Novel Function of Antihemorrhagic Factor HSF as an SSP-Binding Protein in Habu (*Trimeresurus flavoviridis*) Serum

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Abstract

Small serum proteins (SSPs) (molecular mass, 6.5–10 kDa) are high-molecular forms in the serum of Habu (*Trimeresurus flavoviridis*). An SSP-1–binding protein was purified from SSP-deficient serum by affinity chromatography on an SSP-1–HiTrap column and was identified as HSF, a known metalloproteinase inhibitor that suppresses the hemorrhagic activity of several snake venom metalloproteinases (SVMPs). The binding of SSP-1 to HSF was non-covalent and reversible, and the complex formed from these proteins dissociated at acidic pH. The mean dissociation constant of the complex was $2.5 \pm 1.1 \times 10^{-7}$ M as determined by surface plasmon resonance analysis. HSF also bound all other SSPs (SSP-2–SSP-5) with equimolar stoichiometry. SSP-1 had no effect on the inhibition of several SVMPs by HSF. Thus, HSF plays an additional role in Habu serum as a carrier of SSPs.

Introduction

Small serum proteins (SSPs) are low-molecular-mass proteins first found in Habu (*Trimeresurus flavoviridis*) serum[1]. At present, 5 homologs, namely SSP-1–SSP-5, have been isolated[2]. Structural analysis indicated that they belong to the PSP94 (prostatic secretory protein of 94 amino acids) family, which is characterized by a low molecular mass of approximately 10 kDa and 10 strictly conserved cysteine residues that form 5 disulfide bonds[3-5]. The proton NMR spectra of pig and human PSP94s have shown that they are β-sheet proteins composed of 2 domains (N- and C-terminal domains)[6,7]. Although SSP-1 and SSP-2 are composed of about 90 amino acids, including the 10 conserved Cys residues, SSP-5 has only 8 Cys residues. Interestingly, SSP-3 and SSP-4 consist of only 60 amino acids, as they lack the C-terminal domain.

In a previous paper, we reported that all SSPs exist in high-molecular-mass forms in serum[8]. When *T. flavoviridis* serum was analyzed by analytical gel filtration, all the SSPs were eluted as a broad peak with apparent molecular masses of approximately 60–120 kDa. Since SSPs do not self-associate in physiological buffers, they are hypothesized to be present in a protein complex. Like SSPs, human PSP94 exists in complex with a specific protein (PSP94–binding protein) in the blood and with cysteine-rich secretory protein-3 (CRISP-3) in prostate fluid[10].

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Abbreviations: CRISP, cysteine-rich secretory protein; HSF, habu serum factor; PSP94, prostatic secretory protein of 94 amino acids; SSP, small serum protein; SVMP, snake venom metalloproteinase.

In a search for SSP-binding proteins in *T. flavoviridis* serum, we previously isolated a novel protein named serotriflin that shows significant sequence similarity to triflin, a CRISP family protein, in *T. flavoviridis* venom[11]. Although serotriflin was isolated as a binding protein candidate for SSPs, it showed no affinity to any SSP except SSP-2[8].

Here, we report the identification of a novel SSP-binding protein. On performing affinity chromatography using an immobilized SSP-1 column, we isolated a 48-kDa candidate SSP-1–binding protein. Sequence analysis revealed this protein to be HSF, a metalloproteinase inhibitor that was isolated as an antihemorrhagic factor from *T. flavoviridis* serum[12]. Furthermore, analytical gel filtration proved that all SSPs can bind to HSF. Thus, HSF plays a dual role in Habu serum—as a hemorrhagic SVMP defense protein and as an SSP carrier.

Experimental

Materials

Blood from *T. flavoviridis* from the Amami Oshima Islands was collected by decapitation. The serum was separated by centrifugation and stored at -20°C. HSF and 5 SSPs were purified from *T. flavoviridis* serum[2,13]. Low–molecular-weight snake venom metalloproteinases (SVMPs) corresponding to HR2a and HR2b, and high–molecular-weight hemorrhagic enzymes corresponding to HR1A and HR1B were purified from *T. flavoviridis* venom[14,15]. All other reagents were purchased from Wako Pure Chemicals (Osaka).

