名古屋学院大学論集　医学・健康科学・スポーツ科学篇　第１巻　第１号　pp. 27-33

Chemical Modification of Carbohydrate Chain of a Thrombin-Like Serine Protease Isolated from the Venom of *Agkistrodon Halys Brevicaudus Stejneger*

Junichi Sakai[1,3], Yoshihiro Hotta[2], Takahiro Nakano[1], Kenji Saitou[1], Chikashi Yamamoto[1], Yutaka Murase[1], Tadashi Okada[3] and Tadahiro Takeda[4]

Abstract

We have studied the structure-function relationship of a thrombin-like serine protease isolated from the venom of *Agkistrodon halys brevicaudus stejneger*[1–4]. The protease was a single chain glycoprotein, 18% of which was carbohydrate. Both the sugar composition and the glycosylated site were different from those of thrombin. The function of the carbohydrate chain of the isolated protease remains unknown. In this report, the enzymatic property of carbohydrate-modified protease was compared to intact protease in order to elucidate the function of the carbohydrate chain. The chemical modification of the vicinal hydroxy groups on carbohydrate was carried out using PDBA-hydrazide. The intact protease released fibrinopeptide A, B and Bβ1–42. The PDBA-modified protease formed fibrin clots; however, fibrinopeptide A was primarily released while fibrinopeptide B and Bβ1–42 released only slightly. The result led us to propose that access of the fibrinogen Bβ chain to the catalytic site cleft was restricted by the modification of the carbohydrate chain. The carbohydrate chain played an important role in protease activity, especially through its interaction with the protease and macromolecular substrates, such as fibrinogen. The enzymatic characteristics distinct from those of thrombin may be derived from the structural difference of the carbohydrate moiety.

Keywords：thrombin-like serine protease, carbohydrate chain, chemical modification

1 *Faculty of Sports and Health Science, Nagoya Gakuin University, Seto, Aichi 480-1298, Japan*

2 *Department of Pharmacology, Kinjo Gakuin University, Nagoya, Aichi 463-8521, Japan*

3 *Department of Physiology, Aichi Medical University, Nagakute, Aichi 480-1195, Japan*

4 *Keio University, Shibakoen, Minato, Tokyo 105-8512, Japan*

Correspondence to: J. Sakai, NagoyaGakuin University

E-mail: j_sakai@ngu.ac.jp

Received 25 July, 2012

Accepted 20 August, 2012

Introduction

A thrombin-like serine protease, kangshuanmei, was isolated from the venom of *Agkistrodon halys brevicaudus stejneger*[1]. The protease was found to be a single chain glycoprotein, having 236 amino acid residues with a molecular weight of 34 kDa, containing 18% carbohydrates[2]. The carbohydrate chains were asparagine (Asn)-binding oligosaccharides, consisting of bi-, tri-and tetra-lactosamins[3]. Binding positions were located near the catalytic site cleft. However, the function of the carbohydrate chain and its enzymatic property remains unknown. In this report, the fibrinopeptide released by the carbohydrate-modified protease was compared to the intact protease to elucidate the function of the carbohydrate chain.

Materials and Method

Purification of kangshuanmei

Kangshuanmei was purified from the venom of *Agkistrodon halys brevicaudus stejneger* according to the method of Zhung[1].

Chemicals

A carbohydrate modifying reagent, N-amino-6- [(3, 5-di (dihydroxy boryl) phenyl) carbonylamino] hexanamide hydrochloride (PDBA-hydrazide), was purchased from Versalinx, PROLINX. Human fibrinogen was obtained from Biopool, Sweden. The reagents used for peptide sequencing were supplied by PE Corporation Applied Biosystems, USA. Human fibrinopeptide A (FPA) and B (FPB) were purchased from Sigma, USA. The other chemicals used were analytical reagents from commercial sources.

Chemical modification reaction

$NaIO_4$ was added to purified kangshuanmei to obtain a final concentration of 10 mM. The oxidation reaction was kept in ice for 30 min in the dark. The quenching of the oxidation was carried out instantaneously by Na_2SO_3. A modifying reagent, PDBA-hydrazide, in dimethyl sulfoxide was added to the reaction solution. The chemical modification reaction was kept in ice for 4 hours, and the reaction mixture was dialyzed against Tris buffer. PDBA-modified kangshuanmei was purified by reversed-phase liquid chromatography, and lyophilized.

