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STABILITY EVALUATION OF *n*-ALKYL HYALURONIC ACID DERIVATES BY DSC AND TG MEASUREMENT

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The thermal and thermooxidative behavior of sodium salt of hyaluronic acid (HA) and its *n*-hexyl, *n*-decyl, *n*-tetradecyl and *n*-hexadecyl ether derivatives having an equal degree of substitution have been studied by means of differential scanning calorimetry (DSC) and thermogravimetry (TG). Derivatives were prepared by a substitution of H atom at the OH bound to the sixth C of N-acetyl-*D*-glucosamin of HA unit by *n*-hexyl, *n*-decyl, *n*-tetradecyl and *n*-hexadecyl chains. Both thermal and thermooxidative degradation of HA and derivatives resulted in multistep process. The main interest of this work was focused on processes occurring in the course of the first decomposition step. Experimental DSC data showed lower stability of derivatives and, remarkably lower heat evolution in comparison with original HA. On the other hand, TG measurement recorded lower mass loss for derivates which indicated appearance of new types of crosslinking reactions. Oxidative stability was evaluated by means of DSC that provided the induction period and the protection factor determination. Derivates showed remarkably lower stability in comparison with original HA; comparing each other, the highest oxidation stability showed *n*-decyl and *n*-tetradecyl derivates.

Keywords: *DSC, hyaluronic acid, hydrophobization, induction period, TG*

Introduction

Hyaluronic acid (HA), also called hyaluronan, is a high molecular mass naturally occurring linear polymer, belonging to a group of acidic polysaccharides called mucopolysaccharides or glycosaminoglycans. Its monomeric unit is composed of a repeating disaccharide that contains N-acetyl-*D*-glucosamin and *D*-glucuronic acid, interconnected with β-(1-3) and β-(1-4) interglycosidic bonds (Fig. 1).

HA isolating procedure and chemical structure was described by Meyer and Palmer in 1934 [1]. It occurs as the gelling substance of the vitreous humor of eyes, a constituent of the synovial fluid of the joints and an extracellular matrix of all connective tissues. HA and other glycosaminoglycans are of particular interest due to their possible role as cellular recognition sites. Such recognition sites are involved in many fundamental processes, including cellular differentiation, cell adhesion and regulation of cell growth [2, 3]. In addition, some glycosaminoglycans are well-known to have biological functions such as anticoagulant and antithrombotic activity.

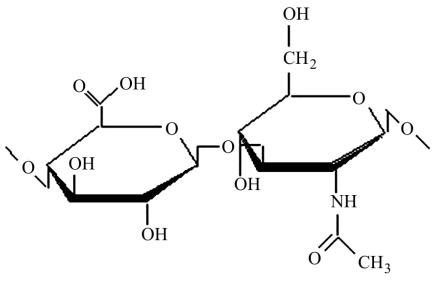

Fig. 1 Monomeric unit of HA

The molecular masses of HA polymers cover the range from around a hundred thousand up to several million Daltons and depend on their source and methods of isolation. Due to the content of glucuronic acid residues, HA is able to bind cations such as Na^+, K^+, Ca^{2+}. Aqueous solutions of HA are pseudoplastic and exhibit shear–dependent apparent viscosity and simultaneous frequency–dependent elasticity [4–7].

Currently HA is one of the most important starting materials for the preparation of new biocompatible and biodegradable polymers that have applications in many medical and cosmetics domains [8, 9]. For the medicinal purposes, HA is used as an anti-adhesive component for a variety of clinical applications such as ocular surgery, viscosupplementation for arthritis, wound healing and plastic surgery.

