A Chondroitin Sulfate Chain of Urinary Trypsin Inhibitor Enhances Protease Inhibitory Activity of the Core Protein

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Abstract: Human urinary trypsin inhibitor (UTI) is a proteoglycan composed of one core protein covalently linked to one glycosaminoglycan, which is a low sulfated chondroitin 4-sulfate. It is used as an anti-inflammatory medicine based on the protease inhibitory activity of the core protein. However, functions of the chondroitin sulfate have not been clarified. Recently, we succeeded in remodeling the UTI chondroitin sulfate to hyaluronan to create hyaluronan hybrid UTI, without changing the core protein. Here, we investigated the effect of the remodeled chondroitin sulfate on the activities of serine proteases. Native UTI showed stronger protease inhibitory activity than hyaluronan hybrid UTI or hydrolyzed glycosaminoglycan UTI. Chondroitin 4-sulfate chains with a small peptide derived from the UTI chondroitin sulfate to hyaluronan (HA) hybrid UTI, without changing the core protein did not show any protease inhibitory activity. These results suggest that the chondroitin sulfate chain linked covalently to core protein enhances protease inhibitor activity of UTI although the chondroitin sulfate chain itself does not.

Key words: urinary trypsin inhibitor, glycosaminoglycan, chondroitin sulfate, hyaluronan, serine protease, hyaluronidase

Human urinary trypsin inhibitor (UTI), otherwise known as bikunin, urinastatin, ulinastatin, and others, is a small proteoglycan that is composed of one core protein with 143 amino acids and one glycosaminoglycan (GAG) chain.1 The GAG chain consists of clustered chondroitin 4-sulfate (Ch4S) disaccharide units and clustered chondroitin (Ch) disaccharide units. The Ch4S units are located at the reducing end of the GAG chain of UTIs via the GlcUAβ1-3Galβ1-3Galβ1-4Xylβ1-Ser10 (GlcUA-Gal-Gal-Xyl-Ser10) or GlcUA-Gal (4-sulfate)-Gal-Xyl-Ser10 linkage regions,10(15) and the number of both types of disaccharide units can vary (Fig. 1).10

UTI is a key anti-inflammatory response mediator inhibiting the activities of many enzymes that facilitate inflammation.18,19 UTI inhibits serine proteases, such as trypsin, chymotrypsin, plasmin, elastase, thrombin, kallikrein, etc., by binding to them at either of the two Kuniz-type domains of its core protein. Based on the protease inhibitory activity, human UTI is used as an anti-inflammatory medicine, for example, in the treatment for acute pancreatitis,10 or Kawasaki disease,11 or for inhibiting threatened premature delivery,12 etc. However, functions of GAG of UTI have not been well studied.

Recently, we remodeled the GAG chain of UTI from low sulfated Ch4S to hyaluronan (HA), with no changes to the core protein to create the HA hybrid UTI (HA-UTI) as a tool for investigating native GAG chain functions as well as the possible superiority of HA-UTI over native UTI.13,14 In the present study, we investigated the effect of the GAG chain on the activities of trypsin, chymotrypsin, plasmin, and pancreatic elastase. It was demonstrated that UTI having a native GAG chain showed stronger inhibitory activity on these proteases compared with HA-UTI or with UTI with only a linkage region as sugar chain moiety. We also found that peptide-GAG did not inhibit protease activity.
EXPERIMENTAL

Human UTI, as a primary material, was from Biotech Center, Shanghai Institute of Pharmaceutical Industry (Shanghai, China) and further purified by DEAE-Cellulose column chromatography and used as a native UTI. The GAG-deficient UTI having just the linkage hexasaccharide between a GAG chain and a core protein (Link-age-UTI), and the GAG-remodeled UTI whose GAG chain was remodeled from low-sulfated Ch4S to HA (HA-UTI), were prepared in our previous study using hydrolysis and transglycosylation of bovine testicular hyaluronidase (BTH, type 1-S, Sigma-Aldrich, St. Louis, MO, USA) (Fig. 1).\(^{13}\) HA (from S. zooepidemicus, M\(_r\)=80,000) was purchased from Food Chemifa Co., Ltd. (Tokyo, Japan). Actinase E (protease from S. griseus) was from Kaken Pharmaceutical Co. (Tokyo, Japan). Trypsin and \(\alpha\)-chymotrypsin, both from bovine pancreas, were purchased from Fujifilm Wako Pure Chemical Corporation (Osaka, Japan), and Nacalai Tesque Co. Ltd. (Kyoto, Japan), respectively.

