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Research review paper

The many ways to cleave hyaluronan

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Abstract

Hyaluronan is being used increasingly as a component of artificial matrices and in bioengineering for tissue scaffolding. The length of hyaluronan polymer chains is now recognized as informational, involving a wide variety of size-specific functions. Inadvertent scission of hyaluronan can occur during the process of preparation. On the other hand, certain size-specific hyaluronan fragments may be desirable, endowing the finished bioengineered product with specific properties. In this review, the vast arrays of reactions that cause scission of hyaluronan polymers is presented, including those on an enzymatic, free radical, and chemical basis.

Keywords: Hyaluronan; Hyaluronidase; Reactive oxygen species; Degradation; Fragmentation; Hydrolysis

Contents

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1. Introduction

Hyaluronan (HA) is a high-molar-mass linear glycosaminoglycan (GAG) found in the extracellular matrix (ECM). This linear polysaccharide can reach a size of 6 to 8 MDa. It is ubiquitous, but is particularly prominent in tissues undergoing rapid growth and repair. The polymer has the structure of poly[(1→3)-2-acetamido-2-deoxy-β-D-glucose-(1→4)-β-D-glucopyranosyluronic acid] (Fig. 1). It has one carboxyl group per disaccharide repeating unit, and is therefore a polyelectrolyte with a negative charge at neutral pH. It is near perfect in chemical repeats, with no known deviations in its simple disaccharide structure with the possible exception of occasional deacetylated glucosamine residues.

High-molar-mass forms of HA are reflections of intact normal tissue, while fragmented forms of HA are usually indications of stress. Indeed, various size HA fractions have an enormous repertoire of functions and constitute an information-rich system (Stern et al., 2006). Because of its novel characteristics of biocompatibility and rheological properties, HA is being used increasingly as a component of artificial matrices and in bioengineering for tissue scaffolding. The size of HA polymers used in bioengineering and biotechnology is obviously of critical importance. Inadvertent degradation of the polymer during the process of its formulation, cross-linking, and other covalent modifications prior to incorporation into scaffolding and matrix devices may have deleterious effects and limit usefulness of the product.

Increased evidence has been gathered that low-molar-mass HA fragments have different activities than the native polymer. Large matrix polymers of HA are space-filling, anti-angiogenic, and immunosuppressive, whereas the intermediate-sized polymers comprising 25–50 disaccharides are inflammatory, immunostimulatory, and highly angiogenic. These low-molar-mass oligosaccharides (OSs) appear to function as endogenous danger signals (Kogan et al., 2007, in press). Certain activities of HA oligomers are associated with a specific size of the fragment and some functions are very sensitive even to subtle changes in the chemical structure of the HA fragment employed. For example, it has been demonstrated that a mixture of HA tetrasaccharide and hexasaccharide can induce complete maturation of human dendritic cells through a Toll-like receptor 4-mediated pathway.

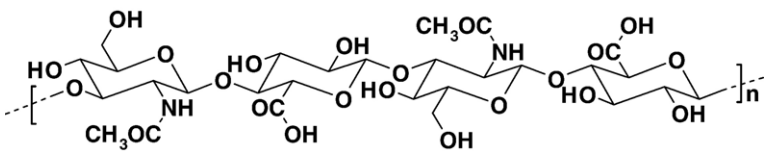

Fig. 1. Fragment of the HA structure.

This is in contrast to high-molar-mass HA that does not possess such an effect (Termeer et al., 2000).

In a canine arthritis model, stressed synovial cells suppress cell death upon treatment with HA tetrasaccharides but not with disaccharides or hexasaccharides (Xu et al., 2002). Fine variations in the chemical structure can also have pronounced effect on the biological properties of HA fragments: HA oligomers produced by digestion of the polymer with vertebrate hyaluronidase elicit significantly greater release of interleukin-12 from leukocytes than do OSs produced by the action of bacterial hyaluronan lyases, though the differences in molecular structure are rather minor (Jobe et al., 2003). Thus, method of preparation of the HA oligomers may affect their size and physico-chemical properties as well as influence their biological activities and properties.

For this reason, we have assembled here the array of known mechanisms utilized for the cleavage of the HA polymer, including both enzymatic and non-enzymatic reactions. We attempt to define the limits for working with HA, pointing out limitations, defining situations and reagents to avoid in order to prevent HA chain scission, and to formulate the precise environment within which it is possible to work.

The enzymes are also tabulated so that they can be used as tools to trim and fashion polymers, to generate precise sizes, as well as to provide desired reducing and non-reducing termini. This may appear to be inordinately refined. However, it is also becoming apparent that such resolution may be necessary. HA fragments of an intermediate size can induce apoptosis in cultured cells (Alaniz et al., 2006), and inflammation *in vivo* (Noble, 2002), while smaller fragments, in the tetrasaccharide range ameliorate such effects by suppressing apoptosis, and by inducing heat shock proteins (Xu et al., 2002). It is entirely reasonable that such small fragments will have effects that are dependent on the nature of the residues that constitute the termini. Such details were not deemed important heretofore, but with the myriad of functions ascribed to HA fragments, attention to structural precision may well become standard procedures in the near future.

The turnover of HA is extremely rapid. It is estimated that of the 15 g of HA in the vertebrate body, 5 g turn over daily. The $t_{1/2}$ of HA in the circulation is between 2 and 5 min. In the epidermis of skin, where one half the HA of the body is found, it is one to two days, and in an apparently inert tissue as cartilage, it is approximately one to three weeks (Stern, 2003). It is evident that when HA is used for tissue scaffolding and matrix supports, modification of the HA is required to avoid the body's robust catabolic system. The various catabolic cascades and techniques are reviewed here. Though these reactions are useful in assembling the biomaterials, the same reactions must subsequently be avoided once the materials are inserted. Techniques to evade such reactions are the subject of a different review.

2. Enzymatic catabolism of hyaluronan

The hyaluronidases (Hyals) are a class of enzymes that degrade predominantly HA. The name is somewhat of a misnomer, since most of these enzymes have the limited ability to also degrade chondroitin (Ch) and chondroitin sulfates (CS). Both the prokaryotic and eukaryotic Hyals generally have the ability to degrade both classes of substrates, though a few bacterial enzymes have absolute specificity for HA.

There is a major difference between the mechanisms that prokaryotic and eukaryotic enzymes utilize in cleaving their HA, Ch, and CS substrates. These differences are outlined below.

2.1. Historical background

Hyaluronidase activity was first identified as a "spreading factor" in extracts from mammalian testes (Duran-Reynals, 1928), and was also observed in bacterial extracts shortly thereafter (Duran-Reynals, 1933). The term "hyaluronidase" was then introduced to denote specifically the enzymes that degrade HA (Chain and Duthrie, 1940; Hobby et al., 1941). Karl Meyer classified these originally into three distinct categories, a scheme based on biochemical analyses of the enzyme reaction products (Meyer, 1971). With the advent of genetic data, it is now recognized that this classification scheme was remarkably accurate. Meyer was able to identify the three principal types of hyaluronidases, the two classes of eukaryotic endoglycosidase hydrolases described below, and the prokaryotic lyase-type of glycosidase.

2.2. Prokaryotic enzymes that cleave HA

2.2.1. Bacterial β-endoglycosidases and their lyase mechanism of action

The bacterial enzymes are lyases that specifically cleave the β-(1→4) linkage in HA and CS. These are β-endoglycosidases that are eliminases with a mechanism of action entirely different from that of the eukaryotic glycoside hydrolases. These enzymes function by β-elimination with introduction of an unsaturated bond (EC 4.2.99.1) (Jedrzejas, 2004; Stern and Jedrzejas, 2006). The generation of the double bond enables to perform a spectrophotometric assay, which is not available for the eukaryotic enzymes, which act hydrolytically. The relative

ease of determination of their activity has provided a wealth of information and thorough characterization (Jedrzejas, 2004; Stern and Jedrzejas, 2006).

The mechanism of action of these enzymes is of an acid/base processive type termed proton acceptance and donation (PAD). This is well established for the *Streptococcal* enzyme, but has not been shown conclusively for other bacterial enzymes. The catalytic mechanism involves several sequential steps (Li et al., 2000; Li and Jedrzejas, 2001; Ponnuraj and Jedrzejas, 2000):

1. binding of the HA substrate to a cleft in the enzyme;
2. acidification of C-5 carbon atom of a glucuronate residue by an enzyme Asn residue that functions as an electron sink;
3. extraction of this C-5 carbon proton by an enzyme His residue, followed by the formation of an unsaturated bond between C-4 and C-5 of the glucuronate on the reducing side of the glycosidic bond;
4. cleavage of the glycosidic bond after a proton has been donated from the enzyme Tyr residue;
5. departure of the HA or CS disaccharide product from the active site and balancing of the hydrogen ions by an enzyme exchange with the water environment.

