Carbohydrate Polymers 82 (2010) 1116–1123

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Cytotoxic and antioxidant effects of unsaturated hyaluronic acid oligomers

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ARTICLE INFO

Article history:
Received 20 May 2010
Received in revised form 14 June 2010
Accepted 18 June 2010
Available online 30 June 2010

Keywords:
Hyaluronate lyase
Unsaturated hyaluronan oligomers
Cytotoxicity
Antioxidant

ABSTRACT

Hyaluronate lyase cleaves hyaluronan chains at a β-D-GalNAc-(1→4)-β-D-GlcA bond, ultimately breaking the polysaccharide down to 3-(4-deoxy-β-D-gluc-4-enuronosyl)-N-acetyl-D-glucosamine. In the present paper, the recombinant hyaluronate lyase (EC 4.2.2.1, HA lyase) of *Streptococcus pyogenes* bacteriophage H4489A was produced, purified and applied to digest hyaluronan producing unsaturated hyaluronan oligomers. The resulted unsaturated hyaluronan oligomers were purified and subjected to anion-exchange high performance chromatography. The cytotoxic activities of naturally hyaluronan, hyaluronate lyase and unsaturated hyaluronan oligomers were tested and evaluated against HEp-2 (human laryngeal carcinoma), Daoy (human medulloblastoma), MCF-7 (human breast adenocarcinoma), and WiDr (human colon adenocarcinoma) tumor cell lines. Also, their antioxidant activities have been examined by the inhibition of lipid oxidation and free radical scavenging assays.

1. Introduction

Hyaluronan (hyaluronic acid, or hyaluronate, HA) is a high-molecular mass polysaccharide of alternating D-glucuronic acid (GlcUA) and N-acetyl-D-glucosamine (GlcNAc) units found in the extracellular matrix (Fig. 1). Under in vivo conditions hyaluronic acid possesses several distinct physiological functions (El Maradny et al., 1997; Evanko & Wight, 1999; Feinberg & Beebe, 1983; Knudson, Bartnik, & Knudson, 1993; Prehm, 2002). The most prevalent degradation of HA in biological environments is catalyzed by a group of enzymes called "hyaluronidases". A detailed knowledge of the different categories of these enzymes can be found in our recent review article published lately (El-Safory, Fazary, & Lee, 2010). Recently, synthetic hyaluronan oligomers with defined structures and their pyridylaminated derivatives were used to investigate the mechanism of hydrolysis of hyaluronan by bovine testicular hyaluronidase (Kakizaki, Ibori, Kojima, Yamaguchi, & Endo, 2010). In 2009, Dr Chen and his co-authors analyzed novel products in hyaluronan digested by bovine testicular hyaluronidase (Chen et al., 2009). Also, an enzymatic degradation method of HA was developed with a novel recombinant hyaluronan lyase and gel permeation chromatography (Guo, Liu, Zhu, Su, & Ling, 2009).

Several analytical techniques have been used to analyze HA oligomers such as the electrospray ion mass spectrometry (ESI-MS) analysis of saturated and unsaturated HA oligomers derived from digestion with testicular hyaluronidase (Mahoney, Aplin, Calabro, Hascall, & Day, 2001; Prebyl, Kaczmarek, Tuinman, & Baker, 2003; Tawada et al., 2002). Off-line liquid chromatography (LC) (Price, Tuinman, Baker, Chisena, & Cysyk, 1997), direct coupled with ESI-MS (Kühn, Raith, Sauerland, & Neubert, 2003; Kühn, Rüttinger, Neubert, & Raith), on-line capillary electrophoresis with ESI-MS (Kühn, Raith et al., 2003), and MALDI time-of-flight (TOF) MS (Prebyl et al., 2003; Volpi, 2007; Yeung & Marecak, 1999) techniques have been used for the separation and identification of HA oligomers and HA-derived oligomers. It was reported that, strong anion-exchange (SAX)-HPLC was used to fractionate glycosaminoglycan and HA-derived oligosaccharide mixtures (Imanari, Toida, Koshiishi, & Toyoda, 1996), as well as, reversed-phase ion pairing (RPIP)-HPLC analytical approach has been applied to the separation and characterization of oligomers derived from heparosan and of highly sulfated species from heparin (Kuberan et al., 2002; Thanawiroon, Rice, Toida, & Linhardt, 2004).

