



Characterisation of separated end hyaluronan oligosaccharides from leech hyaluronidase and evaluation of angiogenesis

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ABSTRACT

Hyaluronan oligosaccharides (o-HAs), especially saturated o-HAs, have attracted intensive attention due to their potential applications in medical treatments. In this study, the hydrolysis process of leech hyaluronidase (LHase) towards the hyaluronan was investigated by HPLC and HPLC/ESI-MS. The proportions of hyaluronan tetrasaccharide (HA4) with hexasaccharide (HA6), end products, were illustrated to have a relationship with the amount of LHase. Higher yield of HA4 was achieved with higher activity of LHase. After optimisation of the packing resin and operation parameters (balanced pH, elution concentration, elution volume and elution flow rate), the highly pure HA4 and HA6 were efficiently separated and prepared by combining ion exchange Q-Sepharose Fast Flow and size exclusion column chromatography. Compared with o-HAs (average *Mr* of 4000 Da), HA4 and HA6 were demonstrated to show higher activity for promoting angiogenesis, which was similar with the corresponding HA4 and HA6 produced by bovine testicular hyaluronidase. The pure HA4 and HA6 that prepared from LHase will attract intensive studies and be used in potential applications in near future.

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1. Introduction

Hyaluronan (HA) is a linear polysaccharide composed of repeating β -1,3-linked *N*-acetyl-D-glucosamine (GlcNAc) and β -1,4-linked D-glucuronic acid (GlcA) disaccharide units, which is widely present in the connective tissues and body fluids of vertebrates (Hascall, Weigel, & Toole, 2016; Kakehi, Kinoshita, & Yasueda, 2003; Kogan, Soltes, Stern, & Gemeiner, 2007; Liu, Sun, Heggeness, Yeh, & Luo, 2004; Vigetti et al., 2014; Viola et al., 2015). Recently, hyaluronan oligosaccharides (o-HAs), especially those produced by bovine testicular hyaluronidase (BTH), have attracted intensive attention basing on their size-dependent functions. Large molecular weight HA which has been demonstrated to maintain water balance and keep issues lubricated is uniquely viscoelastic, being widely used in medicine, cosmetics, food and other industries (Ferguson, Roberts, Moseley, Griffiths, & Thomas, 2011; Meyer & Stern, 1994; Robert, 2015). However, o-HAs (molecular mass < 10,000 Da) can inhibit multidrug resistance to tumours, induce angiogenesis and cause inflammation (Ghatak, Misra, &

Toole, 2002; Ghosh, Hoselton, Dorsam, & Schuh, 2015; Stern, Asari, & Sugahara, 2006; Toole, Ghatak, & Misra, 2008; West & Kumar, 1989).

There are many ways to cleave HA, including on an enzymatic, free radical and chemical basis (Bezáková et al., 2008; Blundell & Almond, 2006; Drimalova, Velebny, Sasinkova, Hromadkova, & Ebringerova, 2005; Stern, Kogan, Jedrzejewski, & Soltes, 2007). HA degrading enzymes are widely used, and have been divided into three groups, represented by BTH (EC 3.2.1.35), leech hyaluronidase (LHase) (EC 3.2.1.36) and microbial hyaluronidase (EC 4.2.2.1) basing on substrate specificity (Meyer, 1971). Saturated o-HAs are produced by BTH and LHase (Linker, Meyer, & Hoffman, 1956). BTH is an endo- β -N-acetylglucosaminidase that degrades HA into (GlcA-GlcNAc)₂ and (GlcA-GlcNAc)₃ by cleaving the β -1,4-N-acetylhexosaminide bonds, while LHase is characterised as a β -endoglucuronidase that degrades HA into (GlcNAc-GlcA)₂ and (GlcNAc-GlcA)₃ by cleaving the β -1,3-glucuronide bonds (Linker, Hoffman, & Meyer, 1957; Meyer & Rapport, 1952). Compared with BTH, LHase has no activity towards chondroitin and chondroitin-4- or -6-sulfate, and does not have transglycosylation properties (Hoffman, Meyer, & Linker, 1956; Linker, Meyer, & Hoffman, 1960). Moreover, the activity of LHase is higher than BTH, even though the content of LHase is 1/10 of BTH (Hovingh & Linker, 1999). As a

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result, o-HAs with glucuronic acid at the reducing end are able to be produced by LHase more effectively.

