



Short Communication

Molecular cloning and fibrin(ogen)olytic activity of a bumblebee (*Bombus hypocrita sapporoensis*) venom serine proteaseYuling Qiu^a, Young Moo Choo^a, Hyung Joo Yoon^b, Byung Rae Jin^{a,*}^a College of Natural Resources and Life Science, Dong-A University, Busan 604–714, Republic of Korea^b Department of Agricultural Biology, National Academy of Agricultural Science, Suwon, Republic of Korea

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ABSTRACT

Although several bee venom serine protease genes have been previously described, fibrin(ogen)olytic activity of these serine proteases has been reported for only two bumblebees to date, *Bombus ignitus* and *B. terrestris*. Here, we cloned venom serine proteases from the other bumblebee species, *B. hypocrita sapporoensis* and *B. ardens ardens*. The venom serine protease genes of *B. h. sapporoensis* and *B. a. ardens* consist of 358 amino acids and 357 amino acids, respectively. We compared the predicted mature protein sequences of these serine protease genes to those previously reported for other bees. A phylogenetic analysis shows that *B. h. sapporoensis* venom serine protease is further immediately close to *B. ignitus* and *B. terrestris* venom serine proteases, excluding the venom serine protease of *B. a. ardens*. Using *B. h. sapporoensis* venom serine protease (Bs-VSP), we identified that Bs-VSP acts as a fibrin(ogen)olytic enzyme. We also found that Bs-VSP activates prothrombin and directly degrades fibrinogen into fibrin degradation products. Our results further define roles for bumblebee venom serine proteases as fibrin(ogen)olytic agents.

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Introduction

Bee venom has been used traditionally for the treatment of various diseases, including arthritis, rheumatism, pain, tendonitis, cancerous tumors, and skin diseases (Hider, 1988; Son et al., 2007). Bee venom contains a variety of enzymes (i.e., serine protease), peptides, and biogenic amines (Hoffman and Jacobson, 1996; Winningham et al., 2004; Hoffman, 2006; Son et al., 2007). Serine proteases, found in many organisms, are of broad interest because they have diverse physiological functions, affecting processes such as digestion, immune response, complement activation, cellular differentiation, and hemostasis (Neurath, 1984; Krem and Cera, 2002). In snake venom, a number of serine proteases exhibit fibrin(ogen)olytic activity (Braud et al., 2000; Matsui et al., 2000; Kini, 2005; Swenson and Markland, 2005). Bumblebee (*Bombus* spp.) venom contains three major components: bombolitin, phospholipase A₂, and serine protease (Xin et al., 2009; Choo et al., 2010a,b; Qiu et al., 2011). Our previous studies provided the first evidence of fibrin(ogen)olytic activity of the bumblebee venom serine proteases that act as a prothrombin activator, a thrombin-like protease, and a plasmin-like protease (Choo et al., 2010b; Qiu et al., 2011).

Although bee venom has been studied intensively as a rich source of pharmacologically active components (Son et al., 2007), serine proteases in bee venom remain poorly understood compared with those in snake venoms. The molecular function of bee venom serine proteases has been reported for *Bombus ignitus* (Choo et al., 2010b) and *B. terrestris* (Qiu et al., 2011), but the properties of these proteins in other bee species have not yet been elucidated. Here, we report on the molecular cloning of venom serine proteases from two bumblebee species, *B. hypocrita sapporoensis* and *B. ardens ardens*, and the fibrin(ogen)olytic activity of *B. hypocrita sapporoensis* venom serine protease (Bs-VSP). The present study demonstrates that Bs-VSP is a fibrin(ogen)olytic enzyme that functions as a prothrombin activator, a thrombin-like protease, and a plasmin-like protease, further defining the fibrin(ogen)olytic roles of bumblebee venom serine proteases.

Materials and methods

Bumblebees

B. hypocrita sapporoensis and *B. ardens ardens* (Hymenoptera: Apidae) bumblebees used in this study were supplied by the Department of Agricultural Biology at the National Academy of Agricultural Science, Republic of Korea. The bumblebees were maintained in continual darkness at 28 °C and 65% relative humidity as previously described (Yoon et al., 2009).

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Gene cloning and sequence analysis

Total RNA was isolated from the venom glands of *B. h. sapporoensis* and *B. a. ardens* worker bees using a Total RNA Extraction Kit (Promega, Madison, WI, USA).

