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Enhanced multiparametric hyaluronan degradation for production of molar-mass-defined fragments

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ABSTRACT

Hyaluronic acid (HA) is known to serve as a dynamic mediator intervening in many physiological functions. Its specific effect has been repeatedly confirmed to be strongly influenced by the molecular size of hyaluronan fragments. However common technological approaches of HA fragments production have their limitations. In many cases, the final products do not meet the strict pharmaceutical requirements, specifically due to size polydispersity and reaction contaminants. We present novel methodology based on combination of unique incidental ability of the plant-derived protease papain to split the glycosidic bonds and an indispensable advantages of biocompatible macroporous material with incorporated ferrous ions serving as carrier for covalent papain fixation. This atypical and yet unpublished highly efficient multiparametric approach allows enhanced HA fragmentation for easily and safely producing molar-mass-defined HA fragments with narrow size distribution. Native polyacrylamide gel electrophoresis (PAGE) and size exclusion chromatography/multi-angle light scattering (SEC-MALS) confirmed the effectiveness of our multiparametric approach.

1. Introduction

Hyaluronic acid (hyaluronan, HA), a common component of synovial fluid and extracellular matrix, is a negatively charged, straight-chain glycosaminoglycan with high molar mass and is composed of alternating (1 → 4)-β linked D-glucuronic acid and (1 → 3)-β linked *N*-acetyl-D-glucosamine residues (Kogan, Soltes, Stern, & Gemeiner, 2007; Kogan, Soltes, Stern, Schiller, & Mendichi, 2008; Maharjan, Pilling, & Gomer, 2011; Segura et al., 2005; Stern, Kogan, Jedrzejas, & Soltes, 2007; Vercruysse, Ziebell, & Prestwich, 1999). It is present in almost all biological fluids and tissues (Kogan et al., 2007; Soltes, Brezova, Stankovska, Kogan, & Gemeiner, 2006). Hyaluronan's high molar mass and its associated unique viscoelastic and rheological properties predispose HA to play important physiological roles in living organisms (Ikegami-Kawai & Takahashi, 2002; Kogan et al., 2007). It already has been confirmed that HA fragments (HAFs) are involved in cell proliferation, differentiation, migration and signal transduction (Ikegami-Kawai & Takahashi, 2002; Kogan et al., 2007; Liu et al., 2004). Modified HA molecules already have found a broad range of biomedical applications even as they are used in cosmetics, pharmaceutics (drug delivery systems, therapeutic reagents) and specialty foods production (DeAngelis, Oatman, & Gay, 2003; Ikegami-Kawai & Takahashi, 2002; Kogan et al., 2007; Kühn, Raith, Sauerland, & Neubert, 2003; Liao, Jones, Forbes, Martin, & Brown 2005; Liu et al., 2004; Stern et al., 2007; Weindl, Schaller, Schäfer-Korting, & Korting, 2004).

Abbreviations: BApNA, Nα-benzoyl-DL-arginine 4-nitroanilide hydrochloride; EDC, 1-ethyl-3(3-dimethylaminopropyl)carbodiimide hydrochloride; EDTA, ethylenediaminetetraacetic acid; HA, hyaluronic acid; HAFs, hyaluronic acid fragments; MBC, magnetic macroporous bead cellulose; M_w, molar mass; NBC, nonmagnetic macroporous bead cellulose; ORD, oxidative–reductive depolymerization; PAGE, polyacrylamide gel electrophoresis; SEC-MALS, size exclusion chromatography/multi-angle light scattering; sulfo-NHS, N-hydroxysulfosuccinimide sodium salt; TBE, Tris/borate/EDTA; TEMED, *N,N,N,N*-tetramethylenediamine.

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Despite HA's uniform and simple primary structure, the exact and defined-molar-mass HAFs is clearly of critical importance due to the various biological effects of HAFs differing in size (Cantor & Nadkarni, 2006; Ikegami-Kawai & Takahashi, 2002; Kogan et al., 2007).

Today's technological approaches to producing HAFs with desired properties typically are based on enzymatic (Stern et al., 2007; Vercruysse, Ziebell, & Prestwich, 1999) or free radical (Matsumura, Herp, & Pigman, 1966; Pigman, Rizvi, & Holley, 1961; Soltes et al., 2007; Uchiyama, Dobashi, Ohkouchi, & Nagasawa, 1990) degrading processes, although we also can use methods that disturb the covalent bonds chemically and/or mechanically (Kubo, Nakamura, Takagaki, Yoshida, & Endo, 1993; Miyazaki, Yomota, & Okada, 2001). In many cases, however, the final products do not meet the strict requirements for pharmaceutical products. Polydispersity as well as reaction residues in the final product (e.g., reactive oxidants or enzymes of animal origin) are the main factors limiting the application of such products. The choice among the applied methods is made in practice according to the intended final purpose or use of the HAFs. Still, it is not always possible to guarantee the stability and homogeneity of those fragments produced.

Oxidative–reductive depolymerization (ORD) is a technique based upon the action of such substances as L-ascorbic acid (Harris, Herp, & Pigman, 1972; Pigman et al., 1961; Wong, Halliwell, Richmond, & Skowroneck, 1981), cupric chloride (Harris et al., 1972; Soltes et al., 2006), sodium hypochlorite (Hawkins & Davies, 1998; Uchiyama et al., 1990), photoexcited riboflavin (Frati et al., 1997), cysteine and ferrous salt, in the absence or presence of hydrogen peroxide (Akeel, Sibanda, Martin, Paterson, & Parsons, 2013; Harris et al., 1972; McNeil, Wiebkin, Betts, & Cleland, 1985; Roberts, Roughley, & Mort, 1989; Soltes et al., 2006). Unfortunately, this approach leads to fragments with significant polydispersity in size (McNeil et al., 1985). A frequently neglected fact, moreover, is that final products may be contaminated by such ingredients as metal ions, which not only can adversely affect the immune system but also pose a potential risk for undesirable further decrease in molar mass and subsequent change in the properties of HAFs (Kogan et al., 2007). Thus, subsequent purification processes are necessary.