Protein quantification

The concentration of pure samples was determined using a NanoVue spectrophotometer (GE Healthcare), and the molar extinction coefficients were calculated at 280 nm for SSP-1 (9,105 $M^{-1}\cdot cm^{-1}$), SSP-2 (10,595 $M^{-1}\cdot cm^{-1}$), SSP-3 (8,855 $M^{-1}\cdot cm^{-1}$), SSP-4 (12,865 $M^{-1}\cdot cm^{-1}$), SSP-5 (13,450 $M^{-1}\cdot cm^{-1}$), and HSF (23,670 $M^{-1}\cdot cm^{-1}$)[16].

Measurement of proteolytic activity

Proteolytic activity was measured in a solution containing 5 mM $CaCl_2$ and 50 mM Tris-HCl (pH 8.5) using fluorescein isothiocyanate- labeled casein as the substrate[17]. The increase in fluorescence was analyzed on an FP-550A spectrofluorometer (Jasco) at 520 nm with excitation at 490 nm.

Electrophoresis

SDS-PAGE was carried out on 12 % gels using Laemmli's method[18]. A prestained XL-ladder marker (Apro) was used as the molecular-weight marker. The gel was stained using 0.1 % Coomassie brilliant blue R-250 and destained with 10 % acetic acid.

Preparation of the SSP-deficient serum

Habu serum was fractionated with ammonium sulfate into P_{30}–P_{60} fractions as described previously[13]. Fraction P_{60} (500 mg) was dissolved in a 20 ml solution containing 50 mM NaCl and 50 mM citrate buffer (pH 3.5), and the solution was centrifuged at 7,000 rpm for 20 min. The supernatants were transferred to ultrafiltration tubes with a molecular weight cut-off of 30,000 (Ultracel YM-30, Millipore). The tubes were centrifuged at 4,000 × *g* for 30 min, and the solution retained on the membrane was dialyzed against PBS. The protein concentration was adjusted to 20 mg/ml.

Affinity chromatography

An affinity adsorbent was prepared by reacting an *N*-hydroxy- succinimide-activated HiTrap column (1 ml, GE Healthcare Bio-Science) with purified SSP-1 (5.5 mg), according to the manufacturer's instructions. The SSP-deficient serum (1 ml) was applied to the column, and the column was washed with 20 mM phosphate buffer (pH 7.4) containing 0.15 M NaCl The absorbed materials on the column were then eluted with 0.1 M Gly-HCl buffer (pH 3.0) containing 0.5 M NaCl and, and 1-ml fractions were collected.

Column chromatography

Analytical gel filtration was carried out using a TSKgel G3000SW column (0.75 × 30 cm, Tosoh) equilibrated with a 50 mM phosphate buffer (pH 7.0) containing 0.2 M NaCl at a flow rate of 1.0 ml/min. Elution was monitored at 280 nm using a 807-IT integrator. The column was calibrated using alcohol dehydrogenase (150 kDa), bovine serum albumin (67 kDa), ovalbumin (46 kDa), and soybean trypsin

inhibitor (20.5 kDa). Analytical reverse-phase HPLC was performed on a SepaxBio-C8 column (0.46 × 25 cm, Sepax Technologies Inc.) with a linear gradient of acetonitrile in 0.1 % trifluoroacetic acid at a flow rate of 1.0 ml/min. Elution was monitored at 220 nm.

Sequence analysis

The amino acid sequences of proteins were determined using an automatic protein sequencer PPSQ 21 (Shimadzu).