Venom serine protease cDNAs of *B. h. sapporoensis* and *B. a. ardens* bees were RT-PCR-amplified using the following primer sets designed from the *B. ignitus* venom serine protease gene sequence (Choo et al., 2010b): forward primer, 5'-ATGACGGGCTCCAAGATGCTGTTC-3' and reverse primer, 5'-TTATTGCATCGCTGGGAGAATAAA-3'. The nucleotide sequences of PCR products were determined using a BigDye Terminator cycle sequencing kit and an automated DNA sequencer (Perkin-Elmer Applied Biosystems, Foster City, CA). The sequences were compared using the DNASIS and BLAST programs provided by NCBI (<http://www.ncbi.nlm.nih.gov/BLAST>). MacVector (ver. 6.5, Oxford Molecular Ltd., Oxford, UK) was used to align the deduced amino acid sequences of venom serine protease genes. Phylogenetic relationships among bee venom serine protease sequences were obtained by the neighbor-joining method using MEGA Version 4.0 (Tamura et al., 2007).

Bee venom preparation and serine protease purification

B. h. sapporoensis venom was collected from venom sacs dissected from worker bees as previously described (Choo et al., 2010b; Qiu et al., 2011). The complete sting apparatus was removed from *B. h. sapporoensis* worker bees after inducing anesthesia by chilling. Venom sacs were cut free and immersed in phosphate-buffered saline (PBS; 140 mM NaCl, 27 mM KCl, 8 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.4), and the supernatant was collected after centrifugation at 10,000 ×g for 10 min. Bs-VSP was purified from the supernatant by size-exclusion column chromatography. Size-exclusion chromatography was performed at a flow rate of 0.5 ml/min using a HiPrep 16/60 Sephacryl S-100 column (Amersham Biosciences) equilibrated with 0.05 M sodium phosphate buffer (pH 7.2) containing 0.15 M NaCl. Purified Bs-VSP was identified with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Prothrombin activation assay

A prothrombin activation assay was performed as previously described (Choo et al., 2010b; Qiu et al., 2011). Human prothrombin (2 µg, Sigma) was incubated at 37 °C with 2 ng of Bs-VSP in 50 mM Tris-HCl buffer (pH 8.0) containing 100 mM NaCl and 5 mM CaCl₂. Aliquots were removed at various time points and analyzed using 14% SDS-PAGE.

Fibrin(ogen)olytic cleavage assay

A fibrin(ogen)olytic cleavage assay was performed as previously described (Choo et al., 2010b; Qiu et al., 2011). Human fibrinogen (10 µg, Sigma) was incubated at 37 °C with 0.2 µg of Bs-VSP in 50 mM Tris-HCl buffer (pH 8.0). Aliquots were removed at various time points and analyzed using 14% SDS-PAGE.

Fibrin plate assay

The fibrin plate assay was performed with 5 ml human fibrinogen (0.5%) clotted with three units of thrombin (Sigma). Varying concentrations of *B. h. sapporoensis* and *B. terrestris* crude venom were dropped onto the fibrin plate and incubated at 37 °C for various periods of time. Fibrinolytic activity was determined by examining the formation of a clear area (Choo et al., 2010b; Qiu et al., 2011).

Results and discussion

Cloning and sequence analysis of venom serine protease genes

To characterize the serine proteases in bumblebee venom, we cloned two venom serine protease genes from *B. h. sapporoensis* and *B. a. ardens* worker bees. Bs-VSP and *B. a. ardens* venom serine protease (Ba-VSP) consist of 358 amino acids and 357 amino acids, respectively (GenBank accession numbers: JF979158 for Bs-VSP and JN542539 for Ba-VSP). Database searches using the protein sequence of mature Bs-VSP showed similarity to members of the bee venom serine protease family, such as that from *B. ignitus* (97% protein sequence identity), *B. terrestris* (97% protein sequence identity), *B. a. ardens* (81% protein sequence identity), and *B. pennsylvanicus* (66% protein sequence identity) (Fig. 1A). In addition, the predicted Bs-VSP and Ba-VSP proteins contain features consistent with the bee venom serine proteases, including a catalytic triad (His, Asp, and Ser) in the serine protease domain (Fig. 1A). These results indicate that both Bs-VSP and Ba-VSP possess a catalytic domain that is structurally similar to other bee venom serine proteases (Hoffman and Jacobson, 1984; Hoffman et al., 2001; Winningham et al., 2004; Choo et al., 2010b; Qiu et al., 2011). Collectively, these data suggest that Bs-VSP and Ba-VSP belong to the bee venom serine protease family.