Similarly to other biopolysaccharides, HA could be degraded chemically using acid or alkaline hydrolysis. However, chemical hydrolysis proceeds in a random fashion and gives rise to a statistical mixture of oligo- and monosaccharides (Kuo, Swann, & Prestwich, 1991; Tokita & Okamoto, 1995).

Depolymerization involving specific scission of the glycosidic linkages is recommended for preparing HAFs. The extent of the reaction can be easily controlled by means of pH, temperature and reaction time. Hyaluronidases, chondroitinases and hexosaminidases are specific endoglycosidases with the ability to degrade glycosaminoglycans efficiently (Frost, Csoka, & Stern, 1996; Furukawa et al., 2013; Highsmith, Garvin, & Chipman, 1975; Kreil, 1995; Maksimenko, Schechilina, & Tischenko, 2003; Stern et al., 2007). The animal origin of all of these combined with their high prices and risk of viral contamination has strongly limited their utilization in the medical, pharmaceutical and even cosmetic industries.

It should be noted that the choice of method for producing HAFs may affect the physicochemical properties and that the resulting biological properties could be slightly altered or even wholly changed. The biotechnology and pharmaceutical industries need to find the simplest possible manner of HAFs production. In particular, they need a process leading to large yields of molar-mass-defined fragments that are generated reliably, at low cost, within a reasonable time, and, of course, in the desired purity and with a narrow size distribution.

Our multiparametric approach that has not been described heretofore combines the ability of plant-derived papain to split the glycosidic bonds with advantages provided by a carrier to which papain is covalently captured and of ferrous ions incorporated into the macroporous material to accelerate the fragmentation of polymeric chains.

2. Materials and methods

2.1. Chemicals and reagents

Papain from papaya latex (EC 3.4.22.2, papainase, buffered aqueous suspension, 16–40 units mg^{-1}); hyaluronic acid sodium salt from *Streptococcus equi* ($1.5–1.8 \times 10^6$ g mol^{-1}); Nα-benzoyl-DL-arginine 4-nitroanilide hydrochloride (BApNA); ethylenediaminetetraacetic acid (EDTA); L-cysteine; saccharide acid-1,4-lacton; polysaccharide standard for electrophoresis select-HA LoLadder; acrylamide; *N,N′*-methylen-bis-acrylamide; and *N,N,N,N*-tetramethylenediamine (TEMED) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Sodium cyanoborohydride was obtained from Fluka (Buchs, Switzerland). Perloza MT 100 nonmagnetic macroporous bead cellulose (NBC; 80–100 μm) and Perloza MG magnetic macroporous bead cellulose (MBC; 80–100 μm) were supplied by Iontosorb (Ústí nad Labem, Czech Republic). SiMAG-Carboxyl microparticles (1 μm) were purchased from Chemicell GmbH (Berlin, Germany). Sucrose and Alcian blue 8GS were obtained from SERVA electrophoresis GmbH (Heidelberg, Germany) and all other chemicals were of reagent grade and produced by PENTA (Chrudim, Czech Republic).

2.2. Immobilization of papain on macroporous bead cellulose

Following Turková, with slight modification (Turková, 1993), 1 ml of Perloza MT 100 (nonmagnetic form, NBC) or Perloza MG (magnetic form, MBC) was washed 5 times with distilled water and then oxidized using 1 ml of 0.2 M $NaIO_4$. The mixture was stirred for 90 min in darkness at room temperature. Perloza was washed 10 times with 0.1 M phosphate buffer (pH 7.0) with 0.002 M EDTA. Thereafter, 4 mg of papain dissolved in the same buffer were added and the reaction mixture was stirred for 10 min at room temperature. Then 5 mg of sodium cyanoborohydride in 0.1 M phosphate buffer (pH 7.0) was added and the reaction was permitted to occur overnight at 4 °C. The carrier with immobilized papain (papain-MBC; papain-NBC) was washed 5 times with 0.1 M phosphate buffer (pH 7.0) with 0.002 M EDTA, then 5 times with 0.1 M phosphate buffer (pH 7.0) with 1.0 M NaCl to remove non-specifically adsorbed molecules, and again 5 times with 0.1 M phosphate buffer (pH 7.0) with 0.002 M EDTA. The enzyme reactor was stored in fresh 0.1 M phosphate buffer (pH 7.0) with 0.002 M EDTA at 4 °C. The presence of enzyme-active molecules bound to the surface of magnetic particles was verified by a previously published, standard method using the low-molecular-weight substrate BApNA (Bhardwaj et al., 1996; Gaertner & Puigserver, 1992).