The enzyme is then geared for another round of catalysis. For processive degradation of the substrate, HA is translocated by one disaccharide unit toward the reducing end of the chain and endolytically degraded using the PAD mechanism. During the process, the C-4 and C-5 carbon atoms change their hybridization from sp^3 to sp^2 with respective changes in the product conformation of the sugar ring, involving a puckering of the ring. This leads to a distorted half chair type of conformation.

2.2.2. Bacterial β-exoglycosidases

The exoglycosidases, including a β-glucuronidase and a β-*N*-acetyl-hexosaminidase are able to degrade HA, by removing single monosaccharide units. Endolytic cleavage creates new substrate sites for these exoglycosidases. However, the contribution to the overall catabolism of HA in bacteria or in their eukaryotic hosts is not known. The β-glucuronidase from *E. coli* is a *ca.* 290 kDa tetrameric protein with a pH optimum in the neutral range, in marked contrast with the acid-active enzyme from eukaryotes.

2.3. Eukaryotic enzymes that cleave HA

The eukaryotic enzymes that degrade HA have a surprisingly wide range of pH optima. These enzymes are hydrolases and mechanisms of their action have, until recently, defied thorough explication, largely because of the difficulties in assaying their activities.

2.3.1. β-Endoglycosidases

The eukaryotic endoglycosidase types of hyaluronidases were a long neglected class of enzymes (Kreil, 1995). They are hydrolases, adding water across the cleavage site, and their activities are not detectable by spectrophotometry. These problems are now largely overcome, and much data are now accumulating rapidly, in part due to the human genome project and the EST (expressed sequence tag) data bank.

2.3.1.1. Endo-β-n-acetylhexosaminidases. There are six such hyaluronidase-like sequences in the human genome (EC 3.2.1.35), but only three, of their products, Hyal1, Hyal2, and PH-20 are associated with known hyaluronidase enzymatic activities. The other proteins may have enzyme activities, but to date, the conventional *in vitro* assays have failed to detect them.

They may have other activities, functioning perhaps as adhesion proteins. One of these proteins, Hyal2, acts as a cell-surface receptor for certain retroviruses (Rai et al., 2001). In fact, it can be suggested that all of these proteins have functions other than enzymatic activity.

Three of the protein sequences Hyal2, Hyal4, and PH-20 are GPI-(glycophosphatidylinositol-) linked to outer cell-surface membranes. However, they also exist as free processed forms. Some of the processed forms have changes in pH optima (Oettl et al., 2003).

Three sequences, *Hyal1*, *Hyal2*, and *Hyal3* are located on chromosome 3 at 3p21.3, and another three, *Hyal4*, *HyalP1* (a pseudogene, transcribed but not translated in the human), and *SPAM-1* are clustered similarly on chromosome 7 at 7q31.3. Tetrasaccharides are the major catabolic products. The seventh sequence occurs in the mouse at the syntenic region, chromosome 6A2, termed rather unfortunately *Hyal5* (since this is the seventh sequence to be identified, *Hyal7* would be a more suitable appellation). The human genome may be the only mammalian one that lacks this sequence. The Hyal5 protein is present in testicular extracts and may participate in penetration of the cumulus mass and in fertilization (Kim et al., 2005).

The major hyaluronidase activities in somatic tissues are tentatively identified as Hyal1 and Hyal2 (Stern, 2003, 2004). Hyal2 cleaves extracellular matrix HA to approximately 50 disaccharides (20 kDa), and then its activity slows down considerably. Hyal1, an acid-active lysosomal enzyme can accept HA of any size and rapidly cleaves these chains to small fragments. The tetrasaccharide is the predominant cleavage product.

These enzymes may work independently of each other in two separate pathways, or their activities may be coordinated. The relationship between the pathways is unknown. However, both are associated with CD44, the major HA receptor that can exist both extra- and intracellularly (Bourguignon et al., 2004; Harada and Takahashi, 2007).

Based on high primary, secondary, and tertiary homology to the crystal structure of bee venom hyaluronidase and site directed mutagenesis of the human PH-20 enzyme, it is proposed that degradation by human hyaluronidase hydrolases may proceed *via* a double-displacement mechanism, with retention of HA substrate conformation. Such a mechanism involves one Glu amino acid residue as a hydrogen atom donor and a carbonyl oxygen of *N*-acetyl group of the HA performing the function usually assigned to a carboxyl group of another amino acid. The usual two-carboxylic-acids mechanism can be modified to reflect the different nucleophilic residues. The steps involved in this mechanism are:

(1) binding of the HA substrate to the hyaluronidase;
(2) residues around the catalytic site positioning the carbonyl oxygen nucleophile of the HA's *N*-acetyl group next to the to-be-cleaved β-(1→4) glycosidic bond, attacking the C-1 carbon of the same sugar to form a covalent intermediate between them. This leads to cleavage of the glycosidic bond and also results in the inversion of the anomeric C-1 atom configuration;
(3) at the same time a protonated Glu donates its H (deprotonation, acid function) to the glycosidic oxygen, leaving part of HA (the glycone part) unaltered;
(4) hydrolytic cleavage of the intermediate bond between carbonyl oxygen and C-1 by a water molecule in the active site leads to re-protonation of a Glu, readying it for the next catalytic step, and to the second inversion of the configuration of C-1;
(5) release of the HA product from the hyaluronidase's active site (glycan on the aglycone side of the cleaved glycosidic bond).

The anomeric configuration of the C-1 carbon atom of the substrate is retained throughout the process, being inverted twice during catalysis (in steps 2 and 4 above). The formation of an oxocarbonium ion transition state has been implicated in this process in step 2. Structural evidence from the enzyme-(HA tetrasaccharide) complex suggests that:

(1) as the carbonyl nucleophile moves into place to interact with the C-1 carbon of HA, it results in change in puckering of the pyranose ring of *N*-acetyl-D-glucosamine on the non-reducing side of the bond to be cleaved, from regular chair to distorted boat and consequently
(2) in moving the glycosidic bond into nearly an equatorial position, which results in its positioning closely to a Glu to allow for the donation of its H atom to this glycosidic oxygen as the bond is being cleaved.

2.3.1.2. β-Endoglucuronidases. The hyaluronidases that are β-endoglucuronidases (EC 3.2.1.36) cleave the β-(1→3) glycosidic bond. No sequence data are available, thus it is not possible to compare enzymatic mechanisms, nor is it understood why the β-(1→3) is a more susceptible bond for cleavage than the β-(1→4) bond. They generate tetrasaccharides as the predominant catabolic end-products, but also hexasaccharides. These enzymes, characteristic of annelids such as the leech *Herudo medicinalis* and certain crustaceans, utilize the hydrolysis mechanism. They thus resemble the vertebrate hyaluronidases more closely than the prokaryotic enzymes.

2.3.2. β-Exoglycosidases

There are two acid-active enzymes that remove single sugar units sequentially from the non-reducing termini of HA chains, both of which are lysosomal enzymes. The endolytic cleavage by the hyaluronidases generates increasing numbers of non-reducing termini as substrates for these exoglycosidases. The relative contribution of exo- and endoglycosidases to overall HA catabolism has not been established. The exoglycosidases appear to be in vast excess over the endoglycosidase enzymes. It is highly likely that such activities can contaminate impure preparations of hyaluronidases, and generate spurious results.

2.3.2.1. β-Exoglucuronidase. The activity of *β*-glucuronidase increases in many pathological conditions including liver inflammation, cholestatic jaundice, cirrhosis of the liver, inflammations of other organs, tuberculosis, sarcoidosis and also in a number of neoplasms. Increased activity is also a sensitive indicator of aberrations in cell signaling networks.

2.3.2.2. Exo-β-N-acetylglucosaminidase. The human protein has no apparent signal sequence, supporting the idea that this enzyme is localized in the cytosol. A gene database survey reveals the occurrence of enzyme homologues in the fruit fly, *Drosophila melanogaster*, in the nematode, *Caenorhabditis elegans*, and in plants indicates the broad occurrence of the enzyme in eukaryotes. The gene is expressed in the lysosomes of a variety of human

tissues, suggesting that it is involved in basic biological processes in eukaryotic cells, including the trimming of OSs in the cytosol of cells (Suzuki et al., 2002).

2.4. Substrate specificity of the hyaluronidases

The hyaluronidases of all origins do not have absolute substrate specificity, but have the ability to utilize Ch and CS as substrates as well. These two GAGs are far less effective substrates, and the reactions proceed more slowly (Rigden and Jedrzejas, 2003). There is a natural binding affinity between HA and CS (Turley and Roth, 1980). It may be that these two materials can exist in nature as a complex, and that the enzyme is adapted to degrading the two polymers simultaneously, at approximately a speed that reflects their relative abundance in nature.

