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Contributed Paper

Identification of Bioactive Moleculesfrom Thai Centipede, Scolopendrasubspinipes

Identification of Bioactive Molecules from Thai Centipede, *Scolopendra subspinipes dehaani*, Venom

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ABSTRACT

Centipede venoms are rich sources of bioactive molecules with various actions. These bioactive molecules obtained from Thai centipede, *S. subspinipes dehaani,* venom have been investigated by proteomic approach and bioactivity assay. Protein profile analysis by SDS-PAGE technique showed 7 major bands with a molecular weight ranging under 66 kDa. After resolved in 2D-PAGE gel, numerous spots of proteins were found in centipede venom. The trains of major spots were observed at 22 and 45 kDa. The 10 major protein spots were analyzed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). The presence of protease and phospholipase activities in centipede venom was tested by zymographic method an don activity indicator plate (PCY plate), respectively. These molecules were classified into 4 groups including cysteine-rich venom protein, protease, hyaluronidase and phospholipase.

Keywords: bioactive molecules, *Scolopendra subspinipes dehaani*, 2D-PAGE, LC-MS/MS

1. INTRODUCTION

Several bioactive molecules can be found in natural products. The bioactive molecules found in animal venoms are interesting sources which can be used for further applications like pharmacology, because their strong and various activity. Many venoms from animals which can cause serious health problem to human such as snake, scorpion, bee, hornet, wasp, spider, ant, jelly fish and some of fish, have been widely studied [1, 2, 3, 4, 5]. Here, this research focuses on bioactive molecules found in Thai centipede venom where limited knowledge is available.

Centipedes are predatory arthropods classified in class Chilopoda which are widely found in tropical area. *Scolopendra subspinipes dehaani* is a representative species of centipedes in Thailand. They have elongated and segmented body separated into head and thorax. Each thorax has a pair of legs. The first pair of walking legs is modified into venomous appendages called poison claws, forcipules, toxicognaths, or maxillipeds, which is equipped with a venom gland [6, 7, 8]. Venom from this gland composed of a variety of toxic substances such as peptide toxins and enzymes. This venom is used to paralyze their prey and also in the defense against predators [9, 10]. The long list of common symptoms of centipede bite was described such as pain, tissue swelling, redness, swollen and painful lymph nodes. Although symptoms of centipede bitten are usually not serious as such to cause death against human, severe allergy or anaphylactic shock could occur if a large amount of venom is injected [8, 11]. However, venom is a source of useful biologically active molecules, therefore purification, identification and characterization of these molecules may be useful for many types of application such as in fields of novel therapeutic agents, pesticides and antibacterial molecules [3, 12].

Early studies of centipede venomous properties grouped compounds of venom into non-protein including 5-hydroxytryptamine, histamine, lipids, polysaccharides and protein molecules and sub-grouped this second into enzymes and non-enzymatic molecules [7, 10]. Molecules in enzyme groups include esterases such as alkaline phosphatases and phospholipase A2 [13], proteases such as metalloproteases, serine proteases [14] and hyaluronidases whereas non-enzymatic proteins include myotoxins, cardiotoxins, neurotoxins and cytotoxins [9, 10, 15, 16, 17].

Recently, 2 studies [6, 17] used proteomic approach to study the biological component and properties of Brazilian centipede venoms. Two-dimensional chromatography (2D-LC) showing protein size ranging from 3 kDa to 21 kDa was found in *Scolopendra viridicornis nigra* (62 molecular masses) and from 1.3 kDa to 22.6 kDa in *Scolopendra angulata* (65 molecular masses). Using SDS–PAGE, protein sizes ranging from 7 to 205 kDa were found in three different Brazilian centipede venoms (*Otostigmus pradoi, Cryptops iheringi* and *Scolopendra viridicornis)*. Most of these protein enzymes were metalloproteinases. Hyaluronidase activity was observed only in *S. viridicornis* and *O. pradoi* venoms. No phospholipase A2 activity was found in *C. iheringi* venom.

In addition, studies combining proteomic and transcriptomic approach have been performed on centipede venoms [18, 19]. Due to the small amount of venom collected, proteomic studies of centipede venom are challenging. Adding transcriptomic studies of venom gland to the proteomic approach is a powerful strategy to overcome the complexity of centipede venom. These two studies showed that venom of centipede were composed of various enzyme and toxins. In particular, Liu.*et al* showed the importance of neurotoxins in composition of *S. subspinipes dehaani* venom [18].