In the current reports, the preparation, purification and characteristics of o-HAs produced by BTH have been well studied (Grimshaw et al., 1996; Kogan et al., 2007; Linker et al., 1956; Meyer & Rapport, 1952). However, the o-HAs prepared by LHase were rarely studied. Recently, by combining the strategies of protein engineering and high-density culture, a high activity LHase (8.42×10^5 U/mL) has been prepared with the yeast *Pastoris pichia* using the secretory expression system, which has produced o-HAs more effectively (Jin, Kang, Zhang, Du, & Chen, 2014). Moreover, o-HAs with low polydispersity produced by LHase were achieved by Yuan et al. (2015). However, further quantitative investigation was required to reveal the hydrolysis process and control the proportions of the end products HA4 and HA6. Moreover, development of an effective and efficient separation method to prepare pure HA4 and HA6 was also imperative for further studies. In addition, it was still completely unknown whether HA4 and HA6 produced by LHase have similar promotion of angiogenesis comparing to those from BTH (Cui et al., 2009; West, Hampson, Arnold, & Kumar, 1985).

2. Materials and methods

2.1. Chemicals and reagents

HA of high purity (from *Streptococcus zooepidemics*, average M_r of 1.21×10^6 Da) was purchased from Sangon Biotech Co. Ltd (Shanghai, China). LHase was prepared from the recombinant *P. pastoris* (Jin et al., 2014) with a specificity value of 4.0×10^5 U/mL. BTH (type 1-S) and VEGF were obtained from Sigma-Aldrich (St Louis, MO, USA). Growth factor reduce-Matrigel™ (Product #356231) was purchased from BD Biosciences (San Jose, CA, USA). Masson dye was obtained from Nanjing Jiancheng Biological Technology, Inc (Nanjing, China). Other reagents were obtained from Sinopharm Chemical Reagent Co. Ltd.

2.2. Preparation of o-HAs

The thermal stability of LHase has been investigated (Jin et al., 2014) and LHase kept high activity when incubated at 38 °C for several hours. As a result, one gram HA was hydrolysed by a series of doses of LHase (4000–32,000 U/mL) in 100 mL distilled water with different incubation time at 38 °C, respectively. 1 mL sample was taken out at 0.5, 1, 2, 4, 8, 12, 16, 24, 32 and 40 h, and the enzymatic treatment for HA was stopped by boiling the samples for 20 min. The samples were centrifuged by ultracentrifugal filters with a cut-off molecular weight of 3000 Da (from Millipore, Billerica, MA, USA) to separate o-HAs with those molecular weight larger than 3000 Da, and the ultracentrifugation filtrate were collected (Schmaus et al., 2014). o-HAs of different incubation time were then lyophilised, and dissolved in 1 mL of distilled water to give the final products. o-HAs with large molecular weight were obtained by four grams HA treated with 4.0×10^4 U/mL LHase in 100 mL distilled water at 45 °C for a few hours. o-HAs (M_r = 4000 Da) were qualitatively analysed by HPLC-SEC-RI (Yuan et al., 2015). Furthermore, HA was hydrolysed by BTH as the previously reported (Tawada et al., 2002). Pure HA4 and HA6 were obtained by combining ion exchange Dowex 1 × 2 column and Sephadex G-10 column.

