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Preparation of the methyl ester of hyaluronan and its enzymatic degradation

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Abstract—A methyl ester of hyaluronan in which the carboxyl groups were fully esterified was prepared using trimethylsilyl diazomethane. This derivative, while not depolymerized by hyaluronan lyases or hyaluronan hydrolases, was a substrate for both chondroitin ACI lyase (EC 4.2.2.5) from *Flavobacterium heparinum* and chondroitin ACII lyase (EC 4.2.2.5) from *Arthrobacter aurescens*. The major product isolated in these depolymerization reactions was methyl α-L-*threo*-hex-4-enepyranosyluronate-(1→3)-2-acetamido-2-deoxy-α,β-D-glucopyranoside as determined by ^{1}H NMR spectroscopy and MALDITOF mass spectrometry.

Keywords: Methyl ester of hyaluronan; Chondroitin AC lyase; Chondroitinase; *Flavobacterium heparinum*; *Arthrobacter aurescens*

1. Introduction

Hyaluronan is a linear glycosaminoglycan with a repeating core of disaccharide structure comprised of a D-glucopyranosyluronic acid glycosidically linked to a 2-acetamido-2-deoxy-D-glucopyranose residue.[1–3] Hyaluronan is the simplest of the glycosaminoglycans, the only one not covalently linked to a core protein, not synthesized in the Golgi, and not sulfated.[1–3] Despite its lack of branching and the monotony of its saccharide composition, hyaluronan has a great number of diverse and important biological and physiological functions.[4–7] Hyaluronan promotes cell motility,[8] regulates cell–cell and cell–matrix adhesion,[9] promotes proliferation,[10] and suppresses processes such as embryological development and morphogenesis,[11] promotes wound healing,[12] repair and regeneration,[13] and is involved in inflammation.[14] Hyaluronan levels increase in response to severe stress,[15] as well as in tumor progression and invasion.[16] Recent studies have indicated that hyaluronan can also exist intracellularly, however, the intracellular functions of hyaluronan are unknown.[17] Chondroitin has a structure similar to that of hyaluronan in which the 2-acetamido-2-deoxy-D-glucopyranose residue of hyaluronan is replaced with a 2-acetamido-2-deoxy-D-galactopyranose residue.

While a single enzyme is now recognized as being able to synthesize hyaluronan,[18] hyaluronidases fall into three classes of enzymes based on the structure of their reaction products:[19–21] (1) Bacterial hyaluronidases (EC 4.2.99.1) are *endo*-β-acetyl-hexosaminidases that function as eliminases affording disaccharide products.[20,22] In contrast to their eukaryotic counterparts, bacterial hyaluronidases are specific for hyaluronan. (2) Hyaluronidases (EC 3.2.1.36) found in leeches, crustanceans, and some parasites, are *endo*-β-glucuronidases that function as hydrolases, generating tetrasaccharide and hexasaccharide products.[20,23] (3) Mammalian hyaluronidases (EC 3.2.1.35) are *endo*-β-acetylhexosaminidases that function as hydrolases, affording tetrasaccharide as their major product.[24,25] Mammalian hyaluronidases lack substrate specificity and can act at

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a slow rate on chondroitin sulfates. In addition, the mammalian hyaluronidases also display transglycosidase activity, and can generate complex cross-linked hybrid chains in vitro; however, this transglycosidase activity has not been documented in vivo. While polysaccharide hydrolases have been studied intensely and reaction mechanisms of these enzymes are well characterized,[26,27] the reaction mechanisms for polysaccharide lyases are relatively less understood.

Chondroitin lyases are also capable of acting on hyaluronic acid. Chondroitin AC lyase (EC 4.2.2.5) from *Flavobacterium heparinum* (described as 'chondroitin lyase ACI Flavo' in this paper), degrades chondroitin, chondroitin 4-sulfate (CS-A), chondroitin 6-sulfate (CS-C), and hyaluronan.[28,29] The action pattern for this enzyme has been established as random endolytic.[29,30] Chondroitin AC lyase (EC 4.2.2.5) from *Arthrobacter aurescens* (chondroitin lyase ACII Arthro) acts in an exolytic fashion on chondroitin, CS-A, CS-C, and hyaluronan.[31] Dermatan sulfate containing L-idopyranosyluronic acid has an inhibitory effect on both these AC lyases.[32] While there is no absolute requirement of a metal ion for chondroitin AC lyase activity, various mono- and divalent metals have been shown to enhance enzyme activity.[29]

The current study demonstrates for the first time that both chondroitin lyase ACI Flavo and chondroitin lyase ACII Arthro can act on the methyl ester of hyaluronan. While the action of chondroitin lyase ACI Flavo on the methyl ester of chondroitin has been previously reported, chondroitin lyase ACII Arthro was not observed to act on this substrate.[33] The discovery of this new substrate, the methyl ester of hyaluronan, and the activity of both chondroitin lyase ACI Flavo and chondroitin lyase ACII Arthro is significant in that it demonstrates that the negatively charged D-glucopyranosyluronic acid residue is not required by either enzyme, thus, further clarifying its role on the activity of both chondroitin lyases.