2.3. Immobilization of papain on the SiMAG-Carboxyl microparticles

Covalent coupling of papain was performed by the common one-step carbodiimide method using zero-length cross-linker EDC and sulfo-NHS as described by Hermanson (1996), with slight modification. One milligram of SiMAG-Carboxyl was washed 5 times with 1 ml of 0.1 M phosphate buffer (pH 7.3) with 0.002 M EDTA. Then, 7.5 mg of EDC, 1.25 mg of sulfo-NHS and 3 mg of papain in the 0.1 M phosphate buffer (pH 7.3) with 0.002 M EDTA were added and the reaction mixture was stirred at room temperature for 6 h or at 4 °C overnight. Immobilized papain (papain-SiMAG)

was washed 10 times with 0.1 M phosphate buffer (pH 7.3) with 0.002 M EDTA, which was used also as storage buffer. The presence of enzyme-active molecules bound to the surface of magnetic particles was verified by a previously published, standard method using the low-molecular-weight substrate BApNA (Bhardwaj et al., 1996; Gaertner & Puigserver, 1992).

2.4. Determination of papain activity by low molecular-weight substrate BApNA

Determination of papain activity was carried out in accordance with Gaertner and Puigserver (1992) and Bhardwaj et al. (1996), with slight modification. Soluble or immobilized papain (25 μl of sedimented Perloza MT 100 or Perloza MG or 100 μg of SiMAG-Carboxyl) was first activated using 0.02 M L-cysteine in the presence of 0.004 M EDTA for 30 min at 37 °C. The reaction mixture with immobilized papain was stirred. After that, 1 ml of 0.1 M Tris–HCl buffer (pH 7.8) and 0.02 ml of 0.055 M BApNA dissolved in N,N-dimethylformamide were added and the mixture was stirred 30 min at 37 °C. The reaction was stopped with 0.2 ml of 30% acetic acid. The absorbance was measured spectrophotometrically at 405 nm.

2.5. Multiparametric fragmentation of hyaluronic acid

The quantity 1.5 mg of hyaluronic acid sodium salt from *S. equi* ($1.5–1.8 \times 10^6$ g mol^{-1}) was dissolved in 1 ml 0.1 M phosphate buffer (pH 7.0) with 0.1 M NaCl and 1.5 mM saccharide acid-1,4-lacton. The HA solution was stirred overnight at 4 °C for swelling.

One milliliter of sedimented carrier with immobilized papain was washed 5 times with the buffer of composition mentioned above. After the washing steps, 1 ml of swelled HA diluted in 1 ml of the same buffer was mixed with 1 ml of washed carrier. After incubation at 37 °C (using different incubation intervals) under gentle stirring, the supernatant was then separated and HAFs were analyzed by native polyacrylamide gel electrophoresis (PAGE) with specific Alcian blue-silver staining and using size-exclusion chromatography/multi-angle light scattering (SEC-MALS). All steps were carried out at atmospheric pressure.

2.6. Native PAGE analysis of HA molecules

Native PAGE analysis followed by the specific Alcian blue with silver staining was performed according to a working procedure that was adapted for our conditions by combining previously published methods (Cowman et al., 1984; Ikegami-Kawai & Takahashi, 2002; Min & Cowman, 1989). Polyacrylamide gel containing 15% acrylamide and 0.5% N,N-methylenebisacrylamide in 0.1 M TBE buffer (0.1 M Tris/0.1 M borate/0.001 M EDTA; pH 8.3) was used as a separating gel while a polyacrylamide gel containing 5% acrylamide and 0.16% N,N-methylenebisacrylamide in 0.1 M TBE buffer was used as a stacking gel. Electrophoretic separation was performed at 4 °C first at 125 V and 20 mA/gel for 20 min and then at 200 V and 40 mA/gel for approximately 45 min. The specific Alcian blue and silver staining method was employed for visualizing HAFs. After PAGE separation, the gel with separated HAFs was fixed in 0.05% aqueous solution of Alcian blue (which needs to be boiled before use) for 30 min in darkness. After destaining in water (8 times, 5 min each with stirring), the gel with separated HAFs was subjected to silver staining. The gel was placed in 0.03 M potassium dichromate with 0.03 M nitric acid for 5 min and washed with distilled water (6 times, 5 min each). After that, the gel was soaked in 0.01 M silver nitrate for 20 min and then washed with distilled water. The fragments of HA in gel were immediately developed using 0.3 M sodium carbonate with 0.02% formaldehyde. The reaction was stopped with 5% aqueous acetic acid solution. The gel images were captured using a digital camera.

2.7. SEC-MALS analysis of molar-mass-defined HA molecules

Molecular weights (M_w) of hyaluronic acid or hyaluronan fragments were assigned using SEC-MALS. Samples were dissolved overnight in a mobile phase (aqueous 50 mM sodium phosphate containing 0.02% sodium azide). The chromatographic system consisted of an Alliance e2695 separation module, 2414 refractive index detector and 2489 UV-VIS detector (Waters, Milford, MA), and a miniDAWN TREOS light scattering photometer (Wyatt Technology Corporation, Santa Barbara, CA). The injection volume was 100 μl. Each sample was filtered through a 0.22 μm MS nylon syringe filter. The flow rate of the mobile phase was 0.8 ml min^{-1}. Data acquisition and molecular weight calculations were performed using ASTRA software (version 5.3.4, Wyatt Technology Corporation). The specific refractive index increment of 0.155 ml g^{-1} was used for HA (Podzimek, Hermannova, Bilerova, Bezakova, & Velebny, 2010).

3. Results and discussion

The goal was to develop a technology platform for efficient and controllable production of HAFs. As already noted, physical as well as chemical methods of HA polymers degradation lead to producing fragments with high size polydispersity while the enzymatic methods using hyaluronidase of animal origin significantly increase the price of the final products. Additionally, their possible contamination by viral agents can be a risk for biomedical and biotechnological applications. Accordingly, the use of a plant-derived enzyme with the ability to split the glycosidic bond is an eligible alternative.

