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A novel serine protease from the snake venom of *Agkistrodon blomhoffii ussurensis*

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Abstract

A novel serine protease, ABUSV-SPase, was isolated to homogeneity for the first time from Chinese *Agkistrodon blomhoffii ussurensis* snake venom, and its enzymatic and structural properties were characterized by multiple techniques. ABUSV-SPase is a stable monomeric protein with a molecular mass of 26,752.6 a.m.u. It reacts optimally with its substrate N_α-tosyl-L-arginine methyl ester (TAME) at pH 7.0 and 41 °C. ESI-MS/MS analysis indicates that ABUSV-SPase is a new serine protease, sharing peptide homologies with various snake venom serine proteases, especially the snake venom thrombin-like enzymes of this group, and serine protease precursors. It is a zinc-containing protein, and although zinc is not essential for activity, its replacement by various divalent metal ions, including Mg²⁺, Mn²⁺, and Ca²⁺, increases the TAME hydrolysis activity of the enzyme. The intrinsic fluorescences of Tyr and Trp residues of ABUSV-SPase have emission wavelengths red-shifted by 12.8 nm and 3.6 nm from those of free Tyr and Trp, respectively. The zinc ion increases the hydrophobicity of the environment of the Trp residues, increases the thermostability of the protein, and affects the protein secondary structure to stabilize the enzyme, but appears to have no direct role in its esterase hydrolysis activity.

Keywords

Serine protease; Snake venom; Characteristics; Structure

1. Introduction

The venoms from most species in crotalidae and viperidae snake families are particularly rich in a wide spectrum of hydrolytic enzymes, including several types of proteolytic enzymes, which result in toxic effects such as hemorrhage and blood-clotting disorders (Braud et al., 2000; Castro et al., 2004; Castro and Rodrigues, 2006; Kornalik, 1990; Liu et al., 2006). High-

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Conflict of interest

The authors declare that there are no conflicts of interest.

throughput proteomic/transcriptomic snake venom analyses have proved the presence of various SPases in the venoms of crotalidae and viperidae snakes (Calvete et al., 2007; Fox et al., 2006; Guerranti et al., 2008; Li et al., 2004; Serrano et al., 2005).

Serine proteases with high specificity for arginine ester hydrolysis activity that can be inhibited by DFP and PMSF but not by the thrombin inhibitor heparin are widely distributed in crotalidae and viperidae snake venoms (Gaffney, 1977; Jin et al., 2007; Muanpasitporn and Rojnuckarin, 2007; Oshima et al., 1969; Shier and Mebs, 1990). These SPases have been considered to play important roles in blood-clotting disorders (Braud et al., 2000; Castro et al., 2004; Castro and Rodrigues, 2006; Jin et al., 2007; Kornalik, 1990; Liu et al., 2006). Considerable attention has been attracted by these particular enzymes because of their potential therapeutic usage in myocardial infarction and thrombotic diseases by acting on fibrinogen to lead to the defibrinogenation of blood, which results in a consequent decrease in blood viscosity (Farid et al., 1989; Jin et al., 2007; Muanpasitporn and Rojnuckarin, 2007). The consistency and flow properties of blood are improved, and a marked decrease in the fibrinogen level is obtained (Ouyang et al., 1992; Jin et al., 2007). Various thrombin-like enzymes (TLEs), kallikrein-like enzymes, fibrinogenases, and other enzymes possessing arginine ester hydrolysis activity have been isolated and characterized from different snake venoms (Bjarnason et al., 1983; Gaffney, 1977; Guedes et al., 2008; Jin et al., 2007; Liu et al., 2006; Markland and Damus, 1971; Muanpasitporn and Rojnuckarin, 2007; Oshima et al., 1969; Oyama and Takahashi, 2006; Pirkle et al., 1986; Sant'Ana et al., 2008; Toom et al., 1970).

In this paper, an ABUSV-SPase, a novel stable monomeric SPase with arginine ester hydrolytic activity, was isolated to homogeneity from ABUSV and analyzed by multiple techniques to characterize its enzymatic and structural characteristics.

2. Materials and methods

2.1. Materials

DEAE Sephadex A-50 (A-50), Sephadex G-75 (G-75), acrylamide, Bis, SDS, TEMED, DTT, and iodoacetamide were purchased from GE Healthcare. Trifluoroacetic acid (TFA), formic acid (FA), and TAME were from Sigma.

