Functional characterization of a novel rHellerin (recombinant cysteine-rich secretory protein) from *Crotalus oreganus helleri*

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Introduction

While many snake venom toxins have been comprehensively characterized, other toxins such as snake venom cysteine-rich secretory proteins (svCRiSPs) remain largely unexplored. svCRiSPs have garnered a new spotlight as possible inflammatory and cell signaling cascade modulators (Table 1). Bj-CRP's, a CRiSP from the venom of *Bothrops jararaca,* has been shown to induce profound inflammatory responses in local tissues through the production of IL-6.[1] Other studies showed CRiSP from *Echis carinatus sochureki* inhibited MAPK Erk1/2 signaling pathway in vascular endothelial cells.[2] At the same time, CRiSP from Cobra venom was also demonstrated to interact with endothelial cells by interfering with cell adhesion molecules, which may disrupt the wound healing process during envenomation.[3]

svCRiSPs are low abundant and difficult to purify with traditional methods as large amounts of venom is needed. Expression of a recombinant protein that retains its biological function allows for the collection of the protein in large quantities, which can accelerate research into further understanding the molecular biology of svCRiSPs.

Table 1 Targets and biological effects of cysteine-rich secretory proteins from venom.[4]

Animals	Name	Species	Target (Interaction Molecules)	Biological Effect (or Related Investigation)	Accession Numbers	Ref.
Snake						
(Vipers)	Ablomin	*Gloydius blomhoffi*	N.D.	Ca^{2+} channel blocker-like properties	Q8JI40	[60]
	Piscivorinc	*Agkistrodon piscivorus*	N.D.	Ca^{2+} channel blocker-like properties	AY181982	[61]
	Catrin	*Crotalus atrox*	N.D.	Ca^{2+} channel blocker-like properties	AY181983	[61]
	Triflin	*Protobothrops flavoviridis*	N.D.	Ca^{2+} channel blocker-like properties	Q8JI39 1WVK, 6IMF (with inhibitor)	[60]
	Stecrisp	*Trimeresurus stejnegeri (Viridovipera stejnegeri)*	N.D.	No proteolysis activity (unlikeTex31)	P60823 1RC9	[62]
	Bj-CRP	*Bothrops jararaca*	Component C3 and C4	Bind and cleaved to component C3 and C4 Lack of effect of K^+ channel blockage activity (1.0 μM) (Kv1.1 to Kv1.6, Shaker-IR, Kv3.1, Kv7.1, Kv7.2, Kv7.4 and Kv10.1)	N.D. (partial sequence)	[63]
	Hellerin	*Crotalus oreganus helleri*	N.D.	To increase trans-epithelial permeability Cytotoxicity against HUVEC (cytotoxic concentration CC50 = 2.3 μM)	C9DCH4	[64]
	EC-CRISP	*Echis Carinatus Sochureki*	N.D.	Binding to HUVEC cell Anti-angiogenic Activities (10-20 μg/mL, <1 μM)	P0DMT4	[65]
	Crovirin	*Crotalus viridis viridis*	N.D.	Anti-protozoan activity against *Trypanosoma cruzi* and *Leishmania amazonensis*	N.D (partial sequence)	[66]

In this study, a novel recombinant CRiSP (rHellerin) cloned from the venom of *Crotalus oreganus helleri*, was expressed in *E. coli* strain Origami 2(DE3) and purified. rHellerin was characterized and compared with native CRiSP from *C. scutulatus scutulatus* venom in the hopes of elucidating functionally active residues and subsequent molecular targets/interactions, which can further the development of new therapeutic strategies to prevent death and disability from snakebite.