The ability of such proteases as pepsin (Kumar, Varadaraj, & Tharanathan, 2007; Tao, Wei, Mao, Zhang, & Xia, 2005), papain (Lin, Wang, Xue, & Ye, 2002; Muzzarelli, Terbojevich, Muzzarelli, & Francescangeli, 2002; Terbojevich, Cosani, & Muzzarelli, 1996; Vishu Kumar, Varadaraj, Lalitha, & Tharanathan, 2004;), pronase E (Vishu Kumar & Tharanathan, 2004), bromelain (Muzzarelli et al., 2002), ficin (Pantaleone, Yalpani, & Scollar, 1992), and pancreatin (Yalpani & Pantaleone, 1994) also to split the glycosidic bonds of polysaccharides has been demonstrated many times. In addition, the use of an enzyme in an immobilized form allows continuous monitoring process of HA fragmentation and stopping the reaction at a defined time point. Papain, as a plant-derived enzyme, appears to offer a valuable alternative with an acceptable price and bearing no risk of viral or bacterial contamination.

The first phase of this study was to develop the carrier with immobilized papain. Various types of magnetic microparticles originating in natural or synthetic materials and characterized by high stability in a wide range of reaction conditions were tested. In order to achieve maximum activity and stability of the carrier, the reaction conditions of enzyme immobilization were optimized. The covalent binding of papain onto macroporous bead cellulose was performed via a Schiff base formation between the primary amine groups of the enzyme and the carbonyl functional groups of oxidized cellulose beads. The one-step carbodiimide technique with zero-length cross-linker EDC in combination with sulfo-NHS was applied for papain immobilization to the SiMAG-Carboxyl microparticles as an alternative nonporous carrier. The efficiency of the immobilization procedure, the total amount of papain, and the papain's hydrolytic activity were evaluated by the standard method using low-molecular-mass BApNA substrate.

The carriers' operational characteristics and storage stability were repeatedly tested. Papain immobilized on the magnetic form of macroporous bead cellulose shows high stability with repeated

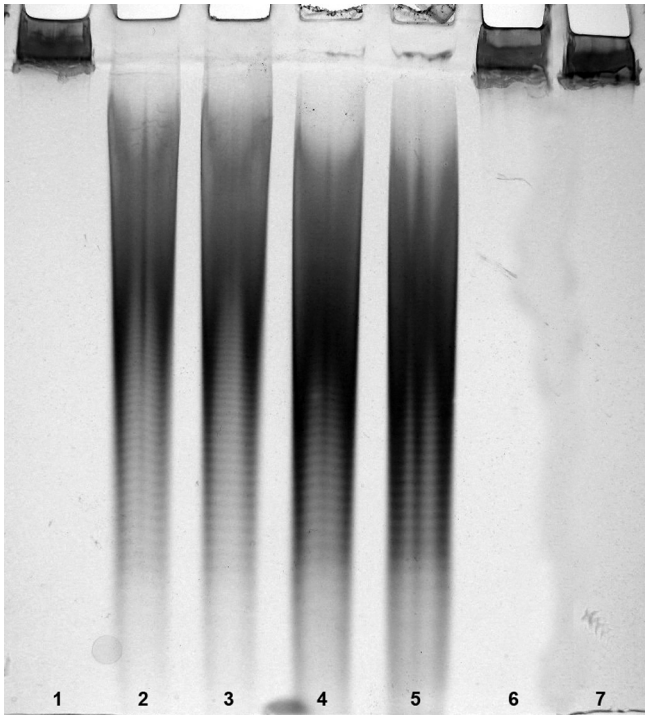

Fig. 1. Native PAGE analysis of HA fragments obtained using immobilized papain (under stirring at room temperature) on various carriers: 1 – native HA without fragmentation, 2 – MBC for 5 h, 3 – MBC for 10 h, 4 – MBC for 24 h, 5 – MBC for 48 h, 6 – SiMAG-Carboxyl for 48 h, 7 – NBC for 48 h; 15% PAA separation gel, 5% focusing PAA gel and Alcian blue-silver staining.

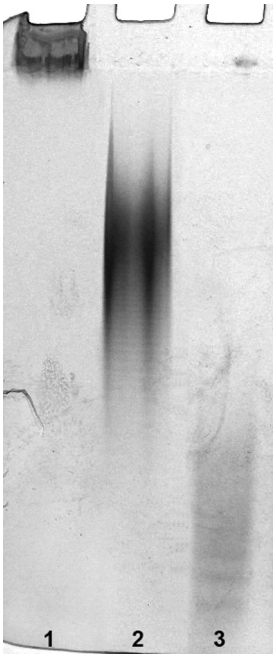

Fig. 2. Native PAGE analysis of HA molecules after incubation while using MBC without or in the presence of ferrous ions (under stirring at 37 °C, 48 h): 1 – MBC, 2 – MBC and 10 mM Fe^{2+}, 3 – MBC and 100 mM Fe^{2+}; 15% PAA separation gel, 5% focusing PAA gel and Alcian blue-silver staining.

use, as well as during long-term storage. Based on these results, we confirmed that the carrier can be used at least eight times without significant decrease in its activity. Moreover, the bioactive carrier could be stored for more than one month (data not shown) without any significant decline in its activity.

The efficiency of HA polymer chains fragmentation was evaluated by native PAGE, and SEC-MALS was applied for precisely estimating molar mass distribution and monitoring the kinetics of HA polymer degradation.