Hyaluronan oligomers have been attracted consideration due to their variety of medicinal activities such as potential stimulators to angiogenesis (Cui et al., 2009; Gao, Yang, Mo, Liu, & He, 2008), innate alloimmune agonists (Tesar et al., 2006), induce many kinds of inflammatory factors (Horton, Burdick, Strieter, Bao, & Noble, 1998), elastogenic effects on vascular smooth muscle cells (Joddar & Ramamurthi, 2006), potential anticancer therapeutic (Sugahara, Hirata, Murai, & Miyasaka, 2004; Taylor et al., 2004; Toole, Ghatak, & Misra, 2008). During the last decade, two interesting articles appeared outline the vast range of activities of hyaluronan (Rössler & Hinghofer-Szalkay, 2003; Stern, Asari, & Sugahara, 2006). These reports suggest that, nonsulfated oligosaccharides and low-molecular-mass derivatives besides HA may acquire new activities and functions after depolymerization. A search in literature data showed that no cytotoxic or antioxidant activities of unsaturated

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doi:10.1016/j.carbpol.2010.06.042

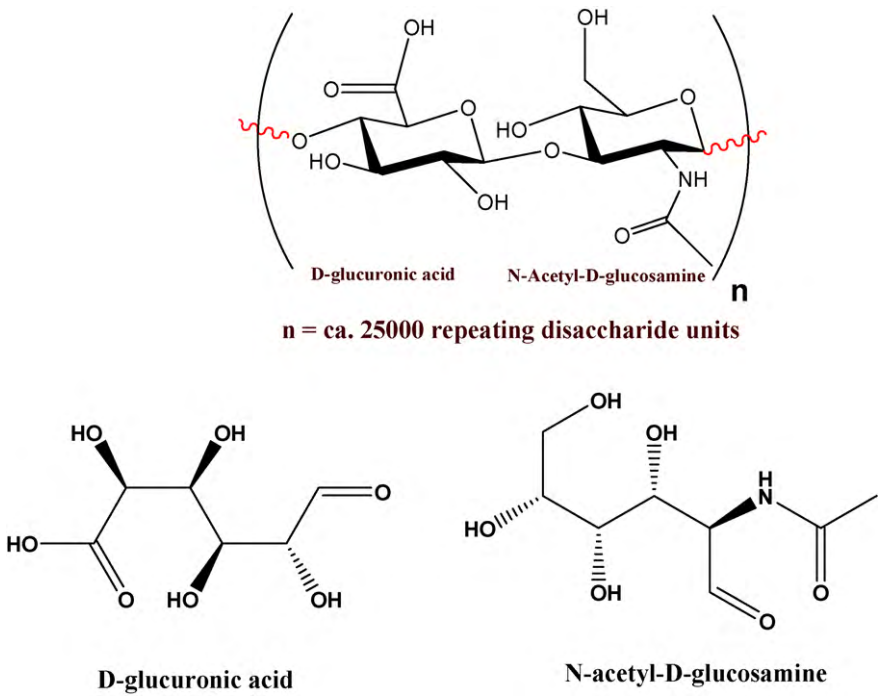

Fig. 1. Chemical structures showing the repeating disaccharide unit of hyaluronan.

hyaluronan oligomers resulted from the digestion of hyaluronan by HA lyases of *Streptococcus pyogenes* bacteriophage H4489A. Therefore, the present work illustrates the production and purification of recombinant HA lyase of *S. pyogenes* bacteriophage H4489A. As well, the preparation, purification and HPLC analysis of unsaturated HA oligomers resulted from the enzymatic digestion of hyaluronan chains by the recombinant HA lyase have been described in this paper. More consideration has been taken on the cytotoxic and antioxidant activities of the hyaluronan, HA lyase, and unsaturated HA oligomers.

2. Experimental

2.1. Materials

Plasmid pSF49, containing the HA lyase hylP gene of *S. pyogenes* bacteriophage H4489A, was kindly supplied by Dr Wayne L. Hynes (Department of Biological Science, Old Dominion University, Norfolk, VA). PCR pfu, dNTP and T4 Ligation enzymes were purchased from New England Biolabs, UK. DNA makers, plasmid vector pET30b were purchased from Novagen, UK. PCR purification and gel extraction kits were purchased from Qiagen, USA. Plasmid miniprep kit was purchased from Viogene, USA. *E. coli* strain DH5α, BL21 (DE3) and DNA markers bring from Eastern Biotech. A protein marker was purchased from Fermentas. Isopropyl-β-D-thiogalactopyranoside (IPTG) and kanamycin were purchased from Sigma, USA. Sodium hyaluronate was obtained from Fine Chemical Division of Q.P. Co. (Tokyo, Japan). 1-Stearoyl-2-linoleoyl-sn-glycerol-3-phosphocholine, fluorescence probe 3-[*p*-(6-phenyl)-1,3,5-hexatrienyl] phenylpropionic acid, and all other chemicals and solvents used in this study are analar grade and were purchased from Acros, Merck and Sigma.

2.2. Phage HA lyase vector construct

An open reading frame (ORF) fragment of *S. pyogenes* bacteriophage H4489A encoding functional hylP (GenBank® accession number M19348.1) was amplified from plasmid pSF49 using a forward primer containing an NdeI site and a reverse primer containing a NotI site by polymerase chain reaction (PCR). The forward primer (5′-CCGCATATGACTGAA AATATACCATTAAGAGTC-3′) and the reverse primer (5′-CCGGCGGCCGCTC AATGATGATGATGATGATGTT TTTTTAGTATGAGTTTTTTTAA-3′) generated a product of 1.08 kb in length. The hylP gene fragment was purified by PCR extraction kit. After digestion with NdeI and NotI using standard procedures, the hylP gene fragment was ligated into a pET30b vector. The product (pET30b-hylp) was transformed into *E. coli* DH5α and selected on Luria–Bertani (LB) medium plates containing 50 μg/ml kanamycin. DNA sequencing confirmed the homogeneity of the sequence. The plasmid pET30b-hylp was then transformed into the expression host, *E. coli* BL21 (DE3) for HA lyase protein production.