2.2. ABUSV-SPase purification

ABUSV-SPase was purified by a three-step column purification. Crude venom was dissolved in 0.05 M Tris-HCl (pH 7.6) and centrifuged at 10,000 rpm for 10 min at 4 °C. The supernatant was loaded onto an A-50 column (1.6 × 64 cm). The column was washed with 0.05 M Tris-HCl, pH 7.6, and the protein eluted by a linear (0–0.6 M) NaCl gradient, with an elution flow rate of 0.40 mL/min. Fraction sizes of 4 mL/tube were collected. The active fractions were concentrated, loaded onto a G-75 column (1.6 × 96 cm), and the protein eluted by 0.05 M Tris-HCl, pH 7.5, with a flow rate of 0.3 mL/min and fraction sizes of 3 mL/tube. ABUSV-SPase was further separated by a second A-50 column (1.2 × 48 cm), eluted with Tris-HCl buffer (pH 7.5, 0.05 M) and a linear (0–0.3 M) NaCl gradient, with an elution flow rate of 0.3 mL/min and fraction sizes of 2.5 mL/tube.

2.3. Esterase hydrolysis activity assay

The TAME hydrolysis activity was determined by the method of Roberts (Liu et al., 2006; Roberts, 1958). One unit of the esterase activity is defined as the amount of the enzyme that hydrolyzes 1.0 μmol of TAME/min.

2.4. PAGE and gel filtration chromatography

SDS-PAGE and native PAGE were performed using 15% and 12% polyacrylamide gels. Gel filtration chromatography was performed on an S-300 (1.0 × 120 cm) column eluted with 25 mM Tris-HCl (pH 7.5, 1.0 mM DTT, 0.15 M NaCl). The column was calibrated with thymoglobulin (670,000 Da), bovine gamma-globulin (158,000 Da), chicken ovalbumin (44,000 Da), equine myoglobin (17,000 Da), and vitamin B₁₂ (1350 Da). Protein molecular weight was calculated according to the following equation:

$$\text{Log}(M_w) = 8.25 - 0.056 \times V$$

where V is the elution volume for the sample.

2.5. MALDI-TOF mass spectrometry analysis

Experimental methods were as previously reported (Liu et al., 2006; Sun et al., 2006). The concentration of ABUSV-SPase was 3 μM. Sinapinic acid was served as the matrix. The mass spectrum was obtained from an accumulation of 200 single shots. The error in molecular mass is less than 0.05%.

2.6. Protein identification of ABUSV-SPase by nESI-MS/MS, sequence analysis and phylogenetic tree construction

ABUSV-SPase was digested in-gel by trypsin (Liu et al., 2006; Sun et al., 2006). Tryptic peptides were then extracted from the gel by two cycles of 50% ACN, 1% FA. Nanoflow multi-dimensional chromatography (Ettan MDLC) and an LTQ mass spectrometer (Thermo) combined platform were used for separation of peptides and MS/MS analyses (America et al., 2006; GE, 2006). Mobile phase A consisted of HPLC-grade water with 0.1% FA while mobile phase B was 84% HPLC-grade ACN with 0.1% FA. Peptide analysis was performed using data-dependent acquisition of an MS scan (400–2000 m/z) followed by an MS/MS scan. MS/MS spectra were analyzed using TurboSEQUENT (Bio-works) (Crockett et al., 2005). Peptide cross-correlation (Xcorr) scores of 1.9, 2.5, and 3.75 were set for charge states 1, 2, and 3. Protein probability was set below 10^{-3} .

Sequence similarity between ABUSV-SPase and other SVSPases sequences was accessed using ClustalW and the multi-sequence alignment was conducted by CLUSTX2 (Thompson et al., 1994; Larkin et al., 2007). Phylogenetic tree was constructed by using the software of MEGA 4 (Tamura et al., 2007).

2.7. Determination of optimal pH and temperature

pH and temperature effects on enzyme activity were measured between pH 2 and pH 11, and 20 °C and 80 °C. The following buffers (50 mM) were used: glycine-HCl (pH 2.0–4.0), acetic acid-sodium acetate (4.5–6.0), Bis-Tris (6.0–7.0), Tris-HCl (7.0–9.0) and glycine-sodium hydroxide buffer (9.5–11.0). Protein concentration of ABUSV-SPase was 2 μM. Tris-HCl buffer (0.05 M, pH 7.0) was used as the solvent for the optimum temperature determination.