To improve the physico-chemical properties and stability of HA, the chemical modification as well as molecular cross-linking is often employed [10, 11]. As this biomacromolecule is soluble in water, modifi-

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cations are required in the non-soluble form, when it is to be applied in medicine, for instance as a matrix in drug delivery systems or as a sheet to prevent tissue adhesion. Typical alteration of HA properties involves derivatization of chemical groups such as carboxylic acid and/or the alcohol groups of its backbone. The carboxylic acid groups have been modified by esterification [12] and cross-linked via dihydrazide [13], dialdehyde [14, 15], or disulfide [16] cross-linking agents. Furthermore, HA has been photocross-linked by modifying the carboxylic acid groups with methacrylamide [17]. The alcohol groups have been modified using divinyl sulfone [18] and diglycidyl ethers [19]. To control cellular interactions with the hydrogels, cell adhesion sites have been introduced to HA by modification with the integrin binding peptide RGD [20] or by coating HA gels with adhesion proteins including collagen, laminin and fibronectin [18]. Alternatively, irradiation by ultraviolet light can induce surface modifications that subsequently promote cell adhesion [21].

The interest is focused on the development of derivates without affecting the polymerisation degree of the polymer, while preserving biologically and physiologically important side chain groups, i.e. acetylamide and negatively charged carboxylate groups. Therefore a procedure of substitution of the H atom at the OH group bound on sixth C of N-acetyl-*D*-glucosamin unit has been developed [22–24].

An alteration of the HA chemical structure brings about different changes in its physico-chemical behavior. Due to the increasing number of HA derivates applications, an experimental study on a relationship between the chemical structure and thermodynamic properties is of great interest.

Thermal behavior of sodium hyaluronate has already been described [25]. FTIR was employed to characterize the residual products of thermal treatment in nitrogen atmosphere at 150, 280, 350, 450 and 500°C that corresponded to the degradation reactions. At 150°C the same bands and intensity as at room temperature were observed which suggested the only moisture evaporation. At 280°C bands of exocyclic groups (C–O) and NH stretching disappeared. That indicates the cleavage of β-(1-4) glycosidic bond in backbone. Simultaneously, a new band stable up to 450°C appeared and corresponded to the C=N stretching. The intensity of carbonyl stretching bands decreased in the range of 350–450°C. At 500°C, FTIR spectra showed the bands associated with a formation of cyclic structures.

The aim of this work was to evaluate the thermal and thermooxidative stability of *n*-hexyl, *n*-decyl, *n*-tetradecyl and *n*-hexadecyl hydrophobic derivates of HA and especially to focus on the initial phase of degradation. Since the degradation reactions ordinarily occur under oxidative conditions, the atmosphere of oxygen was also employed. To evaluate the resistance of HA and *n*-alkyl derivates *vs*. oxidation, a method of induction period (IP) determination was employed. The main idea is that many processes exhibit IP, i.e. the stage preceding the main process, where seemingly no chemical or physical action takes place. After the termination of IP, the quality of tested sample often dramatically changes. The oxidation of solid materials belongs to this group. Recently introduced and verified method of IP determination was employed in this paper [26].

Experimental

Materials and chemicals

The sample of HA (Na salt, average molecular mass $1.15 \cdot 10^6$ Da) was supplied by Contipro (Ústí nad Orlicí, Czech Republic). Solvents (pyridine, dimethylsulphoxide, anhydrous ethanol) and the molecular sieve (Union Carbide Type 3 Ĺ) were products of Fluka Chemie AG. KOH, CaH_2 and alcohols (*n*-hexanol, *n*-decanol, *n*-tetradecanol and *n*-hexadecanol) were purchased from Sigma-Aldrich.

Synthesis

For the alkylation procedure, the selective method was used [22–24]. In the first step, alkyl sulphate and the polymer alkoxyde form an alkoxy – sulphonyloxy – complex. Under strict reaction control this reaction is selective for primary alcohols [27]. 0.5 g of HA (sample C0) was dissolved in 40 mL of pyridine, which was previously distilled over CaH_2 and the molecular sieve. After 10 min an equivalent amount of *p*-toluene sulphochloride (TSC) was added and the mixture was gently stirred for 90 min under inert atmosphere and controlled temperature (0–5°C). Then TSC-HA was precipitated with the cold anhydrous ethanol and dried. In the second step, the TSC complex and the equivalent amount of linear alcohols were added to KOH and stirred in 40 mL of DMSO (predistilled over CaH_2 and the molecular sieve) overnight (for approximately 17 h) at the laboratory temperature, resulting in the alkylation of the polymer chain [28]. Alkylated polymer was precipitated with the cold anhydrous ethanol and dried. The synthetic procedure is schematically shown in Fig. 2. This procedure leads to production of derivates C6 (*n*-hexyl derivate), C10 (*n*-decyl), C14 (*n*-tetradecyl) and C16 (*n*-hexadecyl). Samples were stored in refrigerator and prior to the analysis put into the dessicator for 24 h.