Fig. 1 demonstrates the efficiency of HA degradation by papain immobilized on 3 carriers differing in certain characteristics. These are magnetic macroporous bead cellulose (papain-MBC) at different times (lanes 2–5), SiMAG (papain-SiMAG) (lane 6), and nonmagnetic macroporous bead cellulose (papain-NBC) (lane 7). After 24 h of incubating HA with carriers, we observed almost zero fragmentation activity of papain immobilized to nonporous SiMAG microparticles (Fig. 1, lane 6) and essentially the same inactivity was obtained from papain immobilized to the nonmagnetic form of macroporous bead cellulose (Fig. 1, lane 7). By contrast, papain-MBC effectively degraded the HA polymers. This proves that the molecular weight of HA polymer chains decrease with increasing incubation time (Fig. 1, lanes 2–5). The efficient fragmentation was confirmed by the presence of discrete ladder-like bands in the gel, which indicate HAFs differing in the numbers of their disaccharide units.

Next, experiments were performed to confirm the supposed positive effect of free soluble ferrous ions added to the reaction medium during the HA fragmentation. Obtained results clearly document the well-defined auxiliary effect of metal ions in relation to fragmentation efficiency (Fig. 2). Nevertheless, for efficient HA fragmentation high concentration of ferrous ions (namely 100 mM) is necessary which leads to an undesired contamination of the final product and thus it is not appropriate for large scale biotechnological exploitation. For this purpose the use of macroporous bead cellulose with accessible ferrous ions fixed within the highly porous particles is convenient option.

Additionally we had supposed there to be also a mechanical factor incurred by porous character of MBC involved in the HA fragmentation. The experiments evaluated by native PAGE are presented in Fig. 3. Gel A is for samples of HA fragmented over time (3.5–49 h) by naked MBC and gel B is for samples of HA fragmented over time by the carrier MBC with immobilized papain. Gel A clearly shows the synergy effect of mechanical disentanglement and oxidative–reductive depolymerization reaction due to the carrier that was the magnetic form of macroporous bead cellulose. Even after 32 h of incubation, we still can observe a large amount of long polymer chain that is not penetrating into the gel (Fig. 3A, lanes 1–5). On the contrary, the multiparametric approach combining enzymatic, oxidative–reductive and mechanical effects (gel B) provide intermediate fragments of HA even within 7 h, and their size is reduced with the increasing incubation time. While the presented results definitively prove the positive effect of papain on HA degradation, it is nevertheless important to emphasize that the highest fragmentation efficiency can be obtained using papain along with the supporting effect due to the porous character of the beads, which are produced from a biocompatible, highly hydrophilic material saturated with ferric and ferrous ions accessible for catalysis.

Finally, based on SDS-PAGE analysis used for continuous optimization steps, selected fractions showing the most effective degradation of HA molecules were analyzed by SEC-MALS to obtain more detailed information about degradation efficiency and its dynamics in time and also size polydispersity rate of HAFs. The change of molar mass distribution in the course of degradation is shown in Fig. 4. The distribution curves show no major change in the distribution pattern and polydispersity during time course degradation.

Fig. 5, which show the reaction kinetics, characterized by significant decline in M_w at the beginning of degradation and an approximately linear decrease from the reaction time of about 10 h. The most effective fragmentation that is observed in the initial

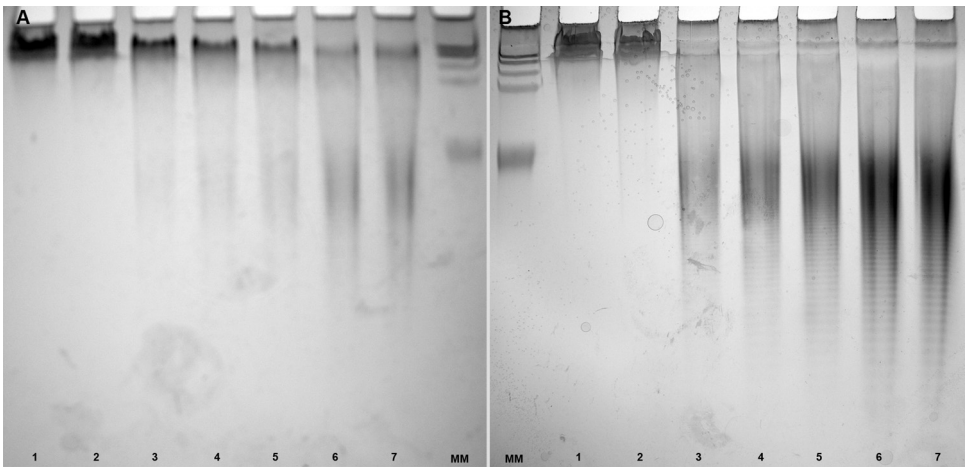

Fig. 3. Native PAGE analysis of HA fragments acquired using MBC without (A) and with (B) papain (papain-MBC) at varying time periods (under stirring at 37 °C, 48 h): (A) 1 – 3.5 h, 2 – 7 h, 3 – 24 h, 4 – 28 h, 5 – 32 h, 6 – 48 h, 7 – 49 h, MM – select-HA LoLadder, (B) MM – select-HA LoLadder, 1 – 3.5 h, 2 – 7 h, 3 – 24 h, 4 – 28 h, 5 – 32 h, 6 – 48 h, 7 – 49 h; 15% PAA separation gel, 5% focusing PAA gel and Alcian blue-silver staining.

phase of the reaction is in accordance with the depolymerization efficiency described by Terbojevich et al. (1996). Mentioned plot allow simple estimation of the fragmentation time needed to obtain HA fragments of requested *Mw*.