2.8. ABUSV-SPase intrinsic fluorescence and fluorescence quenching study

Synchronous fluorescence (Liu et al., 2006; Sun et al., 2006) was used to analyze the fluorescence of ABUSV-SPase to distinguish between the overlapped fluorescences of Trp and Tyr residues. The concentration of ABUSV-SPase was 2 μM and both excitation and emission slits were 5 nm. The quenching effects of acrylamide and iodide were analyzed using the modified Stern-Volmer equation (Lackowicz, 1983):

$$\frac{F_0}{\Delta F} = \left(\frac{1}{f_a K_{SV} [Q]} \right) + \frac{1}{f_a}$$

where F_0 is the fluorescence intensity without quencher, f_a is the fraction of the initial fluorescence which is accessible to quencher, $[Q]$ is the concentration of quencher, K_{SV} is the Stern–Volmer quenching constant, and ΔF is the difference between the fluorescence intensity in the absence and presence of quencher ($F_0 - F$).

2.9. Metal ion determination and the effects of metal ions on enzyme activity

ICP-AES system (Thermo Jarrell Model ICAP 61E) was used for metal ion measurement. Five readings were obtained to enable statistical treatment of results. To remove the zinc, the protein was dialyzed extensively against 0.02 M Tris–HCl buffer, pH 7.5, containing 0.050 M EDTA and Chelex-100 beads. The resulting apo-ABUSV-SPase had a ratio of $[Zn^{2+}]/[enzyme] < 0.03$. Then Ca^{2+} , Mg^{2+} , Zn^{2+} , Fe^{2+} , Cu^{2+} , Co^{2+} , Ni^{2+} , Mn^{2+} , Cd^{2+} , or Tb^{3+} were added to the enzyme solution (4 μ M) to give a final metal ion concentration of 0.02 M. The mixtures were incubated at 4 °C for 24 h before enzyme activities were measured.

2.10. Thermal denaturation measurement by UV-visible absorption and far-UV CD spectroscopy

ABUSV-SPase (1.0 mg) was dissolved in 4 ml of 0.02 M Tris–HCl (pH 7.5), and aliquots of 1.8 mL enzyme solution were added into two 1 cm quartz cuvettes. One cuvette served as the reference; the other cuvette was heated by a flowing-water heating incubator attached to the UV 922 spectrometer (Kontron). The changes of absorbance at 280 nm (A_{280}) were then recorded at temperature intervals of 2–3 °C.

CD spectra were acquired from 190 nm to 260 nm with a Jasco J-720 spectropolarimeter employing a scan speed of 50 nm/min, a band-pass of 1 nm, and a quartz cell of 0.5-cm path length. The native ABUSV-SPase and apo-ABUSV-SPase were prepared in 10 mM Tris–HCl (pH 7.0) with final concentrations of 0.08 mg/mL. Thermal denaturation experiments were carried out by monitoring the CD changes at 220 nm as the temperature was varied from 20 °C to 80 °C at a ramping rate of 2–3 °C/min (Liu et al., 2006; Pace and Scholtz, 1997; Sun et al., 2006).

3. Results and discussions

3.1. ABUSV-SPase purification results

ABUSV-SPase was purified to homogeneity by a three-step chromatographic process, as shown in the isolation flow chart (Fig. 1A). SDS-PAGE result indicates that the purified ABUSV-SPase has an apparent molecular weight of about 27 kDa (Fig. 1B). An accurate molecular mass of ABUSV-SPase was obtained by MALDI-TOF mass spectrometry with a relative error below 0.03% (Fig. 1C) (Liu et al., 2006; Sun et al., 2006). The mass ion signal peak ($m/z = 26,752.6$) is the molecular ion $[M + H]^+$ of ABUSV-SPase, and the peaks $m/z = 13,359.6$, 53,727.1, and 80,432.6 are the doubly-charged molecule $[M + 2H]^{2+}$, dimer ion $[2M + H]^+$, and trimer ion $[3M + H]^+$, respectively. No ion peaks of impurities are observable, testifying to the high purity of the ABUSV-SPase. To this point, a protein with TAME hydrolytic specific activity of 276.6 μ mol/mg min was purified 98.8-fold with 0.16% recovery of protein and 15.8% recovery of activity (Table 1).