Methods

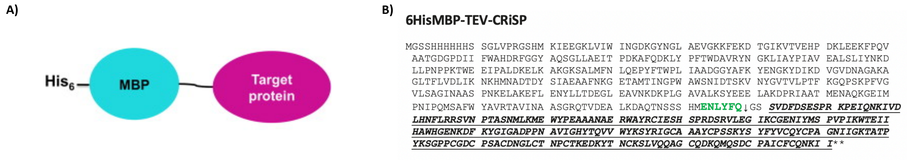


Fig. 1 A) Schematic representation of the organization of the MBP fusion proteins and His-tagged proteins. B) The full-length amino acid sequences of CRiSP from *C. helleri* (italicized letters) in a 6XHis-MBP expression vector. TEV = tobacco etch virus. The TEV protease recognition site is indicated in green. Arrow indicates TEV protease cleavage site.

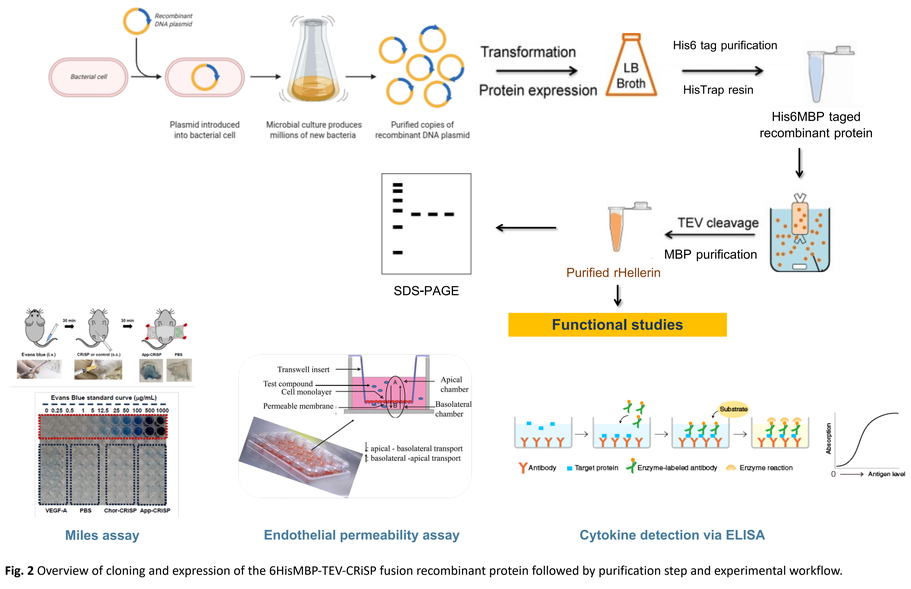
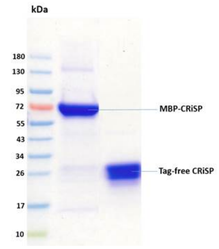


Fig. 2 Overview of cloning and expression of the 6HisMBP-TEV-CRiSP fusion recombinant protein followed by purification step and experimental workflow.

Results

Purification of rHellerin



- We have cloned and expressed a new recombinant CRiSP from *C. o. helleri* (rHellerin).
- Native CRiSP from the venom of *C. s. scutulatus* has 100% amino acid sequence identity to rHellerin and was used as a control for comparison purpose.

Fig. 3 SDS-PAGE of purified rHellerin expressed from *E.coli* using an MBP fusion technique.

Effect of rHellerin on vascular permeability using Miles assay

We examined the activity of tag-free rHellerin in an *in vivo* model of vascular permeability. Subcutaneous injection of rHellerin (170 ng/mouse) produced a rapid increase (40%) in the trans-capillary leakage of an intravascular dye (Evans blue) compared to the saline control (Fig. 4).

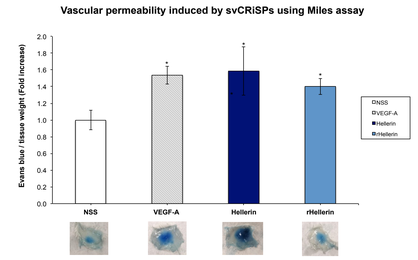


Fig. 4 Quantification of an *in vivo* permeability assay was performed using the modified Miles assay. Mice were injected intravenously with 100 μL of Evans blue dye, followed immediately by a subcutaneous injection of saline, native CRiSP, rHellerin (170 ng/mouse), or VEGF-A (170 ng/mouse). Results were normalized on tissue weight (mean ± SE). *p < 0.05, compared with saline control.