Analysis of release of fibrinopeptide fragments

Two milligrams of human fibrinogen was dissolved in 1 ml of 1/15 M phosphate buffer (Na_2HPO_4-KH_2PO_4, pH 7.4). Human fibrinogen solution (100 μl) was mixed with 100 μl of kangshuanmei (63.4

μg/ml) or PDBA-modified kangshuanmei (equal concentration), and dissolved in the same phosphate buffer. The mixture was incubated at 25℃ for 15 min. The fibrin clot which formed was removed with a 0.45 μm filter membrane by centrifugation at 6,000 rpm for 5 min. An aliquot (25 μl) of the fluid phase was applied to reversed-phase liquid chromatography.

Reversed-phase liquid chromatography (RPLC)

The sample for RPLC was filtrated using a 0.45 μm filter (Ultrafree-MC, Millipore, USA), and then applied to a RPLC system, 130A Separation System (PE Corporation Applied Biosystems, USA). The RPLC column used was an Aquapore RP-300, C8, 2.1 mm×22 cm (Brownlee, USA). Solvent A was 0.1% trifluoroacetic acid (TFA) in water, and solvent B was 0.1% TFA in acetonitrile. The following gradient was used: 5 min isocratic hold at 100% solvent A, followed by a linear gradient to 100% solvent B over 45 min. The flow rate was kept at 200 μl/min. The oven temperature was kept at 30℃, and UV absorption was monitored at 220 nm with an integrator, Chromato-Integrator model D-2500 (Hitachi, Japan).

Measurement of molecular mass of fibrinopeptides

The molecular mass of fibrinopeptides was determined using an electrospray ionization (ESI)/ion trapped type mass spectrometer, model LCQ (Finnigan Mat, ThermoQuest, USA). The fragment fraction of RPLC was lyophilized and then dissolved in 0.1% acetic acid, and the sample solution was applied to an ESI-mass spectrometer. Fifty percent methanol in water was used as an elute solvent, and the elution was performed at a flow rate of 50 μl/min. The positive mass spectra of the fragment were measured by scanning from 50 to 2,000 m/z. The molecular mass $[M + H]^+$ of the fibrinopeptide fragment was determined by deconvoluting the detected multiply charged ions, using LCQ BioWorks™ deconvolution software version 1.0 for Windows NT (ThermoQuest, USA).

Amino acid sequencing of fibrinopeptides

The fibrinopeptides were desalted by RPLC and lyophilized for sequencing. These samples were dissolved in 15 μl of 50% acetonitrile in water containing 0.1% TFA, and were then applied to a sequencer, Peptide Sequencer model 491 (PE Corporation Applied Biosystems, USA).

Results

Both kangshuanmei and PDBA-modified kangshuanmei formed fibrin clots. Three fragments were released from fibrinogen during clot formation, and were identified as FPA, FPB and Bβ1–42, by amino acid sequencing and its mass measurement. Kangshuanmei released FPA, FPB and Bβ1–42 from human fibrinogen during clot formation. PDBA-modified kangshuanmei also released these fragments.

RPLC profiles of released peptide fragments are shown in Fig. 1. The release of FPB and Bβ1−42 by PDBA-modified kangshuanmei was decreased to 29.1% and 40.8%, respectively (Table 1).

Discussion

The central event in hemostasis is the thrombin-catalyzed conversion of fibrinogen to fibrin. Fibrinogen is composed of three polypeptides, Aα-, Bβ-, and γ-chains, which are interconnected by disulfide bonds. When the fibrin clot is formed by thrombin, thrombin digests the peptide bond of arginine (Arg) 16- glycine (Gly) 17 of the fibrinogen Aα chain, and the FPA is released. Subsequently, thrombin cleaves the bond of Arg14-Gly15 of the fibrinogen Bβ chain and the FPB is released.