The phylogenetic relationship of bee venom serine proteases is depicted in Fig. 1B. The tree shows that the bumblebee venom serine proteases of *B. h. sapporoensis*, *B. terrestris*, and *B. ignitus* form further immediately close group, excluding those *B. a. ardens* and *B. pennsylvanicus*. Bs-VSP showed a high similarity to *B. ignitus* and *B. terrestris* venom serine proteases, whereas Ba-VSP showed a comparatively low similarity to them. This result is similar to data characterizing bumblebee mitochondrial 16S rRNA sequences, which show a high similarity among the bumblebee group of *B. ignitus*, *B. h. sapporoensis*, and *B. terrestris* species (Yoon et al., 2004).

Fibrin(ogen)olytic activity of Bs-VSP

To prepare Bs-VSP from *B. h. sapporoensis* venom, the venom components were separated using fast protein liquid chromatography techniques. The active form of Bs-VSP in *B. h. sapporoensis* venom was detected as a 34-kDa protein (Fig. 2A). Considering that Bs-VSP is a bumblebee venom serine protease, we investigated whether Bs-VSP exhibits fibrin(ogen)olytic activity. First we assessed whether Bs-VSP activates prothrombin by determining the time course of human prothrombin cleavage and found that Bs-VSP activated the conversion of prothrombin into thrombin (Fig. 2B). Based on the cleavage pattern observed during prothrombin activation, the activity of Bs-VSP strongly resembles the activity of bumblebee venom serine proteases from *B. ignitus* and *B. terrestris* (Choo et al., 2010b; Qiu et al., 2011). This result demonstrates that Bs-VSP activates prothrombin, indicating that Bs-VSP has a role in thrombin activation.

Next, we assessed whether Bs-VSP possesses direct fibrin(ogen)olytic activity by analyzing the time course of the degradation of human fibrinogen into fibrin degradation products (FDPs) (Fig. 2C). The results revealed that the A α , B β , and γ chains of human fibrinogen disappeared and were replaced by FDPs. These findings show that Bs-VSP not only converts fibrinogen into fibrin, but it also degrades fibrin into FDPs, indicating that Bs-VSP acts as both a thrombin-like protease and a plasmin-like protease, as reported for *B. ignitus* and *B. terrestris* venom serine proteases (Choo et al., 2010b; Qiu et al., 2011). This result further characterizes bumblebee venom serine proteases as fibrin(ogen)olytic enzymes.

We also examined the fibrinolytic activity of *B. h. sapporoensis* and *B. terrestris* crude venoms using the fibrin plate method (Fig. 2D). The fibrin plate assay showed that the addition of *B. h. sapporoensis* and *B. terrestris* crude venoms led to the formation of a clear area, with the

