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Properties and cDNA cloning of an antihemorrhagic factor (HSF) purified from the serum of *Trimeresurus flavoviridis*

Masanobu Deshimaru [a,*], Chie Tanaka [a], Kazuya Fujino [a], Narumi Aoki [a], Shigeyuki Terada [a], Shosaku Hattori [b], Motonori Ohno [c]

[a] *Department of Chemistry, Faculty of Science, Fukuoka University, 8-19-1 Nanakuma, Jonan-ku, Fukuoka 814-0180, Japan*
[b] *Institute of Medical Science, University of Tokyo, Oshima-gun, Kagoshima 894-1531, Japan*
[c] *Department of Applied Life Science, Faculty of Engineering, Sojo University, 4-22-1 Ikeda, Kumamoto 860-0082, Japan*

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Abstract

Habu serum factor (HSF) is a metalloproteinase inhibitor that is isolated from the serum of habu snake (*Trimeresurus flavoviridis*), and it can suppress snake venom-induced hemorrhage. In the present study, the inhibitory property and fundamental structure of HSF were analyzed in detail. HSF inhibited all the hemorrhagic and most of the non-hemorrhagic metalloproteinases tested from the venoms of *T. flavoviridis* and *Gloydius halys brevicaudus*. HSF was extremely stable in a broad range of temperature and pH, and the treatments with a temperature of 100 °C or pH ranging from 1 to 13 barely affects its reactivity against *G. halys brevicaudus* H6 protease. Gel filtration chromatography revealed that HSF binds to the H6 protease with a 1:1 molar ratio. A secondary structure profile of HSF that was monitored by circular dichroism spectrum remained unvaried up to 2 M urea. The activity of HSF was stoichiometrically abolished by chemical modification with 2,4,6-trinitrobenzene sulfonic acid and *N*-bromosuccinimide; this indicates that Lys and Trp residues in its sequence play a role in the inhibitory mechanism. In this study, the amino acid sequence of HSF that was obtained by cDNA cloning was identical to that reported previously, except for five substitutions. We concluded that these discrepancies reflect a difference in the places of capture of the snake specimens.

Keywords: Snake venom; Snake serum; Metalloproteinase; Antihemorrhagic

1. Introduction

All venoms of the Viperidae snake have been shown to contain zinc metalloproteinases, many of which cause hemorrhage (Bjarnason and Fox, 1994). Snake venom metalloproteinases (SVMPs) are grouped into the following four classes according to the domain structure: P-I, composed of a metalloproteinase domain; P-II, metalloproteinase and disintegrin domains; P-III, metalloproteinase, disintegrin-like, and Cys-rich domains; and P-IV, containing an additional C-type lectin sequence at the C-terminal (Hite et al., 1994).

Although a snake bite is a serious medical problem for humans, certain warm-blooded animals and snakes exhibit

Abbreviations used HSF, habu serum factor; FTC, fluorescein isothiocyanate-labeled; H6, brevilysin H6; TNBS, 2,4,6-trinitrobenzene sulfonic acid; NBS, *N*-bromosuccinimide; MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; SVMP, snake venom metalloproteinase; CD, circular dichroism; UTR, untranslated region; PLI, phospholipase A_2 inhibitor.

* Corresponding author. Tel.: +81 92 871 6631; fax: +81 92 865 6030.
E-mail address: deshi@fukuoka-u.ac.jp (M. Deshimaru).

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a remarkable resistance to envenomation of snakes (Domont et al., 1991; Thwin and Gopalakrishnakone, 1998; Pérez and Sánchez, 1999). Naturally occurring factors that neutralize snake venom hemorrhagins or decrease the sensitivity to its localized and lethal effects have been found in the sera of opossums, hedgehogs, mongooses, raccoons, squirrels, wood rats, and various types of snakes as well as in the venoms of the snakes themselves (Pérez et al., 1978; Yamakawa and Omori-Satoh, 1992; Huang et al., 1998). Since hemorrhagins are metalloproteinases, the inhibitors of such enzymes have been considered as excellent candidates for new drugs to prevent the hemorrhage (Catanese and Kress, 1992).

Two antihemorrhagic proteins, namely, HSF (Yamakawa and Omori-Satoh, 1992) and BJ46a (Valente et al., 2001), have been purified from the sera of venomous snakes, *Trimeresurus flavoviridis* and *Bothrops jararaca*, respectively. These proteins are acidic glycoproteins and have no proteolytic activity. On the basis of their primary structures, they were classified as members of the fetuin superfamily that possesses a double-headed cystatin domain and an extra domain. Despite the presence of cystatin domains, HSF does not inhibit papain or cathepsin B (Yamakawa and Omori-Satoh, 1992).