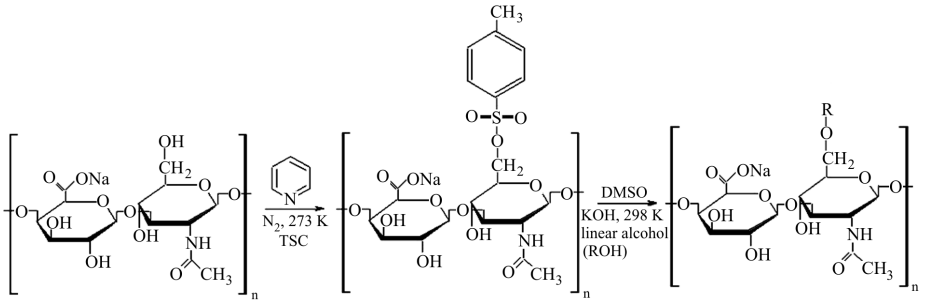

Fig. 2 Scheme of modification of HA chain. ROH responds to linear alcohol chain with different lengths

Thermal analysis

Differential scanning calorimeter DSC-60 Shimadzu and thermogravimeter DTG-60 Shimadzu were employed. The temperature and heat scale of DSC were calibrated using In and Zn standards. Before the measurement, each sample was carefully and mildly homogenized in an agate mortar. Sample mass used in all cases was about 3 mg. DSC and TG measurements were carried out in open aluminum crucibles at various heating rates from room temperature to 500 and 600°C, respectively. Oxygen and nitrogen were used to keep oxidative and inert atmosphere, respectively; flow rate of gases was 20 mL min^{-1} for both oxygen and nitrogen. Standard deviation of a single DSC and TG measurement, determined from three repeated measurements was less than 0.2°C and 1.5%, respectively.

Non-isothermal experiments for induction period (IP) determination were performed in temperature range from 25 to 600°C at heating rates (β) of 1, 3, 5, 7 and 10°C min^{-1} in oxygen atmosphere (flow rate 20 mL min^{-1}). Detailed mathematical description of IP determination was presented previously [26, 29]. In principle, the method represents a number of non-isothermal TA measurements carried out at different heating rates and the onsets of degradation are determined. Briefly, considering the linear increase of temperature, the combination of general kinetic equation of chemical reaction and Arrhenius equation gives after separation of variables and upon integration following equation:

$$\beta = \int_0^{T_i} \frac{dT}{A\exp(B/T)} \quad (1)$$

where T_i is the end of induction period, β stands for the coefficient of temperature increase (scan), T is the absolute temperature, and constants A and B are given as:

$$A = \frac{F(\alpha_i) - F(0)}{A_k} \quad (2)$$

$$B = \frac{E_a}{R} \quad (3)$$

where A_k is the pre-exponential factor, E_a is the (effective) activation energy, R stands for the gas constant. Since the conversion α_i corresponding to the end of IP is assumed to be independent of temperature, the value of the integrated function $F(\alpha)$ at the point α_i, $F(\alpha_i)$, is constant.

The temperature dependence of IP under isothermal conditions can be expressed as

$$t_i = A\exp(B/T) \quad (4)$$

where t_i is the length of IP.