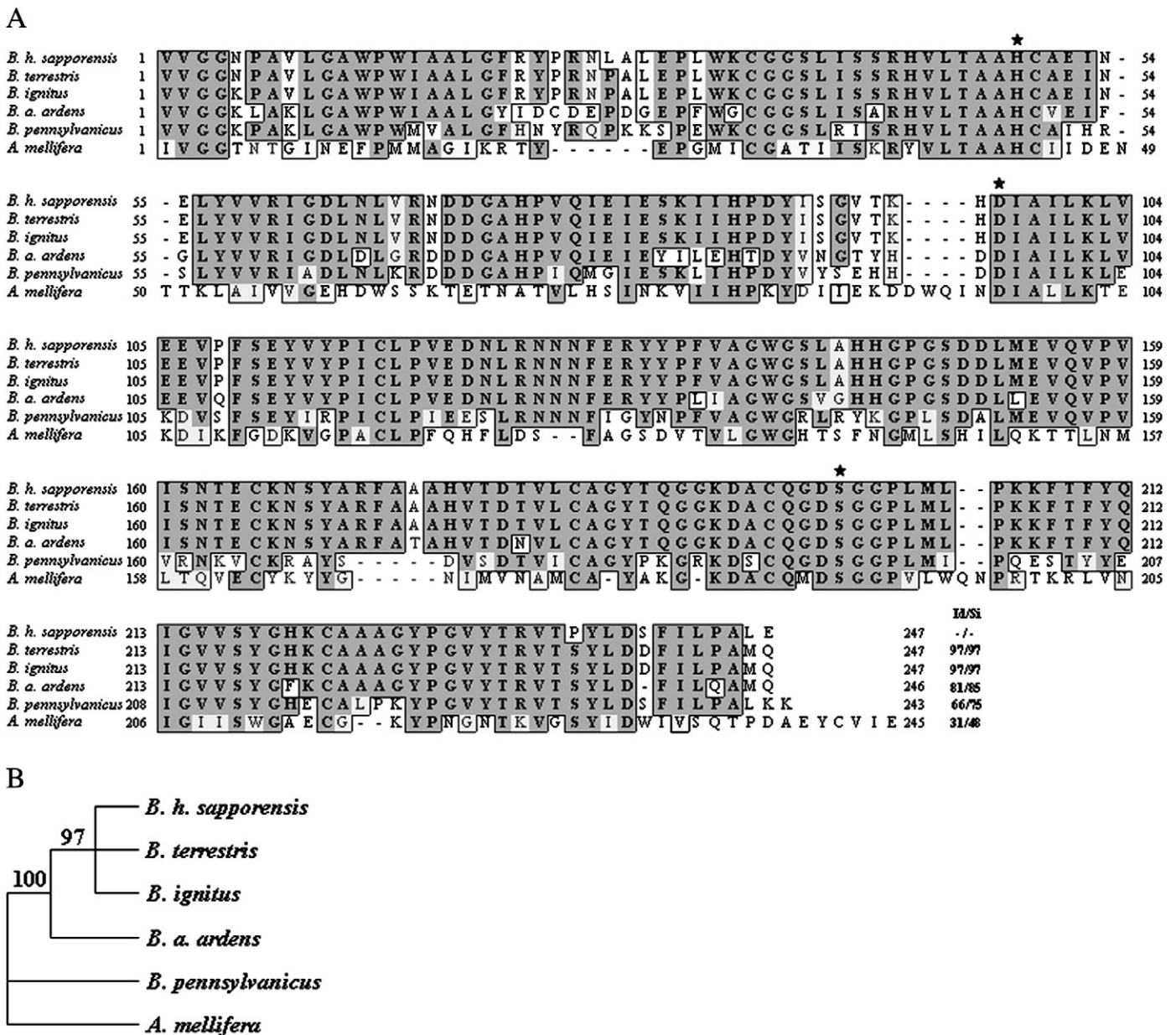


Fig. 1. Predicted amino acid sequences of Bs-VSP and Ba-VSP. (A) The alignment of the amino acid sequences of Bs-VSP and Ba-VSP with known bee venom serine proteases. Identical residues are shown in solid boxes. The dashes represent gaps that were introduced to preserve the alignment. The residues in the catalytic triad of the serine protease domain (H, D, and S) are indicated by asterisks. The sources for the aligned sequences were *B. h. sapporensis* (this study, GenBank accession no. JF979158), *B. a. ardens* (this study, GenBank accession no. JN542539), *B. terrestris* (GenBank accession no. HQ257242), *B. ignitus* (GenBank accession no. FJ159443), *B. pennsylvanicus* (UniProtKB/TrEMBL accession no. Q7M413), and *A. mellifera* (GenBank accession no. AY127579). The Bs-VSP sequence was used as a reference for the identity/similarity (Id/Si) values. (B) Phylogeny of bee venom serine proteases. The phylogenetic tree was constructed from the bee venom serine protease sequences aligned in (A) by the neighbor-joining method using the *A. mellifera* venom serine protease sequence as an outgroup.

size of the area dependent on the venom dose. This result demonstrates that bumblebee venom exhibits fibrinolytic activity, which may be due to the constituent amount of venom serine protease.

Overall, we have demonstrated that Bs-VSP functions as a fibrin (ogen)olytic enzyme similar to other bumblebee venom serine proteases (Choo et al., 2010b; Qiu et al., 2011) and snake venom serine proteases (Braud et al., 2000; Matsui et al., 2000; Kini, 2005; Swenson and Markland, 2005). Our results define roles for Bs-VSP as a thrombin-like protease, as a plasmin-like protease, and as a prothrombin activator. The observed roles for Bs-VSP suggest that the prothrombin activation and fibrin(ogen)olytic protease activities of Bs-VSP may cooperate to effectively decrease the concentration of blood fibrinogen (Koh et al., 2001; He et al., 2007) and thereby

facilitate the spread of bee venom components throughout the bloodstream in mammals, as demonstrated for snake venom fibrin(ogen)olytic enzymes (Koh et al., 2001; Swenson and Markland, 2005; He et al., 2007). Our results show that, as for *B. ignitus* and *B. terrestris* venom serine proteases (Choo et al., 2010b; Qiu et al., 2011), the bumblebee venom serine protease Bs-VSP affects the hemostatic system not only through prothrombin activation but also through thrombin-like and plasmin-like protease activities. The finding that Bs-VSP possesses fibrin(ogen)olytic activity similar to that of other bumblebee venom serine proteases (Choo et al., 2010b; Qiu et al., 2011) provides strong evidence that bumblebee venom serine proteases are hemostatically active proteins that are potentially promising therapeutic agents.