Endothelial cell permeability activity of rHellerin

rHellerin was able to induce a significant trans-endothelial permeability in both human dermal lymphatic (HDLEC) and blood (HDBEC) endothelial cells in comparison with that of cells treated with the PBS control (Fig. 5).

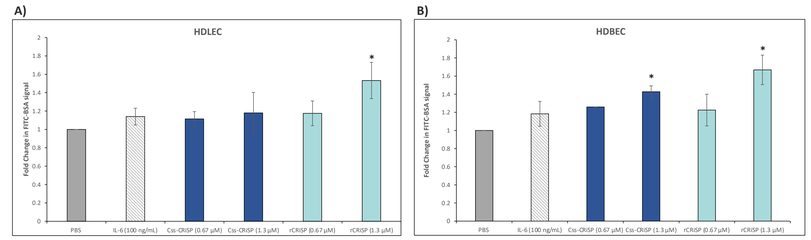


Fig. 5 Effect of rHellerin on monolayer barrier function of HDLECs (A) and HDBECs (B). At 72 h after visual confluence was obtained, cells were treated with rHellerin or native CRiSP (0.67 μM and 1.3 μM) for 60 min. Data expressed as mean ± SD of two individuals experiments (n = 3). *p < 0.05, compared with untreated control.

Effect of rHellerin on cytokine production in HDBECs using ELISA

To assess the effect of rHellerin in the production of proinflammatory cytokines, the cytokine measurements in the supernatants of HDBECs were performed. rHellerin increased the production of IL-8 at 3 and 24 h in comparison to non-stimulated cells, while no significant levels of the cytokine IL-6 was detected at 24 h (Fig. 6).

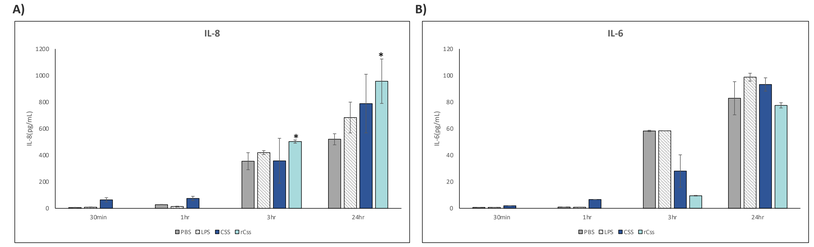


Fig. 6 Concentration of IL-8 (A) and IL-6 (B) produced in HDBECs after various incubation times of culture in the presence and absence of rHellerin. Cells were stimulated with rHellerin (1 μM) and the culture supernatants were collected at various incubation times. Cytokines in the supernatants were measured by sandwich ELISA, according to the manufacturer's suggested protocols. Data expressed as mean ± SD of two individuals experiments (n = 3). *p < 0.05, compared with untreated control.

Conclusions

- A rHellerin was expressed in *E. coli* as soluble form.
- rHellerin showed vascular and cellular permeability and pro-inflammatory responses similar to that of native CRiSP.
- rHellerin was able to induce vascular leakage in vivo and cellular permeability.
- rHellerin was also able to induce increased production of the cytokines IL-8 but not IL-6 in human blood endothelial cells.
- The production of cytokines in HDBECs and HDLECs treated with rHellerin at different incubation times will be further investigated.
- These findings can provide a straightforward method of obtaining biologically viable svCRiSPs identical to the native form, which can accelerate research into further understanding the molecular biology of svCRiSPs by elucidating functionally active residues and subsequent molecular targets/interactions.
- rHellerin can represent potential in the development of new therapeutic strategies to prevent death and disability from snakebite.

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