Results and discussion

Thermal behavior

DSC, TG and derivative TG (DTG) of hyaluronic acid (HA) and *n*-alkyl derivates C6 and C10 in nitrogen atmosphere are given in Figs 3 and 4, respectively. Since C14 and C16 derivates showed similar thermoanalytical records as C10, they are not shown. The data obtained from DSC, TG and DTG runs are summarized in Table 1. TG data concerning the decomposition steps are recalculated for moisture-free samples. Since the data published previously indicated HA thermal resistance at least up to 150°C [25] the moisture content shown in Table 1 was calculated

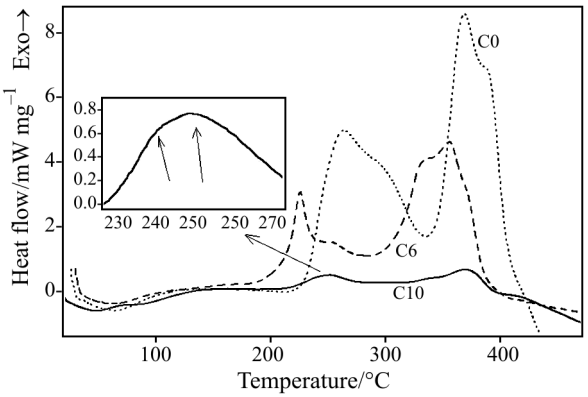

Fig. 3 Comparison of DSC records of C0, C6 and C10 in atmosphere of nitrogen, flow rate 20 mL min^{-1}, heating rate 10°C min^{-1}, open aluminum pans

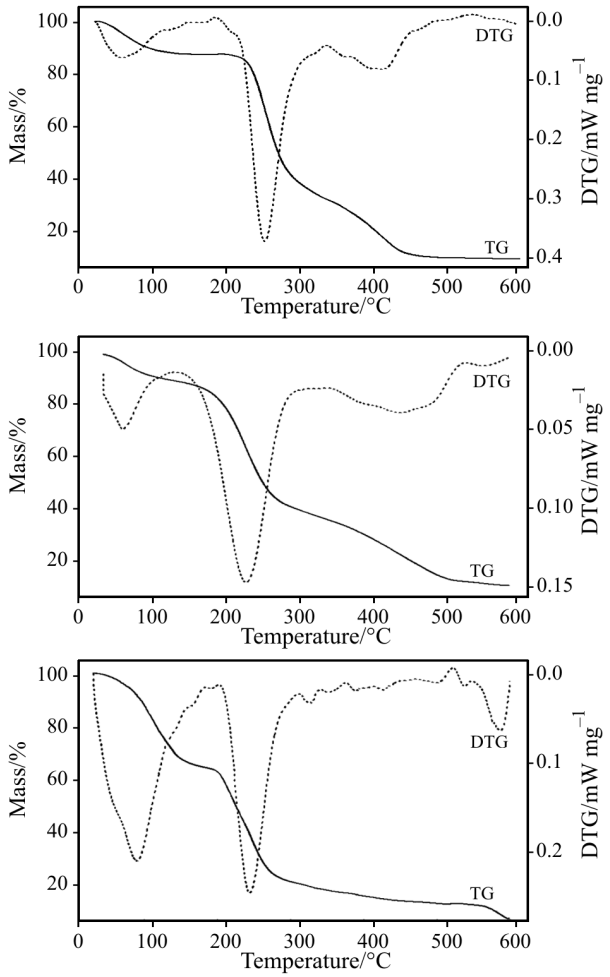

Fig. 4 Comparison of TG and DTG records of C0, C6 and C10 in atmosphere of nitrogen, flow rate 20 mL min^{-1}, heating rate 10°C min^{-1}, open aluminum pans

from TG curve as a mass difference determined by the onset and endset of the first peak given by DTG.

DSC run of thermal decomposition of HA exhibits in two endothermal peaks and two exothermal peaks. Both of the exotherms showed two maxima. TG/DTG measurement allowed screening of the sample mass in dependency on temperature program. Taking into account FTIR results publicized by Viletti [25], the first intense mass loss (seen on DSC record as the first endotherm) can be attributed to the moisture evaporation. Significant amount of moisture content was observed in C10 and C14 derivates (Table 1); that was not removable by storing under moisture-free conditions for 24 h. With respect to the storage conditions given above, it can be assumed either different sample hygroscopicity or a trapping of a portion of solvent in course of synthesis or both. A detail study of DTG peak indicates a gradual moisture evaporation proceeding in several steps. Moreover, ill-defined DTG peak registered also by DSC as the second endotherm was recorded before the onset of first exothermal peak. This indicates different types of water bound on HA molecular skeleton as well as trapped inside the secondary HA structure.