However, there is still few information available on centipede venom composition compared to other venomous animals such as snake. Finding out of bioactive molecule is better chance to well-known with relationship of clinical symptoms. These present studies therefore have attempted to screen and identify the components of centipede venom by using combination of proteomic approach such as SDS-PAGE, 2D-PAGE, LC-MS/MS technique and activity assay. The venoms from Thai Scolopendra species, *Scolopendra subspinipes dehaani* was investigated to better understand the component and complexity of this venom which can be useful for future applications.

2. MATERIALS AND METHODS

2.1 Sample Preparation and Venom Collection

S. subspinipes dehaani centipedes were obtained from suburban areas around Khon Kaen city, Thailand. They were cold-anesthetized on ice and crude venom was collected with a micropipette tip after electric shock directly on forcepules using a stimulator with 100 volt, 1-msec duration at 10 Hz frequency [20]. Crude venom was dissolved in 1X phosphate buffered saline (PBS buffer), pH7.0 and stored at -70 ^{0}C until used. The protein concentration of the venom was determined by Bradford dye assay using bovine serum albumin (BSA) as standard.

2.2 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis Analysis (SDS-PAGE)

Crude venom (30 μg) was solubilized in 2X solubilizing buffer (0.5 M Tris-HCl, pH 6.8, 10% (v/v) glycerol, 10% (w/v) sodium dodecyl sulfate (SDS) and 1% (w/v) bromophenol blue) under reducing condition (β-mercaptoethanol ratio 1:1 (v/v)) and was applied on 15% (w/v) acrylamide separating gel. Electrophoresis was performed at 150 volt for 1 h. The protein bands were visualized by 0.1% (w/v) Coomassie brilliant blue R-250 in water: methanol: acetic acid (5:4:1) and silver solution. Phosphorylase B (97 kDa), bovine serum albumin (66 kDa), chicken ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20 kDa) and α–lactalbumin (14.4 kDa) were used as standards.

2.3 Protein Identification

2.3.1 Two-dimensional polyacrylamide gel electrophoresis analysis (2D-PAGE)

Crude, *S. subspinipes dehaani*, venom (100 μg) was dissolved in rehydration sample buffer (7 M urea, 2 M thiourea, 2% CHAPS, 7 mg/2.5 mL dithiothreitol (DTT), 2 % IPG buffer and 1% bromophenol blue). The first dimension was performed on isoelectric focusing (IEF) by using immobilized pH gradient (IPG) dry strip gel (pH 3-11 non-linear (nL), 7 cm; GE Healthcare). The strip was focused for 9250 Volt-hour using EttanTM IPGphor (Amersham Bioscience). After rehydration for 12 h, isoelectric focusing (IEF) was carried out by applying 500 V for 30 min, 1000 V for 30 min and 5000 V for 1 h and 40 min at 50 mA/strip. After focusing, the strip was soaked for 15 min in reduction solution with 2% DTT, followed by 15 min in alkylation solution with 5% iodoacetic acid (IAA). Then, the strip was performed on 15% (w/v) acrylamide gel for the second step electrophoresis at a constant current 20 mA. The proteins were visualized by colloidal Brilliant Blue G-250 and analyzed by 2D-image platinum program (GE Healthcare, Sweden).

2.3.2 Liquid chromatography coupled with mass spectrometry analysis (LC/MS-MS)

The 10 major protein spots from 2D gel were analyzed by mass spectrometry (LC-MS/MS) at the Genome Institute, Thailand (http://gi.biotec.or.th/GI). The excised spots were washed twice with MilliQ water and destained with 50 mM ammonium bicarbonate and 50% methanol and dehydrated with 100% acetonitrile (ACN). Samples were digested with trypsin solution (20 ng trypsin in 50% ACN/10 mM ammonium bicarbonate) overnight at 37°C. The resulting peptides were extracted by washing the gel pieces three times with 30 μL of 50% ACN/0.1% formic acid. The supernatants containing the peptides were then vacuum-dried in an incubator at 40 °C for 3-4 h and stored maintained at -20°C until mass spectrometry analysis.