3.2. ABUSV-SPase quaternary structure

Since the results from SDS-PAGE and MALDI mass spectrometry only reflect the molecular mass and homogeneity of protein under denatured conditions, native PAGE and S-300 column chromatography were performed to investigate the quaternary structure of the ABUSV-SPase in aqueous solution. Native gels were obtained for various concentrations (1.0 μ M, 5.0 μ M, 20.0 μ M, 50 μ M) of ABUSV-SPase (Fig. 1D); and a single band was consistently observed at the same position, indicating that ABUSV-SPase exists in a single quaternary state. By using an S-300 column, the molecular weight of ABUSV-SPase was estimated to be 24.5 kDa and 27.5 kDa in two separate assays (Fig. 1E). Considering that the accurate molecular mass of ABUSV-SPase is $26,752.6 \pm 13.4$ a.m.u., ABUSV-SPase exists as a stable monomer.

3.3. ESI-MS/MS analysis and protein identification of ABUSV-SPase

MS/MS spectra were searched against the NCBI nr database using TurboSEQUEST Bioworks. Nine peptides, IIGGDECNI-NEHR, TLCAGILEGGKDTCT, SIIAGNTDASCPP, TLCAGILEGGK, VFDHLDWIK, FLVALYTSR, VPNEDAQTR, WDKDIMLMR and RFYCGGTLINQEWVLTAAHCDR were identified by matching sequences of two SPases from *Gloydius blomhoffi* and *Trimeresurus flavoviridis* venoms, two snake venom thrombin-like enzymes (SVTLEs) from *Gloydius shedaoensis* and *Gloydius ussurensis* venoms, and a series of SPases of *Viridovipera stejnegeri* (Table 2). And the MS/MS-derived peptides of ABUSV-SPase give a calculated molecular mass of 11,157.7 Da, which covers 41.7% ($11,157.7/26,752.6 \times 100\%$) of the whole sequencing of ABUSV-SPase. Sequence alignment (Fig. 2A) results indicate that the sequences TLCAGILEGGK and VFDHLDWIK are found in nine of these snake venom proteins. For the SVTLE (21309888/AAM46086) obtained from *G. ussurensis*, the above two sequences are replaced by TLCA-GILKGGK and VFDYTEWIK, respectively. The sequences VPNEDAQTR and RFYCGGTLINQEWVLTAAHCDR are only found in an SVTLE from *G. ussurensis*. Another three peptides, TLCAGILEGGKDTCT, SIIAGNTDASCPP, FLVALYTSR, are matched in three sequences and IIGGDECNI-NEHR is matched in six SVSPases sequences (Table 2, Fig. 2A). Thus the ABUSV-SPase contains peptides from different SVSPases with significant sequence differences. As a large number of SPases sequences were matched for the ABUSV-SPase, we conclude it is a new SPase, which we name ABUSV-SPase. And by comparison with published sequences (Castro et al., 2004), the enzyme is identified as a new member of the SVTLE subset of SPases. The evolutionary relationship of ABUSV-SPase with the other 10 SVSPases (Fig. 2A) was analyzed by phylogenetic tree construction (Fig. 2B). In the tree, the SPase KN7 precursor, venom SPase 1, VSP2_TRIFL and a TLE from *G. ussurensis* forms a cluster, while the four SPase precursors obtained from the same *V. stejnegeri* forms another cluster (Fig. 2B). ABUSV-SPase forms a cluster with VSP1_AGKHA and TLE (*G. shedaoensis*) by indicating that the ABUSV-SPase was evolutionarily related with the later two SVSPases. Considerable attention has been attracted by arginine ester hydrolases from various snake venoms because of their potential therapeutic use in myocardial infarction and thrombotic diseases (Farid et al., 1989; Ouyang et al., 1992). ABUSV-SPase, as a new SVTLE addition to this treasury bank, may benefit the treatment of various thrombotic diseases.