In conclusion, by covalent immobilization of the enzyme papain onto magnetic macroporous particles 80–100 μm in size and made from nontoxic and even biocompatible cellulose we prepared an efficient carrier with multiparametric effect promoting the degradation of hyaluronan polymer molecules. This reusable heterogeneous and low-cost biocatalyst enables obtaining a final product in the desired purity in comparison with animal-source enzyme hyaluronidase. Bead cellulose with a defined porosity and permeated by metal ions seems to be the best carrier for mechanical disentanglement and bursting of long HA polymer chains while promoting yields of shorter molecules of interest. The magnetic carrier used in this study contains ferrous and ferric ions homogeneously distributed and strongly fixed within the cellulose. It is known that iron oxides can interact with components of the liquid medium and thereby support long polymer chain degradation through so-called oxidative–reductive depolymerization reaction (ORD) via a mechanism that has been thoroughly described (Uchiyama et al., 1990).

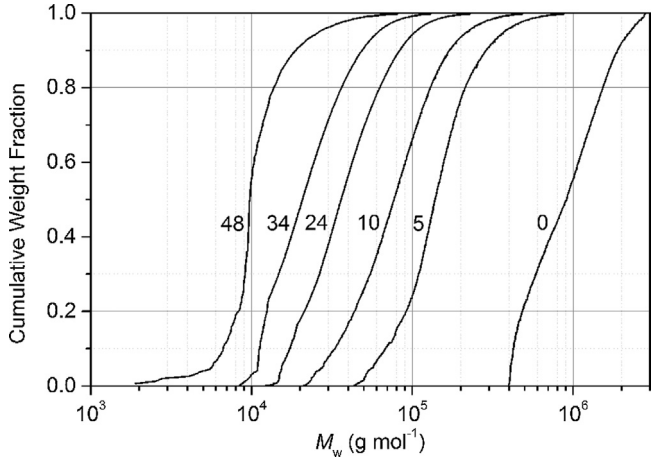

Fig. 4. Chromatogram from size-exclusion chromatography/multi-angle laser light scattering (SEC-MALS) analysis, which proves change in the molar mass distribution of hyaluronan fragments in the course of degradation; the numerical values close to the curve correspond to the reaction time. Hyaluronic acid ($1.5–1.8 \times 10^6$ g mol^{-1}) was fragmented with papain-MBC under stirring at 37 °C for 48 h.

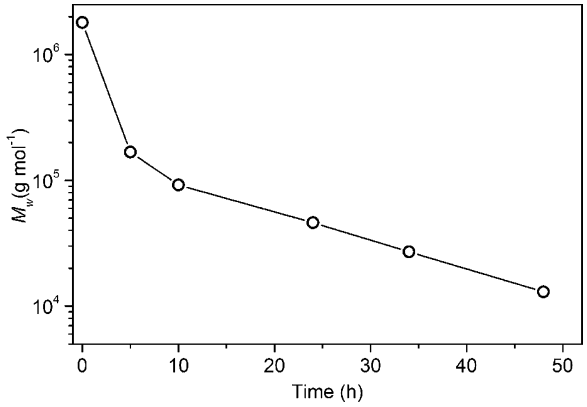

Fig. 5. Weighted-average molar mass (M_w) as a function of degradation time determined using size-exclusion chromatography/multi-angle laser light scattering (SEC-MALS) analysis. Hyaluronic acid ($1.5–1.8 \times 10^6$ g mol^{-1}) fragmented by papain-MBC under stirring at 37 °C for 48 h.

We confirmed that the co-interaction of these three factors (mechanical disentanglement of the long polymer chain due to the macroporous character of used carrier, ORD caused by iron oxides inside the pores of MBC, together with hydrolytic activity of immobilized papain) leads to the efficient and controllable fragmentation that provides molar-mass-defined HAFs.

4. Conclusion

In summary, we have demonstrated an enhanced multiparametric approach for safe and controlled production of molar-mass-defined HAFs with relatively low polydispersity. We do not question the fact that enzyme fragmentation using hyaluronidase is more efficient and rapid, but our multiparametric approach fully solves the problem of contamination in final product while the size of the fragments is readily adjustable by means of quenching the reaction at a defined time point. We confirmed that papain immobilized onto macroporous microparticles, and where iron oxides are fixed within the pores of the microparticles, combined with the mechanical impact of soft cellulose beads and the additional effect of reactive oxygen significantly accelerated the cleavage process.

We assume that a magnetically stabilized fluidized bed within which the continuous and dynamic contact of the carrier with viscous HA molecules driven by an outside magnetic field could

also enhance the fragmentation efficiency. In this case, such an arrangement can be used even for large-scale production in the pharmaceutical or cosmetic industry.

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References

Akeel, A., Sibanda, S., Martin, S. W., Paterson, A. W. J., & Parsons, B. J. (2013). Chlorination and oxidation of heparin and hyaluronan by hypochlorous acid and hypochloride anions: Effect of sulfate groups on reaction pathways. *Free Radical Biology and Medicine*, *56*, 72–88.

Bhardwaj, A., Lee, J., Glauner, K., Ganapathi, S., Bhattacharyya, D., & Butterfield, D. A. (1996). Biofunctional membranes: An EPR study of active site structure and stability of papain non-covalently immobilized on the surface of modified poly(ether) sulfone membranes through the avidin-biotin linkage. *Journal of Membrane Science*, *119*, 241–252.

Cantor, J. O., & Nadkarni, P. P. (2006). Hyaluronan: The Jekyll and Hyde molecule. *Inflammation & Allergy: Drug Targets*, *5*(4), 257–260.

