J. Appl. Glycosci., **67**, 63–66 (2020)
doi: 10.5458/jag.jag.JAG-2019_0021

Note

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

(Received December 27, 2019; Accepted February 12, 2020)
(J-STAGE Advance Published Date: February 21, 2020)

Yu Teshigahara,[1] Ikuko Kakizaki,[2,†] Wataru Hirao,[2] Kanji Tanaka,[3] and Ryoki Takahashi[4]

[1]*Department of Glycotechnology, Center for Advanced Medical Research, Hirosaki University School of Medicine* (*5 Zaifu-cho, Hirosaki 036–8562, Japan*)
[2]*Department of Glycotechnology, Center for Advanced Medical Research, Hirosaki University Graduate School of Medicine* (*5 Zaifu-cho, Hirosaki 036–8562, Japan*)
[3]*Department of Obstetrics and Gynecology, Hirosaki University Graduate School of Medicine* (*5 Zaifu-cho, Hirosaki 036–8562, Japan*)
[4]*MELSMON Pharmaceutical Co., Ltd.* (*3–12–18 Kamiaoki, Kawaguchi 333–0844, Japan*)

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 native UTI 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-Ser[10] (GlcUA-Gal-Gal-Xyl-Ser[10]) or GlcUA-Gal (4-sulfate)-Gal-Xyl-Ser[10] linkage regions,[2)3)4)5)] and the number of both types of disaccharide units can vary (Fig. 1).[6)7)]

UTI is a key anti-inflammatory response mediator inhibiting the activities of many enzymes that facilitate inflammation.[1)8)9)] 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-UTIs 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.

† Corresponding author(Tel.+81–172–39–5542, Fax.+81–172–39–5016, E-mail: kaki@hirosaki-u.ac.jp, ORCID ID: 0000-0002-6702-5830).
Abbreviations: UTI, urinary trypsin inhibitor; GAG, glycosaminoglycan; ChS, chondroitin sulfate; Ch4S, chondroitin 4-sulfate; Ch, chondroitin; HA, hyaluronan; Ch6S, chondroitin 6-sulfate; BTH, bovine testicular hyaluronidase; Xyl, D-xylose; Gal, D-galactose; GlcUA, D-glucuronic acid; GalNAc, *N*-acetyl-D-galactosamine; GlcNAc, *N*-acetyl-D-glucosamine.

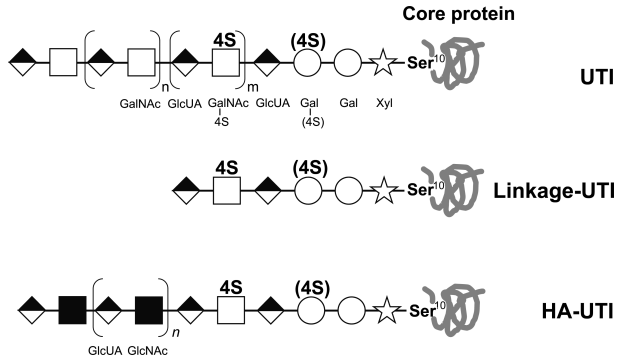

Fig. 1. Structures of UTI and GAG chain remodeled UTIs.

The repeating numbers "n" and "m" of UTI are 12–17, and 2–8, respectively.[7] Total GAG chain length of HA-UTI including the "*n*" repeating units were similar to that of UTI.[13] GlcUA, glucuronic acid; GalNAc, *N*-acetyl-D-galactosamine; GlcNAc, *N*-acetyl-D-glucosamine; Gal, D-galactose; Xyl, D-xylose; Ser, serine, 4S, 4-*O*-sulfate.

EXPERIMENTAL

Human UTI, as a primary material, was from Biotech Center, Shanghai Institute of Pharmaceutical Industry (Shanghai, China) and further purified by DEAE-Cellulofine 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 (Linkage-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 α-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 chymotrypsin activity was measured by a SensoLyte® Protease Assay Kit (AnaSpec, Inc., Fremont, CA, USA) using 1 μg/mL of the bovine pancreatic trypsin or chymotrypsin, described above, respectively. Plasmin activity was measured by a SensoLyte® Rh110 Plasmin Activity Assay Kit (AnaSpec, Inc., Fremont, CA, USA) using 0.1 μg/mL of human plasmin. Pancreatic elastase activity was measured using a SensoLyte® Green Elastase Assay Kit (AnaSpec, Inc., Fremont, CA, USA) using 8 μg/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. UTI 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™ FL, Thermo Fisher Scientific Inc., Yokohama, Japan) at ex/em=485 nm/538 nm.