Thermal degradation processes of C6, C10, C14, and C16 *n*-alkyl derivates showed similar behavior as described for C0. The comparison given in Fig. 3 shows that thermal behavior of C0 and *n*-alkyl derivates differs mainly in intensity of released heat during decomposition in the first step and also in intensities of peak maxima. In fact, decomposition of C0 resulted in higher heat evolution. Two peak maxima appeared also in C0 DSC data. Moreover the intensity ratio of first peak maxima significantly differed. C10, C14 and C16 DSC records of derivates showed higher intensity of the second maxima, which is opposite to C0 and C6. Although Fig. 3 shows the shift of derivatives decomposition onsets to higher temperatures in comparison with C0, TG measurement results given in Table 1 show the highest mass loss during the first decomposition step for C0. On the other hand, *n*-alkyl derivates C6, C10, C14 and C16 seem to be better protected against thermal degradation. As it can be seen from Table 1, peak temperature (determined with regard to the DTG or DSC curves) increase in order C6<C14~C10<C16<C0. Likely alkyl chains are not primarily involved in the first step decomposition processes. Similar behavior has been observed on cellulose fibers after substitution of cellulose hydroxyls with organic acids [30]. When the substituent size

Table 1 DSC, TG and DTG data of thermal degradation of HA C0 and its *n*-alkyl derivates C6, C10, C14 and C16

Sample	TG/DTG				DSC			
	Moisture/%	Mass loss*/% 1st peak	DTG peak temperature/°C	Residue at 600°C/%*	Peak temperature/°C			
					1st peak		2nd peak	
C0	13	41	255	21	265	289	369	389
C6	11	38	237	22	226	249	339	356
C10	34	36	245	39	243	251	348	384
C14	34	35	244	37	243	253	364	408
C16	12	29	248	18	243	259	422	456

*recalculated for moisture-free samples

reached a length of C10 or C12 there appeared side chain crystallization of fully esterified derivates of cellulose. Hence the formation of new-ordered structures in substituted regions can be expected and those play an important role in decomposition reactions at higher temperatures.

According to DTG results of Viletti *et al*. [25], temperatures above 200°C cause degradation of hyaluronic acid sodium salt. It was stated that the first part of processes occurring in this temperature region is probably related, among others, to changes in the polysaccharide conformation prior to the main degradation reaction. On the other hand, molecular dynamics calculations revealed three different types of water molecules located in the vicinity of the HA molecules by more than one H-bond and/or by coulombic interactions [31]. Two types of such water were classified as the so-called non-freezing water because they are separated from the normal freezing water weakly captured in the vicinity of carboxyl groups through a coulombic attraction. It was suggested that particularly non-freezing water contributes to the structural stability of HA chains [31]. This knowledge can partly explain a tenuous DSC endotherm preceding HA decomposition (indiscernible on figure given here). Furthermore, in this temperature range the N-acetyl group decomposition was previously recorded [25], captured water was released and its evaporation could affect the results reported in [25].

Figure 2 shows the reaction scheme where the hydrogen of OH group is substituted by alkyl chain forming ether on C6 of N-acetyl-*D*-glucosamin unit. Since the mass loss and heat evolved of derivates is remarkably lower in comparison with C0, it is likely that alkyl substitution induced a new type of cross-linking reactions/interactions involving alkyl chains. HA represents a polymer with a very marked capability to form meshwork [32]. It was shown [32], that there are present extensive hydrophobic regions of about eight CH groups on single HA helices in HA secondary structures. The hyaluronan twofold helix, previously demonstrated to be present in solution [32], was shown to be sterically capable of extensive duplex formation. This 'stickiness' is postulated to be the basis of the network-forming and laterally aggregating behavior of hyaluronan [33]. The extension of hydrophobic regions by *n*-alkyl substitution seems to affect the *n*-alkyl HA derivates stability not only in solutions. Moreover it is well recognized that thermal cross-linking reactions frequently occur in the initial stages of polymers degradation [34]. Pyrolysis of HA at 280°C brought the appearance of new C=N bond stable up to 450°C and further, formation of cyclic structures at 500°C [25]. In addition, the significant difference in comparison of the decomposed mass of C0 and derivatives, the shape of DSC peaks and amount of heat liberated in this step support the idea of introducing of new type of cross-linking reactions in which *n*-alkyl chains took place. Nevertheless, the detailed treatment of that is beyond the scope of this work.

