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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 This atypical and yet unpublished highly efficient multiparametric approach allows enhanced HA fragmentation for easily and safely producing molar-mass-defined HA fragments with narrow size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)