Surface plasmon resonance analysis of binding kinetics

Kinetic measurements of the interaction between SSP and HSF were performed using a Biacore 2000 instrument (Biacore). The flow cells of CM5 sensor chips were activated with 100 μl of 0.2 M 1-ethyl-3-(3- dimethylaminopropyl) carbodiimide and 0.05 M *N*-hydroxysuccinimide at a flow rate of 10 μl/min. SSP-1 at a concentration of 4.0 μM in 10 mM sodium acetate buffer (pH 4.0) was injected to reach 350 resonance units (RU). The unreacted groups were blocked with 20 μl of 1 M ethanolamine (pH 8.5). The analytes, diluted to various concentrations in a solution containing10 mM Hepes, 0.15 M NaCl, 3 mM EDTA, and 0.005 % surfactant P20 (pH 7.4), were injected for 90 s during the association phase at a constant flow rate of 20 μl/min. The dissociation was subsequently followed for 180 s at the same flow rate. The surface of the sensor chip was regenerated using a 10 mM Gly-HCl buffer (pH 2.0) after analyte binding. The sensograms were corrected by subtraction of the signal from the negative control surface and used to calculate the rate and affinity constants using BIAevaluation 4.1 (Biacore AB) and Origin 5.0 (Microcal).

Results

Identification of the SSP-1 binding protein in *T. flavoviridis* serum

In order to identify the SSP-1–binding protein, purified SSP-1 immobilized to a HiTrap column was used for affinity chromatography. The serum from *T. flavoviridis* was first filtered through an ultrafiltration membrane with a nominal molecular weight cut-off of 30,000 in acidic conditions to remove SSPs. The SSP-deficient serum was then applied

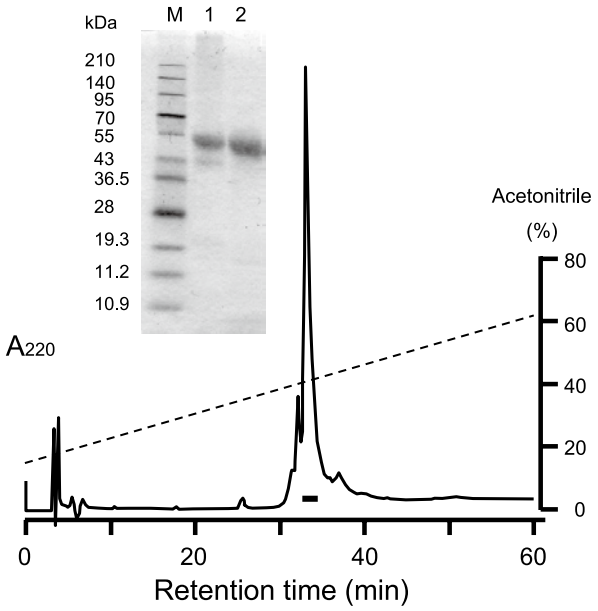

Fig. 1. Purification of the affinity-purified serum fraction by reverse-phase HPLC on a column of SepaxBio-C8 column (0.46 × 25 cm). Acetonitrile gradient of 20–50 % is shown in a dashed line. Fractions shown by a bar were pooled. SDS-PAGE analysis of HPLC-purified protein is shown in the inset. M, molecular weight markers; lane 1, SSP-1–HiTrap-purified material; lane 2, HPLC-purified protein in the main peak.

to the column. The column was washed with the starting buffer, the absorbed materials were eluted with a buffer with a pH of 3.0 (data not shown). The eluted protein was not homogenous, and SDS-PAGE showed 2 bands at 48 kDa and 43 kDa (Fig. 1, lane 1 in the inset). The affinity-purified sample was then subjected to reverse-phase HPLC on a C8 column (Fig. 1). The major peak corresponded to a protein with a molecular mass of 48 kDa, which was consistent with the SDS-PAGE results (Fig. 1, lane 2 in the inset). The exact mass of the major protein was 48,026.3 Da. The amino acid sequence revealed by sequence analysis—SQVRGDLEXD DKEAKNWADD—identical to 20 HSF N-terminal residues, an antihemorrhagic factor isolated from *T. flavoviridis* serum[12,19].