The digested peptides were separated by nanoscale LC using a NanoAcquity system (Waters Corp., Milford, MA) equipped with a Symmetry C_{18} (5 μm, 180-μm x 200-μm) Trap column and a BEH130 C_{18} (1.7 μm, 100-μm x 100-μm) analytical reversed phase column (Waters Corp., Milford, MA) at a flow rate of 350 μL/

min. Water and acetonitrile, both containing 0.1% formic acid, were used as solvents A and B, respectively. All samples were analysed in triplicate. The analysis of tryptic peptides was performed using a SYNAPT^{TM} HDMS mass spectrometer (Waters Corp., Manchester, UK). All analyses were performed using positive nanoelectrospray ion mode. The time-of-flight analyser of the mass spectrometer was externally calibrated with [Glu^1] fibrinopeptide B from m/z 50 to 1600 with the acquisition lock mass corrected using the monoisotopic mass of the doubly charged precursor of [Glu^1] fibrinopeptide B. The quadrupole mass analyser was adjusted such that ions from m/z 200 to 1990 were efficiently transmitted. Bioworks 3.2 software (Thermo Electron) was used to process the data and convert it into a Mascot Generic File. *De novo* sequence was performed using the *De novo* software tool (Applied Biosystems). Theoretical mass analysis and pI determination were performed by Expasy software online (http://br.expasy.org/cgi-bin/peptide-mass.pl?P14790).

2.3.3 Database searching

Peptides were identified using a local Mascot server (Matrix Science) and the following search parameters: a specified trypsin enzymatic cleavage with one possible missed cleavage; +/- 0.6 Da mass tolerances for MS/MS; a peptide tolerance of 1.2 Da; 1+, 2+, 3+ ions; methionine oxidation variable modification; carbamidomethyl (C) fixed modification; monoisotopic mass; and 20 responses. Database homology searches of all peptide sequences were conducted using the Basic Local Alignment Search Tool (BLAST). (http://www.ncbi.nlm.nih.gov/) [21]

2.4 Bioactivity Assay

2.4.1 Protease activity

Crude, *S. subspinipes dehaani*, tissue extracted and venom (20 μg) was dissolved in 2X solubilizing buffer (0.5 M Tris-HCl, pH 6.8, 10% (v/v) glycerol, 10% (w/v) SDS and 1% (w/v) bromophenol blue) under non-reducing condition. Samples were incubated at 65^{0}C for 5 min and subsequently applied on 12% (w/v) acrylamide separating gel containing 1 mg/ml of gelatin. The electrophoresis was performed at 90 volt for 1h. SDS reagent was removed by using 2.5% (v/v) Triton X-100 and, then the gel was incubated in activity buffer (50 mM Tris–HCl, pH 7.6, 150 mM NaCl, and 10 mM $CaCl_2$) at 37^{0}C for 16 h. The protease activity was observed as white transparent band and visualized by staining with 0.1% (w/v) Coomassie brilliant blue R-250 in water: methanol: acetic acid (5:4:1) and destained in water: methanol: acetic acid (47:5:2) until reveal clear zones [22, 23, 24].

2.4.2 Phospholipase activity

Zymographic detection and PCY plate assay were performed to determine phospholipase activity. Crude, *S. subspinipes dehaani*, venom (20 μg) was mixed with solubilizing buffer (1 mM Tris-HCl, pH 7.4, 10% (v/v) glycerol and 2 mM EDTA) and applied onto 15% (w/v) acrylamide separating gel containing 5% (w/v) lecithin (native-PAGE). Electrophoresis was performed at the constant current 25 mA at 4^{0}C for 1 h. The phospholipase activity was visualized by 0.12% (w/v) Rhodamine 6G in 0.1 M Tris-HCl, pH 7.4, 20 mM $CaCl_2$ at 37^{0}C for 12 h [25] and 0.1% (w/v) Coomassie brilliant blue R-250 in water: methanol: acetic acid (5:4:1). Wasp venom and double distilled water were used as positive and negative control, respectively.