2.3. Expression and purification of HA lyase

The recombinant strain *E. coli* BL21 (DE3)/pET30b-hylp was grown overnight at 37 °C in LB medium containing 50 μg/ml kanamycin. Overnight cultures were diluted (1:100) by fresh LB kanamycin media and incubated at 37 °C with 200 rpm shaking. HA lyase expression was induced by adding IPTG to give a final concentration of 1 mM when the culture absorbance at 600 nm reached 0.8. After 3 h induction at 30 °C, the cells were harvested by centrifugation (11,000 rpm). Then, the cells pellets were washed twice using 0.1 M phosphate buffer pH 7.4, and re-suspended in 20 mM phosphate, 500 mM NaCl buffer pH 7.4, contain 20 mM imidazole, 1 mg/ml lysosyme, 0.1% Triton X-100 and 1 mM PMSF and keep in ice for 30 min before sonication. The sample was in an ice-bath, and sonication was carried out in short bursts in order to avoid overheating the mixture ultrasonic cell disruptor (Microson model XL200). The lysate was centrifuged at 11,000 rpm for 20 min at 4 °C. The expression level was analyzed by gel analysis software after SDS-PAGE analysis. For purification, the supernatant of the cell lyses was loaded onto Histrp FF column for immobilized metal affinity chromatography (IMAC) purification. The target enzyme was eluted by stepwise gradient elution of imidazole from 20 to 500 mM use AKTA Chromtography System (Pharmacia, Sweden).

2.4. Protein content and activity assay of HA lyase

The protein concentration was determined by Bradford protein assay using bovine serum albumin as standard (Bradford, 1976). SDS-PAGE analysis was carried out using a 12% polyacrylamide gel under reducing conditional. After electrophoresis protein was stained with modified coomassie brilliant blue G-250. To determine the HA lyase activity quantitatively, the enzyme was incubated with 2 mg/ml HA solution 0.1 M phosphate, 0.1 M NaCl buffer (pH 6.0) at 37 °C BSA final concentrate 0.05 mg/ml. The degradation products were then determined as N-acetyl-glucosamine (NAG) equivalents by modified Elson–Morgan method (Reissig, Storminger, & Leloir, 1955). The rate of release of unsaturated oligomers by the enzyme from HA, measured as the rate of increase in absorbance at 585 nm, was measured by Kc4 micro plate reader spectrophotometer. The amount of the HA lyase that releases 1 μmol of the unsaturated oligomers product per minute at 37 °C was considered as one enzyme unit.

2.5. Preparation, purification and HPLC analysis of HA oligomers via enzymatic digestion of HA

One hundreds milliliter of HA (5 mg/ml) in acetate buffer was mixed with 500 μl HA lyase solution and incubated at 37 °C for 3 h. The hydrolytic reaction was stopped by heating the mixture in a boiling water bath for 20 min. After being cooled down to 4 °C, the hydrolyzate was centrifuged at 12,000 rpm for 10 min, then, the supernatant was freeze dried. The lyophilized white powder was dissolved in 2 ml of 0.05 M NH_4HCO_3, and then subjected to a Bio-gel P6 column chromatography (95 cm × 1.5 cm, Bio-Rad Laboratories). NH_4HCO_3 (0.05 M) was used as eluent at a flow rate of 12 ml/h. Fractions (2 ml) were collected. The salt NH_4HCO_3 was removed by repeated evaporation under diminished pressure at 60 °C. After a second bio-gel P6 column chromatography using the same conditions, the recovered unsaturated HA oligomers were freeze dried, and then solublizied at a concentration of 5 mg/ml in 150 mM Na_2SO_4 and 25 mM NaH_2PO_4. Anion-exchange chromatography-HPLC (HPLC equipment from Hitachi L-6250 intelligent pump and Hitachi L-4000 UV detector UV-1570, Luna-NH2 column (4 mm × 250 mm)) was performed using mobile phase composed of a 150 mM Na_2SO_4 and 25 mM NaH_2PO_4 adjusted to pH 6.0, under constant flow (1 ml/min) at 35 °C. The injection volume was 20 μl.