Thermoxidative behavior of hyaluronic acids and n-alkylderivates

The experiments performed in oxygen atmosphere showed that HA and *n*-alkyl derivates degraded through two main stages as in the case of nitrogen atmosphere. Experimental data are shown in Figs 5 and 6 and corresponding data are summarized in Table 2. Since C10, C14 and C16 derivates showed similar DSC and TG records, in Figs 5 and 6 is only C10 record given. The thermoxidative decomposition of C0 shown in Fig. 5 resulted in one endothermal and two exothermal peaks. A tenuous endotherm preceding the main degradation step seen at DSC measurement in nitrogen atmosphere was not recorded. Three main steps were recorded also by TG/DTG measurement which supported observations done by DSC. Due to oxygen presence, the heat evolution seen as DSC peak area and the mass loss given by TG showed higher intensity than in the case of inert atmosphere.

Alkyl derivates of HA showed different DSC and TG profile in oxygen atmosphere in comparison with previously described thermal behavior in nitrogen. The first decomposition step of C6 resulted in a DSC peak with two maxima. The mutual ratio of intensities of peak shoulders is similar to C0 but differs in comparison with C10, C14, and C16. Those derivates gave similar DSC records but less complicated comparing with C0 and C6. TG results showed, after moisture evaporation, significant decrease of mass during first degradation step (Table 2).

The second oxidation step was registered in the temperature region 300–500°C. While DSC run gave an exothermal peak with shoulders, TG measurement registered a small one step mass loss in this temperature area.

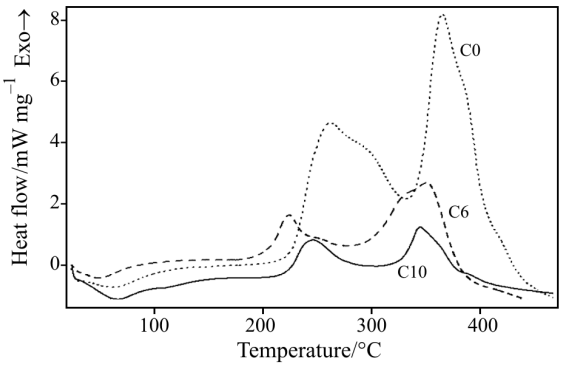

Fig. 5 Comparison of DSC records of C0, C6 and C10 in atmosphere of oxygen, flow rate 20 mL min^{-1}, heating rate 10°C min^{-1}, open aluminum pans