Trypsin or \(\alpha\)-chymotrypsin activity was measured by a SensoLyte\textsuperscript{®} Protease Assay Kit (AnaSpec, Inc., Fremont, CA, USA) using 1 \(\mu\)g/mL of the bovine pancreatic trypsin or chymotrypsin, described above, respectively. Plasmin activity was measured by a SensoLyte\textsuperscript{®} Rh110 Plasmin Activity Assay Kit (AnaSpec, Inc., Fremont, CA, USA) using 0.1 \(\mu\)g/mL of human plasmin. Pancreatic elastase activity was measured using a SensoLyte\textsuperscript{®} Green Elastase Assay Kit (AnaSpec, Inc., Fremont, CA, USA) using 8 \(\mu\)g/mL of porcine pancreatic elastase. These assays were performed according to their manufacturer’s instructions. In order to investigate the effects of UTIs on the activities of these proteases, UTIs were added to the reaction mixture. Trypsin and HA-UTI, both treated with actinase E, were also used to examine the effect of ChS or HA without the intact core protein only with a small peptide. Enzymatic activities were assessed by measuring the fluorescence intensity of cleaved fragments from fluorescently labeled substrate for each protease. The relative fluorescence units (RFU) were measured with a fluorescent microplate reader, (Fluoroskan Ascent\textsuperscript{TM} FL, Thermo Fisher Scientific Inc., Yokohama, Japan) at ex/em=485 nm/538 nm.

All UTIs having an intact core protein showed inhibition in a concentration-dependent manner and the maximum inhibition rates of UTI, linkage-UTI and HA-UTI were 86, 84, and 84 \% for trypsin, 90, 88, and 89 \% for chymotrypsin, and 89, 80, and 73 \% for plasmin at the highest concentration (10 \(\mu\)g/mL by protein concentration) (Fig. 2A–C). The above maximum inhibition rates of native UTI and those for elastase, described below, supported previous reports.\(^{9,13}\) UTI and HA-UTI, both treated with actinase E, did not inhibit activities of these proteases. These results supported that the core protein of UTI has protease inhibitory activity toward a broad range of enzymes previously reported by others.\(^{9,13}\) However, at 1.0 \(\mu\)g/mL of UTIs, for example, stronger inhibition by UTI for trypsin, chymotrypsin, and plasmin, was observed than by linkage-UTI and HA-UTI (Figs. S1A–C; see J. Appl. Glycosci. Web site).
% respectively even at the highest concentration (3,000 μg/mL by protein concentration) in the case of elastase (Fig. 2D). We also investigated the effect of the GAG chain prepared from native UTI by actinase E treatment on the pancreatic elastase activity. UTI, linkage-UTI, and HA-UTI significantly inhibited elastase activity by 39.7, 17.8, and 24.4 %, respectively (Fig. S1D; see J. Appl. Glycosci. Web site). It is noteworthy that UTI treated with actinase E, which corresponds to GAG moiety of UTI, also showed significant inhibition of 22.4 %. This experiment also showed inhibition by UTI was slightly stronger than that of HA-UTI.

It was found that the differences of degree of inhibition due to structural differences in GAG chains are diminished by higher concentrations of UTIs. The enhancement of ChS structures with core protein of UTI on inhibition was obvious at the lower concentrations of UTIs. These results suggested that the ChS structure of UTI contributes to the pharmaceutical efficacy of low-dosage native UTI. It was reported that the ChS chain of UTI increases in chain length and decreases in the degree of sulfation according to the severity of the inflammatory disease such as sepsis. Therefore, it is presumable that the contribution of GAG structures on the inhibition of serine proteases leads to the enhancement of anti-inflammation properties. Investigating the effect of sulfation patterns of UTI ChS on the protease inhibition will be valuable as a future study. For such analysis we should use our glycotechnology to prepare GAG remodeled UTIs with variations of ChS structures.

In conclusion, an intact structure of UTI as proteoglycan is vital for inhibiting the activities of serine proteases that lead to anti-inflammatory effects. ChS chains of UTI may play an important role along with the core protein structure in protease inhibition activity.

CONFLICTS OF INTEREST

The authors declare no conflict of interests.

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Table 1. IC_{50} values of UTIs for inhibition on serine proteases.

| Protease          | Uti              | Trypsin  | Chymotrypsin | Plasmin | Pancreatic elastase |
|-------------------|------------------|----------|--------------|---------|--------------------|
| UTI (native)      | 0.406            | 0.556    | 1.36         | N.D.    |                    |
| Linkage-UTI       | 1.458            | 1.586    | 2.72         | N.D.    |                    |
| HA-UTI            | 1.543            | 1.537    | 3.88         | N.D.    |                    |
| UTI treated with actinase E | N.D.            | N.D.     | N.D.         | –       |                    |
| HA-UTI treated with actinase E | N.D.            | N.D.     | N.D.         | –       |                    |

N.D., not determined; –, not done.

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