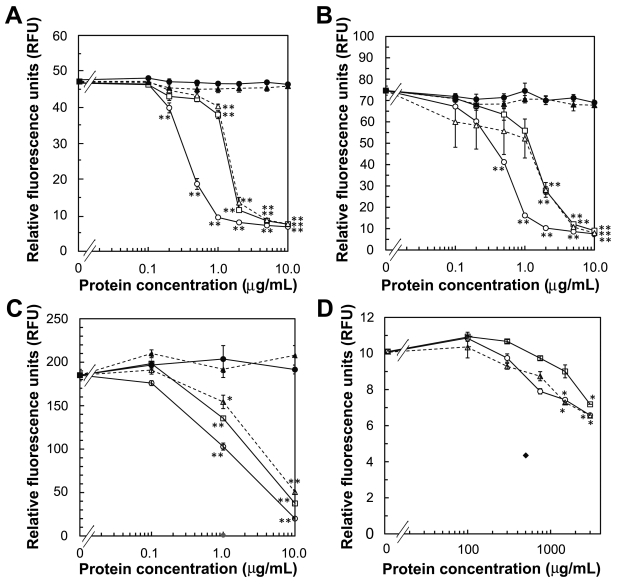

Fig. 2. The effect of UTIs on the activities of serine proteases.

Trypsin (A), chymotrypsin (B), and plasmin (C) activities were assessed in the absence or presence (0.1, 0.2, 0.5, 1, 2, 5 and 10 μg/mL by protein concentrations) of UTIs. The pancreatic elastase activity (D) was assessed in the absence or presence (100, 300, 750, 1,500, and 3,000 μg/mL by protein concentrations) of UTIs. Elastase inhibitor (500 μg/mL of MeOSuc-Ala-Ala-Pro-Val-CMK, closed diamond) was used as a positive inhibition control. The plots show the mean values ± SD (n = 3). *p < 0.05 and **p < 0.01 *versus* in the absence of UTIs (Tukey's test). Open circle, UTI; open square, linkage-UTI; open triangle, HA-UTI, closed circle, UTI treated with actinase E, closed triangle, HA-UTI treated with actinase E. Data for 1.0 μg/mL concentration only in A–C is shown in Fig. S1A–C (see J. Appl. Glycosci. Web site).

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 μg/mL by protein concentration) (Fig. 2A–C). The above maximum inhibition rates of native UTI and those for elastase, described below, supported previous reports.[8][9][15] 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.[1] However, at 1.0 μg/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), suggesting that ChS moiety of UTI enhances protease activity of the core protein of UTI, and HA does not. This was apparent from the values of 50 % inhibitory concentration (IC_{50}) of UTI (Table 1), which were lower than those of the linkage UTI and HA-UTI, which had similar values to each other. As a pharmaceutical, the inhibition activity of UTI toward pancreatic elastase is reported to be much weaker than toward other enzymes such as trypsin, chymotrypsin, and plasmin, according to the manufacturer's information. Indeed, the maximum inhibition rates of UTI, linkage-UTI and HA-UTI were only 34.9, 28.9, and 35.1

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

UTI	IC_{50} (μg/mL)			
	Protease			
	Trypsin	Chymotrypsin	Plasmine	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.

%, 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 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.[16)17)] 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.

ACKNOWLEDGMENTS

This work was supported by the Regional Innovation Strategy Support Program (City Area Type) and JSPS KAENHI Grant numbers 26462470 and 17K11262 for I.K. from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