3.4. Optimum pH and temperature of TAME hydrolysis

The rate of hydrolysis of TAME by ABUSV-SPase (relative to the maximum hydrolysis rate at pH 7.0) was determined between pH 2.0 and pH 11.0. ABUSV-SPase exhibits optimal activity between 5.0 and 9.0 (Fig. 3A). Comparing to other kinds of snake venom proteases having esterase hydrolytic activity (Liu et al., 2006; Mori and Sugihara, 1989; Viljoen et al., 1979), ABUSV-SPase shows a similar optimal pH range to those enzymes (Mori and Sugihara, 1989; Viljoen et al., 1979) but a broader optimal pH range than the plasminogen activator from

ABUSV (Liu et al., 2006), indicating a range of enzymatic characteristics among the different snake venom enzymes.

ABUSV-SPase exhibits maximal hydrolysis of TAME at 41 °C (Fig. 3B) and optimal hydrolytic activity between 30 °C and 50 °C. At temperatures below 27 °C and above 52 °C, its hydrolytic activity decreases dramatically, which implies that ABUSV-SPase is heat labile, as confirmed by thermal stability analysis using UV-visible and CD spectroscopy techniques (see below).

3.5. Zinc content and influences of divalent metal ions on hydrolytic activity of ABUSV-SPase

The accurate molecular mass of ABUSV-SPase was determined by MALDI-TOF mass spectrometry and protein concentration by the method of Lowry. Five readings were obtained for each sample and the results treated statistically (Liu et al., 2006; Sun et al., 2006). Results from ICP-AES measurements indicate that the ABUSV-SPase is a zinc-binding protein with a stoichiometry of 1:1 [Zn^{2+}]:[ABUSV-SPase] (Table 3), which is consistent with our result that snake venom plasminogen activators, including the SVSPases, are potential zinc-containing proteins (Liu et al., 2006). The zinc ions were observed in the crystals of human prostate-specific antigen (PSA or KLK3) (Menez et al., 2008) and human tissue kallikrein 4 (KLK4, Debela et al., 2006) that belong to a 15-member family of closely related SPases, and experimental results indicated that their biological activities of these proteins are modulated by the zinc. However, until now zinc has been reported in only one other snake venom serine protease (Liu et al., 2006).

Since the apoenzyme is as active as the Zn^{2+} -containing enzyme, the Zn^{2+} is not catalytically essential. With Mn^{2+} , Mg^{2+} , and Ca^{2+} , the ester hydrolytic activity of ABUSV-SPase increases 2.5-, 2.3-, and 1.8-fold (Table 4). In comparison, Mg^{2+} , Mn^{2+} , and Ca^{2+} increased the rate of hydrolysis only about 50% for a number of related enzymes (Mori and Sugihara, 1989; Viljoen et al., 1979). Tb^{3+} , which has a similar charge/radius ratio as Ca^{2+} , has been widely used as a substitute for Ca^{2+} in probing calcium-containing proteins (Basu et al., 2003; Xu et al., 2002). In this case, however, the TAME activity of the Tb^{3+} ABUSV-SPase is about the same as the Zn^{2+} or apo-ABUSV-SPase and less than the Ca^{2+} ABUSV-SPase, suggesting that Tb^{3+} may bind to the enzyme more weakly (Basu et al., 2003; Xu et al., 2002) than Ca^{2+} . The only metal ion to show a negative effect on activity was Cd^{2+} , which resulted in a 20% less active enzyme. Like Zn^{2+} , Cu^{2+} , Co^{2+} , and Ni^{2+} show no effect on the hydrolytic activity of ABUSV-SPase. The results suggest that metals preferring hard (oxygen) donor ligands and capable of high coordination numbers result in highest activity, likely because they lead to small changes in protein conformation.

Zinc ion does not change the activity of ABUSV-SPase for hydrolysis of TAME, which is similar to our previous result for a plasminogen activator having one homologous peptide to the SPase from *Trimeresurus jerdonii* (Liu et al., 2006). This suggests that the zinc ion most probably plays a similar role in ABUSV-SPase by helping to maintain the protein's structural integrity.

3.6. Effect of zinc ion on ABUSV-SPase structure

3.6.1. Synchronous fluorescence—In comparison to the fluorescence for free Tyr and Trp, synchronous fluorescence results reveal red shifts of 12.8 nm and 3.6 nm for the maximum emission wavelengths of the Tyr and Trp residues of ABUSV-SPase, which suggests that the Tyr and Trp residues in ABUSV-SPase localize in relatively polar and hydrophilic environments (Liu et al., 2006; Sun et al., 2006).