2. Experimental

2.1. Chemicals and instruments

Hyaluronan sodium salt (MW 20,000) from *Streptococcus zooepidemicus* was purchased from Kibun Food Chemipha Co., Tokyo, Japan. Hyaluronidases from *Streptomyces hyalurolyticus* (lyase, EC 4.2.2.1), from bovine testis (hydrolase, EC 3.2.1.35) and from *Streptococcus dysgalactiae* (lyase, EC 4.2.2) for hyaluronic acid oligosaccharide preparation was purchased from Seikagaku Kogyo Co. (Tokyo, Japan), Calbiochem (Darmstadt, Germany) and Seikagaku Kogyo, respectively. Chondroitin lyase ACI Flavo (*F. heparinum*, EC 4.2.2.5), chondroitin lyase ACII Arthro (*A. aurescens*, EC 4.2.2.5) and chondroitin lyase ABC (*Proteus vulgaris*, EC 4.2.2.4) were from Seikagaku Kogyo. The conditions used for the degradation of hyaluronan and derivatized hyaluronan samples were according to the procedures provided by each company. Briefly, each enzyme was used for degradation of 1 mg substrate in 1.0 mL buffer under conditions as follows: 10 TRU [1 TRU (Turbidity Reducing Unit) is that amount of enzyme which causes 50% of decrease in absorbance at 660 nm in 30 min at 60 °C] *S. hyalurolyticus* hyaluronidase at 60 °C for 24 h in 0.04 M NaOAc buffer (pH 6.0); 0.1 U (1 U is defined as the quantity of the enzyme that liberates 1 μmol of the unsaturated disaccharide from hyaluronan per minute at 37 °C, pH 6.2) *S. dysgalactiae* hyaluronidase at 37 °C for 24 h in 50 mM sodium phosphate buffer (pH 6.2); 100 TRU [1 TRU (Turbidity Reducing Unit) is that amount of enzyme that causes 50% of decrease in absorbance at 600 nm in 30 min at 37 °C, pH 5.3] sheep testicular hyaluronidase at 37 °C for 24 h in 0.1 M sodium phosphate buffer (pH 5.3) containing 0.15 M NaCl; 0.1 U (1 U is defined as the quantity of the enzyme that catalyzes the formation of 1 μmol of the unsaturated disaccharide from chondroitinase C from shark cartilage per minute at 37 °C, pH 8.0) chondroitin lyase ABC at 37 °C for 24 h in 0.1 M Tris–acetate buffer (pH 6.2); 0.1 U (1 U is defined as the quantity of the enzyme that catalyzes the formation of 1 μmol of the unsaturated disaccharide from chondroitinase C from shark cartilage per minute at 37 °C, pH 7.3) chondroitin lyase ACI Flavo at 37 °C for 24 h in 0.4 M Tris–acetate buffer (pH 6.0); 0.1 U (1 U is defined as the quantity of the enzyme which catalyzes the formation of 1 μmol of the unsaturated disaccharide from chondroitinase C from shark cartilage per minute at 37 °C, pH 6.0) chondroitin lyase ACII Arthro at 37 °C for 24 h in 0.4 M acetate buffer (pH 6.0). All other chemicals were of analytical reagent grade.

The CE system was assembled with a Beckman capillary electrophoresis system (P/ACE 5010) equipped with a UV detector and an operating system using version 0.4 P/ACE station on an IBM-compatible PC, from Beckman, USA. JEOL GSX500A and ECP600 NMR instruments, equipped with a 5-mm field-gradient tunable probe with standard JEOL software, were used for ^{1}H NMR experiments at 60 °C on 500 μL of each sample.

2.2. MALDITOF MS

MALDITOF mass spectra were collected as follows: Mass analysis was carried out in negative/positive linear and reflector mode using an Axima™ (Shimadzu Kratos Inc, Kyoto) instrument equipped with a 337-nm nitrogen laser. The acceleration voltage was set to 19 kV, and the delay time was 450 ns. A total of 100 mass spectra were acquired and summed for each sample spot.