Cowman, M. K., Slahetka, M. F., Hittner, D. M., Kim, J., Forino, M., & Gadelrab, G. (1984). Polyacrylamide-gel electrophoresis and Alcian Blue staining of sulphated glycosaminoglycan oligosaccharides. *Biochemical Journal*, *221*, 707–716.

DeAngelis, P. L., Oatman, L. C., & Gay, D. F. (2003). Rapid chemoenzymatic synthesis of monodisperse oligosaccharides with immobilized enzyme reactors. *Journal of Biological Chemistry*, *278*, 35199–35203.

Frati, E., Khatib, A.-M., Front, P., Panasyuk, A., Aprile, F., & Mitrovic, D. R. (1997). Degradation of hyaluronic acid by photosensitized riboflavin in vitro. Modulation of the effect by transition metals, radical quenchers, and metal chelators. *Free Radical Biology and Medicine*, *22*(7), 1139–1144.

Frost, G. I., Csoka, T., & Stern, R. (1996). The hyaluronidases: A chemical, biological and clinical overview. *Trends in Glycoscience and Glycotechnology*, *8*, 419–434.

Furukawa, T., Arai, M., Garcia-Martin, F., Amano, M., Hinou, H., & Nishimura, S.-I. (2013). Glycoblotting-based high throughput protocol for the structural characterization of hyaluronan degradation products during enzymatic fragmentation. *Glycoconjugate Journal*, *30*(2), 171–182.

Gaertner, H. F., & Puigserver, A. J. (1992). Increased activity and stability of poly(ethylene glycol)-modified trypsin. *Enzyme and Microbial Technology*, *14*, 150–155.

Harris, M. J., Herp, A., & Pigman, W. (1972). Metal catalysis in the depolymerization of hyaluronic acid by autoxidants. *Journal of the American Chemical Society*, *94*(21), 7570–7572.

Hawkins, C. L., & Davies, M. J. (1998). Degradation of hyaluronic acid, poly- and monosaccharides, and model compounds by hypochlorite: Evidence for radical intermediates and fragmentation. *Free Radical Biology and Medicine*, *24*(9), 1396–1410.

Hermanson, G. T. (1996). *Bioconjugate technique*. San Diego: Academic Press (Part II – Bioconjugate reagents).

Highsmith, S., Garvin, J. H., Jr., & Chipman, D. M. (1975). Mechanism of action of bovine testicular hyaluronidase. *Journal of Biological Chemistry*, *250*(18), 7473–7480.

Ikegami-Kawai, M., & Takahashi, T. (2002). Microanalysis of hyaluronan oligosaccharides by polyacrylamide gel electrophoresis and its application to assay of hyaluronidase activity. *Analytical Biochemistry*, *311*, 157–165.

Kogan, G., Soltes, L., Stern, R., & Gemeiner, P. (2007). Hyaluronic acid: A natural biopolymer with a broad range of biomedical and industrial applications. *Biotechnology Letters*, *29*, 17–25.

Kogan, G., Soltes, L., Stern, R., Schiller, J., & Mendichi, R. (2008). Hyaluronic acid: Its function and degradation in in vivo systems. In Atta-ur-Rahman (Ed.), *Studies in natural products chemistry: Bioactive natural products. Part D* (vol. 34) (pp. 789–882). Amsterdam: Elsevier.

Kreil, G. (1995). Hyaluronidases: A group of neglected enzymes. *Protein Science*, *4*(9), 1666–1669.

Kubo, K., Nakamura, T., Takagaki, K., Yoshida, Y., & Endo, M. (1993). Depolymerization of hyaluronan by sonication. *Glycoconjugate Journal*, *10*(6), 435–439.

Kühn, A. V., Raith, K., Sauerland, V., & Neubert, R. H. H. (2003). Quantification of hyaluronic acid fragments in pharmaceutical formulations using LC-ESI–MS. *Journal of Pharmaceutical and Biomedical Analysis*, *30*, 1531–1537.

Kumar, B., Varadaraj, M., & Tharanathan, R. (2007). Low molecular weight chitosan – Preparation with the aid of pepsin, characterization and its bacterial activity. *Biomacromolecules*, *8*(2), 566–572.

Kuo, J. W., Swann, D. A., & Prestwich, G. D. (1991). Chemical modification of hyaluronic acid by carbodiimides. *Bioconjugate Chemistry*, *2*(4), 232–241.

Liao, Y.-H., Jones, S. A., Forbes, B., Martin, G. P., & Brown, M. B. (2005). Hyaluronan: Pharmaceutical characterization and drug delivery. *Drug Delivery*, *12*(6), 327–342.

Lin, H., Wang, H., & Xue Ch Ye, M. (2002). Preparation of chitosan oligomers by immobilized papain. *Enzyme and Microbial Technology*, *31*, 588–592.

Liu, H., Mao, J., Yao, K., Yang, G., Cui, L., & Cao, Y. (2004). A study on a chitosan–gelatin–hyaluronic acid scaffold as artificial skin *in vitro* and its tissue engineering applications. *Journal of Biomaterials Science, Polymer Edition*, *15*, 25–40.

Maharjan, A. S., Pilling, D., & Gomer, R. H. (2011). High and low molecular weight hyaluronic acid differentially regulate human fibrocyte differentiation. *PLOS ONE*, *6*(10), e26078.

Maksimenko, A. V., Schechilina, Y. V., & Tischenko, E. G. (2003). Role of the glycosaminoglycan microenvironment of hyaluronidase in regulation of its endoglycosidase activity. *Biochemistry (Moscow)*, *68*(8), 862–868.