2.6. Cytotoxic assay

The cells for assay was cultured in RPMI-1640 medium supplemented with a 5% CO_2 incubator at 37 °C. The cytotoxicity assay depends on the binding of methylene blue to fixed monolayers of cells at pH 8.5, washing the monolayer, and releasing the dye by lowering the pH value. Samples and control standard drugs were prepared at a concentration of 150 μg/ml. After seeding 2880 cells/well in a 96-well microplate for 3 μl, 20 μl of sample or standard agent was placed in each well and incubated at 37 °C for 3 days. After removing the medium from the microplates, the cells was fixed with 10% formaldehyde in 0.9% saline for 30 min, then dyed with 1% (w/v) methylene blue in 0.01 M borate-buffer (100 μl/well) for 30 min. The 96-well plate was dipped into a 0.01 M borate-buffer solution four times in order to remove the dye. Then, 100 μl/well of EtOH–0.1 M HCl (1:1) was added as a dye eluting solvent, and the absorbance was measured on a microtiter plate reader (Dynatech, MR 7000) at a wavelength of 650 nm. The IC_{50} value was defined by a comparison with the untreated cells as the concentration of test sample resulting in 50% reduction of absorbance. Each experimental variable was run three times, and the results were expressed via mean value and standard deviation. A two-tailed P value of less than 0.05 was considered to be statistically significant.

2.7. Antioxidant activities

2.7.1. Lipid oxidation inhibition assay

A mixture containing 5 μmol of 1-stearoyl-2-linoleoyl-sn-glycerol-3-phosphocholine and 0.015 μmol of the fluorescence probe 3-[*p*-(6-phenyl)-1,3,5-hexatrienyl] phenylpropionic acid was dried under vacuum using a rotary evaporator. The resulting lipid film was suspended in 500 μl of a solution containing NaCl (0.15 M), EDTA (0.1 mM), and MOPS (0.01 M) and subjected to 10 freeze–thaw cycles using a dry ice/EtOH bath. The buffer was previously treated with chelating resin, Chelex 100 (5 g/100 ml buffer), to remove trace metal ion. The lipid-buffer suspension was then extruded 29 times through a Liposo-Fast extruder containing a polycarbonate membrane with a pore size of 100 nm to produce unilamellar liposomes. A 20 μl aliquot of this liposome suspension was diluted to 2 ml in Chelex 100-treated buffer containing 200 mM NaCl and 100 mM HEPES buffer at pH 7.0 and incubated for 5 min at room temperature, followed by incubation for another 5 min in the thermostatic cuvette holder (23 °C) of the spectrofluorometer. Peroxidation was then initiated by the addition of 20 μl of 0.5 mM stock $FeCl_2$ solution to achieve a final concentration of 0.5 μM of Fe^{2+} in the absence or presence of test compounds. The control sample did not contain either Fe^{2+} or any test compound. Fluorescence intensity of these liposome solutions at an excitation wavelength of 384 nm was recorded every 3 min on a fluorescence spectrofluorometer over a period of 21 min. The decrease in relative fluorescence intensity with time indicates the rate of preoxidation. The percent inhibition of the lipid oxidation was calculated using the equation:

$$\text{percent inhibition} = \frac{(F_{\text{rel}})_{\text{Pl}} - (F_{\text{rel}})_{\text{Fe}}}{(F_{\text{rel}})_{\text{C}} - (F_{\text{rel}})_{\text{Fe}}} \times 100$$

where $(F_{\text{rel}})_{\text{Pl}}$ is the relative fluorescence for the Fe(II) and test samples at the end of 21 min, $(F_{\text{rel}})_{\text{C}}$ is the relative fluorescence for the control sample at 21 min, and $(F_{\text{rel}})_{\text{Fe}}$ is the relative fluorescence for the Fe(II)-containing sample at the end of 21 min. Each experimental variable was run three times, and the results were expressed via mean value and standard deviation. A two-tailed P value of less than 0.05 was considered to be statistically significant.

2.7.2. DPPH radical scavenging assay

Antiradical activities of the tested compounds (HA, HA lyase, and HA oligomers) were measured using the stable radical, 2,2-diphenyl-1-picrylhydrazyl-hydrate (DPPH) based on the method reported in literature (Gadow, Joubert, & Hansmann, 1997). 50 μg/ml, 100 μg/ml, 150 μg/ml, and 250 μg/ml methanolic solutions of gallic acid and tested compounds were placed in 1 cm cuvettes, and 2 ml methanolic solutions of DPPH (6×10^{-5} mol/l) were added. Absorbance measurements commenced immediately. The decrease in absorbance at 515 nm was determined continuously with data acquisition at 2 s intervals with a spectrophotometer until the absorbance stabilized (16 min). The absorbance of the DPPH radical without antioxidant, i.e. the control, was measured daily. Special care was taken to minimize the loss of free radical activity of the DPPH radical stock solution as recommended (Blois, 1958). All determinations were performed in triplicate. The percent inhibition of the DPPH radical by gallic acid and tested compounds was calculated according to the formula:

$$\text{percent inhibition} = \frac{A_{C(0)} - A_{C(t)}}{A_{C(0)}} \times 100$$

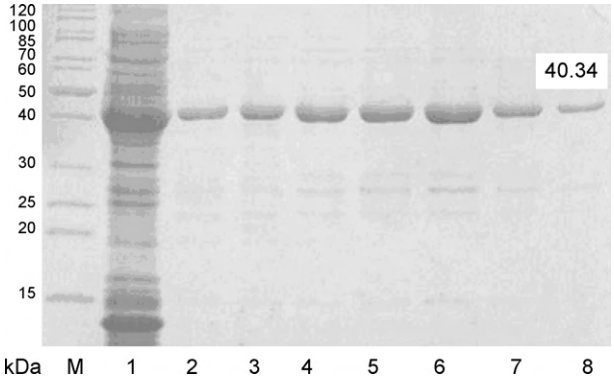

Fig. 2. SDS-PAGE analysis of recombionant HA lyase. Lane M: molecular mass standard protein; lane 1: crude protein; lanes 2–8: elution fractions resulted from IMAC purification step. Electrophoresis conditions: 150 V, 1 h.