In a previous paper, we described a new and rapid method for purifying HSF that consisted of ethanol precipitation, reverse-phase HPLC, and gel filtration on a Sephacryl S-200HR column (Deshimaru et al., 2003b). The purified protein showed a band of 68 kDa on SDS-PAGE, while its molecular mass, which was determined by MALDI-TOF MS, was 47,810. HSF bound to brevilysin H6, a hemorrhagic component of *G. halys brevicaudus* venom (Fujimura et al., 2000), with l:1 stoichiometry. The dissociation constant (K_d) was calculated as 3.7×10^{-9} M. Here, we describe the detailed properties and cDNA cloning of HSF. Further, chemical modification of HSF was carried out to investigate the mechanism of inhibition toward SVMPs.

2. Materials and methods

2.1. Materials

The blood of Japanese habu snakes (*T. flavoviridis*) from the Amami Oshima islands was collected by decapitation, and the serum was separated by centrifugation. HSF was purified from the serum as described previously (Deshimaru et al, 2003b). The N-terminal amino acid sequence of purified HSF was determined by an automatic protein sequencing system PPSQ21 (Shimadzu, Kyoto). The venom of *T. flavoviridis* was also collected, lyophilized, and stored at −20 °C. The low molecular weight metalloproteinases corresponding to the H2-proteinase, HR2A, and HR2B were purified from the venom (Takahashi and Ohsaka, 1970; Omori-Satoh and Sadahiro, 1979). High molecular weight hemorrhagic enzymes corresponding to HR1A and HR1B were also purified from the same venom. Brevilysins H2, H3, H4, L4, L6, and H6 were prepared from *Gloydius halys brevicaudus* venom as described previously (Deshimaru et al., 2003a; Fujimura et al., 2000, 1995; Terada et al., 1999). FTC-casein was prepared in accordance to the method described by Twining (1984). All the other reagents were purchased from Wako Pure Chem. (Osaka).

2.2. Measurement of the inhibitory activity

Inhibition of proteolytic activity by HSF was measured in 5 mM $CaCl_2$–50 mM Tris–HCl (pH 8.5) using 0.2% FTC-casein, as described previously (Deshimaru et al., 2003b). The enzyme concentration ranged from 20 to 40 μg/ml. The increase in fluorescence was analyzed using a FP-550A spectrofluorometer (Jasco, Tokyo) at 520 nm with excitation at 490 nm. Unless otherwise noted, brevilysin H6 (20 μg/ml) was used as the enzyme.

2.3. Analytical gel filtration

The sample was dissolved in 5 mM $CaCl_2$–50 mM Tris–HCl (pH 7.4), and applied to a TSKgel G4000SW column (0.75×30 cm, Tosoh, Tokyo). Elution was performed with 0.1 M NaCl–0.1 M Tris–HCl buffer (pH 7.4) at a flow rate of 1 ml/min and monitored at 280 nm. The column was calibrated with apoferritin (443 kDa), bovine serum albumin (67 kDa), and ovalbumin (45 kDa).

2.4. Thermal and pH stability

Thermal stability was tested in a 50 mM Tris–HCl (pH 7.4). The HSF solution (240 μg/ml) was heated in a block incubator (ASTEC, Fukuoka) at 40–l00 °C and then cooled in ice. The solutions were diluted with equal volumes of 50 mM Tris–HCl (pH 8.5), and the residual inhibitory activity was measured as described above. The stability of HSF was examined in various buffers, namely, 20 mM KCl–HCl buffer (pH 1–3), 20 mM acetate buffer (pH 4–5), 20 mM phosphate buffer (pH 6–7), 20 mM Tris–HCl (pH 8–9), and 20 mM Gly–NaOH (pH 10–13). The HSF solutions (240 μg/ml) in the above buffers were maintained at 37 °C for 1 h, and then equal volumes of 0.5 M Tris–HCl (pH 8.5) were added. The residual activity was measured as described above.

2.5. Chemical modifications

All the reactions were carried out at room temperature with $5\times10^{\times7}$ M HSF, and were terminated by quenching the excess reagent by adding a corresponding amino acid. Subsequently, the reaction mixtures were directly used to measure the residual inhibitory activity of HSF as described above.