There is one class of prokaryotic hyaluronidases that does, however, have absolute specificity for HA, and these are the enzymes from *Streptomyces hyalurolyticus* (Ohya and Kaneko, 1970; Shimada and Matsumura, 1980). This enzyme is entirely different from other bacterial hyaluronidases. It is used for structural studies, because of its absolute specificity. In many viscoelastics, CS and HA are combined, as in phaco-emulsifiers used in cataract surgery (*e.g.* Hutz et al., 1996). The specific and non-specific hyaluronidases may be useful complementary tools for probing contributions of individual components of such preparations.

2.5. Provisos for the hyaluronidase reactions

There are a number of problems that will be encountered in working with the hyaluronidase enzymes, and in attempting to control their reactions. These are listed below:

a) Effects of pH can be wide ranging. The pH optimum of the expressed human Hyal1 is pH 3.6, which is far more acidic than any cellular compartment. The pH of lysosomes is estimated to be pH 4.8. The *in vitro* assay reaction may be far different from the catalysis that takes place *in vivo*. Hyaluronidases undergo processing, and their apparent pH optimum can undergo changes in parallel. This is demonstrated for PH-20 (Oettl et al., 2003).

b) Protein effects have not been thoroughly examined. The characterization of the hyaluronidases is classically carried out *in vitro* utilizing highly purified enzymes and substrates. This is far removed from the situation *in vivo*. A wide range of HA-binding proteins, termed hyaladherins have been documented, ranging from tightly associated proteoglycan core proteins such as the amino terminal of aggrecan and link protein, to a loose association with albumin. Presentation of substrate to enzyme occurs most likely in the presence of such hyaladherins. How the enzyme is able to recognize, bind, and catabolize HA decorated with an array of varying proteins is a complete mystery.

Albumin has long been identified as an HA-binding protein (Johnston, 1955; Niedermeier et al., 1966), as has fibrinogen (LeBoeuf et al., 1986). The presence of albumin, fibrin, or fibrinogen in preparations of matrices for tissue engineering most likely modulates enzymatic activity of hyaluronidases.

c) Salt effects have been documented, which may result from a "salting out" of associated proteins, and their attendant effects. Albumin similarly can ameliorate salt effects on hyaluronidase activity, as has been shown for the liver-derived enzyme (Gold, 1982).

d) Stability of hyaluronidase enzymes from eukaryotic sources is enhanced in the presence of detergents. The first somatic enzyme from a vertebrate source was dependent on the constant presence of detergents (Frost et al., 1997). However, it is well established that detergents can also function as protease inhibitors, and the detergent effect may be precisely that. The hyaluronidases in vertebrate tissues are potent enzymes that are present in exceedingly low concentrations. Protease inhibition may be the actual function of detergents on such highly active enzyme proteins present at extremely low concentrations. Recent isolation of Hyal1 was apparently conducted without detergents (Hofinger et al., 2007).

2.6. The transglycosylation reaction

An additional proviso for working with the hyaluronidases is the mysterious transglycosylation reaction (Hoffman et al., 1956). This poorly understood reaction takes on intensity as the individual HA and CS chains decrease in size. It is not certain whether this reaction occurs in nature or whether it is an artifact of the reaction *in vitro*. It is capable of cross-linking individual HA chains, or forming hybrid chains composed of HA and CS. Neither has it been established whether such hybrid molecules exist in nature, nor whether they have biological activity. However, it must be kept in mind that such unwanted side-reactions may occur in preparing tissue matrices, in which transglycosylation may even predominate over the cleavage reaction.

2.7. Inhibitors of hyaluronidases

A further limitation for working with hyaluronidases, particularly in the presence of tissue-derived materials, is the ubiquitous presence of hyaluronidase inhibitors

(Mio et al., 2000; Mio and Stern, 2002). These inhibitors are present in vast excess, as becomes obvious in the purification of these enzymes. The total units of enzyme activity increase after the initial steps in the purification, once inhibitors are separated from enzyme. Working with eukaryotic hyaluronidases in preparing tissue matrices must keep these provisos in mind.

A number of compounds with intrinsic inhibitor activity have also been described (Mio and Stern, 2002). Many of these are derivatives of ascorbic acid (Botzki et al., 2004; Hofinger et al., 2007). These vitamin C-based inhibitors have the ability to inhibit bacterial as well as the vertebrate enzymes (Spickenreither et al., 2006).

Heparin is an extraordinarily potent inhibitor of vertebrate hyaluronidases (Glick and Sylven, 1951), while the prokaryotic enzymes are impervious (R. Stern unpublished observation), as is the leech endoglucuronidase type of hyaluronidase (Jones and Sawyer, 1989). Other sulfated OSs are also effective inhibitors (Salmen et al., 2005). Among these are dextran sulfate (Lishanti et al., 2004), and probably sulfated proteoglycans, such as the syndecans (Tkachenko et al., 2005; Fears and Woods, 2006). The opposing activities of HA and syndecans in many systems in suppressing and promoting differentiation, respectively, should be kept in mind when preparing materials for artificial matrices and tissue engineering. The presence of heparin and heparan sulphate, therefore, can have major effects on the ability to modulate HA-based tissue matrices using the various hyaluronidases.

Finally, hyaluronidase action on HA results in formation only of even-numbered OSs having glucuronic acid at the non-reducing end and *N*-acetylglucosamine moiety at the reducing terminus (testicular hyaluronidase) or a reverse sequence of these two component monosaccharides (leech hyaluronidase) (Weissmann et al., 1954; Linker et al., 1960). Thus, preparation of the odd-numbered HA oligomers or even-numbered ones having an alternative structure would require additional treatment of the HA oligomers produced by hyaluronidase hydrolysis with chemical or enzymatic methods (Blundell and Almond, 2006).

3. Non-enzymatic reactions that degrade HA

3.1. Acidic and alkaline hydrolysis

Similarly to other polysaccharides, HA can be degraded by acid or alkaline hydrolysis (Weissmann et al., 1953; Jeanloz and Jeanloz, 1964; Inoue and Nagasawa, 1985). However, chemical hydrolysis proceeds in a random fashion and gives rise to a statistical mixture of oligo- and monosaccharides that can hardly be used for any specific purpose. Moreover, even short-term treatment of HA polymers at acidic or alkaline conditions can result in degradation, including "peeling" from the reducing end and β-elimination, characteristic for the uronic acid-containing poly- and OSs (Kiss, 1974). Similarly to other polysaccharides containing 4-substituted or non-reducing glycuronic acids, HA under alkaline conditions degrades with the formation of unsaturated glucuronic acid units — 4-deoxy-hex-4-enoglucuronate (Yang and Montgomery, 2001). A "peeling" reaction occurs concomitantly, and leads to the formation of saccharinic acid (Whistler and BeMiller, 1958). At the same time, the *N*-acetylglucosamine component of HA is also degraded with alkaline treatment, giving rise to furan-containing species (BeMiller and Whistler, 1962). The authors warn that since HA is readily degraded in alkaline solutions, exposure to alkaline conditions during isolation should be avoided.

In comparison to the effect of alkaline treatment, effects of lower pH conditions on HA have been much less investigated, and the results are even more ambiguous (Reed et al., 1989). No decrease of molar mass is observed at pH values below 2, and actual increase is described (Hofmann et al., 1979). It appears that HA solutions in acid media undergo random degradation (Reed and Reed, 1989; Lapčík et al., 1998) or racemization, so that the products of acidic hydrolysis also contain structural components different from those comprised in the native HA.

In a complex study of the hydrolytic fragmentation of HA, Tokita and Okamoto (1995) suggest mechanisms for acidic and alkaline hydrolysis based on the data obtained by ^{13}C NMR spectroscopic investigation and theoretical quantum chemical calculations. Conclusions have been made that:

a) hydrolytic degradation of HA occurs *via* random chain scission and obeys first-order kinetics;
b) acidic hydrolysis affects the glucuronic acid moiety, while base-catalyzed hydrolysis occurs on the *N*-acetylglucosamine unit;
c) cleavage of the glycosidic bond takes place in one step at acidic hydrolysis, whereas under alkaline conditions it may involve two steps.

Schematic illustrations of both reaction pathways are provided in Figs. 2 and 3.