Phospholipase activity with PCY plate assay was detected directly from crude, *S. subspinipes dehaani*, venom and other animal venom (20 ug) as comparative samples including king cobra venom, cobra venom, wasp venom, centipede tissue extracted and ant venomon the PCY plate. Phospholipase activity was observed on the modified PCY agarose gel

for 24h, showing a transparent clear zone as a halo (gel-diffusion assay). The modified PCY gel contained 50 mM Tris–HCl, pH 8.0, 1% (w/v) NaCl, 0.8% (w/v) phosphatidylcholine from fresh lechitin, 0.25% (w/v) taurocholic acid and 20 mM $CaCl_2$ in 1% (w/v) agarose gels (separately autoclave) [26].

3. Results and Discussion

Centipede venom composed of complex mixtures of biochemically and pharmacologically active molecules which are mostly found to be peptides and proteins with various molecular weights. The most of bioactive molecules found in Thai centipede venom (*S. subspinipes dehaani*) was observed under 66 kDa (Figure 1) which is less than other reported species ranging from 7 to 200 kDa [6]. 2D-PAGE analysis revealed that there were approximately 50 protein spots (Figure 2). Most of protein spots were found at isoelectric point (pI) values ranging from 3.4 to 10.2 with molecular weight (MW) between 12 and 50 kDa. The 10 major protein spots were excised for protein identification and referred as SC1 to SC10, respectively. To identify bioactive proteins, LC-MS/MS technique was used to investigate the protein spots SC1 to SC10 (Table 1). BLAST homology searching revealed that peptides sequenced by LC-MS/MS matched with protein related with venom. They were classified into 3 different groups of enzyme including cysteine-rich venom protein (SC1 and SC2), protease (serine protease: SC4, SC6, SC8 and SC9, cysteine protease: SC5 and metalloprotease: SC7) and hyaluronidase (SC10).

In particular, the intense protein band around 45 kDa on SDS-PAGE composed of several spots with different isoelectric points on 2D-PAGE. Interestingly, the biological assay showed a strong protease activity on the

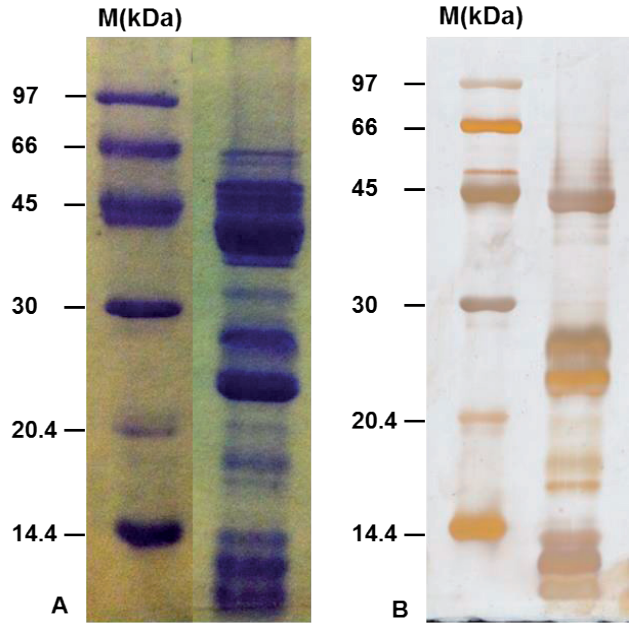

Figure 1. SDS-PAGE analysis of crude venom protein (30 ug protein) of Thai centipede, *S. subspinipes dehaani*. Representative of 15% (w/v) acrylamide separating gel were stained with Coomassie Brilliant Blue R-250 (A) and silver staining (B). Molecular weight marker (M, kDa) shows in the number on left: 97, Phosphorylase B; 66, bovine serum albumin; 45, chicken ovalbumin; 30, carbonic anhydrase; 20.4, trypsin inhibitor; 14.4, α–lactalbumin.