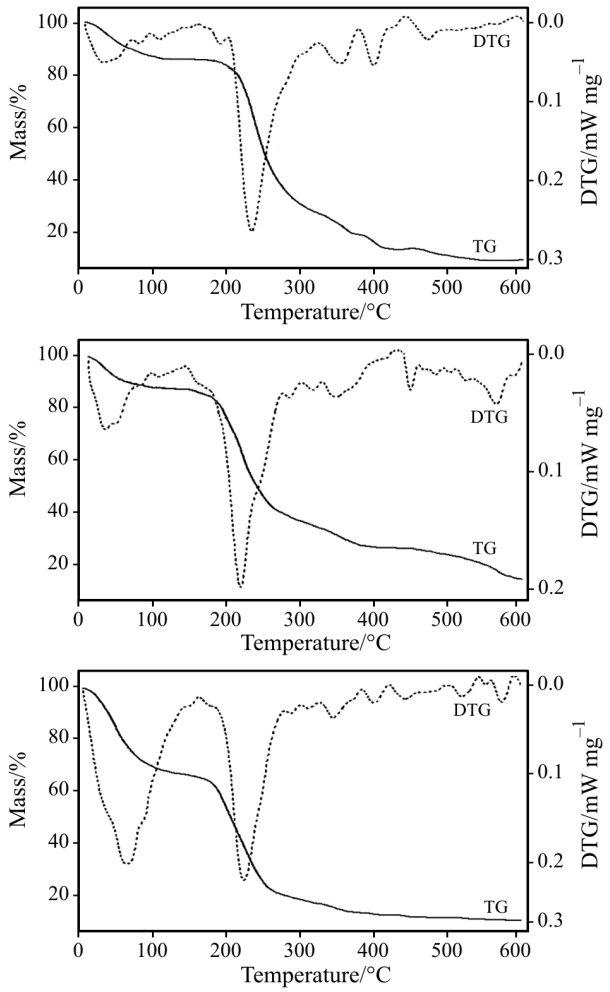

Fig. 6 Comparison of TG and DTG records of C0, C6 and C10 in atmosphere of oxygen, flow rate 20 mL min^{-1}, heating rate 10°C min^{-1}, open aluminum pans

As expected, the oxidation of HA and *n*-alkyl derivates induced new type of reactions in comparison with pyrolysis experiments. That can be easy deduced from shapes, peak temperatures and heat evolution seen on DSC curves and mass losses recorded by TG. The oxygen presence causes a partial oxidation and/or autocatalytic reactions accelerating the collapse of polysaccharide structure. In fact, HA contains oxidized functionalities COOH and OH that are readily to decompose to form free radicals. Evolution of free radicals in HA responsible for degradation of HA during freeze drying caused by secondary reaction of hydroxyl radicals derived from carboxyl radicals was described by Tokita *et al*. [31].

The extension of mass loss at first oxidation step is remarkably higher only in the case of C0 whereas of alkyl derivates is comparable in both measurements (Table 2). On the other hand, onset temperatures of oxidation of alkyl derivates are shifted to lower temperatures in comparison with C0. That suggests, the presence of *n*-alkyl chains primarily accelerates collapse of HA structure but secondarily protects molecules against additional degradation i.e. mass loss. Likely, as mentioned in previous section, a new type of conformation arrangements was introduced. The substitution of OH group on HA skeleton by *n*-alkyl chain likely altered the original H-bonds system. Van der Waals forces are known to bind together non-polar molecules or molecular parts [35]. H-bonding is considered to be relatively strong linkage since involves an energy gain from 10 to 20 kJ mol^{-1} in comparison with the van der Waals bonds [36]. The influence of weak interactions on TG and DTA or DSC record was demonstrated recently [37, 38]. Therefore, an alteration of original system of H-bonds caused a decrease of the resistance of HA molecular skeleton *vs*. thermal and oxidation attack. The results concerning the thermooxidative stability are discussed below.

Thermooxidation stability

In this work, the stage preceding the first degradation step was employed for IP calculation based on principle of isoconversional methods. Although the parameters providing by the latter should be taken only as 'effective', they enable to model the process without a deeper insight into its mechanism [39].

In comparison with pyrolysis experiments, where no clear onset peak was seen, the oxidation records

Table 2 DSC, TG and DTG data of thermooxidative degradation of HA and *n*-alkyl derivates C6, C10, C14 and C16

Sample	TG/DTG			DSC			
	Mass loss/% 1st peak*	DTG peak temperature/°C	Residue at 600°C/%*	Peak temperature/°C			
				1st peak		2nd peak	
C0	56	256	15	263	292	367	419
C6	40	228	27	225	255	330	353
C10	39	245	43	240	248	346	389
C14	38	243	42	241	249	347	391
C16	40	243	27	241	259	365	401

*recalculated for moisture-free samples

Table 3 Parameters A and B of IP calculation, extrapolated IP length for 37°C and protection factor (PF); oxidative atmosphere

Sample	C0	C6	C10	C14	C16
A	3.30E-20	1.40E-16	1.12E-20	2.77E-20	2.43E-17
B	22420	17890	22440	22100	18890
IP at 37°C (years)	1 476 786	2 875	535 060	442 431	12 507
PF	1	1.95E-3	3.62E-1	3.00E-1	8.47E-3