Matsumura, G., Herp, A., & Pigman, W. (1966). Depolymerization of hyaluronic acid by autoxidants and radiations. *Radiation Research*, *28*(4), 735–752.

McNeil, J. D., Wiebkin, O. W., Betts, W. H., & Cleland, L. G. (1985). Depolymerisation products of hyaluronic acid after exposure to oxygen-derived free radicals. *Annals of the Rheumatic Diseases*, *44*, 780–789.

Min, H., & Cowman, M. K. (1989). Combined Alcian blue and silver staining of glycosaminoglycans in polyacrylamide gels: Application to electrophoretic analysis of molecular weight distribution. *Analytical Biochemistry*, *155*, 275–285.

Miyazaki, T., Yomota, C., & Okada, S. (2001). Ultrasonic depolymerization of hyaluronic acid. *Polymer Degradation and Stability*, *74*, 77–85.

Muzzarelli, R. A. A., Terbojevich, M., Muzzarelli, C., & Francescangeli, O. (2002). Chitosans depolymerized with the aid of papain and stabilized as glycosylamines. *Carbohydrate Polymers*, *20*, 69–78.

Pantaleone, D., Yalpani, M., & Scollar, M. (1992). Unusual susceptibility of chitosan to enzymic hydrolysis. *Carbohydrate Research*, *237*, 325–332.

Pigman, W., Rizvi, S., & Holley, H. L. (1961). Depolymerization of hyaluronic acid by the ORD reaction. *Arthritis Rheumatology*, *4*, 240–252.

Podzimek, S., Hermannova, M., Bilerova, H., Bezakova, Z., & Velebny, V. (2010). Solution properties of hyaluronic acid and comparison of SEC-MALS-VIS data with off-line capillary viscometry. *Journal of Applied Polymer Science*, *116*, 3013–3020.

Roberts, C. R., Roughley, P. J., & Mort, J. S. (1989). Degradation of human proteoglycan aggregate induced by hydrogen peroxide. *Biochemical Journal*, *259*, 805–811.

Segura, T., Anderson, B. C., Chung, P. H., Webber, R. E., Shull, K. R., & Shea, L. D. (2005). Crosslinked hyaluronic acid hydrogels: A strategy to functionalize and pattern. *Biomaterials*, *26*, 359–371.

Soltes, L., Brezova, V., Stankovska, M., Kogan, G., & Gemeiner, P. (2006). Degradation of high-molecular-weight hyaluronan by hydrogen peroxide in the presence of cupric ions. *Carbohydrate Research*, *341*, 639–644.

Soltes, L., Kogan, G., Stankovska, M., Mendichi, R., Rychly, J., Schiller, J., et al. (2007). Degradation of high-molar-mass hyaluronan and characterization of fragments. *Biomacromolecules*, *8*(9), 2697–2705.

Stern, R., Kogan, G., Jedrzejas, M. J., & Soltes, L. (2007). The many ways to cleave hyaluronan. *Biotechnology Advances*, *25*, 537–557.

Tao, H., Wei, W., Mao, Y., Zhang, S., & Xia, J. (2005). Study on degradation characteristics of chitosan by pepsin with piezoelectric quartz crystal impedance analysis technique. *Analytical Sciences*, *21*, 1057–1061.

Terbojevich, M., Cosani, A., & Muzzarelli, R. A. A. (1996). Molecular parameters of chitosans depolymerized with the aid of papain. *Carbohydrate Polymers*, *29*, 63–68.

Tokita, Y., & Okamoto, A. (1995). Hydrolytic degradation of hyaluronic acid. *Polymer Degradation and Stability*, *42*(2), 269–273.

Turková, J. (1993). *Bioaffinity chromatography*. Amsterdam: Elsevier (Chapter 6).

Uchiyama, H., Dobashi, Y., Ohkouchi, K., & Nagasawa, K. (1990). Chemical change involved in the oxidative reductive depolymerization of hyaluronic acid. *Journal of Biological Chemistry*, *265*(15), 7753–7759.

Vercruysse, K. P., Ziebell, M. R., & Prestwich, G. D. (1999). Control of enzymatic degradation of hyaluronan by divalent cations. *Carbohydrate Research*, *318*, 26–37.

Vishu Kumar, A. B., & Tharanathan, R. N. (2004). A comparative study on depolymerization of chitosan by proteolytic enzymes. *Carbohydrate Polymers*, *58*, 275–283.

Vishu Kumar, A. B., Varadaraj, M. C., Lalitha, R. G., & Tharanathan, R. N. (2004). Low molecular weight chitosans: Preparation with the aid of papain and characterization. *Biochimica et Biophysica Acta*, *1670*, 137–146.

Weindl, G., Schaller, M., Schäfer-Korting, M., & Korting, H. C. (2004). Hyaluronic acid in the treatment and prevention of skin diseases: Molecular biological, pharmaceutical and clinical aspects. *Skin Pharmacology and Physiology*, *17*, 207–213.

Wong, S. F., Halliwell, B., Richmond, R., & Skowroneck, W. R. (1981). The role of superoxide and hydroxyl radicals in the degradation of hyaluronic acid induced by metal ions and by ascorbic acid. *Journal of Inorganic Biochemistry*, *14*(2), 127–134.

Yalpani, M., & Pantaleone, D. (1994). An examination of the unusual susceptibilities of aminoglycans to enzymatic hydrolysis. *Carbohydrate Research*, *256*, 159–175.