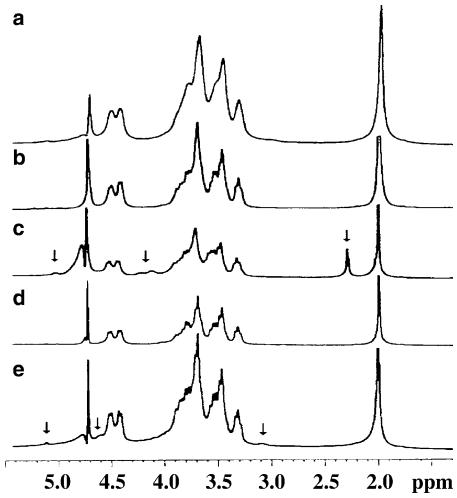

Fig. 5. ^{1}H NMR spectra (in D_2O) of HA after US for 120 min at (a) pH 7.5, (b) pH 3/0.4%H_2O_2, and (c) pH 9/0.09% NaOCl, after (d) MW at pH 3 for 120 min, and (e) CH at pH 3 for 480 min. The corresponding M_r values for *a–e* were as follows: 107, 42, 68, 31, and 5.5 kDa.

from the higher susceptibility of the GlcNAc residues to decomposition (Tokita & Okamoto, 1995).

The ^{1}H and ^{13}C-NMR spectra of representative samples from the US, MW and CH degradation experiments are shown in Figs. 5 and 6. Their spectral pattern resembled that of the hitherto published HA spectra (Cowman, Hittner, & Feder-Davis, 1996; Kwam, Atzori, Toffanin, Paoletti, & Biviano, 1992; Uchiyama et al., 1990). At $M_w > 100$ kDa, the spectra showed no signs of chemical modification, independently on the depolymerisation method used. With further decrease of M_w weak but distinct resonances appeared, particularly at acidic pH, in the anomeric region corresponding to H-1 and C-1 resonances of the reducing end sugar units of both GlcNAc and GlcA constituents. After US at oxidative conditions in both acid and alkali media as well as after MW at pH 3 for 240 min, additional signals occurred in the upfield shift range (Figs. 5 and 6), indicating sugar destruction reactions. Based on kinetic studies of hydrolytic degradation of HA, Tokita & Okamoto (1995) suggested that hydrolysis occurs in acid solution on the GlcA residue and the hemiacetal ring remains. In alkaline medium, the destruction of GlcNAc residue takes place, starting by cleavage between C-1 and C-2 and resulting in formation methylene groups. However, there were no additional signals in the low-field region ($\delta > 5.6$ and δ 140–170) of the ^{1}H and ^{13}C NMR spectra of the most degraded HA samples, which would indicate the presence of unsaturated structures (Tjan, Voragen, & Pilnik, 1974). Therefore, the possible chemical modification of HA by the applied depolymerisation methods was further tested by UV spectroscopy.

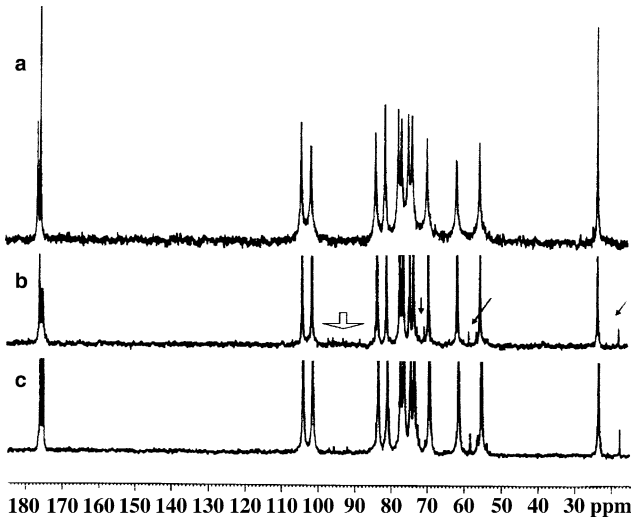

Fig. 6. ^{13}C NMR spectra (in D_2O) of HA after (a) US at pH 7.5 for 120 min, (b) US at pH 3/0.4%H_2O_2 for 120 min, and (c) MW at pH 3 for 240 min.

The spectra of representative US-degraded HA samples compared to that of the parent HA are shown in Fig. 7. During US at acidic and acidic/oxidative conditions, the absorption band at ~210 nm, attributed to carboxyl groups, increased, and a band at ~240 nm appeared after 120 min sonication, indicating the presence of unsaturated structures. Its intensity increased with increasing treatment time (not shown). In this region, the 4,5-unsaturated uronic acid (HexA) formed by β-eliminative cleavage of HA chains, is known to absorb (Ermolenko, 1959). However, also other unsaturated structures might be produced by reactions of oxygen-derived radicals generated during the treatment (Uchiyama et al., 1990). A similar effect was observed after MW and CH degradations at lower pH (Fig. 8). US degradation at alkaline pH (Fig. 9) and, particularly, in the alkaline/oxidative medium (Fig. 7) led to formation of new