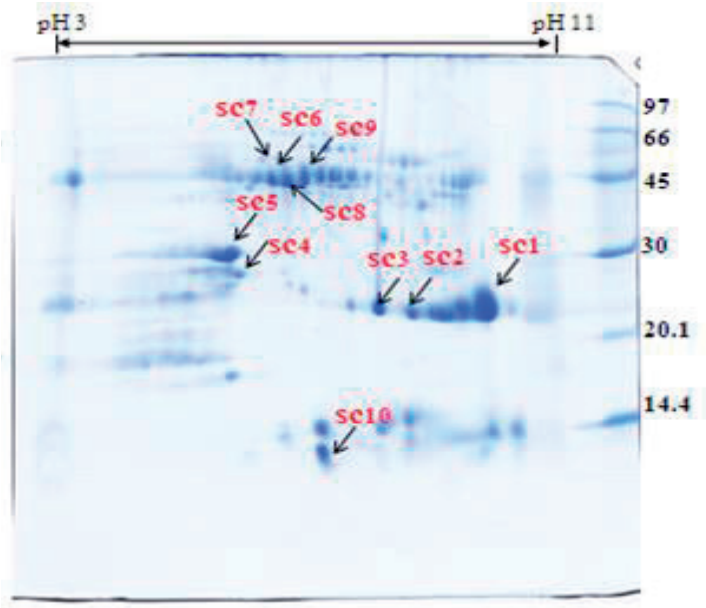

Figure 2. 2D-PAGE analysis of centipede, *S. subspinipes dehaani*, venom proteins. Representative of 15% (w/v) SDS-gel was stained with colloidal Coomassie Brilliant Blue G-250. The proteins were resolved with IPG dry strip gel (pH 3-11 nL, 7 cm; GE Healthcare) The identified proteins were analyzed by LC-MS/MS followed by *de novo* sequencing (indicated by arrows).

gelatin-zymogram gel at approximately 45 kDa (Figure 3). Most of reported protease around 45 kDa found in centipede venom has been described to be metalloprotease with caseinolytic, gelatinolytic and fibrinolytic activity blocked by phenanthroline [7]. However, the three of four excised (SC6, SC8 and SC9) spots around 45 kDa were identified as serine proteases by LC-MS/MS. Another weak protease activity was detected in the band ranging between 20-30 kDa relating with molecular weight of reported serine protease found in *O. pradoi*, *S. viridicornis* and *C. iheringi* [17]. Moreover, LC-MS/MS analysis identified as serine protease spots SC4 which has a compatible molecular weight of 30 kDa with protease activity band.

The other bioactive molecule found in Thai centipede, *S. subspinipes dehaani*, venom was detected as phospholipase by both phospholipase activity assay, lecithin-zymogram gel and on PCY plate. Phospholipase activity was strongly detected on lecithin-zymogram (Data not shown) and exhibited different appearance comparing with other venom phospholipases on PCY plate (Figure 4). The difference on appearances of the PCY gel was caused by the different phospholipases in venom. There are 4 different types of phospholipase including phospholipase A1, A2, C and D which showed different appearances: phospholipase A1 and A2 showed transparent halo, phospholipase C showed cloudy halo and phospholipase D did not exhibit on PCY gel [26]. We can imply that only phospholipase A1 or A2 activity can be detected in centipede venom as transparent halo was exhibited. Comparison with two kinds of snake venom (king cobra venom and cobra venom) and two kinds of insect venom (wasp venom and ant venom) showed similarity between phospholipase activities of different venoms. The observed halo of centipede venom was more similar to halo of snake venom than halo

Table 1. Identifications of putative proteins from Thai centipede, *S. subspinipes dehaani*, venom obtained from LC-MS/MS analysis..

Protein spot no	M_r/pI 2D-PAGE		Peptide sequence	Peptide mass	Protein name [Accession no]	Organism	Identification
SC1	23	9.5	SHHWNDELAANAQR	825.51	Venom allergen 5 [C0ITL3.1]	*Pachycondyla chinens*is	Cysteine-rich venom protein
SC2	22	7.9	NSYLMCQYGPGGNYR	899.15	Venom allergen 5 [Q05109.1]	*Polistes annularis*	Cysteine-rich venom protein
SC3	22	7.1	HRGALAGTTVGR	599.65	-	-	ND
SC4	26	5.4	VDDNDLMLLK	588.59	Kallikrein-14 [Q8CGR5.1]	*Mus musculus*	Serine protease
SC5	28	5.3	MGELEYNSQPAK	693.07	Cysteine peptidase [XP_003872328]	*Leishmania mexicana*	Cysteine protease
SC6	44	5.5	RDDGWGSQLSGWGNTR	897.71	Transmembrane protease serine 5 [Q9ER04.3]	*Mus musculus*	Serine protease
SC7	47	5.5	YTEQENGELVDLLKYK	971.24	Carboxypeptidase A [ABV60311.1]	*Lutzomyia longipalpis*	Metallo protease
SC8	43	5.7	MLYGWGPNVSGWGNTR	898.27	Trypsin-4 [P12788.1]	*Rattus norvegicus*	Serine protease
SC9	43	5.9	FPNEMYEGQVAK	715.13	Serine protease snake [XP_001649319.1]	*Aedes aegypti*	Serine protease
SC10	13	6.3	LKSPNGSQEASDAERK	859.19	Hyaluronidase [AAD32195.1]	*Lutzomyia longipalpis*	Hyluronidase