3.2. Ultrasonic degradation

Contrary to acid and alkaline hydrolysis, ultrasonication (US) degrades HA in a non-random fashion, resulting

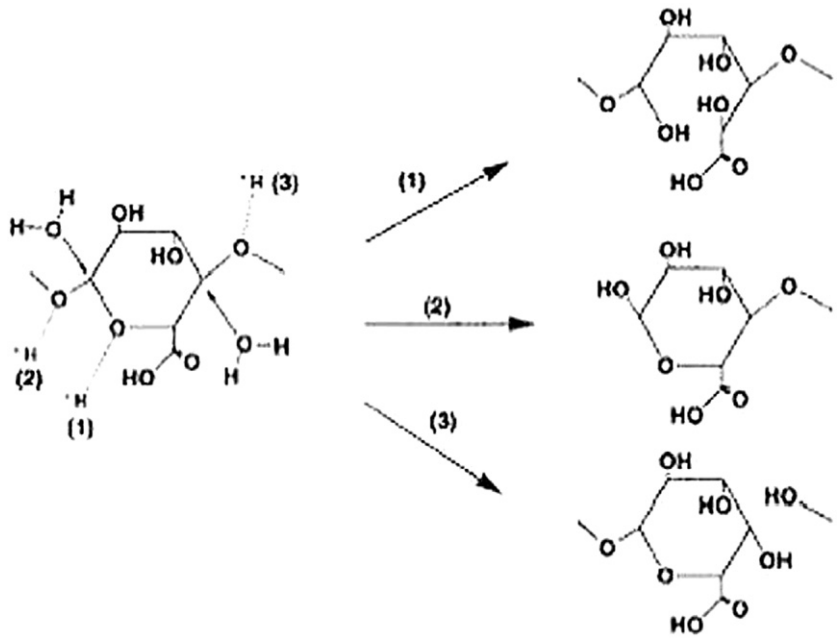

Fig. 2. Mechanism of the hydrolysis of HA under acidic conditions. Three possible sites of proton attack on the glucuronic acid unit and the corresponding degradation pathways are marked with numbers (1)–(3). Adapted from Tokita and Okamoto (1995) with the publisher's permission.

in a bimodal molar mass distribution (Vercruysse et al., 1995). Kinetic studies demonstrate that high-molar-mass HA chains are degraded more slowly than low-molar-mass molecules. Also ultrasonic degradation of different HA samples applying different ultrasound energies never lead to complete degradation into monomers (Kubo et al., 1993; Reháková et al., 1994; Vercruysse et al., 1995; Šoltés et al., 1996). As no increase of the absorbance at 232 nm is observed after sonication, a conclusion is reached that depolymerization is not occurring *via* an elimination reaction (Kubo et al., 1993). Using three HA samples from human umbilical cord (400 kDa), rooster comb (1000 kDa), and that produced by *Streptococcus zooepidermicus* (1200 kDa), it is demonstrated that the final depolymerized products have molar mass values of 11, 3, and 60 kDa, respectively. No further degradation is observed, even with prolonged sonication (Kubo et al., 1993). Dřímalová et al. (2005) compared ultrasonic degradation of HA with the action of conventional heating and microwave (MW) irradiation and monitored the degradation process and the produced fragments using viscometry, UV, NMR, and FT-IR spectroscopies, as well as with size-exclusion chromatography coupled to low-angle light scattering detector. The authors conclude that US is preferable to the other two applied degradative procedures in that it is able to efficiently reduce molar mass down to 100 kDa without significant chemical modification of the primary structure.

3.3. Thermal degradation

Rheological studies show that HA solutions retrograde even in distilled water: at higher temperatures, viscosity of the solutions decreases in time exponentially as a function of temperature (Lowry and Beavers, 1994). Bottner et al. (1988) studied two high-molar-mass HA samples that were extensively degraded at 128 °C in an autoclave. The decline of molar mass was determined using laser light scattering and related to a limiting viscosity number. Treatment of the data according to the Mark–Houwink equation indicates that thermal degradation occurs in agreement with the random-scission mechanism. Reháková et al. (1994) report that treatment of different HA samples at temperatures from 60 to 90 °C for 1 h results in only moderate degradation and a small increase of polydispersity.

3.4. Degradation by oxidants

Many human diseases are associated with harmful action of reactive oxygen species (ROS) (Halliwell and Gutteridge, 1989). These species are involved in the

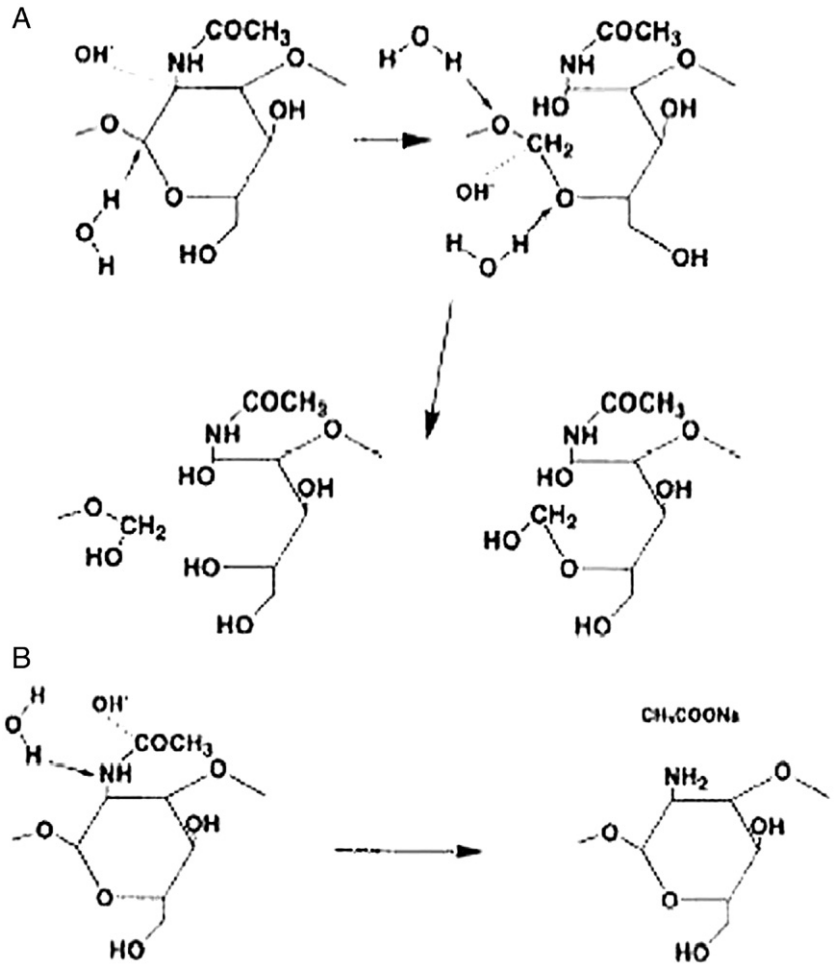

Fig. 3. Mechanism of the hydrolysis of HA under alkaline conditions. A two-step pathway is shown according to Tokita and Okamoto (1995). Reproduced with the publisher's permission.

degradation of essential tissue or related components. One such component is synovial fluid (SF) of the joint that contains high-molar-mass HA. A healthy joint allows smooth, frictionless, pain-free movement. However, when damaged or affected by arthritis, joints become stiff and painful. Of the many known arthritic diseases, osteoarthritis (OA) and rheumatoid arthritis (RA) are the most common chronic conditions, which affect particularly the elderly population. While OA is a degenerative disease of cartilage and bone, resulting in pain and stiffness of the affected joints, RA is classified as a systemic inflammatory disease in which pain of the joint (s) is often accompanied with degenerative changes in other organs, such as lungs, heart, and blood vessels (Henrotin et al., 2003; Yamazaki et al., 2003).

Although the etiology and pathogenesis of RA are as yet unknown, a progressive degradation of polymeric carbohydrates, including HA, occurs in synovial fluid in the course of the disease. In acute phases, large numbers of neutrophils accumulated in the patient's SF and their products, especially ROS, can contribute to the destruction of joint structures. Due to chronic inflammation, the ROS alter or destroy the joint structure to such an extent that it is no longer functional. The altered tissues are recognized as "foreign", and subsequently autoimmune reactions promote the disease and make RA a systemic ailment affecting the entire body.

The observed reduction of HA molar mass in the synovial fluid of patients suffering from rheumatic diseases has led to *in vitro* studies of HA degradation by reactive oxygen species (Parsons et al., 2002). The earliest investigation was carried out by Pigman et al. (1961) and since then, numerous studies have been reported, in which the action of various ROS on HA was investigated. Some of the reported studies are listed in

Table 1. Here, we will briefly describe basic reactions, by which individual ROS are produced and the probable mechanisms of their degradative action on HA.