3.6.2. Intrinsic fluorescence quenching study—Solute fluorescence quenching reactions of tryptophan residues in protein and other biological assemblies are an important tool for studying the location of fluorescence groups, and conformational changes and dynamics of protein molecules (Eftink and Ghiron, 1987; Muanpasitporn and Rojnuckarin, 2007). Plotting the fluorescence intensity data according to the modified Stern–Volmer relationship resulted in a linear dependence for acrylamide (Fig. 3C), which suggests that the fluorescence quenching takes place on a simple collisional basis. The value of $1/f_a$ is 0.93, indicating that all of enzyme fluorescence is quenched by acrylamide for the selected range of acrylamide concentration, and $K_{SV} = 33 \pm 1/\text{M}$. Quenching by iodide ion, at concentrations up to 1 M, also follows a Stern–Volmer relationship with $f_a = 0.63$, while NBS, which oxidizes Trp residues, quenches ~50% of the fluorescence at an [NBS]:[ABUSV-SPase] ratio of 2.0, after which no further quenching occurred (data not shown). These results suggest that all the Trp residues are accessible to acrylamide and partially accessible to iodide and NBS as certain Trp residue is not on the surface and thus not accessible to NBS. The larger K_{SV} for acrylamide suggests that it is better access to all of the Trp residues than the negatively charged iodide. Both types of Trp are, however, situated in polar environments as no blue-shift was observed for the maximum emission wavelength for ABUSV-SPase fluorescence during any of the fluorescence quenching experiments (Eftink and Ghiron, 1987; Zhu et al., 1997).

3.6.3. The effect of zinc on the intrinsic fluorescence of ABUSV-SPase—The presence of the zinc ion was found to change both the emission fluorescence intensity and maximum emission wavelength for ABUSV-SPase, as shown in Fig. 3D. The presence of Zn^{2+} ion changes the conformational environment by increasing the fluorescence emission yield for Trp residues, resulting in a maximal fluorescence intensity of the native form ABUSV-SPase (line b, Fig. 3D), 42.6% higher than that of the apo-ABUSV-SPase (line a). In addition, the maximal emission wavelengths for the native and apo-ABUSV-SPases were 350.5 nm and 354.5 nm, respectively; the 4.0-nm-blue-shift indicates that Zn^{2+} ion increases the hydrophobicity of the environment of the Trp residues in ABUSV-SPase. The results suggest that the zinc ion probably affects the tertiary structure of ABUSV-SPase.

3.7. UV and CD evidence for zinc affecting the thermal stability of ABUSV-SPase

The thermal stabilities of the native form ABUSV-SPase and apo-ABUSV-SPase were characterized by employing UV-visible and CD spectroscopy, and the apparent denaturation temperatures were obtained for both (Table 5). Data from UV and CD are consistent with each other in indicating that the presence of zinc ion facilitates the thermal stability of ABUSV-SPase. UV spectroscopy results show that the native ABUSV-SPase has an apparent denaturation temperature (T_m) of $71.0 \pm 1.0^\circ\text{C}$ and the apo-ABUSV-SPase has an apparent T_m of $67.5 \pm 1.0^\circ\text{C}$. The 3.5°C difference in the T_m s indicates the importance of the zinc ion in maintaining the thermal stability and structural integrity of ABUSV-SPase (Liu et al., 2006; Rossi et al., 2002; Sun et al., 2006). The removal of the zinc ion from ABUSV-SPase likely changes the secondary structure of ABUSV-SPase, as was further confirmed by studying the thermal denaturation by far-UV CD. The CD results indicate that the native ABUSV-SPase has a T_m of $69.5^\circ\text{C} \pm 1.0^\circ\text{C}$, 3.5°C higher than that of apo-ABUSV-SPase (Table 5). Since the far-UV CD signal reflects changes in the secondary structure of monomeric protein species, especially the α helical content (Liu et al., 2006; Pace and Scholtz, 1997; Sun et al., 2006) the T_m difference between the native- and apo-forms of ABUSV-SPase reflects and confirms an important role of the zinc ion in maintaining the secondary structure of ABUSV-SPase.