REFERENCES

1) M.J. Pugia, R. Valdes, Jr., and S.A. Jortani: Bikunin (urinary trypsin inhibitor): structure, biological relevance, and measurement. *Adv. Clin. Chem.*, **44**, 223–245 (2007).
2) H. Toyoda, S. Kobayashi, S. Sakamoto, T. Toida, and T. Imanari: Structural analysis of a low-sulfated chondroitin sulfate chain in human urinary trypsin inhibitor. *Biol. Pharm. Bull.,* **16**, 945–947 (1993).
3) S. Yamada, M. Oyama, Y. Yuki, K. Kato, and K. Sugahara: The uniform galactose 4-sulfate structure in the carbohydrate-protein linkage region of human urinary trypsin inhibitor. *Eur. J. Biochem*., **233**, 687–693 (1995).
4) J.J. Enghild, I.B. Thogersen, F. Cheng, L.A. Fransson, P. Roepstorff, and H. Rahbek-Nielsen: Organization of the inter-alpha-inhibitor heavy chains on the chondroitin sulfate originating from Ser(10) of bikunin: posttranslational modification of IalphaI-derived bikunin. *Biochemistry* **38**, 11804–11813 (1999).
5) M. Ly, F.E. Leach, 3rd, T.N. Laremore, T. Toida, I.J. Amster, and R.J. Linhardt: The proteoglycan bikunin has a defined sequence. *Nat. Chem. Biol*., **7**, 827–833 (2011).
6) Y. Yuki, K. Nomura, M. Kirihara, M. Shimomura, H. Hiratani, R. Nishimura, and K. Kato: Charge isomers of urinary bikunin (trypsin inhibitor). *Biochim. Biophys. Acta*, **1203**, 298–303 (1993).
7) I. Kakizaki, R. Takahashi, N. Ibori, K. Kojima, T. Takahashi, M. Yamaguchi, A. Kon, and K. Takagaki: Diversity in the degree of sulfation and chain length of the glycosaminoglycan moiety of urinary trypsin inhibitor isomers. *Biochim. Biophys. Acta*, **1770**, 171–177 (2007).
8) C. Umeadi, F. Kandeel, and I.H. Al-Abdullah: Ulinastatin is a novel protease inhibitor and neutral protease activator. *Transplant. Proc.*, **40**, 387–389 (2008).
9) C.C. Nduaguibe, K. Bentsi-Barnes, Y. Mullen, F. Kandeel, and I. Al-Abdullah: Serine protease inhibitors suppress pancreatic endogenous proteases and modulate bacterial neutral proteases. *Islets*, **2**, 200–206 (2010).
10) T. Tsujino, Y. Komatsu, H. Isayama, K. Hirano, N. Sasahira, N. Yamamoto, N. Toda, Y. Ito, Y. Nakai, M. Tada, M. Matsumura, H. Yoshida, T. Kawabe, Y. Shiratori, and M. Omata: Ulinastatin for pancreatitis after endoscopic retrograde cholangiopancreatography: a randomized, controlled trial. *Clin. Gastroenterol. Hepatol.*, **3**, 376–383 (2005).
11) S. Iwashima, M. Seguchi, T. Matubayashi, and T. Ohzeki: Ulinastatin therapy in kawasaki disease. *Clin. Drug. Investig.*, **27**, 691–696 (2007).
12) N. Kanayama, E. el Maradny, A. Halim, K. Maehara, Y. Kajiwara, and T. Terao: Urinary trypsin inhibitor suppresses premature cervical ripening. *Eur. J. Obstet. Gynecol. Reprod. Biol.*, **60**, 181–186 (1995).
13) I. Kakizaki, R. Takahashi, M. Yanagisawa, F. Yoshida, and K. Takagaki: Enzymatic synthesis of hyaluronan hybrid urinary trypsin inhibitor. *Carbohydr. Res.*, **413**, 129–134 (2015).

14) S. Tanaka, I. Kakizaki, K. Tanaka, T. Kodama, A. Ito-Fukuyama, S. Suto, R. Takahashi, and Y. Yokoyama: Development of new therapeutic agents for preterm birth by glycosaminoglycan chain remodeling of urinary trypsin inhibitor. *Hypertens. Res. Pregnancy*, **7**, 27–35 (2019).
15) H. Kobayashi, H. Shinohara, K. Takeuchi, M. Itoh, M. Fujie, M. Saitoh, and T. Terao: Inhibition of the soluble and the tumor cell receptor-bound plasmin by urinary trypsin inhibitor and subsequent effects on tumor cell invasion and metastasis. *Cancer Res*., **54**, 844–849 (1994).
16) C. Mizon, C. Mairie, M. Balduyck, E. Hachulla, and J. Mizon: The chondroitin sulfate chain of bikunin-containing proteins in the inter-alpha-inhibitor family increases in size in inflammatory diseases. *Eur. J. Biochem*., **268**, 2717–2724 (2001).
17) C. Capon, C. Mizon, J. Lemoine, P. Rodie-Talbere, and J. Mizon: In acute inflammation, the chondroitin-4 sulphate carried by bikunin is not only longer, it is also undersulphated. *Biochimie*, **85**, 101–107 (2003).