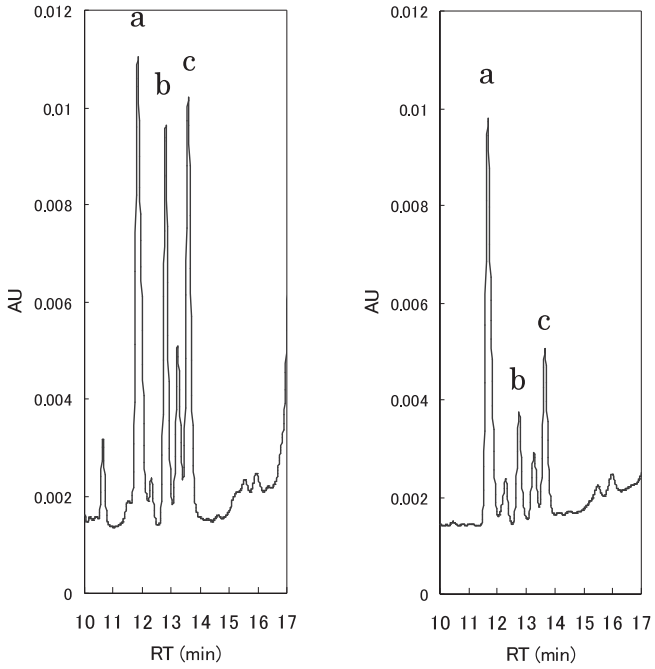

Fig. 1 RPLC profiles of fibrinopeptide fragments released by intact kangshuanmei (left) and PDBA-modified kangshuanmei (right). Peaks a, b and c are FPA, FPB and Bβ1−42, respectively. RT is retention time.

Table 1 Comparison of released fibrinopeptide fragment contents

	FPA	FPB	Bβ1-42
kangshuanmei	132,407	79,578	95,188
PDBA-kangshuanmei	109,962 (88%)	23,162 (29.1%)	38,805 (40.8%)

Values indicate peak area of fragment on chromatogram.

Various thrombin-like proteases have been isolated and characterized from the venom of snakes of the crotalidae family, such as *Agkistrodon bilineatus*[5], *Agkistrodon rhodostma*[6], *Trimeresurus albolabris*[7], and *Bothrops andianus*[8]. Most of the thrombin-like proteases derived from snake venoms have been reported to release only FPA, and lack FPB release during clot formation[9], while thrombin, bilineobin[10], and kangshuanmei[1] have been shown to release both FPA and FPB. The thrombin-like serine protease molecule possesses an active site cleft consisting of a catalytic triad and a substrate recognition portion. The hydrophobic pocket flanked by the catalytic triad is an important site for substrate recognition[11].

The binding carbohydrate chains of kangshuanmei were Asn-binding oligosaccharides, which consisted of bi-, tri-and tetra-lactosamins at 81, 99, 148, and 229[3]. The glycosylated at Asn99 is located in the insertion loop near the hydrophobic pocket. The N-acetyl neuraminic acid (NeuAc) residue shares a space at the non-reductive terminal of the binding carbohydrate chain.

In this report, the NeuAc of the carbohydrate chain was chemically modified by PDBA-hydrazide. The PDBA-modified kangshuanmei formed fibrin clots; however, FPA was primarily released, while the release of FPB declined (Fig. 1). This result led us to a proposal: the access of the aromatic amino acid residues, phenylalanine (Phe) 10-Phe11, from the fibrinogen Bβ chain to the hydrophobic pocket, was restricted by the modification of the carbohydrate chain. Consequently, the complex formation of the catalytic triad with the hydrolysis region Arg14-Gly15 of the fibrinogen Bβ chain was impaired, and the release of FPB was reduced. The binding carbohydrate chain played an important role in protease activity, especially in the interaction between kangshuanmei and macromolecular substrates, such as fibrinogen. Modification of the NeuAc of the carbohydrate chain may cause structural alteration of the hydrophobic pocket of kangshuanmei. Therefore, the binding carbohydrate chain is critical for maintaining the active site cleft of the kangshuanmei molecule.

Conclusion

The carbohydrate chain of kangshuanmei was chemically modified by PDBA-hydrazide. FPA was primarily released while release of FPB was reduced by PDBA-modified kangshuanmei. The result led us to propose that the access of the fibrinogen Bβ chain to the active site cleft *via* the hydrophobic pocket of kangshuanmei was restricted by the modification of the carbohydrate chain. The carbohydrate chain is critical for protease activity, especially through its maintenance of the active site cleft of the kangshuanmei molecule.

References

1. Shujuan Zhang, Bion Ma, Junichi Sakai, Hiroyuki Shiono, Takuya Matsui, Isamamu Sugie, and Tadashi

Okada. Characterization of a thrombin-like serine protease, kangshuanmei, isolated from the venom of a Chinese snake, *Agkistrodon halys brevicaudus stejneger*. J Natural Toxins, 2001; 10: 221–238.