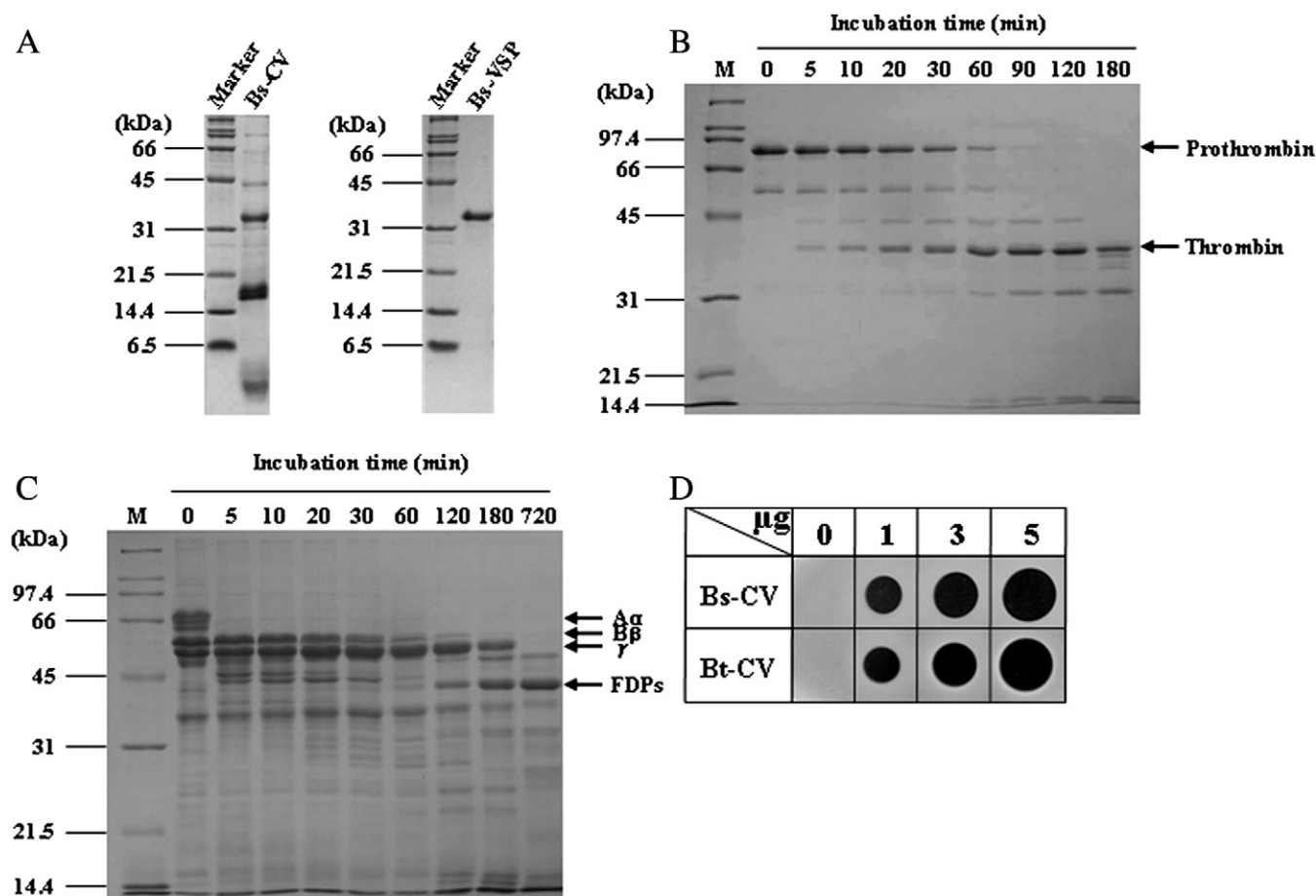


Fig. 2. Fibrin(ogen)olytic activity of Bs-VSP. (A) Purification of Bs-VSP. SDS-PAGE of Bs-VSP (right) purified from *B. h. sapporoensis* crude venom (Bs-CV, left). (B) Prothrombin activation of Bs-VSP. Human prothrombin activation of Bs-VSP was analyzed by SDS-PAGE. The number indicates the time (minutes) that the prothrombin was incubated with the Bs-VSP. Prothrombin and thrombin are shown. (C) SDS-PAGE analysis of human fibrinogen hydrolysis by Bs-VSP. The number indicates the time (minutes) that fibrinogen was incubated with Bs-VSP. Human fibrinogen chains and FDPs are shown. (D) Detection of the fibrinolytic activity of *B. h. sapporoensis* and *B. terrestris* crude venoms on fibrin plates. *B. h. sapporoensis* and *B. terrestris* crude venoms (1, 3, or 5 μg) were dropped onto fibrin plates and incubated at 37 °C for 12 h.

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