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# Enzymatic synthesis of hyaluronan hybrid urinary trypsin inhibitor

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ABSTRACT

Human urinary trypsin inhibitor is a proteoglycan that has a single low-sulfated chondroitin 4-sulfate chain at the seryl residue in position 10 of the core protein as a glycosaminoglycan moiety, and is used as an anti-inflammatory medicine based on the protease inhibitory activity of the core protein. However, the functions of the glycosaminoglycan moiety have not yet been elucidated in detail. In the present study, the glycosaminoglycan chains of a native urinary trypsin inhibitor were remodeled to hyaluronan chains, with no changes to the core protein, using transglycosylation as a reverse reaction of the hydrolysis of bovine testicular hyaluronidase, and the properties of the hybrid urinary trypsin inhibitor were then analyzed. The trypsin inhibitory activitiy of the hyaluronan hybrid urinary trypsin inhibitor was similar to that of the native type; however, its inhibitory effect on the hydrolysis of hyaluronidase were not as strong as that of the native type. This result demonstrated that the native urinary trypsin inhibitor possessed hyaluronidase inhibitory activity on its chondroitin sulfate chain. The hyaluronan hybrid urinary trypsin inhibitors obtained affinity to a hyaluronan-binding protein not exhibited by the native type. The interactions between the hyaluronan hybrid urinary trypsin inhibitors and phosphatidylcholine (abundant in the outer layer of plasma membrane) were stronger than that of the native type. Hyaluronan hybrid urinary trypsin inhibitors may be useful for investigating the functions of the glycosaminoglycan chains of urinary trypsin inhibitors and hyaluronan, and our hybrid synthesizing method may be used widely in research for future medical applications.



## 1. Introduction

Human urinary trypsin inhibitor (UTI)[‡], otherwise known as bikunin, urinastatin, ulinastatin, mingin, and human inhibitor 30 (HI-30),[1] is a proteoglycan (PG)[2] that was originally purified from human urine in its complete form[3] and is considered to be derived from inter-α-trypsin inhibitor.[4] The core protein of UTI consists of 143 amino acids and has a single low-sulfated chondroitin 4-sulfate (Ch4S) chain covalently linked to the residue Ser-10 as a glycosaminoglycan (GAG) moiety through a GlcUAβ1-3Galβ1-3Galβ1-4Xylβ1-Ser (GlcUA-Gal-Gal-Xyl-Ser) or GlcUA-Gal(4-sulfate)-Gal-Xyl-Ser linkage region.[5–7] Clustered sulfated units (Ch4S disaccharides) are located at the reducing terminus of the GAG chain linked to the linkage region, while clustered non-sulfated units (chondroitin disaccharides, Ch) are located at the non-reducing terminus, and the number of both units can vary in UTI.[8–10]

UTI has been used as an anti-inflammatory medicine for pancreatitis, threatened premature delivery, and other conditions based on the protease inhibitor activity of its core protein.[11] Structural changes in the chondroitin sulfate (ChS) chains of UTI have been observed in the urine of patients with inflammatory

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‡ The abbreviations used are: UTI, urinary trypsin inhibitor; PG, proteoglycan; GAG, glycosaminoglycan; ChS, chondroitin sulfate; Ch4S, chondroitin 4-sulfate; Ch6S, chondroitin 6-sulfate; Ch, chondroitin; BTH, bovine testicular hyaluronidase; HA, hyaluronan; HABP, hyaluronan binding protein; PVDF, polyvinylidene fluoride; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; GlcUA, glucuronic acid; Gal, galactose; Xyl, xylose; PA, 2-pyridylamine.

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diseases.[12,13] However, the functions of the GAG moiety of UTI have not yet been elucidated in detail. Therefore, the development of tools for functional investigations on the GAG moiety of UTI is eagerly anticipated.

Bovine testicular hyaluronidase (BTH) is an endo-β-N-acetyl-D-hexosaminidase (EC 3.2.1.35) that acts on hyaluronan (HA) and ChSs at β1, 4-N-acetylhexosaminide bonds.[14] BTH exhibits transglycosylation activity as a reverse reaction of hydrolysis in vitro[15–18] in the same manner as many glycosidases. Using the transglycosylation activity of BTH, we previously developed a remodeling method for ChS and HA[19–22] and successfully remodeled the ChS chains of PG, without changes to the core protein.[23]

In the present study, the low-sulfated Ch4S chains of native UTI were remodeled to HA chains to create hyaluronan hybrid UTIs (HA-UTIs) as a tool for investigating ChS chain functions as well as the possible advantages of HA-UTIs over native UTI.

## 2. Materials and methods

### 2.1. Materials

UTI (Biotech Center, Shanghai Institute of Pharmaceutical Industry, China) was further purified by DEAE-Cellulofine column chromatography.[3] HA (from *Streptococcus zooepidemicus*, $M_r$, 80,000) was purchased from Food Chemifa Co., Ltd. (Tokyo, Japan). BTH (type 1-S) and cellulase (from *Aspergillus niger*) were from Sigma–Aldrich (St. Louis, MO, USA). Ch4S (from whale cartilage; $M_r$, 34,000), chondroitin (Ch, chemically desulfated Ch6S; $M_r$, 10,000), HA lyase (from *Streptomyces hyalurolyticus*), chondroitin ABC lyase (from *Proteus vulgaris*), and biotinylated hyaluronan binding protein (HABP) were obtained from Seikagaku Biobusiness Co. (Tokyo, Japan). Actinase E (protease from *Streptomyces griseus*) was obtained from Kaken Pharmaceutical Co. (Tokyo, Japan). Peroxidase-conjugated goat anti-rabbit immunoglobulins and peroxidase-conjugated streptavidin were from Dako Japan (Tokyo, Japan). CNBr-activated Sepharose 4 Fast Flow resin was from GE Healthcare, Japan (Tokyo, Japan). Amicon Ultra-15 was from Merk Millipore Ltd. (Tullagreen, Ireland), and polyvinylidene fluoride (PVDF) filters were from Millipore (Billerica, MA, USA). 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC, 18: 1) and1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE, 18: 1) from Wako Pure Chemical Industries (Osaka, Japan), and mineral oil (density 0.81 g/ml) from Nacalai Tesque (Kyoto, Japan) were used for preparing cell-sized droplets. Other reagents were of analytical grade and obtained from commercial sources.