Binding analysis between HSF and SSP-1

In order to confirm that HSF is an SSP-1 carrier

protein in *T. flavoviridis* serum, we analyzed the binding between HSF and SSP-1 by analytical gel filtration. HSF and SSP-1 were eluted at 7.28 and 9.98 min, respectively (Fig. 2A). The molecular weight of HSF was estimated to be approximately 100,000 by using a calibration curve; this indicates that HSF is dimeric. The mixtures of HSF and various amounts of SSP-1 in 50 mM phosphate buffer (pH 7.0) containing 0.2 M NaCl were analyzed by gel filtration. When the molar ratio of SSP-1/HSF was less than 1, the SSP-1 peak was not present, but a shoulder was observed at 7.11 min. When more than 1 equivalent of SSP-1 was mixed with HSF, the 7.11-min peak was prominent, and a peak appeared at 9.98 min because of excess SSP-1. When the eluate containing the 7.11-min peak shown in Fig. 2A was recovered and directly subjected to reverse-phase HPLC, both SSP-1 and HSF were detected (Fig. 2B). Thus, the 7.11-min peak was attributable to SSP-1 and HSF binding. The molecular weight of the complex was estimated to be approximately 120,000 on the basis of a calibration curve. This value agreed well with the complex formation between an HSF dimer and 2 molecules of SSP-1, indicating a stoichiometry of 1 : 1 on the basis of subunits.

Kinetics of complex formation between HSF and SSP-1

The SSP-1–HSF complex is stable and does not dissociate during gel filtration, as described above. The affinity between SSP-1 and HSF was further examined by continuous monitoring of association and dissociation by means of surface plasmon resonance technology. The affinity was evaluated as illustrated in Fig. 2C. Sensograms revealed that HSF has a high affinity for SSP-1, as indicated by a low dissociation rate. Global evaluation using the 1 : 1 Langmuir binding model yielded kinetic binding parameters (Table 1). The association rate (k_a) of SSP-1 for HSF was considerably higher than the dissociation rate (k_d). The calculated mean dissociation constant (K_D) was 2.5×10^{-7} M (SD 1.1×10^{-7}), suggesting that the interaction between HSF and SSP-1 is highly specific.

Binding of HSF to other SSPs

Sequence similarity between the 5 SSPs suggested that HSF could also bind to other SSPs. When a slight excess of each of SSP-2, SSP-3, SSP-4, or SSP-5 was mixed with HSF, the 7.28-min peak corresponding to HSF disappeared and a new peak, attributable to the SSP-HSF complex, appeared at 7.12, 7.15, 7.13, and 7.06 min, respectively (Fig. 3). These results revealed that all of the SSPs are capable of binding to HSF. The binding potency of some SSPs to HSF was also determined by surface plasmon resonance using a Biacore 2000 (data not shown). Affinities between HSF and other SSPs were similar to that between HSF and SSP-1 complex (Table 1).

Effect of HSF–SSP-1 complex on the proteolytic activity of SVMPs

Like HSF, SSP-1 inhibits brevilysin H6, an SVMP isolated from *Gloydius blomhoffi brevicaudus* venom[20]. However, the inhibition was weak and the binding of SSP-1 to H6 was not stoichiometric. To explore the reason behind complex formation

Table 1. Surface plasmon resonance analyses of the binding of HSF to immobilized SSPs.

Ligand	Analyte	k_a (1/Ms)	k_d (1/s)	K_D (M)
SSP-1	HSF	$2.0 \pm 1.1 \times 10^{3}$	$5.3 \pm 3.2 \times 10^{-4}$	$2.5 \pm 1.1 \times 10^{-7}$ ($1.4–4.2 \times 10^{-7}$)
SSP-2	HSF	$1.8 \pm 0.9 \times 10^{3}$	$4.6 \pm 2.0 \times 10^{-4}$	$2.6 \pm 0.3 \times 10^{-7}$ ($2.4–2.8 \times 10^{-7}$)
SSP-3	HSF	2.2×10^{3}	4.6×10^{-4}	2.0×10^{-7}
SSP-5	HSF	0.76×10^{3}	3.5×10^{-4}	4.6×10^{-7}

The kinetics of the interaction between 4 SSPs and HSF was evaluated with a Biacore 2000 instrument. Ligands were immobilized on a surface that was challenged with different concentrations of HSF, and association and dissociation rates (k_a and k_d) were measured. The average values of 2 independent experiments are shown for SSP-1 and SSP-2. The range of the equilibrium dissociation constants (K_D) are given in parentheses.