3.8. Zinc ion and the structural integrity of ABUSV-SPase

ICP-AES measurements indicate that the ABUSV-SPase is a zinc-binding protein with a stoichiometry of 1:1. However, zinc ion is not essential for the hydrolysis of TAME by ABUSV-SPase. We conclude that the zinc ion is involved in maintaining the structural integrity

of ABUSV-SPase based on the experimental evidences listed below. First, Zn^{2+} ion is at least conformationally required for the regional environment for Trp residue(s) of ABUSV-SPase. The presence of Zn^{2+} ion changes both the emission fluorescence intensity and maximum emission wavelength for ABUSV-SPase (Fig. 3D). The maximal fluorescence intensity of the native form ABUSV-SPase is 42.6% higher than that of the apo-ABUSV-SPase. In addition, Zn^{2+} ion induces a 4.0-nm-blue-shift for the intrinsic fluorescence of ABUSV-SPase. Second, Zn^{2+} also enhances the thermal stability of ABUSV-SPase by increasing its T_m by 3.5 °C, implying a more stable structure in the presence of Zn^{2+} (Liu et al., 2006; Rossi et al., 2002; Sun et al., 2006). Third, Zn^{2+} ion at least can change the composition of the secondary structure of ABUSV-SPase. The far-UV CD measurement indicates that the Zn^{2+} changes the secondary structure composition of monomeric ABUSV-SPase, especially the α helical content (Liu et al., 2006; Pace and Scholtz, 1997; Sun et al., 2006). Taken together, the results show that Zn^{2+} enhances the structural integrity of the enzyme.

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Abbreviations

A-50	DEAE Sephadex A-50
ABUSV	<i>Agkistrodon blomhoffii ussurensis</i> snake venom
ACN	acetonitrile
Bis	<i>N,N'</i> -methylenebisacrylamide
CD	circular dichroism
DFP	diisopropylfluorophosphates
DTT	dithiothreitol
FA	formic acid
G-75	Sephadex G-75
MALDI-TOF MS	matrix-assisted laser adsorption time-of-flight mass spectrometry
NBS	N-bromosuccinamide
nESI-MS	nanoelectrospray ionization mass spectrometry
PAGE	polyacrylamide gel electrophoresis
PMF	phenylmethylsulfonyl fluoride
SDS	sodium dodecyl sulfate
S-300	Sephacryl 300
SPase	serine protease
SVSPase	snake venom serine protease
SVTLE	snake venom thrombin-like enzyme
TAME	N_α -tosyl-L-arginine methyl ester
TFA	trifluoroacetic acid
UV	ultraviolet visible absorption spectroscopy

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Fig. 1.
(A) ABUSV-SPase purification flow chart; (B) SDS-PAGE (12%) of ABUSV-PA; (C) MALDI mass spectrum of ABUSV-SPase; (D) native PAGE gel ABUSV-SPase at different concentrations, 1.0 μ M, 5.0 μ M, 20 μ M, and 50 μ M for the lanes from left to right; and (E) molecular weight determination of ABUSV-SPase by S-300 chromatography. Calculated results for the duplicate experiments on ABUSV-SPase are indicated by arrows.

Fig. 2.

(A) Sequence comparison between ABUSV-SPase and other 10 snake venom serine proteases sharing the homologous peptides with ABUSV-SPase; (B) phylogenetic tree of snake venom proteases sharing the homologous peptides with ABUSV-SPase. Protein sequences were aligned by CLUSTLX2 and phylogenetic tree was constructed by MEGA 4. The sequences used in the alignment are MS/MS-derived sequences of ABUSV-SPase, VSP1_AGKHA, 3122187/P81176; TLE, 38146946/AAR11859; TLE, 21309888/AAM46086; VSP2_TRIFL, 13959615/O13057; SPase KN9 precursor, 33307056/AAQ02896; SPase KN10 precursor, 33307065/AAQ02899; SPase KN7 precursor, 33307089/AAQ02907; SPase KN3 precursor, 33307074/AAQ02902; SPase1, 24266790/AAN52346; and SPase KN1 precursor, 33307050/AAQ02894.

Fig. 3.

(A) Optimal pH for ABUSV-SPase activity; (B) optimal temperature for the enzyme activity; (C) the modified Stern–Volmer plot for fluorescence quenching of ABUSV-SPase by acrylamide, slope = 0.0307, intercept = 0.927, $R^2 = 0.9993$; and (D) the effect of Zn^{2+} on the intrinsic fluorescence of ABUSV-SPase, lines a and b are the emission fluorescence spectra of native ABUSV-SPase and apo-ABUSV-SPase, respectively.