2. Junichi Sakai, Shujuan Zhang, Hongmiao Chen, Fukiko Atsumi, Takuya Matsui, Hiroyuki Shiono, Susumu Sanada, and Tadashi Okada. Primary structure of a thrombin-like serine protease, kangshuanmei, from the venom of *Agkistrodon halys brevicaudus stejneger*. Toxicon, 2006; 48: 311–322.
3. Junichi Sakai, Ponlapat Rojnuckarin, Panchalee Jangprasert, Takahiro Nakano, Kenji Saitou, Chikashi Yamamoto, Yutaka Murase and Tadashi Okada. Structure of carbohydrate chain of a thrombin-like protease from the venom of *Agkistrodon halys brevicaudus stejneger* snake. Journal of Nagoya Gakuin University, 2009; 46: 35–43.
4. Junichi Sakai, Takahiro Nakano, Kenji Saitou, Chikashi Yamamoto, Yutaka Murase, Tadashi Okada, Mariko Yato, Tadahiro Takeda. Role of Carbohydrate of a thrombin-like serine protease from the venom of *Agkistrodon halys brevicaudus stejneger*. Journal of Nagoya Gakuin University, 2011; 47: 47–50. (in Japasese)
5. T. Nikai, A. Ohara, Y. Komori, JW. Fox, and H. Sugihara. Primary structure of a coagulant enzyme, bilineobin, from *Agkistrodon bilineatus* venom. Arch Biochem Biophys, 1995; 318: 89–96.
6. W. Burkhart, G. F. H. Smith, J. L. Su, I. Parikh, and H. LeVine III. Amino acid sequence determination of Ancrod, the thrombin-like α-fibrinogenase from the venom of *Akistrodon rhodostoma*. FEBS Lett, 1992; 297: 297–301.
7. Chaunahom Maunpasitporn and Ponlapat Rojnuckarin. Expression and characterization of a recombinant fibrinogenolutic serine protease from green pit nipper (*Trimeresurus albolabris*) venom. Toxicon, 2007; 49: 1083–1089.
8. JA. Valeriano-Zapana, FS. Segovia-Cruz, JM. Rojas-Hualpa, D. Nartins-de-Souza, LA. Ponce-Soto, and S. Marangoni. Function and structural characterization of a new serine protease with thrombin-like activity TLBan from *Bthrops andianus* (Andean Lancehead) snake venom. Toxicon, 2012; 59: 231–240.
9. Hubert Pirkle. Thrombin-like enzymes from snake venoms: an update inventory. Thromb Haemost, 1998; 79: 675–683.
10. Y. Komori, T. Nikai, A. Ohara, S. Yagihashi, and H. Sugihara. Effect of bilineobin, a thrombin-like proteinase from the venom of common cantil (*Agkistrodon bilineatus*). Toxicon, 1993; 31: 257–270.
11. M. C. Guillin, A. Bezeaud, M. C. Bouton, and M. Jandort-Perrus. Thrombin specificity. Thrombosis and Haemostasis, 1995; 74: 129–133.

*Agkistrodon halys brevicaudus stejneger*蛇毒由来トロンビン様セリン酵素の糖鎖の化学修飾

酒井淳一[1,2,3], 堀田芳弘[2], 中野貴博[1],
齋藤健治[1], 山本親[1], 村瀬豊[1],
岡田忠[3] 竹田忠紘[4]

要　旨

*Agkistrodon halys brevicaudus stejneger*の蛇毒に由来するトロンビン様セリン酵素の構造―機能に関して検討してきた[1–4]。精製した本酵素は一本鎖糖蛋白質で糖鎖を18%含有していた。糖鎖構造と糖鎖結合部位はトロンビンと異なっていた。この結合糖鎖の機能に関しては解明されていない。本論文では糖鎖の持つ機能を明らかにする目的で，糖鎖部分を化学修飾し，酵素特性を比較した。糖鎖における隣接する水酸基の化学修飾はPDBAを用いて行った。修飾されていない精製本酵素ではフィブリノペプチドA，BおよびBβ1–42の放出がみられた。PDBA修飾化酵素ではフィブリンクロットは形成されるものの，フィブリノペプチドAの放出が主で，フィブリノペプチドBおよびBβ1–42の放出は僅かであった。この結果は糖鎖が修飾されたことによりフィブリノゲンBβ鎖と酵素活性部位の接近が阻害されたことを示している。結合糖鎖は酵素活性，特にフィブリノゲンの様な巨大分子の基質と酵素との相互作用に重要な役割を果たしていた。精製した蛇毒由来トロンビン様セリン酵素がトロンビンと異なる特性を示すのは糖鎖部分の相違に因るのかもしれない。

キーワード：トロンビン様セリン酵素，糖鎖，化学修飾

1　名古屋学院大学
2　金城学院大学
3　愛知医科大学
4　慶応義塾大学