ND: No identification

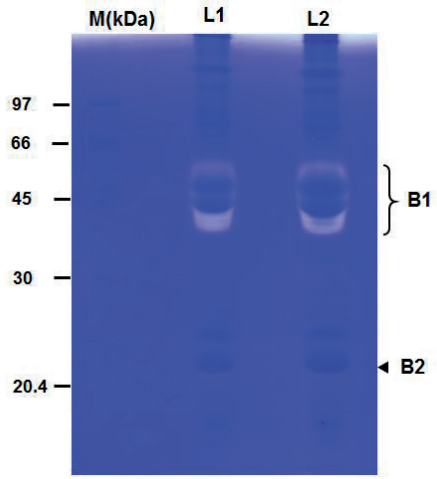

Figure 3. SDS-PAGE analysis with zymographic detection of Thai centipede, *S. subspinipes dehaani*, venom protein for protease activity. Representative of 12% (w/v) SDS-gel containing 1 mg/ml of gelatin was stained with Coomassie Brilliant Blue R-250. M: molecular weight marker, L1-2: crude venom of centipede 20 ug and 30 ug, respective.

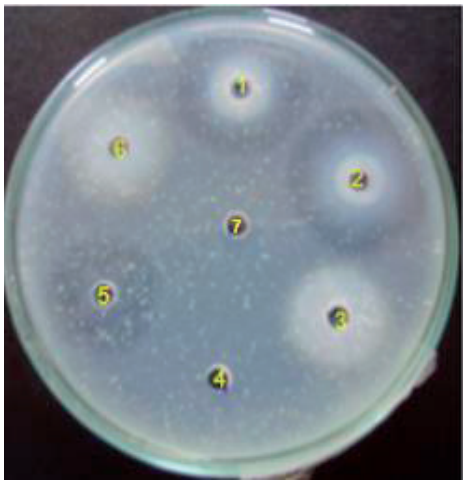

Figure 4. PCY plate analysis of Thai centipede, *S. subspinipes dehaani*, venom and other venom proteins (20 ug) for phospholipase activity. 1: King cobra venom, 2: Cobra venom, 3: Wasp venom, 4: Centipede tissue extracted, 5: Centipede venom, 6: Ant venom, 7: negative control.

of insect venom. This is in accordance with the report that phospholipase of centipede venom are related with cluster of snake phospholipase by homology and phylogenetic analysis [13].

Although no hyaluronidase activity assay has been investigated in this study, but a hyaluronidase was identified in spot SC10 by LC-MS/MS analysis (Table 1). They have been few information of hyaluronidase found in *O. pradoi* and *S. viridicornis* with different molecular weight.

4. Conclusions

In this work, we attempted to identify different proteins which compose venom of *S. subspinipes dehaani* venom by a proteomic approach and activity assay. Enzymatic activity test identified protease and phospholipase activity while mass spectrometry identified cysteine-rich venom protein, protease and hyaluronidase. Protease and phospholipase activity are important component of many venom where they commonly act as hemorrhagic and cytotoxic factor. Cysteine-rich venom protein contributes to hypersensitive reaction and hyaluronidase is known to play a role of spreading factor. These bioactive molecules can be implied in various clinical symptoms and contribute to the long list symptoms of centipede bite [8, 11].

A better known of biochemically composition and activity of centipede venom helps to understand the physiopathology of symptoms caused by centipede bite and to treat injuries caused by centipedes.

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