### 2.2. Remodeling of GAG chains on core proteins of UTI

In order to achieve the hydrolysis and transglycosylation of BTH, a reaction column packed with BTH-immobilized resin (internal diameter, 5.0 cm; high, 1.8 cm; bed vol., 36 ml) was prepared using CNBr-activated Sepharose 4 Fast Flow according to the instructions supplied.

GAG-deficient UTI having a linkage hexasaccharide structure, GlcUA-GalNAc(4-sulfate)-GlcUA-Gal-Gal-Xyl, GlcUA-GalNAc-GlcUA-Gal(4-sulfate)-Gal-Xyl, or GlcUA-GalNAc(4-sulfate)-GlcUA-Gal(4-sulfate)-Gal-Xyl, on the intact core protein (linkage-UTI) was prepared by exhaustive hydrolysis of the GAG chains of native UTI by BTH. Using a BTH-immobilized reaction column, native UTI (230 mg) was incubated in 0.1 M sodium acetate buffer, pH 4.0, containing 150 mM NaCl (optimal for hydrolysis) for 18 h at 37 °C. Reaction products were eluted with 0.1 M sodium acetate buffer, pH 4.0 containing 0.5 M NaCl and fractions having absorbance at 280 nm were collected, desalted, and concentrated by Amicon Ultra-15. This solution was then displaced to distilled water.

HA-UTIs were constructed by transglycosylating HA to the linkage-UTI by BTH. Using a BTH-immobilized reaction column, linkage-UTI (550 μg) as an acceptor and HA ($M_r$=80,000, 82 mg) as a donor were incubated in 0.15 M Tris–HCl, pH 7.0 in the absence of NaCl (optimal for transglycosylation) for 24 h at 4 °C. Reaction products were eluted with 0.1 M Tris–HCl buffer, pH 7.0 containing 0.5 M NaCl, and fractions having absorbance at 280 nm were collected, desalted, and concentrated by Amicon Ultra-15. This solution was then displaced to distilled water. Ch-UTI and Ch4S-UTI were constructed in the same manner using Ch or Ch4S as a donor. HA/ChS hybrid-UTI, which has HA at the non-reducing terminus and original low-sulfated Ch4S at the reducing terminus linked to the linkage region, was constructed using native UTI as an acceptor and HA as a donor in the same manner without the hydrolysis step.

### 2.3. SDS-PAGE, immunoblotting, and immunoblotting-like detection

SDS-PAGE was performed with 15% acrylamide gels by the method of Laemmli.[24] Regarding immunoblotting of the UTI core protein, proteins were transferred to a PVDF filter and detected with a rabbit anti-UTI antibody, which was developed previously, according to the method of Towbin et al.[25] using the ECL system (GE Healthcare Japan, Tokyo, Japan) for detection. Biotinylated HABP and peroxidase-conjugated streptavidin were used instead of antibodies for the detection of transferred HA chains to UTIs in a similar manner to immunoblotting.

### 2.4. Sugar chain analysis

After the proteolysis of UTIs with actinase E, GAG chains, each having a xylose at the reducing terminus, were liberated from UTI core peptides by the endo-β-xylosidase activity of cellulase (from *Aspergillus niger*).[26] These procedures were performed as described previously[27] with a slight modification for the temperature of incubation with cellulase, which was 50 °C. The liberated GAG chains were pyridylaminated and analyzed by HPLC using a TSKgel Amide-80 column.

### 2.5. Measurement of trypsin inhibitory activity

The trypsin inhibitory activities of UTI and HA-UTIs were measured by a method from the Japanese Pharmacopoeia, a modified Kassell's procedure.[28] Briefly, trypsin as an enzyme and *N*-α-benzoyl-L-arginine-4-nitroanilide as a substrate were incubated in 0.25 M of 2, 2′, 2″-nitrilotriethanol-HCl buffer, pH 7.8 in the absence or presence of various doses (1, 3, 10, 30, 100, and 300 μg) of UTI or HA-UTIs for 2 min at 25 °C. After stopping the reaction, absorbance at 405 nm was measured. The trypsin inhibitory activities of UTI and HA-UTIs were then calculated from the standard curve of standard UTI with known inhibitor units.

### 2.6. Measurement of hyaluronidase activity

Hyaluronidase activity was measured by the method of Reissig et al. based on the Morgan-Elson reaction[29] for *N*-acetylhexosamine with free reducing termini. The effects of UTIs on hyaluronidase were assessed by the procedures of Salmen et al.[30] with a slight modification for the pH of incubation with hyaluronidase, which was 4.0, the optimal for the hydrolysis of BTH. Briefly, 12.5 μg of BTH and 150 μg of HA ($M_r$=80,000) were incubated in 50 μl of 0.1 M sodium acetate buffer, pH 4.0 containing 0.1 M NaCl, 1.5 mM saccharic acid 1,4-lacton, and 0.05 mg/ml bovine serum albumin in the absence or presence of UTIs or GAGs for 1 h at 37 °C using a