where $A_{C(0)}$ is the absorbance of the control at $t = 0$ min and $A_{A(t)}$ is the absorbance of the reaction solution at $t = 16$ min. Each experimental variable was run three times, and the results were expressed via mean value and standard deviation. A two-tailed P value of less than 0.05 was considered to be statistically significant.

3. Results and discussions

A PCR product of 1.08 kb was obtained by amplification of the interested gene (hylp). The recombinant plasmid pET30b-hylp which carried by *E. coli* BL21 (DE3) was induced with 1 mM IPTG and expression level analyzed by 12% SDS-PAGE. Time course of protein expression shows that, the maximum level of expression at 3 h after induction. This level of expression remained almost constant. Compared with the molecular mass of bacterial HA lyases (75–130 kDa) (Abramson, 1973; Akhtar & Bhakuni, 2004; Akhtar, Krishnan, & Bhakuni, 2006; Allen et al., 2004; Arvidson, 1983; Gerlach & Köhler, 1972; Hill, 1976; Ingham, Holland, Gowland, & Cunliffe, 1979; Jedrzejas & Chantalat, 2000; Mishra, Akhtar, & Bhakuni, 2006), the phage HA lyase was notably have smaller molecular weight. The expression of recombinant HA lyase was present predominately (>90%) in soluble fraction. The calculated molecular mass from primary amino acid sequence for hylp included 6× His at C-terminal is 40.34 kDa conformity with molecular mass about 40 kDa observed molecular weight observed with SDS-PAGE (Fig. 2). It was purified to at least 90% purity based on SDS-PAGE analysis (Fig. 2). As shown in the purification table (Table 1), a single metal chelating affinity purification step (IMAC) resulted in a 20-fold increase in purity and activity recovery yield of 124%.

Various methods for the production, purification of HA oligomers (Fig. 3) have been described in literature (Mahoney et al., 2001; Tawada et al., 2002). Most of these reports involve the digestion of polysaccharide with endohydrolase or testicular hyaluronidase followed by purification via size-exclusion chromatography (Lesley, Hascall, Tammi, & Hyman, 2000; Tammi et al., 1998) and ion-exchange chromatography (Chai, Beeson, Kogelberg, Brown, & Lawson, 2001; Tammi et al., 1998). Ordinarily, hyaluronidase is used in reducing the molecular weight of HA to prepare low-molecular weight derivatives and to facilitate the isolation and purification of specific HA oligosaccharide components (Mahoney et al., 2001; Tawada et al., 2002). The spectral studies carried out on the purified HA oligosaccharides showing the loss of N-acetylglucosamine from the even-numbered HA oligomers and glucuronic acid from the odd-numbered HA oligomers (Kühn, Raith et al., 2003; Kühn, Rüttinger et al., 2003; Mahoney et al., 2001; Prebyl et al., 2003; Price et al., 1997). It was known that, testicular hyaluronidase (EC 3.2.1.35), digest hyaluronic acid and generates GlcA-(1→3)-[GlcNAc-(1→4)-GlcA]$_n$-GlcNAc with a fully saturated uronic acid at the non-reducing end (Prebyl et al., 2003; Price et al., 1997). Also, odd-numbered HA oligomers have been observed in ESI-MS (Kühn, Raith et al., 2003; Kühn, Rüttinger et al., 2003; Prebyl et al., 2003) and MALDI-MS (Prebyl et al., 2003; Yeung & Marecak, 1999) experimental results. The purification and anion-exchange HPLC separation method employed here allowed the production and purification of about 20 HA oligomers as shown in Fig. 4, but it is expected to obtain species possessing larger homogeneous molecular masses by varying the conditions of the enzymatic treatment.