Table 1

Isolation results for ABUSV-SPase

Steps	Mass (mg)	Protein yield (%) ^a	Activity (μmol/min mg)	Purification factor ^b
Crude ABUSV	500	100	2.8	1
1st A-50	5	1	26.0	9.3
G-75	1.8	0.36	121.8	43.5
2nd A-50	0.8	0.16	276.6	98.8

^a Mass of protein obtained in each step as a percentage of the total mass of crude ABUSV.

^b The specific activity of the enzyme fraction from each step compared to the specific activity of crude ABUSV.

Table 2

Peptides identified in ABUSV-SPase

MS/MS-derived sequence	Measured mass (monoisotopic)	z	Peptide probability
IIGGDEC ^l NINEHR ^{a,c,d}	1526.73	2	2.43E-07
TLC ^l AGILEGGKDTCT ^l Ra,b	1650.85	2	1.54E-09
SIHAGNTDASC ^l PPa,b	1302.63	2	2.84E-04
TLC ^l AGILEGGK ^{a,b,d-j}	1118.62	2	7.97E-03
VFDHLDWIK ^{a,b,d-j}	1172.61	2	7.65E-05
FLVALYTSR ^{b,c}	1069.60	2	5.97E-04
VPNDAQTR ^c	1029.50	2	9.75E-05
WDKDIMLM ^k Ra-d,f,j	1223.59	2	7.37E-04
RFYC ^l GGTLINQEWVLTAAHC ^l DR ^c	2667.32	3	1.10E-08

^aVSP1_AGKHA (*Gloydius blomhoffi*, GI:3122187/P81176 (protein entry number)).^bThrombin-like enzyme (*Gloydius shedaoensis*, GI:38146946/AAR11859).^cThrombin-like enzyme (*Gloydius ussuriensis*, GI:21309888/AAM46086).^dVSP2_TRIFL (*Trimeresurus flavoviridis*, GI:13959615/O13057).^eSerine protease KN9 precursor (*Viridovipera stejnegeri*, GI:33307056/AAQ02896).^fSerine protease KN10 precursor (*Viridovipera stejnegeri*, GI:33307065/AAQ02899).^gSerine protease KN7 precursor (*Viridovipera stejnegeri*, GI:33307089/AAQ02907).^hSerine protease KN3 precursor (*Viridovipera stejnegeri*, GI:33307074/AAQ02902).ⁱVenom serine protease 1 (*Viridovipera stejnegeri*, GI:24266790/AAN52346).^jSerine protease KN1 precursor (*Viridovipera stejnegeri*, GI:33307050/AAQ028940).^kOxidation of met residue.^lCarboxymethylation of cysteine by IAM.

Table 3

Zinc measurement for ABUSV-SPase

Protein mass (μg)	Protein moles ($\times 10^6$)	Zinc mass (μg)	Zinc moles ($\times 10^6$)	Zn/protein
238 ^a	0.0089	0.62	0.0096	1.08
240 ^b	0.0090	0.67	0.0103	1.14
234 ^c	0.0087	0.71	0.0109	1.24
237 ^d	0.0089	0.67	0.0103	1.16

Column 1, triplicate tests (^a, ^b and ^c) and the average result (^d); column 2, protein amount in mole unit; columns 3 and 4, zinc amount in mass and mole; and column 5, stoichiometry for zinc to protein.

Table 4

Effects of metal ions on ABUSV-SPase hydrolytic activity

Ions	Relative activity (%) ^a
Zn ²⁺ (as isolated)	100 ± 3
Apo-enzyme	98 ± 6
Mg ²⁺	230 ± 12
Ca ²⁺	170 ± 10
Mn ²⁺	250 ± 15
Cu ²⁺	96 ± 6
Co ²⁺	95 ± 5
Ni ²⁺	102 ± 4
Cd ²⁺	80 ± 6
Tb ³⁺	98 ± 2

^a Average of three experiments.

Table 5

Thermal stability of ABUSV-SPase

Method	T_m^b (°C) native ABUSV-SPase	T_m (°C) apo-ABUSV-SPase
UV	71.0 ± 1.0^a	67.5 ± 1.0^a
CD	69.5 ± 1.0^a	66.0 ± 0.8^a

^a Average of three experiments.^b T_m , denaturation temperature.