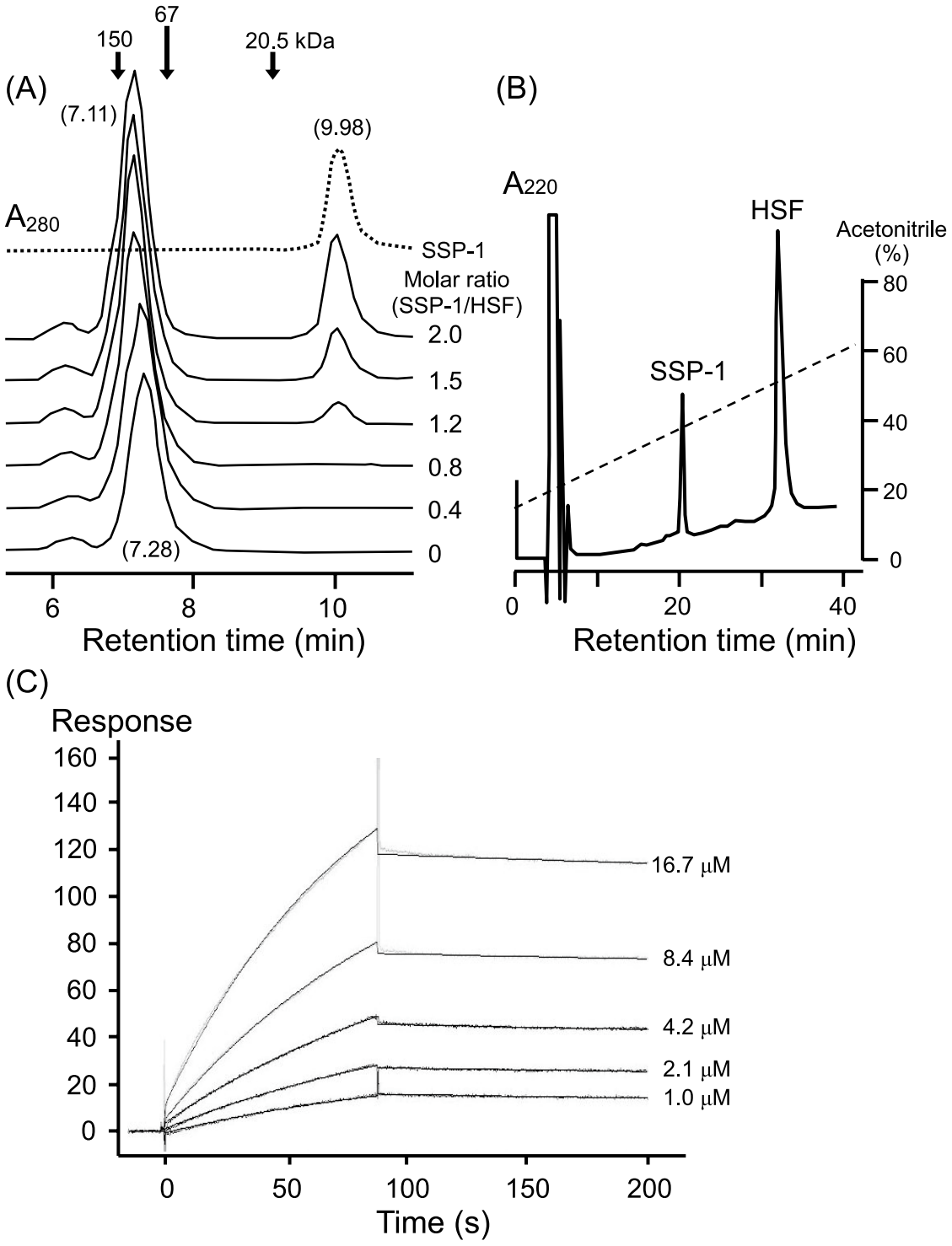

Fig. 2. Binding of SSP-1 to HSF. (A) Analytical gel filtration analysis. A constant amount of HSF (90 μM) was added to varying amounts of SSP-1 and the mixtures were applied to a TSKgel G3000SW (0.75 × 30 cm) column in a 50 mM phosphate buffer (pH 7.0) containing 0.2 M NaCl. Arrows indicate the retention times of molecular-weight marker proteins. Retention times of major peaks are shown in parentheses. (B) Analysis of SSP-1–HSF complex by reverse-phase HPLC on a column of SepaxBio-C8 column (0.46 × 25 cm). (C) Surface plasmon resonance analyses of the binding between SSP-1 and HSF. Representative data of equilibrium binding are shown. Serially diluted HSF (from 500 nM to 31.5 nM) were injected at a flow rate of 20 μl/min through flow cells with SSP-1 immobilized to the sensor chip surface.