Although many studies have been performed in carcinoma models, little is known about the cytotoxicity of unsaturated HA oligomers, native HA and HA lyase. HA is an endogenous polysaccharide exhibiting several and significant structural, rheological, physiological, and biological functions such as migration, adhesion, and proliferation. Biological characterization of hydrogels of hyaluronic acid shows that, HA components are non-cytotoxic (Sbarbati Del Guerra, Cascone, Barbani, & Lazzeri, 1994), and may serve as a carrier molecule for cytotoxic agents in which the addition of HA to cytotoxic agents resulted in rising cellular uptake, improved response rates, reduced toxicity and prolonged survival (Wu & Jin, 2008). It was known that hyaluronidases degrade hyaluronic acid, which promotes metastasis, and it shows a crucial role for the spreading of bacterial infections, toxins, and venoms (Girish, Shashidharamurthy, Nagaraju, Gowda, & Kemparaju, 2004; Kuhn-Nentwig, Schaller, & Nentwig, 2004). Hyaluronidase activity is found in various stages of tissue differentiation (Nathanson, 1990; Sampson, Rochester, Freundlich, & Elias, 1992) and appears to be secreted by tumors, such as Wilms tumor (Coppes, 1993; Stern, Longaker, Adzick, Harrison, & Stern, 1991). Hyaluronidase modulates the pattern of the alternatively spliced HA receptors, which may contribute to cancer cell metastasis (Tanabe, Nishi, & Saya, 1993). In particular, hyaluronidase increases the potency of chemotherapeutic drugs against cancer cells and solid tumors (Beckenlehner et al., 1992; Hobarth, Maier, & Marberger, 1992; Spruss, Bernhardt, Schonenberger, & Schiess, 1995). Unsaturated HA oligomers generated by enzymatic degradation of hyaluronancan stimulate dissimilar tumor behavior (Lokeshwar et al., 2001), induces apoptosis and reduces tumor growth (Ghatak, Misra, & Toole, 2002). In the present work, in vitro cytotoxic activities of native hyaluronic acid, hyaluronate lyase, and unsaturated hyaluronate oligomers were investigated against Hep-2 (human laryngeal carcinoma), Daoy (human medulloblastoma), MCF-7 (human breast adenocarcinoma), and WiDr (human colon adenocarcinoma) tumor cell lines. The results are shown in Table 2. Among the compounds tested, unsaturated HA oligomers exhibited a moderate cytotoxic activity against all four tumor cell lines, while the HA lyase shows a weak cytotoxic activities against the tumor

Table 1
Purification of HA lyase.

Step	Volume[a] (ml)	Total activity (U/ml)	Protein (mg/ml)	Specific activity (U/mg)	Yield (%)	Purification fold
Crude protein	5	0.95	5.23	0.18	100	1
IMAC[b]	6	0.98	0.27	3.6	124	20

[a] Volume of the purified fraction.
[b] Immobilized metal affinity chromatography purification using Histrp FF column.