3.4.1. Superoxide anion radical

$O_2^{\bullet-}$ is formed in neutrophils, monocytes, macrophages, and eosinophils due to the action of NADPH oxidase during a so-called "respiratory burst". NADPH oxidase, a highly regulated enzyme complex composed of a number of proteins, reduces oxygen to a superoxide anion radical according to the reaction:

$$2O_2 + NADPH \rightarrow 2O_2^{\bullet-} + NADP^+ + H^+. \quad (1)$$

Another source of superoxide anion radical is xanthine oxido-reductase, also called xanthinoxidase. This molybdenum- and iron-containing flavoprotein catalyzes oxidation of hypoxanthine to xanthine and then to uric acid. Molecular oxygen is the substrate and the products include superoxide anion radicals (Hancock et al., 2001). Other cellular sources for $O_2^{\bullet-}$ are mitochondria in stressed cells, the formation of met-hemoglobin, and the reduction of oxygen by quinone radicals or by oxidized glutathione anion radicals ($GSSG^{\bullet-}$). Superoxide anion radical is both a one-electron oxidant and a one-electron reductant. It does not have direct toxic effects on living targets; however, upon penetrating to important physiological sites, it is converted to hydrogen peroxide, singlet oxygen ($^1O_2$), and possibly to hydroxyl radicals ($^{\bullet}OH$). Although $O_2^{\bullet-}$ is unable to degrade HA on its own, it can participate in reactions with ions of transition metals to produce highly reactive and damaging species, hydroxyl radical. Superoxide anion radicals play also a decisive role by converting nitrogen monoxide ($^{\bullet}NO$) to the powerful oxidant peroxynitrite anion ($ONOO^-$) (Nauser and Koppenol, 2002).

3.4.2. Hydrogen peroxide

H_2O_2 is produced from two superoxide anion radicals that undergo spontaneous dismutation, producing the molecule of hydrogen peroxide, a non-charged non-radical oxidative species, and a molecule of oxygen

$$O_2^{\bullet-} + O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2. \quad (2)$$

Therefore, once superoxide anion radicals are formed *in situ*, occurrence of hydrogen peroxide becomes inevitable. The reaction (2) occurs spontaneously, especially at low pH values; however *in vivo*, this reaction is catalyzed by a family of enzymes known as superoxide dismutase (SOD). The cytosolic SOD form contains Cu and Zn, whereas a mitochondrial form contains Mn. In addition to SOD, another heme-containing enzyme, catalase, converts hydrogen peroxide to oxygen and water.

Similarly to superoxide anion radical, hydrogen peroxide does not itself cause degradation of HA, however in the presence of transition metals, it can undergo heterolytic fission leading to production of

Table 1
Some reported studies on the degradation of high-molar-mass HA by ROS

ROS	Sources of ROS	References
$O_2^{\bullet-}$	Cellular: by polymorphonuclear leukocytes Enzymatic: xanthinoxidase + xanthine Chemical: using KO_2	Rees et al. (2004)
H_2O_2	Chemical: aqueous H_2O_2 Cellular: by polymorphonuclear leukocytes	Stankovská et al. (2005), Hawkins and Davies (1996)
$^1O_2$	Physical and chemical: UV-irradiated riboflavin	Frati et al. (1997)
$^{\bullet}OH$	Enzymatic: xanthine oxidase + xanthine + transitional metal cation Physical and/or chemical: H_2O lysis by γ-rays H_2O_2 irradiation by the UV light H_2O_2 + transitional metal cation H_2O_2 + ascorbic acid	Mendichi et al. (1995), Myint et al. (1987), Lapčík et al. (1991), Al-Assaf et al. (2000), Li et al. (1997), Orviský et al. (1997), Praest et al. (1997), Al-Assaf et al. (1999), Jahn et al. (1999), Šoltés et al. (2001), Rees et al. (2002), Šoltés et al. (2005, 2006a, 2006b, 2007)
$^{\bullet}NO$	Cellular: endothelial cells Chemical: gaseous $^{\bullet}NO$ (from a gas canister)	Vilar et al. (1997)
$ONOO^-$	Chemical: $NaNO_2$ + (H_2O_2 + HCl) + NaOH or $NaN_3 + O_3$	Li et al. (1997), Parsons et al. (2002), Al-Assaf et al. (2003), Corsaro et al. (2004)
$^-OCl/HOCl$	Chemical: hypochlorite Enzymatic *in vivo*: myeloperoxidase	Baker et al. (1989), Jahn et al. (1999), Rees et al. (2002, 2003), Rees et al. (2004), Arnhold (2004)
$CO_3^{\bullet-}$	Physical and chemical: pulse irradiation of nitrous oxide solution in bicarbonate/carbonate mixture	Al-Assaf et al. (2006)
$Cl_2^{\bullet-}$	Physical and chemical: pulse irradiation of HCl solution in the presence of air	Al-Assaf et al. (2006)

hydroxyl radicals (Fenton reaction (3)) and when present simultaneously with superoxide anion radical within the same microenvironment, they may undergo the so-called Haber–Weiss reaction (4), which also leads to formation of hydroxyl radicals:

$$H_2O_2 + Fe^{2+} \rightarrow OH + Fe^{3+} + HO^- \quad (3)$$

$$H_2O_2 + O_2^- \rightarrow HO + HO^- + O_2. \quad (4)$$

The Fenton reaction (3) is the most frequently used experimental method for production of OH radicals, and is also suggested as being a source of free hydroxyl radicals in biological systems. However, under physiological conditions, the iron ions are firmly bound: in blood they are associated with the protein transferrin, in tears, milk, saliva and other body fluids they are bound in lactoferrin, while in the cells they are stored, linked to the proteins ferritin. Yet under stress conditions, *e.g.* in rheumatoid SF, an increase of the so-called labile iron pool is observed (Gutteridge, 1987).

3.4.3. Singlet oxygen

$^1O_2$ is an oxygen form, in which electrons are excited at a higher energy level compared to the normal triplet oxygen. When returning to the ground state, the molecules of singlet oxygen emit energy that may cause degradation of certain biopolymers including HA. $^1O_2$ is considered to be a major ROS formed with UVA irradiation that causes skin ageing (Baier et al., 2006), and is involved in damaging epidermal collagen and HA.

There are several reactions in which the generation of singlet oxygen molecules occurs. For example, under *in vivo* conditions, $^1O_2$ can be formed due to interaction of hypochlorous acid with hydrogen peroxide (Halliwell, 1982):

$$HOCl + H_2O_2 \rightarrow {}^1O_2 + H_2O + H^+ + Cl^-. \quad (5)$$

Production of singlet oxygen is also shown to be elicited by photosensitization of riboflavin (Frati et al., 1997).

3.4.4. Hydroxyl radical

OH can in principle be the product of homolytic fission of the H_2O_2 molecule; however *in vivo* the direct route (→) of the reaction

$$HO–OH \leftarrow HO + OH$$

is not plausible, whereas under experimental conditions such "lysis" of H_2O_2 is attained by using γ- or UV-irradiation (Myint et al., 1987; Lapčík et al., 1991; Al-Assaf et al., 2000). In *in vivo* systems, hydroxyl radicals can be generated through various pathways, including the Fenton reaction (3) (Walling, 1975; Aust et al., 1985). Alternative pathways for generation of $^{\bullet}OH$ include the iron-catalyzed Haber–Weiss reaction, during which secondary hydroxyl radicals are formed from H_2O_2, and $O_2^{\bullet-}$ produced by oxidation of hypoxanthine by xanthine oxidase (Kaur and Halliwell, 1990; Mendichi et al., 1995). Another possible pathway leading to $HO^{\bullet}$ involves interaction of transition metals (copper or iron ions) with ascorbic acid (Šoltés et al., 2006b). Such redox system occurring *in vivo* or prepared under experimental conditions are shown to effectively degrade HA (Šoltés et al., 2006b; Šoltés et al., 2007).

The $^{\bullet}OH$ radical can be classified as an "ultimate oxidant" capable of oxidizing almost all low- and high-molar-mass organic substances. However, due to its extremely short half-life (ca. 10^{-9} s), the action should be site-specific. The toxicity of $^{\bullet}OH$ radicals results from their ability to abstract electrons from a large variety of compounds

$$R + {}^{\bullet}OH \rightarrow R^{\bullet} + HO^-$$

with the formation of a new radical – $R^{\bullet}$ – which consecutively can oxidize other substances. Hawkins and Davies (1996) use electron paramagnetic resonance (EPR) spectroscopy and computer simulation to study the fragmentation products that arise from the hydroxyl radicals attack on HA. While $^{\bullet}OH$ attack of the glucuronic acid unit occurs randomly at all sites of the carbon ring, the *N*-acetylglucosamine moiety is attacked more specifically. No radicals are formed at the C-2 or methyl carbon of the acetyl group. The formed *C*-centered radicals undergo more or less rapid rearrangements under acid and alkaline conditions, resulting in strand breakage and formation of low-molar-mass fragments, usually containing double bonds.

3.4.5. Nitric oxide

$^{\bullet}NO$, a short-living radical can play a dual role in physiology. By interacting with the iron-containing group of guanylate cyclase, it has a regulatory function as an endothelium-derived relaxation factor. On the other hand, it can be converted to other nitrogen oxides, and thus become a toxic or inflammatory agent.