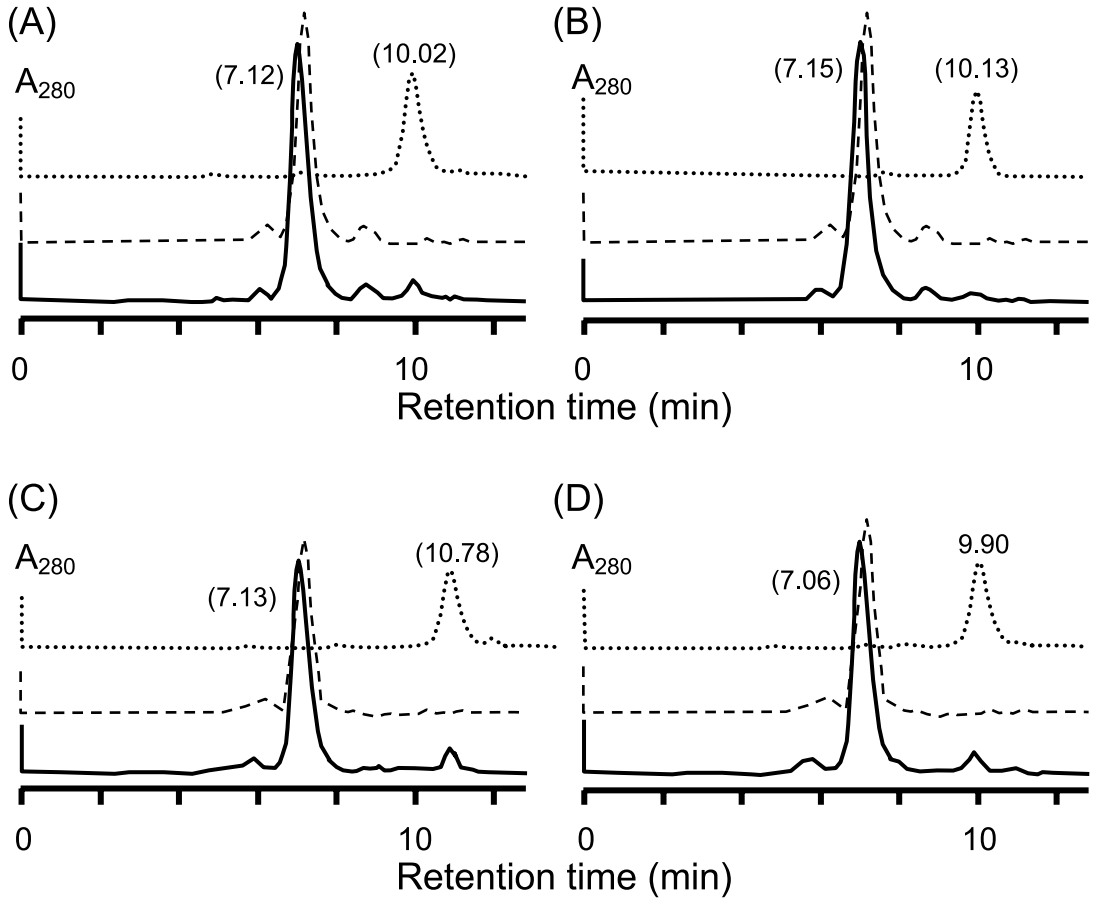

Fig. 3. Binding analysis of HSF to SSP-2 (A), SSP-3 (B), SSP-4 (C), and SSP-5 (D). An equal amount of each of the 4 SSPs (100 μM) was added to 5 μl of HSF (90 μM). The mixtures were analyzed by gel filtration HPLC on a column of TSKgel G3000SW (0.75 × 30 cm) in 50 mM phosphate buffer (pH 7.0) containing 0.2 M NaCl at a flow rate of 1.0 ml/min. Retention times of SSPs and SSP-HSF complexes are shown in parentheses.

between SSP-1 and HSF, we investigated the effect of SSP-1–HSF complex on the proteolytic activity of several SVMPs. SSP-1 alone did not inhibit these enzymes at all (data not shown). As shown in Fig. 4, HSF inhibited the activities of several SVMPs such as HR1A, HR1B, HR2a, and HR2b, but the inhibition was not influenced by the presence of SSP-1.

Discussion

Since SSPs are low-molecular-mass proteins, they may be present in a high-molecular-mass form to prevent excretion in the urine. Therefore, the presence of a binding protein with a high molecular mass was expected. Serotriflin was isolated from *T. flavoviridis* serum as a candidate SSP–binding protein[8]. Structural analysis revealed that serotriflin is a member of the CRISP family. The CRISP family of proteins shows a wide phylogenetic distribution, ranging from plants to mammals[21]. Indeed, SSP-2 forms a noncovalent complex with serotriflin and triflin[1,8]. Recently, we have found that SSP-5 also binds to serotriflin and triflin (unpublished). As discussed in a previous paper[1], SSP-2 seems to prevent self-injury by accidental leaking of venom into the blood since triflin possesses neurotoxin-like activity[11].

Unlike SSP-2 and SSP-5, however, other SSPs could not effectively bind to serotriflin. Since all the SSPs do not aggregate in aqueous buffers but exist in high-molecular-mass forms in the serum, another binding protein may be present. Affinity chromatography of SSP-deficient serum on an SSP-1–HiTrap column revealed that HSF, an