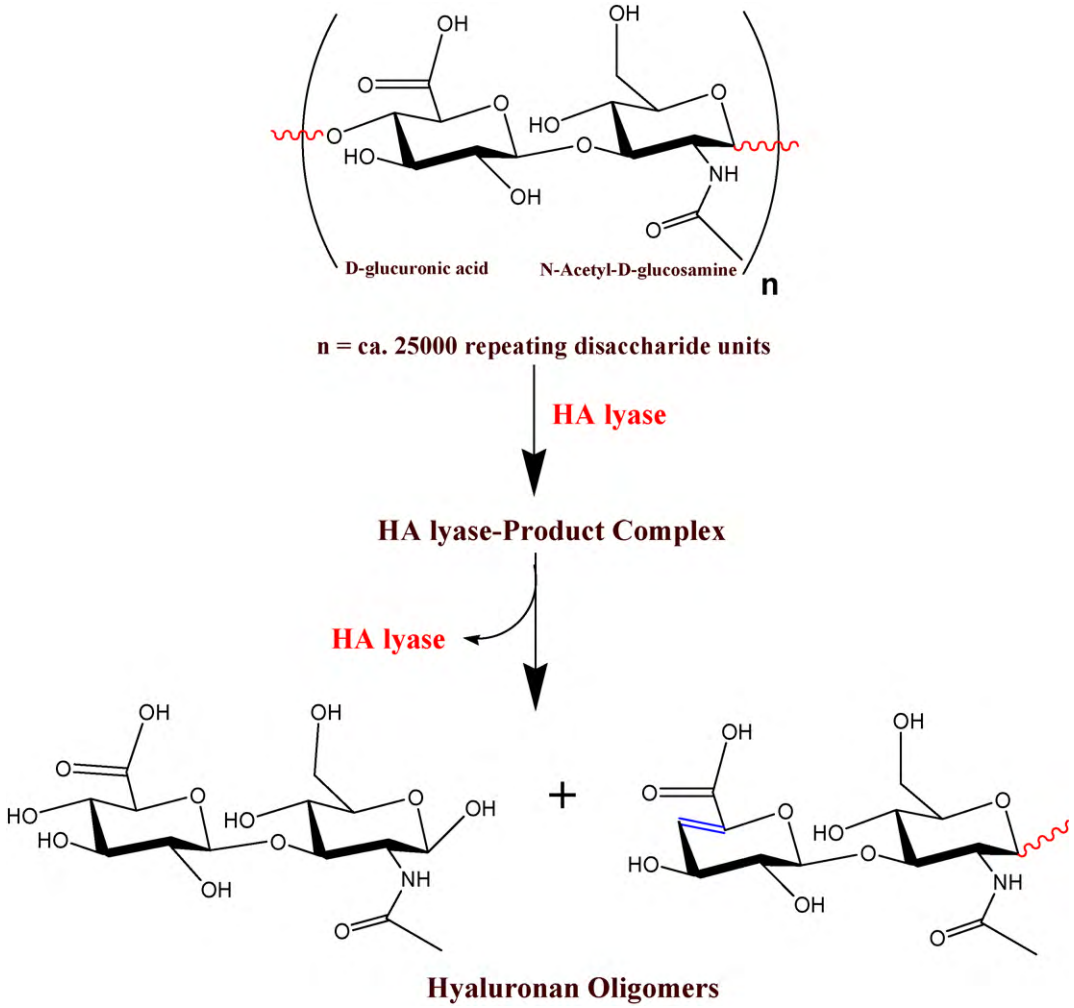

Fig. 3. Preparation of unsaturated HA oligomers.

cell lines. In contrast, naturally HA was not effective as it reveals non-significant cytotoxic effects to all tumor cell types.

Numerous studies have been done recently in the antioxidant activities of hyaluronic acid (Chen, Lv, Chen, & Lin, 2008; Da Rosa, Hoelzel, Viera, Barreto, & Beirão, 2008; Kim et al., 2008). Due to the presence of free carboxyl groups on GlcU units, HA (Fig. 1), has a polyanionic character that allows the formation of associates with cations. Because of the potential advantageous effect of the different cations, several HA-counter ion compounds were prepared for medical purposes (Ialenti & Di Rosa, 1994; Zeng, Toole, Kinney, Kuo, & Stamenkovic, 1998). Many studies suggested that the degradation of HA in several inflammatory diseases (such as rheumatoid and osteoarthritis, chronic wounds, vasculitis, and chronic hepatitis) is mainly due to highly reactive oxygen species (ROS) including OH (hydroxyl radical), O_2^- (superoxide anion), RO_2 (peroxyl radical), and $ONOO^-$ (peroxynitrite) which are thought to be released from activated leukocytes and macrophages both in vitro and during acute and chronic inflammation in vivo (Jahn,

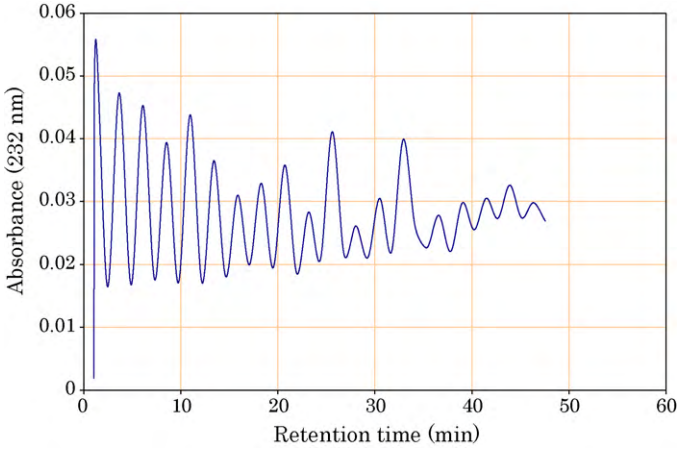

Fig. 4. Anion-exchange HPLC analysis of unsaturated HA oligomers.

Table 2
Cytotoxic activities of hyaluronic acid (HA), hyaluronate lyase (HA lyase), unsaturated HA oligomers against human tumor cells.

Compound	IC_{50} (μg/ml)[a]			
	HEp-2[b]	Daoy[b]	MCF-7[b]	WiDr[b]
HA	3.65 ± 0.31	4.76 ± 0.61	5.89 ± 0.83	4.63 ± 0.51
HA lyase	9.36 ± 0.64	10.49 ± 0.34	11.36 ± 0.17	8.96 ± 0.44
HA oligomers	39.64 ± 0.23	33.65 ± 0.72	28.64 ± 0.73	30.06 ± 0.54

[a] The IC_{50} value is the molarity at which 50% of tumor cell death was observed after 72 h under standard tissue culture conditions. Each experimental variable was run three times, and the results were expressed via mean value and standard deviation. A two-tailed *P* value of less than 0.05 was considered to be statistically significant.

[b] Cancer cell lines: HEp-2 (human laryngeal carcinoma), Daoy (human medulloblastoma), MCF-7 (human breast adenocarcinoma), and WiDr (human colon adenocarcinoma) tumor cell lines.

Table 3
Antilipid oxidation activities of hyaluronic acid (HA), hyaluronate lyase (HA lyase), and unsaturated HA oligomers.