Enhanced $^{\bullet}NO$ synthesis is reported to occur in inflammatory responses initiated by microbial products or autoimmune reactions, and also in the systemic inflammatory response, referred to as sepsis. $^{\bullet}NO$ probably participates in the inflammatory reaction and in the

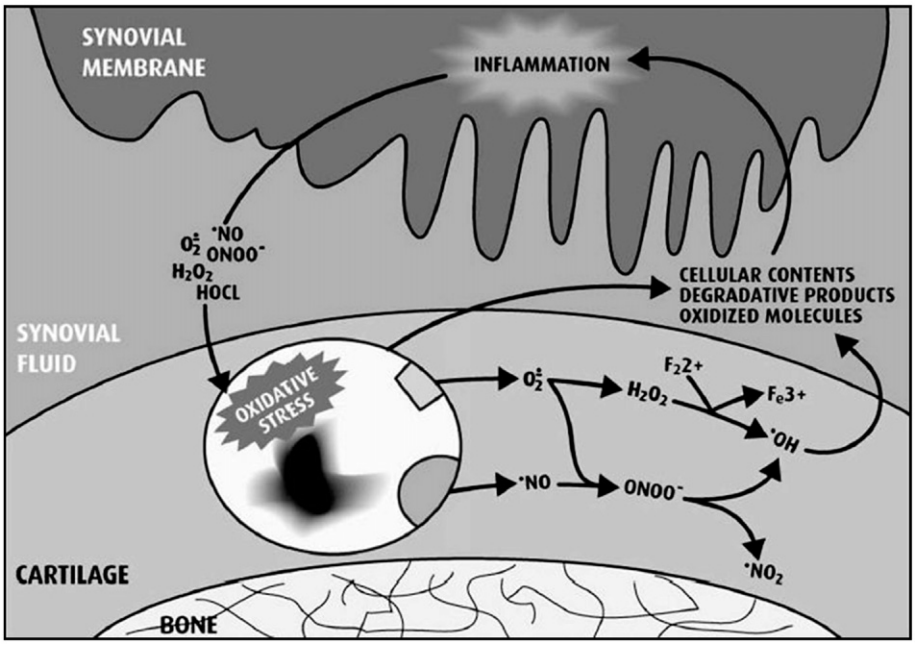

Fig. 4. Involvement of reactive oxygen species in cartilage degradation and related synovium inflammatory reaction. Reproduced from Henrotin et al. (2003) with permission.

subsequent damage to joint tissues in certain types of arthritis. For instance, synovial fluids from patients with arthritis exhibit elevated nitrate concentrations (nitrate is the end-product of the L-arginine–NO synthase pathway). Together with superoxide radical anion, $^{\bullet}NO$ belongs to the main ROS produced by chondrocytes and both of them further generate derivative radicals (including peroxynitrite and hydrogen peroxide) that play major role in cartilage degradation (Henrotin et al., 2003).

$^{\bullet}NO$ is synthesized by $^{\bullet}NO$ synthase enzymes (NOS), the P_{450}-related hemoproteins that oxidize L-arginine to L-citrulline and nitrogen monoxide (Reutov and Sorokina, 1998). Three distinct isoforms of NOS representing three distinct gene products have been isolated and purified. Two of the enzymes are permanently present and termed constitutive NOS (cNOS). The third one is an inducible NOS (iNOS). Stimuli typically include interleukin IL-β, tumor necrosis factor TNF-α, interferon IFN-γ, and lipopolysaccharides (LPS). Inhibition occurs with transforming growth factor TGF-β, IL-4, IL-10 and IL-13 (Henrotin et al., 2003). Once expressed, the enzyme generates large amounts of $^{\bullet}NO$. Thus, nitric oxide does not directly affect HA, however by mediating the production of peroxynitrite, it represents a major factor of *in vivo* degradation of all GAGs including HA (Fig. 4).

3.4.6. Peroxynitrite anion

$ONOO^-$ is formed at sites of inflammation by the rapid diffusion-controlled reaction between superoxide anion radicals and nitric oxide (Nauser and Koppenol, 2002)

$$^{\bullet}NO + O_2^{\bullet -} \rightarrow ONOO^-.$$

$ONOO^-$ is a highly reactive oxidizing species capable of damaging cellular lipids, carbohydrates, proteins, and DNA. The reaction of $ONOO^-$ with tyrosine residues in proteins results in formation of 3-nitrotyrosine, a suggested biomarker of $ONOO^-$ production *in vivo*. Indeed, increased levels of 3-nitrotyrosine have been detected in numerous human diseases, such as rheumatoid arthritis, Parkinson's disease, Alzheimer's disease, and asthma. Direct observation of elevated concentrations of nitrite has been also reported in the SF and serum of RA patients (Farrell et al., 1992).

Peroxynitrite anion in aqueous milieu exists in an acid-base equilibrium with peroxynitrous acid

$$ONOO^- + H^+ \leftrightarrow ONOOH.$$

Peroxynitrous acid is decomposed with a half-life of *ca.* 1 s (Li et al., 1997) and acts as if it had two radical ends, $^{\bullet}OH$ and $ONO^{\bullet}$, and can undergo several

decomposition processes, each of which leads to production of various potent destructive species:

- can decompose by homolytic fission to $ONO^{\bullet} + {}^{\bullet}OH$;
- can decompose by heterolytic fission to $ONO^{+} + HO^{-}$;
- can isomerize, yielding $H^{+} + NO_3^{-}$ *i.e.* HNO_3;
- can react with $O_2^{\bullet -}$:

$$ONOOH + O_2^{\bullet -} \rightarrow ONO^{\bullet} + HO^{-} + O_2$$

- can react with a transition metal ion — *e.g.* ferrous ion:

$$ONOOH + Fe^{2+} \rightarrow ONO^{\bullet} + HO^{-} + Fe^{3+}$$

- can react with CO_2 to yield the nitrating species $ONOOCO_2^{-}$.

Li et al. (1997) report that treatment of high-molar-mass bacterial HA with peroxynitrite leads to formation of macromolecules with reduced molar mass and decreased polydispersity. This conclusion is confirmed by both electrophoretic and viscometric analyses, and is in agreement with a random cleavage mechanism. Later, Al-Assaf et al. (2003) applied a combination of stopped-flow techniques with gel-permeation chromatography and multi-angle light scattering (MALS) to monitor the changes in molar mass distribution of HA treated with peroxynitrite. The kinetic data indicates that there is no detectable bimolecular reaction between ONOOH and HA, which is in agreement with the results of studies involving peroxynitrite and simple carbohydrates (Hogg et al., 1992). Thus, degradation of HA chains is caused by the products of unimolecular decay of peroxynitrite. The intermediate complex of the decay reaction is designated a cage complex

$$ONOOH \rightarrow [ONO^{\bullet}\ {}^{\bullet}OH]$$

and the number of HA chain breaks is shown to be proportional to the number of hydroxyl radicals that escape from the cage. A more detailed investigation of the degradation products using spectroscopic methods as well as mass spectrometry (MS) has been published more recently (Corsaro et al., 2004). Neither NMR nor MS experiments provide any evidence of a peroxynitrite-mediated modification of HA. On the other hand, simultaneously performed EPR experiments give evidence for the generation of *C*-centered carbon radicals, most probably by the way of hydroxyl radical-like reactivity of peroxynitrite.

3.4.7. Hypochlorous acid

HOCl is produced *in vivo* by the heme-enzyme myeloperoxidase (MPO), released from stimulated neutrophils that mediate oxidation of Cl^{-} with hydrogen peroxide (Arnhold, 2004). The following reaction occurs at the oxidation:

$$H_2O_2 + Cl^{-} \rightarrow HOCl + HO^{-} \leftrightarrow {}^{-}OCl + H_2O.$$

MPO is detected in the SF from patients with RA, which supports a hypothesis of its involvement in the degradation of HA (Edwards et al., 1988). The MPO burst is also characteristic of the acute inflammatory reaction. All acute inflammation reactions are characterized by initially infiltrates of neutrophils, generally regarded as the first line of defense.

Hypochlorous acid is a powerful oxidizing and chlorinating species, formed at sites of chronic inflammation, capable of oxidizing proteins, DNA, lipids, *etc.*, and/or chlorinating DNA, cholesterol, lipids, *etc.* This is why HOCl-induced cell death occurs very rapidly, in comparison to that mediated by other ROS. In addition, after activation, MPO is able to oxidize a large variety of small molecules including amino acids, phenols, indoles, sulfhydryls, nitrite, xenobiotics, and other substances, generating different reactive radicals and contributing to progressive damage to biomolecules at sites of inflammation. In cartilage, exposure of GAGs to hypochlorous acid or $MPO–H_2O_2–Cl^{-}$ systems generate long-lived chloramides derived from *N*-acetylglucosamine moieties. Fig. 5 represents the degradative pathway of HA initiated by HOCl action on the *N*-acetyl group (Rees et al., 2004). The results provide evidence for the formation of C-4 carbon-centered radicals on the glucuronic acid units, and on the subsequent scission reaction of these radicals, leading to the cleavage of *N*-acetylglucosamine β-(1→4) glycosidic bonds. Formation of the unstable chloramides and their subsequent decay, accompanied by the production of acetate and formate, is proven by means of ^{1}H- and ^{13}C NMR spectroscopy (Schiller et al., 1994, 1995). Using model compounds and spin-trap EPR spectroscopy, Hawkins and Davies (1998) demonstrate that decomposition of the *N*-acetylglucosamine-derived chloramides proceed through a nitrogen-centered radical, with subsequent rearrangement that leads to the opening of the sugar ring.