antihemorrhagic factor, may be the SSP-1 carrier protein. Furthermore, the analytical gel filtration proved that HSF could bind to all the SSPs, and that HSF is present as a 100-kDa homodimer (Figs. 2 and 3). BJ46a, an HSF protein homolog isolated from the serum of *Bothrops jararaca*, also forms a homodimer[22]. The molecular weight of the SSP-HSF complex suggested that the stoichiometry of HSF and SSP in the complex is 1 : 1 on the basis of subunits. This is in contrast to the 2 : 1 stoichiometry between human PSP94 and CRISP-3. Preliminary experiments showed that MSF, another homolog of HSF in *G. blomhoffi brevicaudus* serum[23], could also bind to all the SSPs in a neutral buffer (data not shown).

The complexes between SSPs and their binding proteins could dissociate in acidic solutions with pH below 4[1]. HSF and SSPs are considerably stable at extreme pH values as well as high temperatures[1,13]. The high stability of SSPs may be responsible for their tight conformation as estimated from the 3D structure of human and porcine PSP94 proteins[7]. The acid stability of both HSF and SSPs facilitated the Biacore experiments; repeated washing of the sensor chip conjugating the SSPs with a buffer at pH 2 did not lead to a decrease in the binding potency to HSF. HSF bound to SSPs with dissociation constants of 2.0×10^{-7}–6.6×10^{-7} M. The affinity was approximately 10 times lower than that of SSP-2 to triflin and serotriflin[8]. This suggests that serotriflin is a better SSP-2 carrier protein than HSF. Since the serum concentration of HSF is much higher than that of serotriflin, SSP-2 seems to be present as a complex with either HSF or serotriflin. However, a preliminary experiment revealed that the SSP-2–serotriflin complex could further bind HSF, and vice versa, to form a ternary complex (data not shown). This indicates that SSP-2 can bind CRISP and HSF at 2 distinct regions.