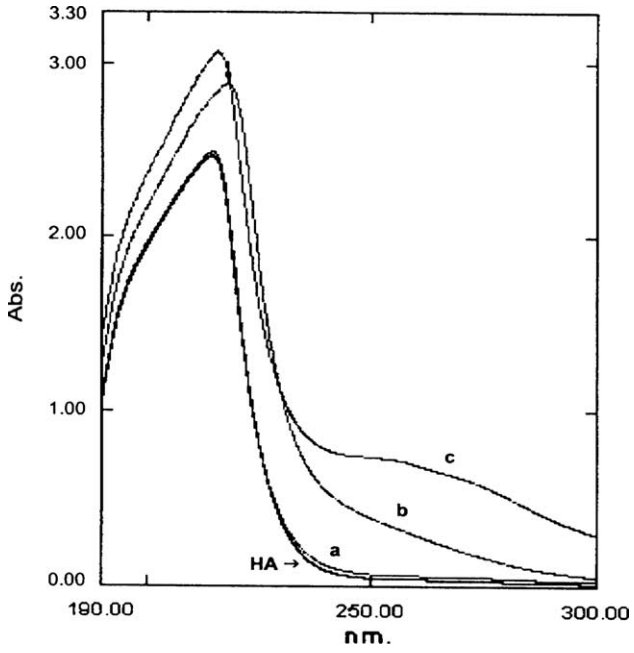

Fig. 7. UV-spectra of HA1 before and after 120 min ultrasonication at different conditions: (a) pH 7.5; (b) pH 4/0.4%H_2O_2; and (c) pH 9/0.09%NaOCl.

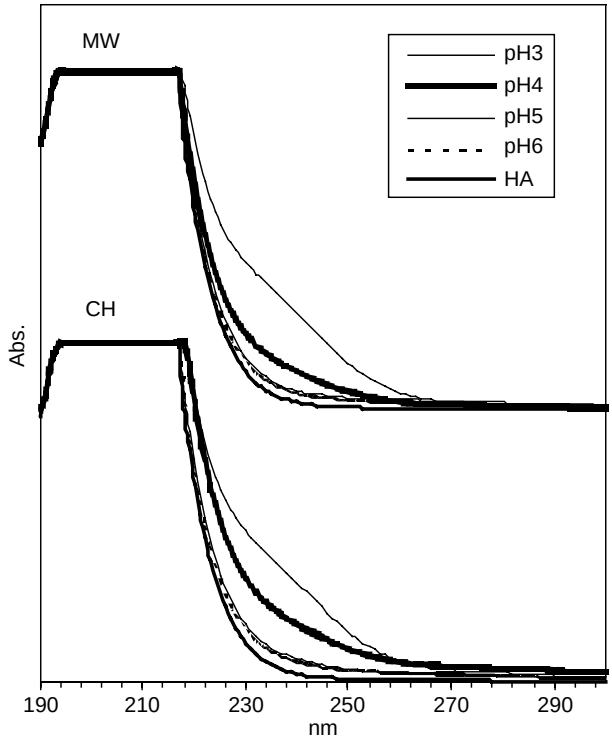

Fig. 8. UV spectra of HA2 before and after 120 min of MW and CH treatments in dependence on pH.

carboxyl groups as well as to conjugated structures absorbing at 260–270 nm. It can be explained by oxidation and alkali-promoted degradation reactions, known to proceed in alkaline sugar solutions (Whistler & BeMiller, 1958), which are enhanced by US and result in chain cleavage and chemical modification of the molecular chains.

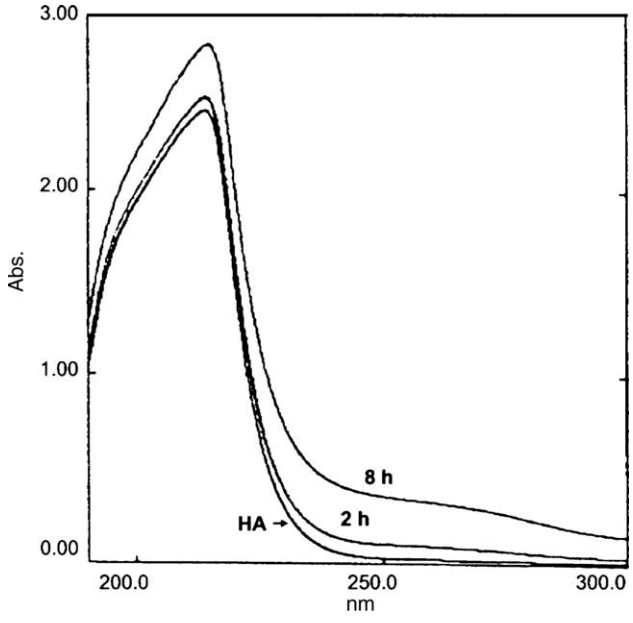

Fig. 9. UV spectra of HA1 depolymerised by US at pH 10 for 2 h (M_r = 255 kDa) and 8 h (M_r = 75 kDa).

Formation of UV_{254}-absorbing unsaturated structures was also observed after prolonged ultrasonication of heteroxylans in alkaline medium at elevated temperature (Ebringerová & Hromádková, 1997; Ebringerová, Hromádková, Hříbalová, & Mason, 1997). However, it is to be mentioned that the observed new UV-absorption bands might originate from some modified sugar units of the macromolecular chains and/or from co-precipitated decomposition products. As the undesired UV-absorption can be suppressed by using appropriate conditions of the HA recovery, i.e. filtration and precipitation (not shown), it originates rather from contaminating substances. In any case, their amount was too low to be unambiguously estimated by the FT-IR and NMR analyses.

4. Conclusions

The results suggested the US-assisted oxidative degradation to be a useful procedure to reduce the molecular mass without significant chemical modification down to M_r ~100 kDa, as the primary structure of the HA macromolecular chains remained unchanged. In contrast to the MW and CH treatments, US at weak alkaline and alkaline/oxidative conditions can be applied at low temperatures to reduce the molecular mass substantially. However, US needs a longer time of treatment than both heating processes. Therefore, suitable combinations of ultrasonication with oxidative treatment or with MW irradiation (Chemat, Lagha, Amar, & Chemat, 2004; Mecozzi, Acquistucci, Amici, & Cardarilli, 2002; Singh et al., 2003) seem to be perspective methods for HA depolymerisation.

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