In contrast to the described mechanism, Jahn et al. (1999) report that NaOCl degradation of HA in physiological buffer solution occurs primarily at glucuronic acid residues and leads to production of *meso*-tartaric acid, which is suggested to be a useful

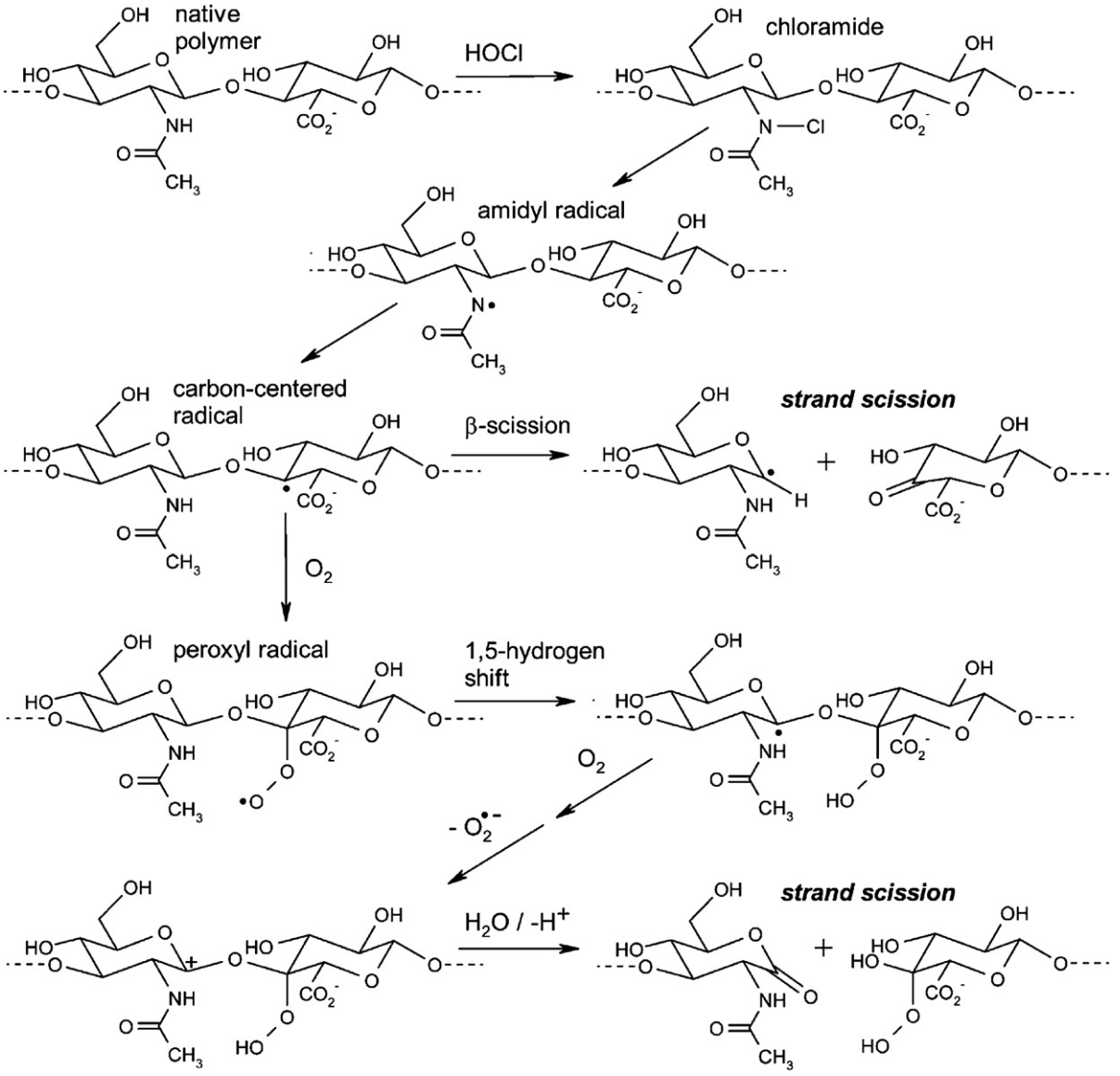

Fig. 5. Degradative pathway of HA initiated by HOCl action on the *N*-acetyl group. Reproduced with permission, from Rees et al. (2004) © the Biochemical Society.

marker of HA oxidation since it is produced by both hypochlorite and Fenton chemistry.

3.4.8. Carbonate radical anion

$CO_3^{\cdot -}$ is produced experimentally by steady-state or pulsed irradiation of nitrous oxide solutions in bicarbonate/carbonate mixtures (Al-Assaf et al., 2006). The relevant reactions taking place under such conditions are as follows:

$$H_2O \rightarrow {}^{\cdot}OH, H^{\cdot}, e_{aq}^{-}, H_2, H_2O_2$$

where e_{aq}^{-} is hydrated electron

$$e_{aq}^{-} + N_2O \rightarrow {}^{\cdot}OH + HO^{-} + N_2$$

$${}^{\cdot}OH + HCO_3^{-} \rightarrow CO_3^{\cdot -} + H_2O$$

$$OH + CO_3^{2-} \rightarrow CO_3^{-} + HO^{-}.$$

The rate of reaction of CO_3^{-} with HA is measured using the pulse radiolysis technique by monitoring the decay of the radical absorbance in the presence and absence of HA. Several concentrations of HA are applied and the first-order decay of the radical species is observed. Different doses of radiation are applied and the dose-related decrease of the molar mass (*i.e.* number of HA chain breaks) is established using the gel-permeation chromatography–MALS setup. The pattern of degradation is characteristic of a random process. The amount of degraded HA is however very small, *ca.* 0.2% and therefore it is impossible to observe a ladder-type pattern as demonstrated by polyacrylamide gel electrophoresis (PAGE) when HA is degraded with HOCl (Rees et al., 2003). The finding that CO_3^{-} causes HA chain scission corroborates its oxidative role in biological systems containing peroxynitrite. Thus, carbonate radical anion may be considered as an additional or alternative factor of oxidative stress in biological systems.

3.4.9. Dichloride radical anion

Cl_2^{-} is produced in acidic solutions in the presence of chloride anion due to its oxidation with hydroxyl radicals (Jayson et al., 1973). The yield of this species is much lower in neutral solutions. In biological systems, Cl_2^{-} may be formed as a consequence of the hypochlorite reduction by transition metals or superoxide radicals (Koppenol, 1994).

Al-Assaf et al. (2006) produced Cl_2^{-} by the irradiation of 0.1 M HCl in the presence of air, suggesting the following pathway:

$$H_2O \rightarrow OH, H, e_{aq}^{-}$$

$$OH + Cl^{-} \rightarrow Cl + OH^{-}$$

$$Cl + Cl^{-} \rightarrow Cl_2^{-}.$$

The authors show that Cl_2^{-} is almost as efficient in producing HA chain breaks as the hydroxyl radical. Much slower rates of reaction with HA could imply that Cl_2^{-} is more selective in its action on the macromolecule, reacting perhaps at only one or two sites in the repeating unit, whereas hydroxyl radicals show similar reactivity towards all eleven sites. Moreover, high extent of degradation found in that study might indicate that similar to HOCl, the dichloride radical anion does not abstract hydrogen from the ring carbons but rather attacks the *N*-acetyl function of *N*-acetylglucosamine moiety.

3.5. Miscellaneous degradations

Several reports on the photodegradation of HA involving microwave, UV, or γ-irradiation have been published (Lapčík et al., 1991; Reháková et al., 1994; Dřímalová et al., 2005). Exposure of sodium hyaluronate solution to the Hg lamp irradiation at the specified wavelengths causes degradation of the macromolecule to smaller fragments that are separated and identified by size-exclusion chromatography. Considerable decreases of molar mass are observed after a prolonged irradiation, while polydispersity increases even after short treatment times (Reháková et al., 1994). The authors also subject HA in solid state and in solution to γ-rays and observe extensive degradation. In both cases, however, the authors suggest that certain recombination of the HA radical fragments may be occurring.