Although SSP-1 is the major component among the 5 SSPs, its physiological function is still speculative. It weakly inhibits brevilysin H6[20] but not other SVMPs. The significance of SSP-1–HSF complex formation is not yet clear. SSP-1 may play a role in the antihemorrhagic activity of HSF as a regulator of its inhibitory activity by either enhancement or suppression. For example, SSP-1 can help the binding of HSF to the P-I class of SVMPs, which are less effective targets of HSF[19]. Another possibility is the inhibition of HSF activity on physiologically important metalloproteinases such as matrix metalloproteinases that are present in the tissues and blood in snakes. If HSF can inhibit the proteolytic activity of these enzymes, it will not be favorable for the animal. SSP-1 may counter or weaken this deleterious effect of HSF.

SSP-1 did not affect HSF-inhibition of SVMPs (Fig. 4). This indicates that HSF functions merely as a carrier of SSP-1 in *T. flavoviridis* serum. It is also likely that the target molecule of the SSP-1–HSF

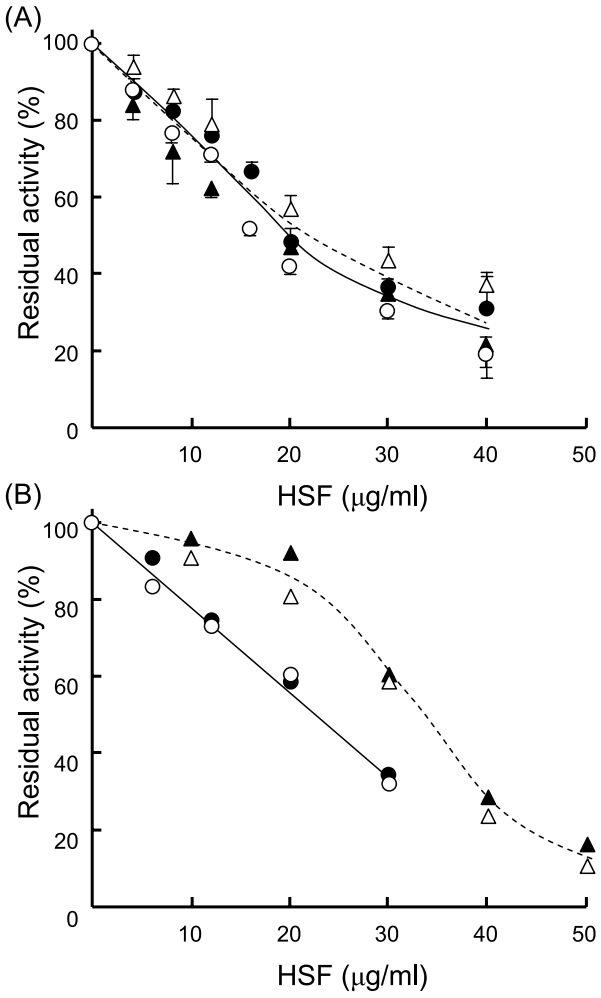

Fig. 4. Effect of SSP-1 on the HSF inhibitory activity to several SVMPs. (A) Inhibitory effect on HR1A (circles, 15 μg/ml) and HR1B (triangles, 15 μg/ml). (B) Inhibitory effect on HR2a (circles, 20 μg/ml) and HR2b (triangles, 10 μg/ml). Open and closed symbols show the results in the absence or presence of SSP-1 (8 μg/ml).

complex is not SVMP. Since many proteins in the serum of crotalid snakes target the toxic substances in their own venom, SSP-1 may interact with some venomous proteins in the free state or in an HSF-bound form.

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