2.3. Optimisation of separation of HA4 and HA6

2.3.1. Optimisation of ion exchangers

The method 'small tube test' was used to optimise ion exchangers (Tian, 2006). The resin were balanced by 0.05 mol/L pH 8 Tris-HCl after pretreatment according the instructions. o-HAs were

then added into small tubes which contained 1 gram resin including 640, DEAE, D730, D750, Q-Sepharose Fast Flow (from Hangzhou ZG Co. Ltd, Hangzhou, China) and Dowex 1 × 2 (from Sigma-Aldrich, St Louis, MO, USA), respectively. The adsorption percentages were calculated through determination of reducing sugar in supernatant by DNS (Zhang, Wang, & Zhou, 2015). Then the resin were eluted by 1 mol/L NaCl (pH 8), and desorption percentages were also calculated through determination of reducing sugar in supernatant.

2.3.2. Optimisation of balanced pH and concentration of eluent

The appropriately balanced pH was chosen from pH 6–8.5 and the optimal concentration of eluent was chosen from 0 to 1 mol/L NaCl (pH 8). The adsorption and desorption percentages were calculated as the method mentioned in Section 2.3.1.

2.3.3. Separation and purification of HA4 and HA6

AKTA Pure equipped with low-pressure anion exchange chromatography on a HiTrap 16/10 Q FF column (column volume: 20 mL, from GE, USA) was used to separate HA4 with HA6 in this study. The o-HAs produced by LHase (16,000 U/mL) for 24 h were injected in the column to the breakthrough point. The column was then balanced by buffer A (0.05 mol/L, pH 8 Tris-HCl) for 10 column volume (CV) before the column was eluted by a linear gradient of 0–0.2 mol/L NaCl (pH 8) for 4, 6, 8 and 10 CV, respectively under a constant flow rate (5 mL/min). The eluent was monitored at 210 nm. The fraction collected of each chromatographic peak was desalted by Sephadex G-10 and lyophilised to give the final products after concentration. Each size-uniformed o-HA fraction was qualitatively analysed by HPLC.

2.4. HPLC analysis and construction of standard curve for HA4 and HA6

HPLC equipped with a YMC-Pack Polyamine II column (4×250 mm, S-5 μ m, 12 nm) was performed on 0.1 mol/L of $\text{NH}_4\text{H}_2\text{PO}_4$ solution containing 10% acetonitrile for protection under 0.5 mL/min for 20–65 min at 30 °C. The sample (25 μ L) was injected and the eluent was monitored at 210 nm (Kakizaki, Ibori, Kojima, Yamaguchi, & Endo, 2010; Tawada et al., 2002).

Pure HA4 and HA6 produced by LHase were analysed by HPLC and the standard peak-concentration curves were drawn. The peak area of HA4 and HA6 displayed a linear relationship with the concentration of glucuronic acid of HA4 and HA6, respectively. The standard curve for HA4 was given by: $y = 15,260x + 115,141$, $R^2 = 0.9998$, where x (g/L) is the concentration of glucuronic acid in the sample and y is the peak area of HA4; the standard curve for HA6 was given by: $y = 13049x + 3056.3$, $R^2 = 0.9999$, where x (g/L) is the concentration of glucuronic acid in the sample and y is the peak area of HA6. The concentrations of glucuronic acid in the pure HA4 and HA6 were analysed using carbazole assay (Song, Im, Kang, & Kang, 2009).

2.5. HPLC/ESI-MS analysis

The representative o-HAs were dissolved in distilled water at a concentration of 1–5 g/L. HPLC separation was performed on a BEH amide column (2.1 × 100 mm, 1.7 μ m) (from Waters, Inc.) at 45 °C. Eluent A was 0.1 mol/L of ammonium formate and eluent B was acetonitrile. The sample (1 μ L) was injected, and a linear gradient (from 0 to 95% eluent B in 10 min) at a flow rate of 0.3 mL/min was used for elution.

The ESI-MS spectra was carried on MALDI Synapt Q-TOF MS (from Waters, Inc.) and the electrospray interface was set in the negative ionisation mode with a detector voltage of 30 V, capillary voltage of 3.0 kV, a source block temperature of 100 °C to obtain the spectrum (50–1,600 Da). The desolvation temperature was 400 °C

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