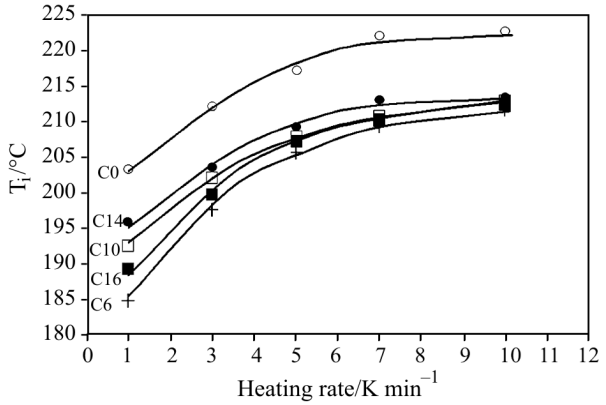

Fig. 7 Experimental and fitted dependences of the onset oxidation temperatures on the scan rates

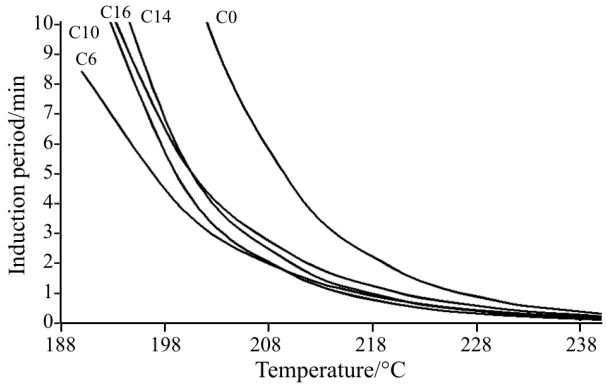

Fig. 8 Temperature dependences of the induction period

gave better definable peak onsets and that could be used for IP calculation. The calculated and measured peak onsets are compared in Fig. 7. Evidently, a very good agreement of experimental and theoretical values was achieved. Dependence of IP on temperature is given in Fig. 8. Table 3 summarizes the calculated parameters A and B and length of IP recalculated for 37°C that corresponds to human body temperature.

As it can be seen in Table 3, lengths of IP extrapolated outside temperature range where processes occurred seem to be quite unrealistic. Elevated temperatures may introduce new types of reactions that do not reflect perfectly the situation at lower temperatures and therefore to change reaction mechanism (conversion function). Thus the extrapolation of absolute values of the lengths of induction periods can lead to non-realistic estimations. A better evaluation can be obtained using the ratio of the lengths of induction periods of original and modified HA since it should be expected that the same structural units are responsible for the degradation. This ratio consisting of a comparison of IP of original and modified HA and it is called the protection factor (PF) [40]. If the value of PF is greater than one, the modification has stabilizing effect on HA. Otherwise, the modification causes destabilization of HA. The lower is the value of PF, the lower is the modification effectiveness of the modification. It follows from Eq. (4) that the length of induction period depends on temperature; hence, the protection factor depends on temperature as well. As can be seen from Table 3, the PF of modified samples is ordered in this way C10>C14>C16>C6. It is evident that *n*-alkyl substitution considerably decreased the resistance of HA against oxidation. Experimental data suggest that for hydrophobic HA derivates the length of alkyl chain should not exceed C14 but should reach at least C10. Nevertheless, such substitution causes decreasing of the PF of HA to values about 0.36.

Conclusions

In this study, thermal and thermooxidative degradation behavior of some partially substituted HA derivates were examined. All *n*-alkyl derivates showed shift to lower decomposition onset temperatures in comparison with the original HA in the first degradation step. On the other hand, modification of HA by *n*-alkyl chains caused a reduction in mass loss during the both thermal and thermooxidative degradation. The reason was attributed to the alteration of the original system of H-bonds which represent more stable arrangement in comparison with hydrophobic interactions likely present in *n*-alkyl derivates. In addition, the extent of mass loss indicates introduction of new type of cross-linking reactions occurring in the first degradation step. Induction period determination was employed in order to evaluate the oxidation stability i.e. protection factor of derivates in comparison with parental HA. Results showed three times lower stability for C10 and C14 and significant thermoxodative destabilization of C6 and C16 derivates.

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