Lapčík et al. (1991) subject HA solutions to UV-irradiation and examine the products using an EPR spin-trapping technique. The data obtained indicate that in the first minutes of irradiation, carbon-centered radicals are formed, whereas upon prolonged irradiation, hetero-atom centered radical adducts are detected, followed by the cleavage of the glycosidic linkages. According to the authors, it is probable that the observed photochemically induced degradation is initiated by the production of reactive hydroxyl radicals during irradiation of the aqueous HA solutions.

Dřímalová et al. (2005) report that MW-assisted HA degradation is more efficient at higher pH values and leads to significant degradation even at short time of application. According to Galema (1997), MW irradiation results in the increased polarization of glycosidic bonds making them more susceptible to hydrolytic cleavage. The NMR and FT-IR spectral analyses indicate that at mild conditions, the backbone of HA is retained, whereas at harsh degradation conditions (long-term treatment, particularly at acidic pH or alkaline pH and in presence of oxidants) degradation is accompanied by destruction of both constituent sugar residues and formation of unsaturated structures detectable by UV-absorption at 230–240 and 260–270 nm.

4. Preparation and application of hyaluronan fragments

HA finds a broad range of biomedical applications, the overwhelming majority of which is due to its viscoelastic properties. Major areas of clinical and cosmetic use of HA and its derivatives have been recently reviewed in

comprehensive studies (Balazs, 2004; Asari, 2004; Shu and Prestwich, 2004; Miller and Avila, 2004; Kogan et al., 2007, in press). For this reason we will focus only on the current state and prospects for preparation and use of HA fragments with decreased molar mass, utilizing their properties not necessarily associated with the viscoelastic/rheological performance.

Because of the increasing demand for this biomaterial in medicine, cosmetics, and specialty foods, the production of HA has been on the rise. The product Hyalgan® (Fidia, Inc., Abano Terme, Italy) is applied topically for the treatment of burns and skin ulcers as early as in the 1960s. Interestingly, despite entering the market first in Europe, HA has never achieved extensive recognition, nor established a stable market similar to that found in the USA or Japan. It has been recently estimated that the current value of the HA market reaches about $300 million in the USA and Japan each, while in Europe it is below $100 million (Chong et al., 2005). The current demand for HA materials approved for applications in human medicine is satisfied primarily by high-molar-mass HAs prepared from animal sources, such as rooster combs. For example, Healon® (Pharmacia & Upjohn, Inc., Peapack, NJ, USA), a major product used in more than 30 million ophthalmological operations, has a mean HA molar mass of about 2.5 MDa. However, as HA from animal tissues HA can be associated with proteins and proteoglycans (Burd, 2004), isolation of high-purity HA is rather costly. Moreover, use of animal-derived preparations for human therapy can be associated with the potential risk of viral and other infections (O'Regan et al., 1994). Also, all HA products derived from rooster combs carry obligatory warnings for those allergic to avian products. Moreover, recent emergence of bird influenza that caused significant number of human deaths stimulated reluctant attitude to the use of the avian-derived products.

Thus, at present, alternative processes for production of HA are being developed, and gradually manufacturing of microbial (fermented) HA is gaining impetus (Chong et al., 2005). Since HA is synthesized as an extracellular capsule by the pathogenic groups A and C streptococci, which can not be prepared by industrial fermentation, the resort is the use of the genetically-modified non-pathogenic bacterial strain, *Bacillus subtilis*, carrying the *HasA* (*HA synthase A*) gene from *Streptococcus equisimilis*. In this way, engineered strains are producing HA with a molar mass of about 1 MDa. The advantage of using *B. subtilis* is that it is easily cultivatable on a large scale and does not produce exo- or endotoxins. Moreover, *B. subtilis* does not produce hyaluronidase that could degrade the synthesized HA (Widner et al. 2005). At present, microbially produced HA has been approved for treatment of superficial wounds as well as for the use in the cosmetic industry.

Although the microbially produced HA has a molar mass lower than that extracted from rooster combs, certain applications require HA fragments in the OS range. Smaller HA fragments are involved in angiogenesis, cell migration, and proliferation, functions distinctly different from those of large HA polymers. Eight- to 50-mer HA OSs were first reported to induce angiogenesis, and were described as 'angiogenic OSs' (West et al., 1985). Six- to 20-mer OSs enhance endothelial cell proliferation (Slevin et al., 2002) and migration (Sattar et al., 1994). They also stimulate the synthesis of type I and type VIII collagens by endothelial cells (Rooney et al., 1993). Sugahara et al. (2004) compiled a table showing extreme versatility of properties and functions of the HA macromolecules in the molar mass range from 1 to 400 kDa. More recently, Stern et al. (2006) published a comprehensive review that summarizes the remarkably vast range of size-specific activities of HA polymers and at the same time warns of the problems associated with preparation and use of the HA fragments. Due to their specific functions, HA fragments of specific size might find application in cancer therapy (Sugahara et al., 2004) and tissue engineering (Ohno et al., 2005a,b).

However the role of HA OSs of distinct size is not entirely established and therefore their clinical applications have yet to undergo systematic study. As yet, they have been applied mostly in functional and structural studies of HA-binding proteins. By competition for HA receptors, OSs have been used to inhibit tumor growth (Ghatak et al., 2002) and adhesion of *Plasmodium falciparum*-infected erythrocytes in placenta (Chai et al., 2001). OSs have been used to establish the size of HA-binding sites in cartilage proteoglycan components (Christner et al., 1979; Nieduszynski et al., 1980; Seyfried et al., 2005), and in combination with NMR, to define the HA-binding site in the Link modules of TSG-6 (Kahmann et al., 2000; Blundell et al., 2003) and CD44 (Teriete et al., 2004). Derivatization of the HA OSs was shown to be a promising tool for production of the novel biomaterials of pharmaceutical importance that promote *e.g.* wound vascularisation and necrosis of tumor cells (DeAngelis et al., 2003). Recently Joddar and Ramamurthi (2006) reported on the elastogenic effect of HA on vascular smooth muscle cells and indicated that the biologic effects of HA are rather specific to HA fragment size, while shorter HA fragments were more cell-interactive than the relatively bio-inert long-chain (MW > 106 Da) form. Low-molar-mass HA OSs can be also with advantage used in the studies of HA properties, especially NMR spectroscopic investigations and the mechanism of degradation, where use of polymeric HA is significantly

hampered by its viscosity (Slaghek et al., 1994; Siciňska et al., 1993). Describing a vast array of potential biomedical applications of HA OSs, Jing and DeAngelis (2004) emphasize the necessity to obtain uniform size-defined HA fragments in order to interpret accurately the various biological functions of HA and to synthesize better HA-containing biomedical products.

As indicated above, degradation and depolymerization processes of high-molar-mass HA, affect primary chemical structures of the HA component units by producing fragments containing double bonds or opened sugar rings. Even some of the enzymatic cleavage mechanisms that are in general more sparing of the HA macromolecule than free radical degradation, can lead not only to scission of glycosidic linkages, but also to abstraction of protons and production of unsaturated HA fragments, particularly by the bacterial Hyals. Since such altered molecules would be most probably recognized by human immune cells as "foreign", an immune reaction might be triggered by such modified biomaterials. This can lead to inflammation, tissue rejection, and other adverse consequences.

Recently, Šoltés et al. (2007) demonstrated that a redox system containing ascorbic acid and transition metal ions (Fe and Cu) is able to degrade high-molar-mass HA, leading to the formation of fragments with a very narrow mass distribution. The system containing ascorbic acid and Cu(II) is known as the "Weissberger system", an efficient OH radical generating system (Weissberger et al., 1943). Since such a system is biogenic, *i.e.* occurs naturally in the human organism, it is reasonable to expect that degradation of HA *in vitro* using this model would lead to fragments similar to those that are produced under *in vivo* conditions. Thus, no or minimal immune responses would be expected to such "biogenically" generated HA fragments. This would facilitate their application in biomedical materials.

5. Concluding remarks

Most degradation and depolymerization procedures applied to HA render fragments with substantially altered primary structure, because of the chemical changes affecting the component monosaccharide units. It is necessary to retain the primary structure of HA for biomedical applications in order to avoid adverse immunological reactions. It seems that enzymatic depolymerization involving exclusively scission of the glycosidic linkages is recommended for preparation of HA fragments of reduced specific molecular size. Alternatively, "biogenic" degradation of HA using the system containing ascorbic acid and transition, especially copper(II) ions can be applied to obtain HA fragments with the structure close to the degradation products found in the synovial fluid. Specific modification of such fragments, particularly the nature of polymer termini can be achieved using specific enzymes or chemical reactions. The subsequent separation and purification of the fragments produced can be performed by a variety of methods that will constitute the subject of a separate review (Kogan et al., 2007, in press).

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