Compound	Lipid oxidation inhibition (%)[a]			
	50 μg/ml[b]	100 μg/ml[b]	150 μg/ml[b]	250 μg/ml[b]
HA	34.36 ± 1.34	37.63 ± 0.67	39.67 ± 0.83	43.43 ± 0.54
HA lyase	14.23 ± 0.98	16.23 ± 0.34	17.91 ± 0.67	19.69 ± 0.46
HA oligomers	47.64 ± 0.81	48.19 ± 0.84	50.01 ± 1.65	57.34 ± 0.84
Gallic acid	75.35 ± 0.64	79.73 ± 0.39	82.64 ± 0.70	87.34 ± 0.91

[a] Each experimental variable was run three times, and the results were expressed via mean value and standard deviation. A two-tailed *P* value of less than 0.05 was considered to be statistically significant.
[b] Concentration.

Table 4
DPPH radical scavenging assay of hyaluronic acid (HA), hyaluronate lyase (HA lyase), and unsaturated HA oligomers.

Compound	DPPH inhibition (%)[a]			
	50 μg/ml[b]	100 μg/ml[b]	150 μg/ml[b]	250 μg/ml[b]
HA	29.45 ± 0.34	29.65 ± 0.51	29.13 ± 0.16	33.29 ± 0.71
HA lyase	19.36 ± 0.43	16.49 ± 0.45	12.36 ± 0.41	17.96 ± 0.36
HA oligomers	41.21 ± 0.45	49.63 ± 0.94	51.21 ± 1.10	59.16 ± 0.49
Gallic acid	83.64 ± 1.34	91.36 ± 0.61	93.94 ± 0.83	96.23 ± 0.54

[a] Each experimental variable was run three times, and the results were expressed via mean value and standard deviation. A two-tailed *P* value of less than 0.05 was considered to be statistically significant.
[b] Concentration.

Baynes, & Spiteller, 1999; Miller & Britigan, 1995). A search in literature survey shows that, no antioxidant activities on hyaluronidases have been appeared (El-Safory et al., 2010). As pointed out in literature, the biological properties of the smaller HA fragments may be quite distinct and even opposite of those of the larger precursor molecules (Camenisch & McDonald, 2000; Koleva, Niederlander, & van Beek, 2000). The capacity of naturally HA, HA lyase protein, and unsaturated HA oligomers to terminate radical chain progression was ultimately evaluated by two methods using two various substrates. Gallic acid is used as a polyphenol antioxidant standard. The use of the free 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical reacting with antioxidant compounds in methanolic solutions was developed during the last few years (Brand-Williams, Cuvelier, & Berset, 1995). Antilipid oxidation activities of naturally HA, native HA lyase protein, and unsaturated HA oligomers at different concentrations (50 μg/ml, 100 μg/ml, 150 μg/ml, and 250 μg/ml) are shown in Table 3. The data (Table 3) revealed that, both naturally HA and unsaturated HA oligomers exhibited a reasonable antilipid oxidation activity. The data shows that, unsaturated HA oligomers gives strong antilipid oxidation activities than naturally HA. Quite the opposite, HA lyase displayed weak antilipid oxidation activities.

Radical scavenging properties of naturally HA, native HA lyase protein, unsaturated HA oligomers were also evaluated against DPPH radicals, and the data have been listed in Table 4. In this context, HA oligomers exhibited a strong radical scavenging activity than HA, in which HA shows a moderate activity. While, it was observed that, HA lyase shows weak radical scavenging activity. It is worth mentioning that, there is slightly increment of antioxidation activities of naturally HA, native HA lyase protein, unsaturated HA oligomers with the increase of their concentrations.

Generally, a variety of digested products of HA have been reported to have extremely different or even opposite biological activities such as (Rooney, Wang, & Kumar, 1993; Slevin, Kumar, & Gaffney, 2002; Stern et al., 2006) angiogenesis (Sattar et al., 1994), and cancer cell growth and metastasis (Ghatak et al., 2002; Sugahara et al., 2003). HA oligosaccharides are small chains or oligomers of HA with molecular weights of less than 10^4 Da (2–40-mers). Generation of this oligomers from the naturally occurring HA polymer is usually mediated by the endoglycosidase, hyaluronidase (HA lyase). HA oligomers may interact with various cells and initiate a program of gene expression leading to cell proliferation, migration, or activation (Noble, 2002; Sherman, Sleeman, Herrlich, & Ponta, 1994), and exhibit biological functions, which are quite distinct from those of the native high molecular weight polymer. Some recent studies shows that, very short HA oligomers (<10 saccharides) are usually obtained using HA hydrolysis catalyzed by testicular HA lyase proteins (Gao, Cao, Yang, He, & Liu, 2006; Suzuki, Toyoda, Toida, & Imanari, 2001; Tranchepain et al., 2006).

In conclusion, we believe that, biological activities of HA change depending on its molecular weight and studies on HA oligomers of exact sizes may have more practical significance for future medicinal applications and biological strategies. Additionally, recent progress in the analytical separation of HA molecular species has revealed novel biological roles of HA. In this study, we have found that the unsaturated HA oligomers (1–20 mers) shows a very strong antioxidant and cytotoxic activities than the naturally hyaluronan and the recombinant HA lyase. Such studies will be a useful tool for understanding of native hyaluronan molecules, and further studies are needed to fully confirm these hypotheses.

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